The diverse role of Ldb1 in cell differentiation and mouse embryonic development

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The Diverse Role of Ldb1 in Cell Differentiation and Mouse Embryonic Development

De veelzijdige rol van Ldb1 in celdifferentiatie en de embryonale ontwikkeling van de muis

Thesis

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to my grandmother...

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List of abbreviations

DNA: Deoxyribonucleic acid
RNA: Ribonucleic acid
ICM: Inner Cell Mass
dpc: days post coitum
VE: Visceral endoderm
ES cells: Embryonic Stem cells
LIF: Leukemia Inhibitory Factor
MEFs: Mouse Embryonic Fibroblasts
SVZ: Subventricular Zone
BL-CFC: Blast Colony Forming Cell
PSp: Para-aortic splanchnopleura
AGM: Aorta Gonads Mesonephros
CFU-S: Colony Forming Unit-Spleen
HSC: Hematopoietic Stem Cell
TGF-β: Transforming Growth Factor-β
BMP: Bone Morphogenetic Protein
LTR-HSC: Long Term Repopulating Hematopoietic Stem Cell
MEL: Mouse Erythroleukemia
LCR: Locus Control Region
HS2: Hypersensitive Site 2
NLS: Nuclear Localization Signal
MLS: Mitochondrial Localization Signal
CFP: Cyan Fluorescent Protein
PCR: Polymerase Chain Reaction
NSC: Neural Stem Cell
CNS: Central Nervous System
hrs: hours

Scope of this thesis

During the development of the mouse embryo tightly regulated differentiation pathways lead to the formation of the tissues and organs of the adult animal. The role of Ldb1 during embryonic development and in particular during hematopoiesis and neural development will be the focus of this thesis.

The **first chapter** of this thesis is a general introduction that covers the initial stages during the formation of the mouse embryo, the onset of hematopoiesis in the extra-embryonic tissues followed by the emergence of HSCs in the intra-embryonic PSp/AGM and the shift of hematopoiesis initially to the fetal liver and finally the bone marrow. In addition the chapter includes an overview of the signalling pathways and transcription factors that are involved in the regulation of hematopoiesis. Finally Ldb1 is introduced as a member of multi-protein complexes that regulate gene expression and cell differentiation and as a facilitator of long range interactions in transcription regulation.

In the **second chapter** the phenotype of the *Ldb1*-/- mouse is described in detail. The deletion of *Ldb1* was found to be detrimental for embryonic development as the resulting phenotype is characterized by early embryonic lethality and severe defects. Mouse expression arrays were used in order to gain insight into which development regulatory pathways and factors were misregulated in the absence of *Ldb1*.

In the **third chapter** the role of *Ldb1* in early hematopoietic development and the emergence of the hemangioblast was investigated in detail with the use of an *Ldb1*-/- ES cell line that was differentiated into embryoid bodies. Mouse expression arrays were used in order to establish which factors act downstream of *Ldb1* in the regulation of hematopoiesis in the mouse embryo.

In the **fourth chapter** the role of *Ldb1* in neural development was investigated with the use of the *Ldb1*-/- ES cell line that was differentiated into Neural Stem Cells (NSCs). Mouse expression arrays were used to determine the position of *Ldb1* in the hierarchy of factors that are involved in the emergence of the NSCs.

In the **fifth chapter** the role of *Ldb1* in cell differentiation and transcription regulation is discussed in detail and applications of the present findings into future directions are proposed.

Chapter 1

Introduction

1. Development of the mouse embryo

The mouse has been extensively used as a model for the study of mammalian embryonic development. The selection of the animal is based on easier housing due to its small size, the large size of the produced litters (6 to 8 animals per litter), the quick gestation time of 19 to 20 days and the sexual maturity time (6-8 weeks after birth).

1.1. Pre-implantation development

Ovulation in mice is initiated under hormonal stimuli on average every 4 days and leads to the preparation of the oocyte for fertilization. The nuclear membrane of the oocyte disappears and the chromosomes move to the periphery of the cell for the first meiotic division. One set of the synthesized homologous chromosomes with the surrounding cytoplasm forms the first polar body and is excluded from the cell. The other set remains at metaphase II. At the end of ovulation the oocyte is released from the ovary. Fertilization requires the successful entrance of one sperm cell into the mature oocyte through the surrounding cumulus mass of follicle cells and the zona pellucida. The incorporation of the sperm cell into the oocyte triggers a set of events to ensure that no more sperm cells will enter the oocyte [1].

Fertilization is followed by the second meiotic division and the formation and exclusion of the second polar body from the now fertilized oocyte. Haploid male and female pro-nuclei are formed around the male and female chromosomes and DNA replication takes place. The pro-nuclei move towards the centre of the oocyte and the surrounding membranes are progressively lost. The chromosomes assemble on the spindle and the first cleavage follows that leads to the two-cell stage approximately 1.5 days after fertilization. At this stage a large number of embryonic genes are switched on while there is extensive degradation of maternal mRNA [2-5]. Subsequent cleavage steps accompanied by compaction steps lead through the 4-cell stage, 8-cell stage and 16-cell stage morulae to the more compact blastocyst 3.5 days after fertilization [1].

The blastocyst consists of the surrounding trophoectoderm, which resembles epithelial tissue, the blastocoel, which is a cavity filled with fluid and the inner cell mass (ICM) from which the embryonic stem cells are derived. Approximately 4.5 days after fertilization the newly formed blastocyst moves down the oviduct and is implanted in the uterus. A group of non-polarized ICM cells generate the primitive endoderm or hypoblast and the rest of the ICM cells give rise to the primitive ectoderm or epiblast (Fig. 1).

1.2. Post-Implantation development

The trophoectoderm cells of the post-implantation blastocyst follow distinct differentiation pathways depending on their position [6]. The cells that surround the blastocoel (mural trophoectoderm) give rise to the primary trophoblastic giant cells, which are large in size, haploid and contain polytene chromosomes, although in the past the were thought to be polyploid instead [7]. The blastocyst trophoectoderm cells that are next to or surround the ICM (polar trophoectoderm) are diploid and they are characterized by an increased

proliferation rate. After implantation a group of polar trophoectoderm cells moves inside the blastocoel cavity forming a column-like structure that forms the extra-embryonic ectoderm. The primitive endoderm (hypoblast) will subsequently differentiate to parietal endoderm at 5.5 dpc and then to visceral endoderm (VE) at 6 dpc. In addition at this time point in development the epiblast cells will form an epithelial layer that surrounds the pro-amniotic cavity (Fig. 1) [8].

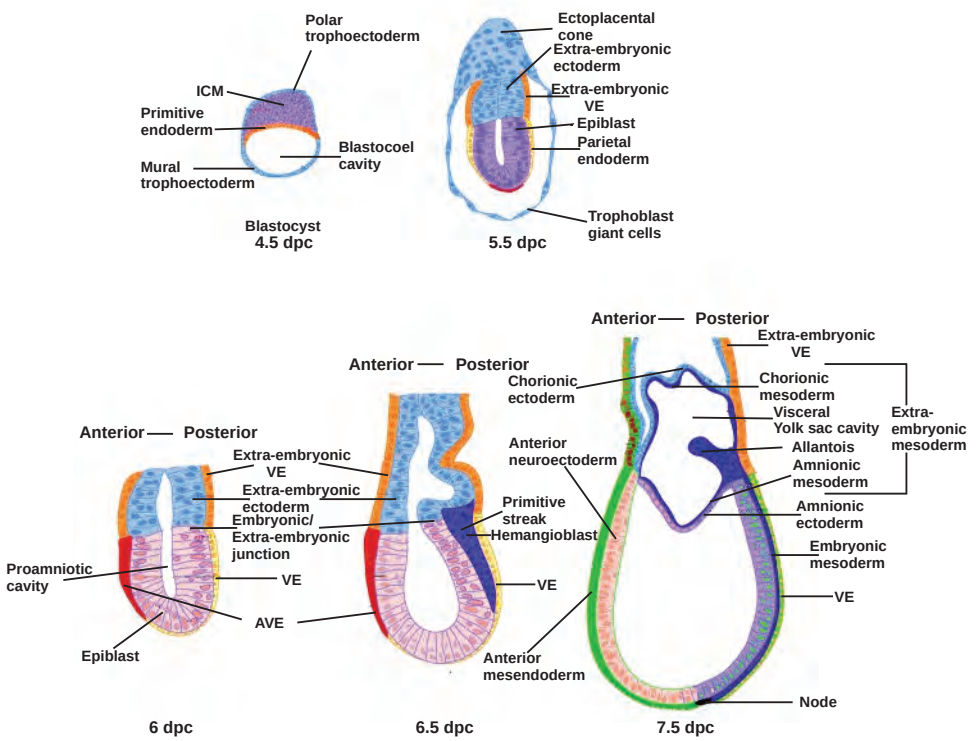

Figure 1: Schematic representation of the mouse embryo post-implantation development. At 4.5 dpc the blastocyst is implanted in the uterus. ES cells can be derived from the ICM at this stage. At 6.5 dpc gastrulation takes place. The primitive streak is formed followed by the formation of the embryonic and the extra-embryonic mesoderm (adapted from [68]).

Gastrulation is initiated at 6.5 dpc and is characterized by an increase in cell proliferation as the primitive streak is formed from a group of epiblast cells next to the embryonic/extra-embryonic junction marking the posterior of the embryo [9]. The emergence of the primitive streak is followed by the formation of the embryonic and extra-embryonic mesoderm, which underlies the epiblast and is surrounded by the VE. The extra-embryonic portion of the mesoderm increases in volume due to the formation of lacunae or small cavities, which will eventually unite and form the exocoelom or visceral yolk sac cavity between the chorion

and the amnion. The visceral yolk sac consists of a layer of mesodermal cells that lines the exocoelomic cavity surrounded on the outside by a layer of visceral endoderm. The mesodermal portion of the visceral yolk sac will give rise to cells of the hematopoietic and endothelial lineages. In addition the mesodermal cells will give rise to the inner mesothelial cell layer that lines the whole exocoelomic cavity between blood islands and serves as the boundary between them and the overlaying visceral endoderm [8]. At the posterior end of the primitive streak the allantois is formed from mesodermal tissue that will grow across the exocoelom and will fuse with the chorion (Fig. 1) [8]. In addition the mesoderm gives rise to the somites.

The most anterior end of the primitive-streak that extends towards the distal tip of the embryo at 7.5 dpc gives rise to the node, which in turn will give rise to the head process and the notochord that underlies the neuroectoderm (Fig. 1). Additionally the anterior portion of the primitive streak gives rise to the definitive endoderm, which extents at the anterior portion of the embryo and will eventually give rise to the gut endoderm, while the posterior portion of the embryo remains associated with primitive endoderm [10]. Early in development the mouse embryo has an inverted U shape and resembles a cup with the endoderm located on the outside and the ectoderm on the inside. Turning takes place between 8.5 and 9.5 dpc and is characterized by changes in the conformation of the embryo that will reverse its positioning.

1.3. Embryonic stem cells

Mouse ES cells are isolated from the ICM of the developing blastocyst (Fig.1) and can be maintained *in vitro* in adherent cultures for an unlimited period of time [11, 12] with the addition of Leukemia Inhibitory Factor (LIF) or on a layer of adherent mouse embryonic fibroblasts (MEFs) [13, 14]. ES sells are characterized by self-renewal and pluripotency as they are able to differentiate into any somatic cell lineage under specific culture conditions. The injection of ES cells into blastocysts and the generation of chimeric mice have demonstrated that these cells can contribute to every cell lineage including the germ line *in vivo* [15, 16]. Other types of pluripotent cell lines, which have also been established and extensively used in research, are embryonal carcinoma cell lines generated from germ line tumours [17, 18]. These cells lines exhibit the same characteristics as ES cells. They can be maintained in culture for long periods of time, they can differentiate into each of the three embryonic germ layers, endoderm, mesoderm and ectoderm and their derivatives. They can contribute to all the cell lineages when they are injected into blastocysts for the generation of chimeric mice [19, 20]. However the tumour identity of these cell lines does not make them a good tool for *in vivo* work or studies into embryonic development.

The injection of genetically manipulated ES cells in blastocysts has been extensively used for the generation of knockout and knockin mice [21, 22]. In a knockout mouse a gene is deleted and the resulting phenotype studied in detail in order to gain insight into the function of the gene. When such a deletion leads to severe developmental defects and embryonic lethality a conditional knockout mouse can be generated, where a gene can

be deleted at a specific time-point in development or in specific tissues. Knockin mice can be used for the over-expression of a gene, which can be indicative of its function especially in the case of a repressor. Additionally the expression of a gene can be coupled with a fluorescent protein in order to gain functional information or a tag for the isolation of a multi-protein complex from the *in vivo* environment and the study of the interactions between its members.

Mouse ES cells are a valuable tool for the study of early mammalian embryonic development. When cultured in suspension and without LIF they form spherical clusters called embryoid bodies that contain differentiated cells of the three germ layers and their derivatives and they resemble the post-implantation embryo [23, 24]. However this comparison has some limitations since embryoid bodies do not have a determined polarity or a body-plan [25]. The generation of embryoid bodies is also an intermediate step in the differentiation of ES cells *in vitro* first to lineage committed progenitors and then to fully mature differentiated cells. In this way the differentiation process of a selected cell type such as erythroid cells from hematopoietic progenitors can be studied in detail.

Other types of stem cells exist in adult tissues and are largely responsible for tissue regeneration. They are able to self-renew and differentiate towards at least one but usually more cell lineages, while the balance between self-renewal and differentiation is subject to tight regulation. Adult stem cells exist in specific micro-environments called niches for their protection and control of their differentiation potential. The importance of a niche has been elegantly demonstrated in experiments where ES cells isolated from the ICM were injected into the centre of blastocysts in a similar environment to the one they were derived from. In this environment the cells maintain their pluripotency and they are able to contribute to all the tissues of the generated chimeras. However when ES cells are injected subcutaneously into mice in an environment very different to their original one they form teratomas that consist of a variety of cell types due to uncontrolled differentiation [26]. Stem cells in the adult mouse exist in protective niches i.e. in the forebrain sub-ventricular zone (SVZ), the hippocampus, the bone marrow, the skin, the bulge of the hair follicle, the crypts of the intestine, the testis [27].

2. Hematopoiesis in embryonic development

Hematopoiesis is the differentiation process through which the mature blood cells are generated from a common progenitor cell. Blood cells are distinguished into red blood cells and white blood cells. Red blood cells have a short life span and are constantly being replaced. They are responsible for the transport of oxygen to tissues and organs. White blood cells are responsible for the immune response. In addition blood contains platelets that are responsible for blood clotting. Blood is transported in every part of the body via a network of vessels assisted by the heart. Arteries carry oxygen to every cell in the body and veins carry the metabolism by-product carbon dioxide to the lungs. The main component of the red blood cells or erythrocytes is hemoglobin, which is a complex molecule that consists of two alpha and two beta polypeptide chains [28]. Each of the

peptide chains folds into eight alpha-helices, which in turn fold again to form an intricate structure that contains a heme group with an iron (Fe^{2+}) ion at its centre responsible for oxygen binding.

Hematopoiesis in the mouse embryo occurs in two waves. First primitive hematopoiesis takes place exclusively in the extra-embryonic yolk sac and gives rise to primitive erythroid cells. It is followed by definitive hematopoiesis, which takes place primarily inside the embryo proper and gives rise to cells of the erythroid, lymphoid and myeloid lineages and to hematopoietic stem cells (HSCs) [29].

2.1. Yolk sac hematopoiesis

The first wave of hematopoiesis in the mouse embryo begins at approximately 7.5 dpc in the yolk sac blood islands [30], which are also the sites where vascular development is initiated. The visceral yolk sac consists of a mesordermal layer and an overlaying endodermal layer. Both the hematopoietic and endothelial lineages originate from cells with mesodermal identity. The commonly used description of the blood island structure states that they consist of primitive erythroblasts surrounded by endothelial cells (Fig. 2) [31]. The primitive erythroblasts present in the yolk sac at the onset of hematopoiesis differ from the mature erythrocytes found later in circulation in that they are large cells with an intact nucleus and they produce mainly embryonic hemoglobins as well a small amount of adult hemoglobins [32-34]. It has been shown however that the primitive erythroblasts do enucleate at approximately 12.5 dpc in a stepwise manner and that their overall number, including nucleated and enucleated cells, remains stable through embryonic development and up to birth [35, 36]. Definitive hematopoiesis is initiated in the yolk sac at 8.25 dpc and gives rise to progenitors of the erythroid and myeloid lineages [37-39]. The definitive hematopoietic progenitors enter the circulation of the embryo from 8.25 dpc, as soon as the newly formed heart begins to contract [37, 40-42].

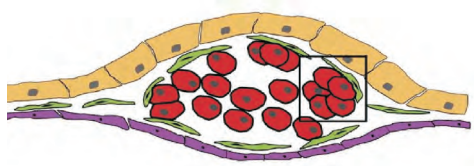

Figure 2: The stages of hematopoiesis in the mouse embryo. Mesoderm cells from the primitive streak form the extra-embryonic yolk sac and the intra-embryonic PSp/AGM region. Primitive hematopoiesis takes place only in the yolk sac following the emergence of the hemangioblast. Definitive hematopoiesis is initiated soon after in the yolk sac and mainly the intra-embryonic hematopoietic PSp/AGM regions. HSCs that emerge in the AGM region seed the fetal liver. There they undergo expansion before finally seeding the bone marrow that remains the main hematopoietic organ after birth. (adapted from Baron 2003; Mikkola, Gekas et al. 2005) [269, 270].

The establishment of a functional circulatory system early in development is an essential step for the survival of the embryo. Vasculogenesis is the formation of the primary vascular plexus through the fusion of the blood islands in the yolk sac [43], the formation of the primary vascular plexus in the embryo proper and the formation of the heart. This is followed by a wave of remodelling, rearrangements and fusions of existing vascular elements in order to establish a secondary and more complex vascular network. During this process there is no expansion of the existing network but rather the generation of larger vessels from the fusion of smaller ones or rearrangements of a few larger vessels into an increased number of smaller ones. The above changes increase the functionality of the newly formed network [44]. An example of remodelling is the establishment of a connection between the yolk sac and the embryo proper that is achieved through the fusion of the vitelline arteries that arise form the dorsal aortae with the yolk sac vessels [45]. The next step in the establishment of a circulatory network in the embryo is angiogenesis, which is the generation of new vessels from already existing ones. The vascular network of the neuroectoderm and central nervous system is formed via this process [46].

As the heart tube is formed and begins to beat and the established vascular networks of the yolk sac and the embryo proper become connected the circulation of the primitive erythroblasts that have originated in the yolk sac begins between 8.25 dpc and 8.5 dpc [47-49]. The number of red blood cells that move from the yolk sac to the embryo proper increases from 8.5 dpc to 9 dpc, however it remains low on average if the total number of these cells that exist in the yolk sac is taken into account, leading to the conclusion that in the early embryo the cardiovascular system may be insufficient for the establishment of a functional circulatory network between the yolk sac and the embryo proper [42].

Research into the initiation of primitive hematopoiesis and vasculogenesis in the yolk sac has been mainly focused into the role of the mesoderm. However the visceral yolk sac also consists of a layer of endoderm that plays an essential part in the above processes, since its removal from *in vitro* cultures of mouse yolk sacs or chick embryo explants leads to a disruption in the formation of blood islands and vasculature [50-52]. In addition immortalized cell lines derived from the mesoderm and the endoderm layers of mouse embryonic yolk sacs were shown to support the proliferation and differentiation of hematopoietic stem and progenitor cells [53, 54]. *Gata-4* is essential for the formation of the VE portion of the yolk sac, which is absent in *Gata-4*-/- embryoid bodies but primitive hematopoiesis and endothelial differentiation are not affected. The conclusion reached was that the VE is not involved in the differentiation of hematopoietic and endothelial progenitors but that it participates instead in the organization of the blood islands and the vascular network [55]. The vascular endothelial growth factor A (*VegfA*) is essential for embryonic and in particular vascular development [56, 57]. It is expressed in the VE and the mesodermal layer of the yolk sac [58]. A series of experiments where chimeric ES cells for a hypomorphic *Vegfa* allele were fused with wild type tetraploid embryos has shown that VE derived *Vegfa* is necessary for correct blood island formation [59]. The disruption of the *Vegfa* receptor *Vegfr2* (*Flk1*) leads to the absence of primitive erythroblasts and

endothelial cells from the yolk sac. The mesodermally derived progenitors programmed to give rise to the hematopoietic and endothelial lineages instead emerge in other mesoderm derived membranes such as the amnion instead of the yolk sac [60]. However the deletion of *Vegfr2* in ES cells showed that the receptor is not essential for the initiation of hematopoiesis as the cells were still able to differentiate into blood cells [61, 62]. It was proposed that Vegfa produced by the yolk sac VE attracts cells with a mesodermal identity from the primitive streak that migrate to the yolk sac where they differentiate towards the hematopoietic and endothelial lineages [59, 60, 63].

2.2. The hemangioblast

Hematopoietic and endothelial progenitors arise simultaneously and in close proximity in the blood islands of the yolk sac suggesting that these two cell lineages originate from a common progenitor cell. This progenitor has been identified as the hemangioblast at the start of the last century [64, 65]. Florence Sabin proposed the hemangioblast as the mesodermally derived progenitor of primitive erythroblasts and endothelial cells from observations into early hematopoiesis in the chick embryo. The blood islands of the chick embryo yolk sac are formed from cells that are derived from the mesoderm and undergo sequential steps of differentiation into primitive erythroblasts and cells of the endothelial lineage [66]. As it was mentioned above the mesodermal layer of the extra-embryonic visceral yolk sac and the mesoderm of the embryo proper originate from the primitive streak during gastrulation (Fig. 1). Signals from the VE regulate the formation of the primitive streak [67, 68] and in addition guide the migration of the mesodermal progenitors of the hematopoietic and endothelial lineages from the posterior of the streak to the yolk sac where they will form the blood islands [69]. Mesoderm progenitors in the primitive streak are organized in a regional and temporal manner based on the cell lineage they will give rise to and the time point that they undergo differentiation [70]. However the regulation of the primitive streak spatial organization and the movement of the mesodermal cells are not fully understood yet. The specification of mesodermal cells to the hemangioblastic fate takes place while the cells are still positioned in the posterior primitive streak prior to the initiation of their emigration to the extra-embryonic yolk sac. In the yolk sac the cells differentiate rapidly into progenitors of the hematopoietic and endothelial lineages [69]. In studies with chick embryos mesodermal cells originating from the posterior part of the primitive streak were found to highly express *Flk1*. The cells were able to give rise to hematopoietic and endothelial colonies in specific culture conditions *in vitro*; however each Flk1+ cell was able to differentiate towards one of the two types of lineages but not both [71, 72]. The *in vitro* equivalent of the hemangioblast has been identified as the Blast Colony Forming Cell (BL-CFC) through the model of differentiation of ES cells into embryoid bodies. BL-CFCs have the ability to differentiate towards the hematopoietic or the endothelial lineages *in vitro* [73, 74]. Flk1 has been extensively used as a marker in order to identify the BL-CFCs from embryoid bodies and the mouse embryo hemangioblasts *in vivo* [75].

Recently it was determined that the hemangioblast differentiates towards hematopoietic cells via the intermediate step of hemogenic endothelium formation. The majority of blast colony cells formed through the differentiation of the Flk1+ BL-CFCs are initially expressing the endothelial marker Tie2 after one day in culture. Between two and four days a population positive for CD41, a marker for hematopoietic progenitors, emerges and increases in size at the expense of the Tie2+ cells. The CD41+ hematopoietic progenitors have the potential to give rise to both primitive and definitive haematopoietic colonies, which are additionally identified through the expression of CD45. *Scl* is involved in the initial step of differentiation from the hemangioblast towards the hemogenic endothelium, identified as Tie2^{hi} c-Kit+ CD41– cells and *Runx1* is involved in the generation of definitive hematopoietic cells from then on [76].

2.3. Intra-embryonic hematopoiesis

Between 7.5 dpc and 8 dpc hematopoietic activity is established in the embryo proper in the para-aortic splanchnopleura (PSp) region, based on the detection of erythroid, lymphoid and myeloid progenitors [77, 78]. This region defines the mesoderm and adjacent endoderm that surround the aorta and will later give rise to the Aorta Gonads Mesonephros or AGM region. At 9 dpc CFU-S (colony forming unit-spleen) multipotent progenitors and HSCs that are able to repopulate neonatal mice are detected in the AGM [79, 80]. At 10.5 dpc the first adult HSCs are detected in the same region [81-83]. Later in development this region will give rise to the liver, the stomach, the kidney, the spleen, the gonads, the omentum and the mesenteries [84]. The colonization of the fetal liver from HSCs and CFU-S progenitors takes place between 10.5 dpc and 11.5 dpc [80]. The hematopoietic potential of multipotent progenitors and HSCs that appear in the PSp/AGM was determined based on their ability to contribute to the hematopoietic system of neonatal and adult irradiated mice. Multipotent progenitors from the 9 dpc PSp were able repopulate neonatal mice only. However HSCs derived from the 10.5 dpc AGM were able to fully repopulate adult irradiated mice [85]. HSCs are also believed to emerge from the vitelline and the umbilical arteries [86, 87] due to the hemogenic activity of the endothelium [88]. The thymus and the spleen are also colonized by multipotent hematopoietic progenitors from 11 dpc and 12.5 dpc respectively [89, 90]. Finally from 15 dpc multipotent hematopoietic progenitors colonize the bone marrow, which is the site where definitive hematopoiesis primarily takes place after birth and throughout adult life [91]. However the ability of the fetal bone marrow to support HSCs comes a little later in development [92]. When the hematopoietic activity of the liver ends a pool of HSCs are maintained in their undifferentiated state in the specialized bone marrow niche, which supports their self-renewal and regulates their differentiation towards the erythroid, myeloid and lymphoid lineages with the assistance of the endosteal surface osteoblasts [93, 94].

3. Signalling pathways in hematopoiesis

The TGF-β signalling pathway

The Transforming Growth Factor-β (TGF-β) family is a large group of highly conserved signalling molecules that participate in the regulation of developmental pathways in the mouse embryo essential for patterning and organogenesis. The family is divided into the BMP (Bone Morphogenic Protein) and the TGF-β/Activin subfamilies of ligands that are able to bind to two types of serine/threonine kinase receptors, type I and type II. The binding leads to the phosphorylation of the ligand specific Smad proteins, which in turn relocate to the cell nucleus and regulate gene expression [95, 96].

The TGF-β signalling pathway regulates axis formation, left-right asymmetry, mesoderm induction [97-104], epithelial to mesenchymal transition, the development of the heart, the lungs, the kidneys, the nervous system [105-108]. The pathway is also involved in the regulation of the cell cycle and cell proliferation [109-113].

Mice deficient for *Bmp4* show severe developmental defects. The phenotype varies in severity from complete lack of mesoderm induction and *brachyury* expression leading to death at 6.5 dpc, to retarded development up to 9.5 dpc and subsequent death characterized by a lack of organization in posterior development, reduced extra-embryonic mesoderm and primitive erythropoiesis in the yolk sac [103]. The addition of Bmp4 to ES cell cultures can induce the formation of ventral mesoderm and the generation of hematopoietic progenitor cells [114].

The disruption of *Smad5* in mouse embryos leads to death between 9.5 dpc and 11.5 dpc and a range of developmental defects, which include lack of vascular development in the yolk sac and initiation of vasculogenesis and hematopoiesis in the amnion [115, 116]. In addition *Smad5* exerts an inhibitory effect in the proliferation of human hematopoietic progenitors, mouse hematopoietic progenitors derived from 9 dpc yolk sacs and BL-CFC cells derived from differentiated embryoid bodies [117, 118]. However *Smad5* is not involved in the regulation of adult hematopoiesis as deficient HSCs don't show any defects in proliferation and differentiation and they are able to successfully repopulate terminally irradiated mice in a similar manner to wild type cells [119].

Deletion of *TGF-β1* in mice leads to a reduced population of erythroid cells in the yolk sac and lack of vascular development due to the defective differentiation of endothelial cells [120]. *TGF-β1* was shown to inhibit the proliferation of LTR-HSCs (Long-Term Repopulating Hematopoietic Stem Cells) *in vitro* [121] but not *in vivo*. *TGFβR1* deficient mice lack TGF-β signalling but the HSCs self-renewal and proliferation are not affected and the cells can repopulate lethally irradiated mice [122, 123].

The WNT signalling pathway

The canonical WNT signalling pathway stabilizes β-catenin, which is normally bound and phosphorylated by a CKIα/GSK3β/APC/Axin complex leading to ubiquitination and subsequent degradation. The binding of a WNT ligand to the transmembrane Frizzled (Fzd) receptor and the LRP (Lipoprotein Receptor-related Proteins)-5/6 coreceptor destroys

the β-catenin degradation complex and stabilizes the molecule, which then travels to the nucleus and interacts with the LEF/TCF transcription factors for the regulation of gene expression [124].

The deletion of β-catenin or γ-catenin (plakoglobin), which can also bind on LEF/TCF and function in a partially redundant manner, in mice leads to embryonic lethality [125, 126]. However WNT signalling independent of β-catenin also exists; as it was shown in HSCs deficient for β- or γ-catenin. These cells still have an active WNT signalling pathway and don't show any defects in their self-renewal, repopulation or differentiation [127]. It is interesting to note that enforced expression of a non-degradable form of β-catenin in the mouse hematopoietic system leads to a block in myeloid, lymphoid and erythroid differentiation at the erythroblast stage and a defect in the repopulating ability of HSCs [128].

WNT signalling contributes to the maintenance and expansion of HSCs as overexpression of the pathway inhibitor axin leads to a halt in their *in vitro* growth and to their ability to repopulate terminally irradiated mice *in vivo*. The expression of *Wnt3a* has a positive effect in the proliferation of HSCs *in vitro* [129]. A similar conclusion was reached through *in vivo* studies as HSCs isolated from *Wnt3a*-/- fetal livers show a defect in their self-renewal capacity and the ability to repopulate irradiated mice. However the cells are still able to generate lymphoid but not myeloid progenitors, which leads to the conclusion that the absence of *Wnt3a* affects the differentiation towards some lineages of mature blood cells but not all [130].

Two other members of the WNT family, *Wnt5a* and *Wnt10b*, were shown to have a positive effect in the proliferation and expansion of hematopoietic progenitor cells from fetal livers [131]. However *Wnt5a*-/- mouse embryos did not exhibit any defects in hematopoietic development [132]. Finally overexpression and deletion of *Wnt4* in mice demonstrated its involvement in the regulation of proliferation and expansion of bone marrow Lin- Sca+ c-kit (hi) (LSK) cells and in thymopoiesis [133].

The WNT signalling pathway together with the Notch signalling pathway is involved in the differentiation of hemangioblast cells towards the primitive erythroid lineage. WNT signalling is active in Flk1+ BL-CFCs during the first hours of differentiation towards blast colonies as the Notch signalling pathway remains inactive through the action of the inhibitor *Numb*. Between 12 and 24 hours in the differentiation process the inhibitory effect that is exerted on the Notch signalling pathway is reduced and the subsequent activation leads to the expression of WNT inhibitors and a block of the WNT pathway [134].

The Notch signalling pathway

Notch signalling is initiated through the binding of the pathway ligands Jagged (Jag-1 and -2) and Delta-like (Dll1, -3, -4) to the Notch receptors (Notch1-4) that consist of an extra-cellular and an intra-cellular domain. The ligand-receptor interaction initiates two successive cleavage steps that lead to the release of the intra-cellular active domain of the Notch receptor (NICD), which moves to the nucleus and functions in transcription

regulation by displacing co-repressors (CoR) from the CSL (CBF1/RBPJk) transcription activator and recruiting co-activators (CoA). The highly conserved signalling pathway is involved in cell fate determination [135, 136].

Notch1 is not required for yolk sac primitive erythropoiesis. However it is required in definitive hematopoiesis as in the *Notch1*-/- embryos PSp region hematopoiesis and angiogenesis are impaired at 9.5 dpc and no HSCs emerge from the AGM region. The phenotype is thought to be caused by a block in the differentiation of the hemogenic endothelial cells towards the hematopoietic lineage. In contrast the deletion of *Notch2*, which similarly to *Notch1* leads to embryonic lethality, is not followed by any defects in hematopoiesis [137].

The deletion of the *Mib1* (*Mind bomb-1*) E3 ubiquitin ligase in mice leads to a halt in the generation of hematopoietic progenitors in the PSp region. *Mib1* is involved in the production of functional Notch signalling ligands. A *Mib1* deficient cell can receive Notch signals but it cannot induce the signalling pathway in its neighbouring cells [138].

The induction of *Notch1* signalling in Lin– Sca-1+ c-kit+ cells isolated from the mouse bone marrow led to the establishment of a hematopoietic cell line that was responsive to the addition of cytokines, could be maintained in culture for an unlimited period of time and was able to differentiate towards the myeloid and lymphoid lineages [139]. *Notch1* is involved in lymphoid differentiation through the regulation of cell fate determination towards the T- or B-cell lineages [140-144].

The Hedgehog signalling pathway

The Hedgehog (Hh) family of secreted proteins has three members in the mouse and human, Sonic Hedgehog (Shh), Indian Hedgehog (Ihh) and Desert Hedgehog (Dhh). The binding of a hedgehog protein on the transmembrane receptor Patched (Ptch) stops the inhibitory effect of the receptor on the signalling molecule Smoothened (Smo), which is then free to interact with the transcription regulatory complex Su (fu)/Fu/Gli/Cos2 leading to gene expression activation [145].*Ihh* is expressed in the visceral endoderm and is involved in the differentiation of hematopoietic and endothelial cells [146]. The deletion of *Ihh* or *Smo* leads to insufficient vascular development due to a block in the process of vascular remodelling. Embryoid bodies deficient for either of the two molecules do not form blood islands. However hematopoietic cells are present in the yolk sacs of *Smo*-/- embryos [147, 148]. *Ihh* is also expressed in the stroma cells of the adult bone marrow and *Shh* is expressed in stroma cells of the spleen and the lymph node. Increased expression of *Ihh* in the bone marrow of SCID (Severe Combined Immunodeficiency) mice can enhance the expansion of hematopoietic cells. Additionally *Ihh* was shown to be involved in the proliferation and differentiation of cells that belong to the lymphoid and myeloid lineages [149]. The Hedgehog signalling pathway is involved in the regulation of cycling, expansion and regeneration of bone marrow hematopoietic cells in adult mice [150].

In the zebrafish embryo it was shown that Hedgehog signalling is not necessary for primitive hematopoiesis in contrast to the phenotype seen in embryoid bodies but that it is required for definitive hematopoiesis and the specification of the dorsal aorta [151].

The FGF signalling pathway

The Fibroblast Growth Factor family consists of 22 members in humans and mice, which bind on four tyrosine kinase FGF receptors (FGFR1-4). The 120 amino acids long receptor interaction domain of the FGF molecules is conserved and can bind to heparin or heparan sulfate proteoglycans (HSPG) to ensure the stabilization of the interaction between an FGF and its receptor. When two FGF-heparin complexes bind on an FGF receptor tyrosine autophosphorylation of the receptor's intracellular domain takes place leading to signal transduction via the following pathways: Ras/MAPK pathway , PI3 kinase/Akt pathway and PLCγ/Ca^{2+} pathway. FGFs are essential for mouse embryonic development and morphogenesis as they are involved in the regulation of cell proliferation, differentiation and migration [87, 152, 153].

Fgf2 is expressed in the bone marrow stroma cells where it is believed to act as a mitogen of platelets and megakaryocytes [154, 155]. It functions as a positive regulator of human and mouse bone marrow myelopoiesis and megakaryocytopoiesis by enhancing the size, proliferation and growth of megakaryocytes [156-158]. Furthermore Fgf2 was shown to downregulate the expression of Stromal cell–derived factor-1 (*Sdf-1*) through the interaction with the receptor FGFR1IIIc. *Sdf-1* supports the survival and growth of hematopoietic progenitors in the bone marrow. An Fgf2 mediated decrease in *Sdf-1* expression results in the impairement of stoma cells support for CD34+ hematopoietic progenitors. Increased levels of *Fgf2* are found in the bone marrow of patients with disorders that result from hematopoiesis impairement [159]. However *Fgf2*-/- mice did not exhibit any defects in hematopoietic development [160]. *Fgf2* as well as *Fgf1* are involved in the regulation of angiogenesis *in vitro* and *in vivo* [161]. FGF receptors are expressed in the LTR-HSCs of the bone marrow and the cells can be maintained and expanded in culture with the addition of Fgf1 [162].

The FGF signalling pathway receptor *Fgfr1* is expressed in endothelial cells but it is not required for vascular development. On the other hand studies performed with embryoid bodies did show that hematopoietic development is halted in the absence of *Fgfr1* [163].

A morphogenic role for FGF signalling has been demonstrated in blood island formation in *Xenopus* embryos. Overexpression of embryonic *FGF* in the ventral region of the embryos blocked the formation of ventral blood islands. On the other hand inhibition of the FGF signalling in the lateral marginal zone, where there should be no hematopoietic activity, led to the emergence of ventral blood islands. The observed phenotype is caused by the misregulation of the Bmp4 downstream targets *Gata-2* and *PV.1*. FGF suppresses the expression of *Gata-2* and halts blood island formation and at the same time it enhances the expression of the erythropoiesis inhibitor *PV.1*. Therefore the FGF and BMP signalling pathways can interact during hematopoiesis [164].

4. Transcription factors in hematopoiesis

GATA family of transcription factors

The GATA transcription factor family includes six members that contain a conserved zinc-finger DNA binding domain that binds the regulatory sequence A/GATAG/A. The GATA binding sequence was initially identified in the promoters of the globin genes and subsequently in the promoters and enhancers of erythroid specific genes [165-169]. The first member of the family to be described was *Gata-1*, which together with *Gata-2* and *Gata-3* are involved in the regulation of hematopoiesis. The other three members of the family *Gata-4*, *Gata-5* and *Gata-6* are mainly expressed in tissues not involved in hematopoiesis such as the heart, the gut and the lungs [170].

Gata-1

Gata-1 is located on the X-chromosome and it is expressed in multipotent hematopoietic progenitors [171], erythrocytes [172], megakaryocytes, mast cells [173], eosinophils [174], dendritic cells [175] and in the testis Sertoli cells [176]. *Gata-1* is also expressed in extra-embryonic mesoderm prior to the onset of blood island formation [177]. The generation of chimeric mice with male *Gata-1*-/- ES cells showed that these cells did not contribute to the mature erythroid lineage [178]. *Gata-1*-/- embryoid bodies gave rise to definitive erythroid colonies only that were arrested at the proerythroblast stage and apoptosis followed soon after. Myeloid differentiation was not affected since macrophages, granulocytes and neutrophils were able to emerge. GATA target genes and the adult globin genes were found to be still expressed in the definitive proerythroblasts that were also characterized by an increase in the expression of *Gata-2* [179].

The deletion of *Gata-1* in mice leads to severe anemia and death in male embryos between 10.5 dpc and 11.5 dpc. The yolk sacs of the male *Gata-1*-/- embryos showed normal vascular development and contained blood islands with hematopoietic cells at 9.5 dpc, in contrast to phenotype seen in the *Gata-1*-/- embryoid bodies. Hemoglobins were expressed in the 9.5 dpc knockout embryos but at decreased levels. The generation of definitive erythroid cells was blocked at the proerythroblast stage similar to the knockout embryoid bodies. Female *Gata-1*+/- mice suffered from anemia at birth but later recovered due to the random inactivation of the X-chromosome [180]. On the other hand over-expression of *Gata-1* in erythroid progenitor cells blocks their differentiation and leads to anemia and embryonic death [181]. *Gata-1* is also involved in the proliferation and differentiation of megakaryocytes and the production of platelets [182].

Gata-1+ VE-cadherin+ cells isolated from yolk sac blood islands at 7.5 dpc were able to generate both primitive and definitive erythroid colonies and a small number of them were also able to differentiate towards the endothelial lineage. Progenitor Gata-1+ cells could partially rescue the block in definitive erythropoiesis of the *Runx1*-/- mice, however the impairment in the hemogenic potential of the endothelium was not recovered [183].

Gata-2

Gata-2 is expressed in pluripotent hematopoietic progenitor cells [184], immature erythroid cells, megakaryocytes [166], mast cells [185], endothelial cells [186] and hematopoietic progenitor cells of the PSp/AGM region where the expression is driven by the hematopoietic cell specific distal promoter. The *Gata-2* expressing cells in the PSp/AGM are also c-kit+ CD34+ CD45+ leading to the conclusion that they most likely are HSCs [187].

Gata-2-/- embryoid bodies give rise to a markedly lower number or primitive and definitive erythroid precursors, a reduced numbers of macrophages and very few mast cells [188]. The deletion of *Gata-2* in mice leads to embryonic lethality between 9.5 dpc and 10.5 dpc due to severe anemia. Primitive and definitive hematopoiesis are reduced in the knockout embryos, although the morphology of the existing primitive erythroid cells is similar to the wild type ones, which concludes that *Gata-2* may be involved in the control of hematopoietic cell proliferation. On the other hand *Gata-2* is not involved in the terminal differentiation and maturation of erythroid cells and macrophages [188, 189]. However overexpression of *Gata-2* was shown to block the differentiation of hematopoietic progenitors and halt their ability to expand after transplantation into lethally irradiated mice, suggesting a dose-dependent element in *Gata-2* function during the regulation of hematopoiesis [190].

Gata-2 together with *Smad5* regulates the expression of *EKLF* in hematopoietic progenitor cells. Prior to the initiation of erythroid differentiation the expression remains at a low level. However as soon as the progenitor cells commit to the erythroid lineage *EKLF* expression is maintained at a high level through the action of *Gata-1* that recruits an Scl containing complex [191].

Gata-3

Gata-3 was initially identified, as was *Gata-2*, due to its homology to *Gata-1*. It is strongly expressed in the placenta of the mouse embryo starting before 10 dpc, in the T-lymphocyte lineage, in the thymus and in erythroid cells [192, 193]. The deletion of *Gata-3* in mice did show that the gene is involved in normal fetal liver definitive hematopoiesis; however it is not involved in the regulation of yolk sac primitive hematopoiesis. The *Gata-3*-/- mouse is embryonic lethal at 11.5 dpc [194].

The generation of chimeric mice with *Gata-3*-/- ES cells showed that in the absence of *Gata-3* differentiation towards the thymocyte lineage and the generation of mature T-cells is blocked [195].

The enforced expression of *Gata-3* in primary mouse HSCs induces the differentiation towards the megakaryocyte and erythrocyte lineages and at the same time it blocks myelopoiesis and lymphopoiesis. As these HSCs differentiate their proliferating potential is negatively affected and the cells loose their capacity to repopulate the hematopoietic system of an adult host mouse after transplantation [196].

Scl

Scl belongs to the bHLH family of transcription factors, the members of which are commonly involved in the regulation of cell fate determination and differentiation [197]. The bHLH transcription factors can form homodimers as well as heterodimers via their interaction domain, which binds on the DNA motif CANNTG termed the E-box [198]. *Scl* was initially identified in a human leukemic cell line as part of the t (1; 14) (p32; qll) translocation [199]. It is expressed in both multipotent hematopoietic progenitors and mature differentiated cells [199]. The *Scl-LacZ* knockin mouse showed that *Scl* is expressed in primitive and definitive hematopoietic cells, in the endothelial cells of the vascular system, in the walls of the dorsal aorta, in the fetal liver, in the central nervous system [200, 201].

A 3' enhancer element is involved in regulating the expression pattern of *Scl*. LacZ reporter assays showed enhancer driven expression in the extra-embryonic mesoderm of the yolk sac at 7.5 dpc, in the allantois, in the heart and blood vessels at 8.5 dpc, in the PSp/AGM region and more specifically in the emerging HSCs at 11 dpc. However enhancer driven expression was rarely seen in mesoderm derived cells of the embryo proper. In the adult mouse the 3' enhancer drives *Scl* expression in multipotent progenitors, erythroid cells, mast cells and megakaryocytes [202].

The deletion of *Scl* in the mouse causes embryonic lethality by 10.5 dpc due to a block in primitive hematopoiesis. The *Scl*-/- embryos are pale and there are no blood islands in their yolk sacs. The knockout embryos show growth retardation, however somitogenesis and organogenesis of the neural tube, the heart, the primitive gut and the allantois appear normal [203]. *Scl*-/- ES cells failed to contribute to any hematopoietic lineage in adult chimeric mice but were able to contribute to non-hematopoietic tissues. *Scl*-/- embryoid bodies were completely devoid of any hematopoietic potential. *Scl* was also shown to be involved in the hematopoietic specification of early mesoderm in the *Xenopus* embryo and *Scl*-/- embryoid bodies [204, 205].

The expression of the hematopoietic transcription factors *Gata-1* and *PU.1* was lost in the yolk sacs of the *Scl*-/- embryos and in the *Scl*-/- embryoid bodies, which concludes that they act downstream of *Scl* during gene expression regulation. Similarly the expression of *Eklf* was severely reduced in *Scl*-/- embryoid bodies [206, 207]. Another direct target of *Scl* is *Mef2c*, which is involved in the regulation of megakaryopoiesis. Conditional knockout mice for both *Mef2c* and *Scl* show the same defects in platelet production that include a reduction in number and an increase in size [208].

The generation of mice expressing a mutated form of *Scl* devoid of DNA binding activity showed that this is not essential for the function of *Scl* in the specification of early hematopoiesis. The homozygous mutant mice suffer instead from a block in the terminal maturation of erythroid cells and die from 14.5 dpc onwards, while some mice were able to survive to adulthood. The phenotype is in part related to the downregulation of the erythroid specific genes *Gata-1*, *glycophorin A*, *p4.2* and *Eklf* seen in Ter119+ cells isolated from 12.5 dpc fetal livers. The expression of *Scl* itself was also reduced in these cells indicating auto-regulation [209].

Runx1

Runx1 (*AML1* or *CBFA2*) was identified through its involvement in human leukemias and belongs to a family of transcription factors characterized by a highly conserved DNA-binding domain called the RUNT domain. Members of the CBFAα family, which also includes *Runx2* (*CBFA1*) and *Runx3* (*CBFA3*), are involved in cell differentiation and organogenesis. Runx1 together with the non-DNA- binding CBFß form the heterodimer core binding factor (CBF) that functions in transcription regulation [210, 211].

Runx1-/- mouse embryos die between 11.5 dpc and 12.5 dpc suffering from severe hemorrhages especially in the central nervous system. Primitive hematopoiesis is not affected; however definitive hematopoiesis is completely impaired. *Runx1*-/- embryoid bodies contained primitive erythroid cells but did not give rise to definitive erythroid or myeloid colonies *in vitro*. Furthermore *Runx1*-/- ES cells did not contribute to the generation of any of the definitive hematopoietic lineages in chimeric mice [212, 213].

Runx1 is expressed in definitive hematopoietic progenitor cells, the AGM, the vitelline and umbilical arteries and the ventral wall of the dorsal aorta. These are sites where HSCs are thought to emerge. *Runx1* expression was identified in endothelial cells on these sites leading to the conclusion that it is involved in the hemogenic activity of the endothelium for the emergence of hematopoietic clusters [214].

The role of *Runx1* in adult hematopoiesis was investigated with the use of a conditional knockout mouse. *Runx1* was found to be involved in the maturation of megakaryocytes and the production of platelets and lymphocytes but not of erythrocytes and neutrophils. Although *Runx1* is expressed in cells of all the definitive hematopoietic lineages [215], its involvement in their differentiation and development in the adult mouse varies. The conditional deletion of *Runx1* in adult mice leads to an increase of hematopoietic progenitors in the expense of myeloid differentiation, similar to leukemias caused by Runx1 fusion proteins that exert a dominant negative effect on normal Runx1 function [216].

Runx1 is expressed in the yolk sac extra-embryonic mesoderm, in blood islands between 7.5 dpc and 8.25 dpc and in embryoid body derived BL-CFCs and blast colonies. Furthermore the *Runx1*-/- embryoid bodies generate fewer blast colonies than their wild type counterparts. These blast colonies were able to differentiate towards the primitive hematopoietic and endothelial lineages, however as expected their definitive hematopoietic potential was impaired [217]. The deletion of *Runx1* affects primitive hematopoiesis in some way, since *Runx1*-/- primitive erythrocytes show abnormal morphology characterized by holes in their membrane and a reduction in *Ter119*, *Gata1* and *Eklf* expression [218].

A 531 bp conserved enhancer element in the first intron of *Runx1* at position +23.5 drives the expression during definitive hematopoiesis in the yolk sac, the PSp at 8 dpc, the HSCs emerging from the AGM and the ventral wall of the dorsal aorta, the vitelline and umbilical arteries and the fetal liver. In addition *Runx1* expression driven by the enhancer was found in the dorsal wall of the dorsal aorta in clonogenic hematopoietic progenitors distinct from the definitive HSCs present in the ventral wall. Conserved GATA and Ets binding motifs are essential for the function of the enhancer in inducing *Runx1* expression [219, 220].

LIM domain proteins

The LIM domain protein group contains a large number of diverse proteins that are characterized by a conserved cysteine-histidine rich zinc finger motif called the LIM domain that does not bind DNA but can facilitate protein-protein interactions instead. The LIM domain proteins can be nuclear or cytoplasmic and can contain one, two or more LIM domains often coupled with other types of regulatory domains. They are an important and interesting protein group to study mainly because they are often involved in the regulation of developmental pathways and cell differentiation. The LIM domain proteins are categorized according to the type and number of LIM domains they have. LIM domains are distinguished into five separate classes (A to E) based on sequence homology [221]. LIM domain proteins can be further distinguished according to other regulatory domains they contain, which can be a homeodomain in the case of LIM-Homeodomain factors (LIM-HD) or a kinase domain in the case of LIM kinases. There is also a group of LIM proteins that do not contain any other regulatory domains and they are called LIM-Only proteins or LMOs [222]. LIM-HD proteins, LMOs and LIM kinases belong to the same group of LIM domain proteins that contain two paired LIM domains, class A and class B, at their amino-terminus. The nuclear LMOs and LIM-HDs are closely related based on the sequence of their LIM domains, while LIM-kinases are only marginally identified as members of the same group and they are the most distant in terms of sequence homology of their LIM domains [223].

The LMO group member *Lmo2* (*Rbtn2*) was identified through its involvement in human leukemias [224]. It is expressed in primitive erythroid cells and in the fetal liver at 14 dpc. Its deletion leads to embryonic lethality by 10.5 dpc and the embryos show growth retardation, short anterior-posterior axis, a delay in turning and lack of blood islands. *Lmo2*-/- embryoid bodies lack hemoglobinized erythroid cells, although they contain macrophages [225].

The *Xenopus* homolog of *Lmo2* induces primitive hematopoiesis and promotes the formation of red blood cells acting synergistically with the Gata-1 and Scl homologs. Its expression is regulated by Bmp4 and follows a similar pattern with *Scl*. *Xlmo2* is present in the ventral mesoderm, the ventral blood islands and the dorsal lateral plate mesoderm where definitive hematopoiesis takes place [226].

Lmo2 forms a complex with Gata-1 and the Scl/E47 heterodimer in MEL (Mouse Erythroleukemia) cells. E47 is one of the two products of the E2A gene, the other being E12 [227]. The Lmo2 containing complex in MEL cells includes the non-DNA binding Ldb1 protein and binds on a bipartite DNA motif that consists of an E-box with the sequence CAGGTG followed closely by a GATA binding site [228]. The overexpression of *Lmo2* in a proerythroblast cell line inhibits the differentiation and maturation of erythroid cells. The same effect was shown to follow the overexpression of *Ldb1* in the same cell line, leading to the conclusion the both factors are responsible for maintaining erythroid progenitor cells in an immature state and can function as positive as well as negative regulators of erythropoiesis [229].

The deletion of the LIM-HD member *Lhx2* leads to embryonic lethality in utero and a variety of developmental defects including severe anemia and a reduction in the size of the liver. The *Lhx2*-/- embryos showed a reduction in the hematocrit but contained mature enucleated erythrocytes leading to the conclusion that erythropoiesis is not completely blocked. However the level at which it functions in these embryos is still not enough to sustain them and this is believed to be the result of a defect in the microenvironment of the fetal liver that supports definitive erythropoiesis [230].
Lhx2 has been used for the generation of immortalized hematopoietic progenitor cell lines derived from differentiated mouse ES cells, which are considered similar in many aspects to primary hematopoietic stem cells. *Lhx2* is thought to regulate self-renewal of the generated cell lines in a nonautonomous manner [231, 232].

5. Ldb1: gene and protein structure

Ldb1 (Clim2 or Nli) is involved in the formation of multi-protein complexes. The screening of a mouse embryo cDNA expression library with a protein probe consisting of the LIM-HD factor Lhx1 (Lim1) two LIM domains identified two members of the *Ldb* family, *Ldb1* and the closely related *Ldb2*. Ldb1 can bind LIM domain factors of the AB group, which includes the nuclear LIM-HDs and LMOs, while it does not interact with LIM kinases or other types of LIM domain proteins. Both LIM domains are essential for high affinity binding. *Ldb1* is ubiquitously expressed in the mouse embryo during development and in many organs of the adult animal [233].
The mouse *Ldb1* gene is located on chromosome 19 (46,107,106-46,119,344) and consists of 11 exons that give two transcripts both of which get translated producing two peptides, one of 375 amino acids in length and 42.7 kDa in size and one of 411 amino acids in length and 46.5 kDa in size (Ensembl release 52, Dec. 2008). Ldb1 has neither DNA binding nor enzymatic activities and its main functional domains are: the LID domain or LIM Interaction Domain, located on the carboxy-terminus from 300 to 338 amino acids, which facilitates the interactions with LMO and LIM-HD factors, the dimerization domain which extends within the first 200 amino acids and facilitates the formation of Ldb1 homodimers, the LCCD domain from 201 to 249 amino acids, which is required for the interaction of Ldb1 with the Ssbp proteins and the NLS or Nuclear Localization Signal (Fig. 3) [234-237].

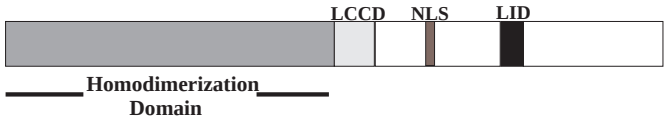

Figure 3: Schematic representation of the Ldb1 protein molecule. The first 200 amino acids form the homodimerization domain, which is essential for the function of Ldb1 as a transcription regulator and a facilitator of long range interactions. The integrity of the Ldb1 molecules in a complex is protected by Ssbp proteins that bind on the LCCD domain. The highly conserved LID domain interacts with LIM domains and its function ensures the diverse role of Ldb1 as a regulator of cell differentiation and development. The nuclear positioning of the Ldb1 protein is ensured through the nuclear localization signal (adapted from Matthews and Visvader 2003) [234].

Ldb proteins exist in many species and are highly conserved. Mammals have two *Ldb* genes, identified as *Ldb1* and *Ldb2*. The human and mouse *Ldb1* genes share a 99% identity. *Drosophila* and *Xenopus* each have one *Ldb* gene that shares a 38% and a 98% identity with the mouse *Ldb1* respectively. The chicken *Ldb1* shares a 96% identity with the mouse ortholog. Finally the zebrafish has four *Ldb* genes, which are thought to have originated from gene duplication events. *Ldb4* is the most closely related to the mouse *Ldb1* as the genes share a 93% identity (Ensembl release 52, Dec. 2008).
Although both LIM domains are required for the efficient binding of Ldb1 to an LMO or a LIM-HD factor, in the case of Lmo2 and Lmo4, the most N-terminal LIM domain, termed LIM1 was able to bind to Ldb1 with higher affinity than the LIM2 domain. The LID domain retains an unfolded conformation in solution, which changes upon interaction with the LIM1 domain of Lmo2 or Lmo4 to an extended conformation stretching across the interacting domain. A β-hairpin next to a highly conserved region of the LIM domains found in LMO and LIM-HD proteins, but not in LIM kinases, interacts strongly with a LID β-strand via the action of main-chain hydrogen bonds. The binding of the LID domain of Ldb1 to a LIM domain is kept stable via the action of electrostatic and hydrophobic side chain interactions, which are essential for the specificity and maintenance of the bond. These side chains are not present in LIM kinases [238]. Overall the interaction between the LID domain of Ldb1 and LIM domains is highly specific, while at the same time being broad enough to cover a wide range of LIM domain proteins. The study of the interaction between the *Xenopus* Xldb and Xlim1 showed that both the LID and the dimerization domains of Ldb1 are required for its efficient function [237].
Spliced isoforms lacking the functional LID domain have been identified for the mammalian and chicken *Ldb1* genes, the *Xenopus Xldb* and the zebrafish *Ldb4*. These isoforms are still localized in the cell nucleus and are able to form homodimers with other full length Ldb molecules but are unable to bind on LIM domains or function as transcription regulators. Truncated spliced isoforms of the *Ldb* genes are proposed to exist as a control mechanism

of their function [239].

Ldb1 was shown to participate in a protein complex together with Scl, E2A, Gata-1 and Lmo2 in MEL cells. The complex was found to bind on a consensus E-box (CAGGTG)/GATA motif via the DNA binding abilities of the Scl/E2A heterodimer and Gata-1. The non-DNA-binding Lmo2 functions as a bridging molecule between Ldb1 and the DNA-binding factors. The efficient assembly of the complex was shown to induce transcription activation in COS-7 cells with the use of luciferase and β-galactosidase reporter assays [228]. However Ldb1 was also shown to inhibit the transcription activation effect of the Lmx1/E47 complex on the rat insulin I mini-enhancer element [236]. The expression of both *Xldb* and *Xlim1* in *Xenopus* animal explants activates the expression of the neural marker *NCAM*, the cement gland marker *XCG7* and the developmental factors *goosecoid* and *chordin*. However mutated forms of *Xlim1* with both LIM domains inactivated can also induce the expression of both *NCAM* and *XCG7*, which demonstrates an inhibitory role for *Ldb1* in transcription regulation [233]. Therefore Ldb1 can function as an activator as well as a repressor in complex with other transcription regulators.

The stoichiometry between Ldb1 and LIM-HD or LMO proteins is very important for the function of the complexes in the regulation of gene expression. Two members of the Single-Stranded DNA-binding protein family (Ssbp), Ssbp2 and Ssbp3, were found to participate in an Ldb1 containing complex together with Scl/E47/Lmo2/Gata-1 for the regulation of *protein 4.2* (*p4.2*) expression in proerythroblast cells. In particular Ssbp2 is able to bind on the LCCD domain of Ldb1 and prevent the RLIM (Rnf12) mediated ubiquitination and subsequent proteosome degradation of both Ldb1 and Lmo2. Thus Ssbp2 and perhaps other members of the Ssbp family can ensure the stoichiometry between Ldb1 and LMOs or LIM-HDs and maintain the stability of the complexes [235]. The *Xenopus* ortholog of *Rnf12* is expressed together with *Xldb* and *Xlim1* in the Spemann-Mangold organizer, where it regulates the stoichiometry of the *Xldb*/*Xlim1* complex by initiating the degradation of the excess *Xldb* molecules not bound by *Xlim1* and thereby participating in the regulation of embryonic development [240].

5.1 Ldb1: involvement embryonic development

Co-injection of *Xlim1* and *Xldb* mRNAs on *Xenopus* embryos causes the formation of a partial secondary axis followed by the ectopic formation of muscle tissue. A similar phenotype follows the injection of a mutated form of *Xlim1*, with inactive LIM domains [233]. *Xlim1* is expressed in the Spemann-Mangold organizer region located at the dorsal part of the *Xenopus* gastrula. It is thought to act as an activator of transcription and it is involved in the development of the amphibian embryo [241]. The Xldb/Xlim1 complex is thought to maintain the expression of the *goosecoid* (*gsc*) gene in the axial mesoderm of the head organizer with the assistance of Otx2 [242]. The mouse *Lim1* is also involved in the function of the head organizer in the developing embryo since it is co-expressed together with *Otx2* and *gsc* in the anterior visceral endoderm. The deletion of either *Lim1* or *Otx2* leads to the truncation of the head structures anterior of the otic vesicle [243, 244].

The *Xenopus* ortholog of Lmo4, *Xlmo4*, is involved in the specification of ventral mesoderm during embryonic development working synergistically with *Gata-2* and its expression in this region is regulated via BMP4 signalling. In the dorsal mesoderm the expression of *Xlmo4* is downregulated probably through the action of *gsc* [245].
The *Drosophila* ortholog of *Ldb1*, *dldb* or *Chi*, is involved in embryo segmentation [246], neuronal development [247], wing development [248] and homozygous mutations lead to a larval lethal phenotype .
Finally a many severe developmental defects follow the deletion of *Ldb1* in the mouse embryo, which are truncation of the anterior head structure similar to the *Lim1* and *Otx2* knockout mouse embryos, small size, lack of a formed heart and specification of the foregut at 8.5 dpc, fusion of somites, duplication along the posterior axis in 40% of the knockout embryos, absence of blood islands and primordial germ cells in the extra-embryonic yolk sac and death after 9.5 dpc [249].
In both the *Drosophila* and the mouse embryos the first *Ldb1* mRNA molecules originate from maternal contribution before the onset of expression in the developing zygote. Furthermore in the *Drosophila* embryo the lack of maternal *Ldb1* leads to a more severe phenotype in comparison to the lack of zygotic expression. These observations strongly support the notion that *Ldb1* is one of the earliest and most essential regulators of embryonic development [246, 249, 250].

5.2 Ldb1: involvement in hematopoiesis

The majority of the transcription factors that are involved in the regulation of hematopoiesis were initially identified through chromosomal rearrangements that cause leukemias. In a similar manner, a fusion product of Ldb1 with Lmo1 was identified in a human leukemia cell line, as the result of the translocations t (11; 14) (p15; q11) or t (11; 14) (p13; q11) [251].
The Ldb1/Lmo2/Scl/E2A/Gata-1 complex identified in MEL cells was recently expanded to include HEB, Lmo4 and the closely related to the Scl factor Lyl1. When the cells were in the non-induced proliferating state this complex was found to interact with another complex consisting of Gata-1/Scl/E47/HEB/Mtgr1/Eto2 as well as with E2-2 and the cell cycle regulator Cdk9, leading to the creation of a large multi-protein complex. Upon cessation of proliferation and induction of differentiation Cdk9 and E2-2 were no longer part of the larger complex, which separated into the two smaller ones. The process coincided with a decrease in the level of Eto2 and an increase in the level of Lmo4. Eto2, Cdk9 and Lmo4 were found to be essential for definitive hematopoiesis in the zebrafish, while Ldb1, Cdk9, E2A, Lmo2, Gata-1 and Eto2 were found to be expressed in the PSp/AGM region of the 9.5 dpc mouse embryo where the HSCs emerge [252]. The above observations together with the established co-expression of Gata-1/Eto-2, Gata-1/Ldb1 and Gata-1/Runx1 in cells of the 9.5 dpc PSp/AGM region leads to the conclusion that the described multiprotein complex is possibly involved in the regulation of definitive hematopoiesis in the mouse embryo.
The Ldb1/Scl/E12/Lmo2 complex with the addition of pRb (retinoblastoma protein) was

shown to assemble in human proerythroblasts and downregulate the expression of c-kit in the maturing erythroblasts from two inverted E-box motifs located on the promoter [253]. Furthermore the expression of the mature erythrocyte membrane protein *glycophorin A* (*GPA*) is regulated by the Ldb1/Scl/E47/Lmo2/Gata-1 complex from an E-box/GATA motif on the promoter [254]. Ldb1 was also shown to bind to an enhancer element of the *Eklf* gene and a hypersensitive site upstream of the *Gata-1* gene together with Scl and Gata-1 in non-induced and induced MEL cells [252]. Finally the Ldb1/Scl/Lmo2/Gata-2 complex together with the Ets factors Fli-1, Elf-1 and Pu.1 was shown to bind *in vivo* on the *Runx1* +23.5 enhancer element that drives its expression during definitive hematopoiesis [219].

5.3 Ldb1: involvement in neural development

Ldb1 together with *Rlim* are widely expressed in the developing neural tube before and after closure. Early in development from 8 dpc to 10 dpc the expression is stronger in the dorsal part. *Clim1* or *Ldb2* was also found to be expressed mainly at the ventral part of the neural tube in the developing embryo [255].

The involvement of *Ldb1* in anterior head development is strongly supported by the phenotype of the knockout mouse and the observation that a similar developmental defect is associated with the deletion of factors that act in complex with Ldb1. The inactivation of *Ssbp1* in the mouse embryo causes the *headshrinker* (*hsk*) phenotype, which is characterized by a truncation of the head structures anterior to the ear. Since members of the *Ssbp* family were shown to protect Ldb1 form degradation, it is expected that their inactivation will lead to a similar phenotype as the one observed in the *Ldb1*-/- mouse [256].

A number of LIM-HD factors are involved in the development of the central nervous system and in neuronal differentiation [257-260]. A tetrameric complex formed by two Lhx3 molecules and an Ldb1 homodimer functions towards the specification of V2 interneurons in the ventral spinal cord. The conversion of the complex to a hexamer through the binding of two Isl1 molecules changes its function and directs it towards the specification of motor neurons again in the ventral spinal cord [261-263]. The LIM-HD factors Lhx1 and Lhx5 together with Ldb1 are involved in the emergence of Purkinje cells in the cerebellum during embryonic development. The deletion of both *Lhx1* and *Lhx5* or *Ldb1* results in similar phenotypes characterized by a sharp reduction in the number of differentiated Purkinje cells [264]. Ldb1 was shown to interact with another member of the LIM-HD family Lhx6.1, the expression of which is strong in the developing forebrain of the mouse. *Ldb1* is more widely expressed in the developing central nervous system, however its expression was found to overlap with that of *Lhx6.1* [265].

5.4 Ldb1: function in long range interactions

Cell fate determination and differentiation during embryonic development is largely dependent on efficient gene expression control in a spatial as well as a temporal manner. Apart from the promoter regions located in close proximity to transcription initiation sites, an abundance of cis- or trans-regulatory elements that function as enhancers or repressors

are located upstream or downstream and are often separated from the initiation site by long distances that can range from a few hundred bases to more than a megabase. The communication between promoters and distant regulatory elements is essential for the regulation of transcription and is described as a long range interaction. These interactions are achieved through molecules that act as a bridge between DNA-binding transcription factor complexes on the promoters and the enhancers or repressors and through the physical bending of the DNA that leads to the formation of a loop. Ldb1 is considered to be a bridging molecule regulating the expression of a variety of genes in an indirect manner.
The *Drosophila* ortholog of *Ldb1* (*dldb* or *Chi*) was identified as a facilitator of enhancer-promoter long range interactions through a screen of mutations that interfere with the activity of the remote wing margin enhancer and reduce the expression of the *cut* gene. The enhancer is located 85kb upstream of the *cut* promoter and it is bound by *scalloped* (*sd*) and *mastermind* (*mam*). The inactivation of both enhancer binding factors as well as the deletion of *Chi* leads to similar cut wing margin phenotypes. In *Drosophila* the gypsy retrovirus is inserted in the genome and when bound by the *suppressor of Hairy-wing* [*su (Hw)*] it can block the communication between an enhancer and a promoter in one direction without physically interacting with either of them. The su (Hw) protein can block the activity of the wing margin enhancer. It was observed that one functional *cut* allele was enough for normal development since flies with a gypsy insertion in one of the *cut* alleles have a wild type phenotype. However the addition of a mutation in one of the *Chi* alleles led to a partial wing margin phenotype, leading to the conclusion that insufficient *Chi* expression affects the transcription of the *cut* locus only when there is a gypsy insertion in one of its alleles. It is interesting to note that flies with reduced expression of *Chi* due to a mutation and a deletion of the wing margin enhancer in one allele show a wild type phenotype. In the absence of *Chi* activity su (Hw) bound on the gypsy insertion in one *cut* allele can interfere with enhancer-promoter communication on the other allele. A similar function for *Chi* as a facilitator of enhancer-promoter interactions has been demonstrated through studying the effect of gypsy insertions in the expression of *Ultrabithorax* (*Ubx*). The *Drosophila Chi* is also required for the regulation of the *eve* gene expression through the activation of seven remote blastoderm stripe enhancers [246, 250].
In *Drosophila* proneural development *Chi* acts as a bridging molecule between the GATA-binding factor *Pannier* and an E-box bound heterodimer formed by the bHLH factors Daughterless (Da) and achaete/scute (ac/sc) for the expression of the *achaete*/*scute* locus. The bHLH heterodimer is bound on E-boxes located on the promoters of *achaete* and *scute* genes, while *Pannier* is positioned on the DC enhancer that is located 4kb from the *ac* promoter and 30kb from the *sc* promoter [266].

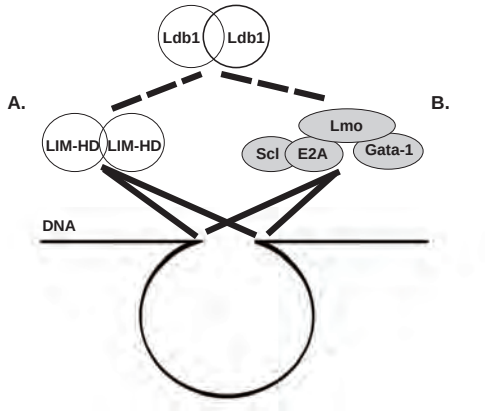

Figure 4: Schematic representation of the way that Ldb1 can function as a facilitator of long range interactions between distant regulatory elements. The Ldb1 homodimer participates in multiprotein complexes that consist of (A.) DNA bound LIM-HD factors or (B.) bHLH (Scl/E2A) factors together with GATA-binding factors. In this case Ldb1 is bound to the complex via LMO proteins. The assembly of the complexes on the DNA will result in the formation of a loop-like structure that will bring distant regulatory elements in close proximity for the initiation of transcription.

The Ldb1/Lmo2/Scl/E2A/Gata-1 multi-protein complex regulates the expression of the *p4.2* gene in MEL cells through binding on an E-box/GATA motif on its proximal promoter. Two such complexes assembled on distinct motifs were shown to interact in solution and this was dependent on the ability of Ldb1 to form homodimers [267].
The Ldb1/Gata-1/Scl/Lmo2 complex was also shown to bind *in vivo* on the human β-globin Locus Control Region (LCR) as well as the mouse β-globin LCR in MEL cells. The strongest binding affinity in the human LCR was observed on HS2 and was dependent on the presence of an E-box/GATA motif. The binding of the complex was essential for the function of HS2 as an enhancer for the transcription of the human ε-globin. In MEL cells the assembly of the complex on the LCR and the βmajor promoter was shown to be necessary for the expression of β-globin. It was proposed that Ldb1 assists the formation of a DNA loop structure bringing the LCR and the promoters of the globin genes in close proximity for efficient transcription [268].

References

1. Kaufman, M.H. and J.B.L. Bard, *The Anatomical Basis of Mouse Development.* Academic Press, 1999.
2. Clegg, K.B. and L. Piko, *Poly(A) length, cytoplasmic adenylation and synthesis of poly(A)+ RNA in early mouse embryos.* Dev Biol, 1983. **95**(2): p. 331-41.
3. Giebelhaus, D.H., J.J. Heikkila, and G.A. Schultz, *Changes in the quantity of histone and actin messenger RNA during the development of preimplantation mouse embryos.* Dev Biol, 1983. **98**(1): p. 148-54.
4. Johnson, M.H., *The molecular and cellular basis of preimplantation mouse development.* Biol Rev Camb Philos Soc, 1981. **56**(3): p. 463-98.
5. Pratt, H.P., V.N. Bolton, and K.A. Gudgeon, *The legacy from the oocyte and its role in controlling early development of the mouse embryo.* Ciba Found Symp, 1983. **98**: p. 197-227.
6. Gardner, R.L., *Origin and differentiation of extraembryonic tissues in the mouse.* Int Rev Exp Pathol, 1983. **24**: p. 63-133.
7. Varmuza, S., et al., *Polytene chromosomes in mouse trophoblast giant cells.* Development, 1988. **102**(1): p. 127-34.
8. Hogan, B., et al., *Manipulating the Mouse Embryo: A Laboratory Manual, Second Edition.* Cold Spring Harbor Laboratory Press, 1994.
9. Snow, M.H. and D. Bennett, *Gastrulation in the mouse: assessment of cell populations in the epiblast of tw18/tw18 embryos.* J Embryol Exp Morphol, 1978. **47**: p. 39-52.
10. Tam, P.P. and R.S. Beddington, *The formation of mesodermal tissues in the mouse embryo during gastrulation and early organogenesis.* Development, 1987. **99**(1): p. 109-26.
11. Evans, M.J. and M.H. Kaufman, *Establishment in culture of pluripotential cells from mouse embryos.* Nature, 1981. **292**(5819): p. 154-6.
12. Martin, G.R., *Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells.* Proc Natl Acad Sci U S A, 1981. **78**(12): p. 7634-8.
13. Smith, A.G., et al., *Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides.* Nature, 1988. **336**(6200): p. 688-90.
14. Williams, R.L., et al., *Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells.* Nature, 1988. **336**(6200): p. 684-7.
15. Bradley, A., et al., *Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines.* Nature, 1984. **309**(5965): p. 255-6.
16. Nagy, A., et al., *Embryonic stem cells alone are able to support fetal development in the mouse.* Development, 1990. **110**(3): p. 815-21.
17. Andrews, P.W., *From teratocarcinomas to embryonic stem cells.* Philos Trans R Soc Lond B Biol Sci, 2002. **357**(1420): p. 405-17.
18. Finch, B.W. and B. Ephrussi, *RETENTION OF MULTIPLE DEVELOPMENTAL POTENTIALITIES BY CELLS OF A MOUSE TESTICULAR TERATOCARCINOMA DURING PROLONGED CULTURE in vitro AND THEIR EXTINCTION UPON HYBRIDIZATION WITH CELLS OF PERMANENT LINES.* Proc Natl Acad Sci U S A, 1967. **57**(3): p. 615-621.
19. Illmensee, K. and B. Mintz, *Totipotency and normal differentiation of single teratocarcinoma cells cloned by injection into blastocysts.* Proc Natl Acad Sci U S A, 1976. **73**(2): p. 549-53.
20. Kleinsmith, L.J. and G.B. Pierce, Jr., *Multipotentiality of Single Embryonal Carcinoma Cells.* Cancer Res, 1964. **24**: p. 1544-51.
21. Thomas, K.R. and M.R. Capecchi, *Targeting of genes to specific sites in the mammalian genome.* Cold Spring Harb Symp Quant Biol, 1986. **51 Pt 2**: p. 1101-13.
22. Thomas, K.R. and M.R. Capecchi, *Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells.* Cell, 1987. **51**(3): p. 503-12.
23. Doetschman, T.C., et al., *The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium.* J Embryol Exp Morphol, 1985. **87**: p. 27-45.
24. Keller, G.M., *In vitro differentiation of embryonic stem cells.* Curr Opin Cell Biol, 1995. **7**(6): p. 862-9.
25. Rippon, H.J. and A.E. Bishop, *Embryonic stem cells.* Cell Prolif, 2004. **37**(1): p. 23-34.
26. Fuchs, E., T. Tumbar, and G. Guasch, *Socializing with the neighbors: stem cells and their niche.* Cell, 2004. **116**(6): p. 769-78.
27. Ohlstein, B., et al., *The stem cell niche: theme and variations.* Curr Opin Cell Biol, 2004. **16**(6): p. 693-9.
28. Bunn, H.F., *Evolution of mammalian hemoglobin function.* Blood, 1981. **58**(2): p. 189-97.

29. Dzierzak, E. and N.A. Speck, *Of lineage and legacy: the development of mammalian hematopoietic stem cells.* Nat Immunol, 2008. **9**(2): p. 129-36.
30. Moore, M.A. and D. Metcalf, *Ontogeny of the haemopoietic system: yolk sac origin of in vivo and in vitro colony forming cells in the developing mouse embryo.* Br J Haematol, 1970. **18**(3): p. 279-96.
31. Maximow, *Untersuchungen uber blut und bindegewebe 1. Die fruhesten entwicklungsstadien der blut und bindegewebszellan bein saugeberembryo, bis zum anfang der blutbilding unden leber.* Arch Mikroskop Anat, 1909. **73**.
32. Barker, J.E., *Development of the mouse hematopoietic system. I. Types of hemoglobin produced in embryonic yolk sac and liver.* Dev Biol, 1968. **18**(1): p. 14-29.
33. Brotherton, T.W., et al., *Hemoglobin ontogeny during normal mouse fetal development.* Proc Natl Acad Sci U S A, 1979. **76**(6): p. 2853-7.
34. Steiner, R. and H. Vogel, *On the kinetics of erythroid cell differentiation in fetal mice. I. Microspectrophotometric determination of the hemoglobin content in erythroid cells during gestation.* J Cell Physiol, 1973. **81**(3): p. 323-38.
35. Kingsley, P.D., et al., *Yolk sac-derived primitive erythroblasts enucleate during mammalian embryogenesis.* Blood, 2004. **104**(1): p. 19-25.
36. Fraser, S.T., J. Isern, and M.H. Baron, *Maturation and enucleation of primitive erythroblasts during mouse embryogenesis is accompanied by changes in cell-surface antigen expression.* Blood, 2007. **109**(1): p. 343-52.
37. Palis, J., et al., *Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse.* Development, 1999. **126**(22): p. 5073-84.
38. Lux, C.T., et al., *All primitive and definitive hematopoietic progenitor cells emerging before E10 in the mouse embryo are products of the yolk sac.* Blood, 2008. **111**(7): p. 3435-8.
39. Rampon, C. and P. Huber, *Multilineage hematopoietic progenitor activity generated autonomously in the mouse yolk sac: analysis using angiogenesis-defective embryos.* Int J Dev Biol, 2003. **47**(4): p. 273-80.
40. Palis, J., et al., *Spatial and temporal emergence of high proliferative potential hematopoietic precursors during murine embryogenesis.* Proc Natl Acad Sci U S A, 2001. **98**(8): p. 4528-33.
41. Wong, P.M., et al., *Hemoglobin switching during murine embryonic development: evidence for two populations of embryonic erythropoietic progenitor cells.* Blood, 1986. **67**(3): p. 716-21.
42. McGrath, K.E., et al., *Circulation is established in a stepwise pattern in the mammalian embryo.* Blood, 2003. **101**(5): p. 1669-76.
43. Risau, W. and I. Flamme, *Vasculogenesis.* Annu Rev Cell Dev Biol, 1995. **11**: p. 73-91.
44. Poole, T.J. and J.D. Coffin, *Vasculogenesis and angiogenesis: two distinct morphogenetic mechanisms establish embryonic vascular pattern.* J Exp Zool, 1989. **251**(2): p. 224-31.
45. Patan, S., *Vasculogenesis and angiogenesis as mechanisms of vascular network formation, growth and remodeling.* J Neurooncol, 2000. **50**(1-2): p. 1-15.
46. Risau, W., *Mechanisms of angiogenesis.* Nature, 1997. **386**(6626): p. 671-4.
47. Navaratnam, V., et al., *Differentiation of the myocardial rudiment of mouse embryos: an ultrastructural study including freeze-fracture replication.* J Anat, 1986. **146**: p. 65-85.
48. Kaufman, M.H., *The role of embryology in teratological research, with particular reference to the development of the neural tube and heart.* J Reprod Fertil, 1981. **62**(2): p. 607-23.
49. Ji, R.P., et al., *Onset of cardiac function during early mouse embryogenesis coincides with entry of primitive erythroblasts into the embryo proper.* Circ Res, 2003. **92**(2): p. 133-5.
50. Wilt, F.H., *Erythropoiesis in the Chick Embryo: the Role of Endoderm.* Science, 1965. **147**: p. 1588-90.
51. Palis, J., K.E. McGrath, and P.D. Kingsley, *Initiation of hematopoiesis and vasculogenesis in murine yolk sac explants.* Blood, 1995. **86**(1): p. 156-63.
52. Miura, Y. and F.H. Wilt, *Tissue interaction and the formation of the first erythroblasts of the chick embryo.* Dev Biol, 1969. **19**(2): p. 201-11.
53. Yoder, M.C., et al., *Murine yolk sac endoderm- and mesoderm-derived cell lines support in vitro growth and differentiation of hematopoietic cells.* Blood, 1994. **83**(9): p. 2436-43.
54. Yoder, M.C., et al., *Murine embryonic yolk sac cells promote in vitro proliferation of bone marrow high proliferative potential colony-forming cells.* Blood, 1995. **86**(4): p. 1322-30.
55. Bielinska, M., et al., *Erythropoiesis and vasculogenesis in embryoid bodies lacking visceral yolk sac endoderm.* Blood, 1996. **88**(10): p. 3720-30.
56. Carmeliet, P., et al., *Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele.* Nature, 1996. **380**(6573): p. 435-9.

57. Ferrara, N., et al., *Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene.* Nature, 1996. **380**(6573): p. 439-42.
58. Miquerol, L., B.L. Langille, and A. Nagy, *Embryonic development is disrupted by modest increases in vascular endothelial growth factor gene expression.* Development, 2000. **127**(18): p. 3941-6.
59. Damert, A., et al., *Insufficient VEGFA activity in yolk sac endoderm compromises haematopoietic and endothelial differentiation.* Development, 2002. **129**(8): p. 1881-92.
60. Shalaby, F., et al., *A requirement for Flk1 in primitive and definitive hematopoiesis and vasculogenesis.* Cell, 1997. **89**(6): p. 981-90.
61. Schuh, A.C., et al., *In vitro hematopoietic and endothelial potential of flk-1(-/-) embryonic stem cells and embryos.* Proc Natl Acad Sci U S A, 1999. **96**(5): p. 2159-64.
62. Hidaka, M., W.L. Stanford, and A. Bernstein, *Conditional requirement for the Flk-1 receptor in the in vitro generation of early hematopoietic cells.* Proc Natl Acad Sci U S A, 1999. **96**(13): p. 7370-5.
63. Shalaby, F., et al., *Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice.* Nature, 1995. **376**(6535): p. 62-6.
64. His, W., *Lecithoblast und angioblast der wirbelthiere.* Abhandl KS Ges Wis Math-Phys, 1900. **22**.
65. Sabin, F., *Studies on the origin of blood vessels and of red corpuscles as seen in the living blastoderm of chicks during the second day of incubation.* Contrib Embryol, 1920. **9**: p. 215-262.
66. Ferkowicz, M.J. and M.C. Yoder, *Blood island formation: longstanding observations and modern interpretations.* Exp Hematol, 2005. **33**(9): p. 1041-7.
67. Beddington, R.S. and E.J. Robertson, *Axis development and early asymmetry in mammals.* Cell, 1999. **96**(2): p. 195-209.
68. Lu, C.C., J. Brennan, and E.J. Robertson, *From fertilization to gastrulation: axis formation in the mouse embryo.* Curr Opin Genet Dev, 2001. **11**(4): p. 384-92.
69. Huber, T.L., et al., *Haemangioblast commitment is initiated in the primitive streak of the mouse embryo.* Nature, 2004. **432**(7017): p. 625-30.
70. Baron, M.H., *Early patterning of the mouse embryo: implications for hematopoietic commitment and differentiation.* Exp Hematol, 2005. **33**(9): p. 1015-20.
71. Eichmann, A., et al., *Ligand-dependent development of the endothelial and hemopoietic lineages from embryonic mesodermal cells expressing vascular endothelial growth factor receptor 2.* Proc Natl Acad Sci U S A, 1997. **94**(10): p. 5141-6.
72. Eichmann, A., et al., *Two molecules related to the VEGF receptor are expressed in early endothelial cells during avian embryonic development.* Mech Dev, 1993. **42**(1-2): p. 33-48.
73. Choi, K., et al., *A common precursor for hematopoietic and endothelial cells.* Development, 1998. **125**(4): p. 725-32.
74. Kennedy, M., et al., *A common precursor for primitive erythropoiesis and definitive haematopoiesis.* Nature, 1997. **386**(6624): p. 488-93.
75. Choi, K., *Hemangioblast development and regulation.* Biochem Cell Biol, 1998. **76**(6): p. 947-56.
76. Lancrin, C., et al., *The haemangioblast generates haematopoietic cells through a haemogenic endothelium stage.* Nature, 2009. **457**(7231): p. 892-5.
77. Cumano, A., et al., *Intraembryonic, but not yolk sac hematopoietic precursors, isolated before circulation, provide long-term multilineage reconstitution.* Immunity, 2001. **15**(3): p. 477-85.
78. Cumano, A., F. Dieterlen-Lievre, and I. Godin, *Lymphoid potential, probed before circulation in mouse, is restricted to caudal intraembryonic splanchnopleura.* Cell, 1996. **86**(6): p. 907-16.
79. Yoder, M.C., et al., *Characterization of definitive lymphohematopoietic stem cells in the day 9 murine yolk sac.* Immunity, 1997. **7**(3): p. 335-44.
80. Medvinsky, A.L., et al., *An early pre-liver intraembryonic source of CFU-S in the developing mouse.* Nature, 1993. **364**(6432): p. 64-7.
81. Muller, A.M., et al., *Development of hematopoietic stem cell activity in the mouse embryo.* Immunity, 1994. **1**(4): p. 291-301.
82. Medvinsky, A. and E. Dzierzak, *Definitive hematopoiesis is autonomously initiated by the AGM region.* Cell, 1996. **86**(6): p. 897-906.
83. de Bruijn, M.F., et al., *Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo.* Embo J, 2000. **19**(11): p. 2465-74.
84. Cumano, A., F. Dieterlen-Lievre, and I. Godin, *The splanchnopleura/AGM region is the prime site for the generation of multipotent hemopoietic precursors, in the mouse embryo.* Vaccine, 2000. **18**(16): p. 1621-3.
85. Durand, C. and E. Dzierzak, *Embryonic beginnings of adult hematopoietic stem cells.* Haematologica, 2005. **90**(1): p. 100-8.

86. Garcia-Porrero, J.A., I.E. Godin, and F. Dieterlen-Lievre, *Potential intraembryonic hemogenic sites at pre-liver stages in the mouse.* Anat Embryol (Berl), 1995. **192**(5): p. 425-35.
87. Haak, E., *The role of the embryonic microenvironment in hematopoietic cell development.* PhD Thesis, 2007.
88. Jaffredo, T., et al., *From hemangioblast to hematopoietic stem cell: an endothelial connection?* Exp Hematol, 2005. **33**(9): p. 1029-40.
89. Delassus, S. and A. Cumano, *Circulation of hematopoietic progenitors in the mouse embryo.* Immunity, 1996. **4**(1): p. 97-106.
90. Godin, I., et al., *Stem cell emergence and hemopoietic activity are incompatible in mouse intraembryonic sites.* J Exp Med, 1999. **190**(1): p. 43-52.
91. Dzierzak, E., A. Medvinsky, and M. de Bruijn, *Qualitative and quantitative aspects of haematopoietic cell development in the mammalian embryo.* Immunol Today, 1998. **19**(5): p. 228-36.
92. Christensen, J.L., et al., *Circulation and chemotaxis of fetal hematopoietic stem cells.* PLoS Biol, 2004. **2**(3): p. E75.
93. Calvi, L.M., et al., *Osteoblastic cells regulate the haematopoietic stem cell niche.* Nature, 2003. **425**(6960): p. 841-6.
94. Zhang, J., et al., *Identification of the haematopoietic stem cell niche and control of the niche size.* Nature, 2003. **425**(6960): p. 836-41.
95. Derynck, R. and X.H. Feng, *TGF-beta receptor signaling.* Biochim Biophys Acta, 1997. **1333**(2): p. F105-50.
96. Johnson, A.N. and S.J. Newfeld, *The TGF-beta family: signaling pathways, developmental roles, and tumor suppressor activities.* ScientificWorldJournal, 2002. **2**: p. 892-925.
97. Hoodless, P.A., et al., *FoxH1 (Fast) functions to specify the anterior primitive streak in the mouse.* Genes Dev, 2001. **15**(10): p. 1257-71.
98. Mishina, Y., et al., *Multiple roles for activin-like kinase-2 signaling during mouse embryogenesis.* Dev Biol, 1999. **213**(2): p. 314-26.
99. Schier, A.F. and M.M. Shen, *Nodal signalling in vertebrate development.* Nature, 2000. **403**(6768): p. 385-9.
100. Tang, S.J., et al., *The Tlx-2 homeobox gene is a downstream target of BMP signalling and is required for mouse mesoderm development.* Development, 1998. **125**(10): p. 1877-87.
101. Tremblay, K.D., N.R. Dunn, and E.J. Robertson, *Mouse embryos lacking Smad1 signals display defects in extra-embryonic tissues and germ cell formation.* Development, 2001. **128**(18): p. 3609-21.
102. Waldrip, W.R., et al., *Smad2 signaling in extraembryonic tissues determines anterior-posterior polarity of the early mouse embryo.* Cell, 1998. **92**(6): p. 797-808.
103. Winnier, G., et al., *Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse.* Genes Dev, 1995. **9**(17): p. 2105-16.
104. Pfendler, K.C., et al., *Nodal and bone morphogenetic protein 5 interact in murine mesoderm formation and implantation.* Genesis, 2000. **28**(1): p. 1-14.
105. Bellusci, S., et al., *Evidence from normal expression and targeted misexpression that bone morphogenetic protein (Bmp-4) plays a role in mouse embryonic lung morphogenesis.* Development, 1996. **122**(6): p. 1693-702.
106. Kim, M.K. and C. Niyibizi, *Interaction of TGF-beta1 and rhBMP-2 on human bone marrow stromal cells cultured in collagen gel matrix.* Yonsei Med J, 2001. **42**(3): p. 338-44.
107. Luo, G., et al., *BMP-7 is an inducer of nephrogenesis, and is also required for eye development and skeletal patterning.* Genes Dev, 1995. **9**(22): p. 2808-20.
108. Monsoro-Burq, A.H., et al., *The role of bone morphogenetic proteins in vertebral development.* Development, 1996. **122**(11): p. 3607-16.
109. Claassen, G.F. and S.R. Hann, *A role for transcriptional repression of p21CIP1 by c-Myc in overcoming transforming growth factor beta -induced cell-cycle arrest.* Proc Natl Acad Sci U S A, 2000. **97**(17): p. 9498-503.
110. Datto, M.B., et al., *Transforming growth factor beta induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism.* Proc Natl Acad Sci U S A, 1995. **92**(12): p. 5545-9.
111. Gomes, W.A. and J.A. Kessler, *Msx-2 and p21 mediate the pro-apoptotic but not the anti-proliferative effects of BMP4 on cultured sympathetic neuroblasts.* Dev Biol, 2001. **237**(1): p. 212-21.
112. Li, J.M., et al., *Transforming growth factor beta activates the promoter of cyclin-dependent kinase inhibitor p15INK4B through an Sp1 consensus site.* J Biol Chem, 1995. **270**(45): p. 26750-3.

113. Warner, B.J., et al., *Myc downregulation by transforming growth factor beta required for activation of the p15(Ink4b) G(1) arrest pathway.* Mol Cell Biol, 1999. **19**(9): p. 5913-22.
114. Johansson, B.M. and M.V. Wiles, *Evidence for involvement of activin A and bone morphogenetic protein 4 in mammalian mesoderm and hematopoietic development.* Mol Cell Biol, 1995. **15**(1): p. 141-51.
115. Chang, H., et al., *Smad5 knockout mice die at mid-gestation due to multiple embryonic and extraembryonic defects.* Development, 1999. **126**(8): p. 1631-42.
116. Yang, X., et al., *Angiogenesis defects and mesenchymal apoptosis in mice lacking SMAD5.* Development, 1999. **126**(8): p. 1571-80.
117. Bruno, E., et al., *The Smad5 gene is involved in the intracellular signaling pathways that mediate the inhibitory effects of transforming growth factor-beta on human hematopoiesis.* Blood, 1998. **91**(6): p. 1917-23.
118. Liu, B., et al., *Disruption of Smad5 gene leads to enhanced proliferation of high-proliferative potential precursors during embryonic hematopoiesis.* Blood, 2003. **101**(1): p. 124-33.
119. Singbrant, S., et al., *Smad5 is dispensable for adult murine hematopoiesis.* Blood, 2006. **108**(12): p. 3707-12.
120. Dickson, M.C., et al., *Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice.* Development, 1995. **121**(6): p. 1845-54.
121. Sitnicka, E., et al., *Transforming growth factor beta 1 directly and reversibly inhibits the initial cell divisions of long-term repopulating hematopoietic stem cells.* Blood, 1996. **88**(1): p. 82-8.
122. Larsson, J., et al., *Quiescence of hematopoietic stem cells and maintenance of the stem cell pool is not dependent on TGF-beta signaling in vivo.* Exp Hematol, 2005. **33**(5): p. 592-6.
123. Larsson, J., et al., *TGF-beta signaling-deficient hematopoietic stem cells have normal self-renewal and regenerative ability in vivo despite increased proliferative capacity in vitro.* Blood, 2003. **102**(9): p. 3129-35.
124. Malhotra, S. and P.W. Kincade, *Wnt-related molecules and signaling pathway equilibrium in hematopoiesis.* Cell Stem Cell, 2009. **4**(1): p. 27-36.
125. Ruiz, P., et al., *Targeted mutation of plakoglobin in mice reveals essential functions of desmosomes in the embryonic heart.* J Cell Biol, 1996. **135**(1): p. 215-25.
126. Haegel, H., et al., *Lack of beta-catenin affects mouse development at gastrulation.* Development, 1995. **121**(11): p. 3529-37.
127. Jeannet, G., et al., *Long-term, multilineage hematopoiesis occurs in the combined absence of beta-catenin and gamma-catenin.* Blood, 2008. **111**(1): p. 142-9.
128. Kirstetter, P., et al., *Activation of the canonical Wnt pathway leads to loss of hematopoietic stem cell repopulation and multilineage differentiation block.* Nat Immunol, 2006. **7**(10): p. 1048-56.
129. Reya, T., et al., *A role for Wnt signalling in self-renewal of haematopoietic stem cells.* Nature, 2003. **423**(6938): p. 409-14.
130. Luis, T.C., et al., *Wnt3a deficiency irreversibly impairs hematopoietic stem cell self-renewal and leads to defects in progenitor cell differentiation.* Blood, 2009. **113**(3): p. 546-54.
131. Austin, T.W., et al., *A role for the Wnt gene family in hematopoiesis: expansion of multilineage progenitor cells.* Blood, 1997. **89**(10): p. 3624-35.
132. Yamaguchi, T.P., et al., *A Wnt5a pathway underlies outgrowth of multiple structures in the vertebrate embryo.* Development, 1999. **126**(6): p. 1211-23.
133. Louis, I., et al., *The signaling protein Wnt4 enhances thymopoiesis and expands multipotent hematopoietic progenitors through beta-catenin-independent signaling.* Immunity, 2008. **29**(1): p. 57-67.
134. Cheng, X., et al., *Numb mediates the interaction between Wnt and Notch to modulate primitive erythropoietic specification from the hemangioblast.* Development, 2008. **135**(20): p. 3447-58.
135. Alva, J.A. and M.L. Iruela-Arispe, *Notch signaling in vascular morphogenesis.* Curr Opin Hematol, 2004. **11**(4): p. 278-83.
136. Tien, A.C., A. Rajan, and H.J. Bellen, *A Notch updated.* J Cell Biol, 2009. **184**(5): p. 621-9.
137. Kumano, K., et al., *Notch1 but not Notch2 is essential for generating hematopoietic stem cells from endothelial cells.* Immunity, 2003. **18**(5): p. 699-711.
138. Yoon, M.J., et al., *Mind bomb-1 is essential for intraembryonic hematopoiesis in the aortic endothelium and the subaortic patches.* Mol Cell Biol, 2008. **28**(15): p. 4794-804.
139. Varnum-Finney, B., et al., *Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling.* Nat Med, 2000. **6**(11): p. 1278-81.
140. Yang, R. and Z.C. Han, *Angiogenesis in hematologic malignancies and its clinical implications.* Int J Hematol, 2002. **75**(3): p. 246-56.

141. Radtke, F., et al., *Deficient T cell fate specification in mice with an induced inactivation of Notch1.* Immunity, 1999. **10**(5): p. 547-58.
142. Stier, S., et al., *Notch1 activation increases hematopoietic stem cell self-renewal in vivo and favors lymphoid over myeloid lineage outcome.* Blood, 2002. **99**(7): p. 2369-78.
143. Wilson, A., H.R. MacDonald, and F. Radtke, *Notch 1-deficient common lymphoid precursors adopt a B cell fate in the thymus.* J Exp Med, 2001. **194**(7): p. 1003-12.
144. Pui, J.C., et al., *Notch1 expression in early lymphopoiesis influences B versus T lineage determination.* Immunity, 1999. **11**(3): p. 299-308.
145. Jiang, J. and C.C. Hui, *Hedgehog signaling in development and cancer.* Dev Cell, 2008. **15**(6): p. 801-12.
146. Dyer, M.A., et al., *Indian hedgehog activates hematopoiesis and vasculogenesis and can respecify prospective neurectodermal cell fate in the mouse embryo.* Development, 2001. **128**(10): p. 1717-30.
147. Byrd, N., et al., *Hedgehog is required for murine yolk sac angiogenesis.* Development, 2002. **129**(2): p. 361-72.
148. Maye, P., et al., *Indian hedgehog signaling in extraembryonic endoderm and ectoderm differentiation in ES embryoid bodies.* Mech Dev, 2000. **94**(1-2): p. 117-32.
149. Kobune, M., et al., *Adenoviral vector-mediated transfer of the Indian hedgehog gene modulates lymphomyelopoiesis in vivo.* Stem Cells, 2008. **26**(2): p. 534-42.
150. Trowbridge, J.J., M.P. Scott, and M. Bhatia, *Hedgehog modulates cell cycle regulators in stem cells to control hematopoietic regeneration.* Proc Natl Acad Sci U S A, 2006. **103**(38): p. 14134-9.
151. Gering, M. and R. Patient, *Hedgehog signaling is required for adult blood stem cell formation in zebrafish embryos.* Dev Cell, 2005. **8**(3): p. 389-400.
152. Eswarakumar, V.P., I. Lax, and J. Schlessinger, *Cellular signaling by fibroblast growth factor receptors.* Cytokine Growth Factor Rev, 2005. **16**(2): p. 139-49.
153. Ornitz, D.M. and N. Itoh, *Fibroblast growth factors.* Genome Biol, 2001. **2**(3): p. REVIEWS3005.
154. Brunner, G., et al., *Phospholipase C release of basic fibroblast growth factor from human bone marrow cultures as a biologically active complex with a phosphatidylinositol-anchored heparan sulfate proteoglycan.* J Cell Biol, 1991. **114**(6): p. 1275-83.
155. Brunner, G., et al., *A T-cell-related proteinase expressed by T-lymphoma cells activates their endogenous pro-urokinase.* Blood, 1992. **79**(8): p. 2099-106.
156. Wilson, E.L., et al., *Basic fibroblast growth factor stimulates myelopoiesis in long-term human bone marrow cultures.* Blood, 1991. **77**(5): p. 954-60.
157. Bruno, E., et al., *Basic fibroblast growth factor promotes the proliferation of human megakaryocyte progenitor cells.* Blood, 1993. **82**(2): p. 430-5.
158. Avraham, H., et al., *Modulation of megakaryocytopoiesis by human basic fibroblast growth factor.* Blood, 1994. **83**(8): p. 2126-32.
159. Nakayama, T., N. Mutsuga, and G. Tosato, *FGF2 posttranscriptionally down-regulates expression of SDF1 in bone marrow stromal cells through FGFR1 IIIc.* Blood, 2007. **109**(4): p. 1363-72.
160. Ortega, S., et al., *Neuronal defects and delayed wound healing in mice lacking fibroblast growth factor 2.* Proc Natl Acad Sci U S A, 1998. **95**(10): p. 5672-7.
161. Bikfalvi, A., et al., *Biological roles of fibroblast growth factor-2.* Endocr Rev, 1997. **18**(1): p. 26-45.
162. de Haan, G., et al., *In vitro generation of long-term repopulating hematopoietic stem cells by fibroblast growth factor-1.* Dev Cell, 2003. **4**(2): p. 241-51.
163. Magnusson, P.U., et al., *Fibroblast growth factor receptor-1 expression is required for hematopoietic but not endothelial cell development.* Arterioscler Thromb Vasc Biol, 2005. **25**(5): p. 944-9.
164. Xu, R.H., et al., *Opposite effects of FGF and BMP-4 on embryonic blood formation: roles of PV.1 and GATA-2.* Dev Biol, 1999. **208**(2): p. 352-61.
165. Evans, T. and G. Felsenfeld, *The erythroid-specific transcription factor Eryf1: a new finger protein.* Cell, 1989. **58**(5): p. 877-85.
166. Orkin, S.H., *GATA-binding transcription factors in hematopoietic cells.* Blood, 1992. **80**(3): p. 575-81.
167. Ponka, P., *Tissue-specific regulation of iron metabolism and heme synthesis: distinct control mechanisms in erythroid cells.* Blood, 1997. **89**(1): p. 1-25.
168. Wall, L., E. deBoer, and F. Grosveld, *The human beta-globin gene 3' enhancer contains multiple binding sites for an erythroid-specific protein.* Genes Dev, 1988. **2**(9): p. 1089-100.
169. Tsai, S.F., et al., *Cloning of cDNA for the major DNA-binding protein of the erythroid lineage through expression in mammalian cells.* Nature, 1989. **339**(6224): p. 446-51.

170. Ohneda, K. and M. Yamamoto, *Roles of hematopoietic transcription factors GATA-1 and GATA-2 in the development of red blood cell lineage.* Acta Haematol, 2002. **108**(4): p. 237-45.
171. Shivdasani, R.A. and S.H. Orkin, *The transcriptional control of hematopoiesis.* Blood, 1996. **87**(10): p. 4025-39.
172. Leonard, M., et al., *Dynamics of GATA transcription factor expression during erythroid differentiation.* Blood, 1993. **82**(4): p. 1071-9.
173. Martin, D.I., et al., *Expression of an erythroid transcription factor in megakaryocytic and mast cell lineages.* Nature, 1990. **344**(6265): p. 444-7.
174. Zon, L.I., et al., *Expression of mRNA for the GATA-binding proteins in human eosinophils and basophils: potential role in gene transcription.* Blood, 1993. **81**(12): p. 3234-41.
175. Gutierrez, L., *Studies on Gata1: Cell Cycle, Survival and Differentiation.* PhD Thesis, 2005.
176. Yomogida, K., et al., *Developmental stage- and spermatogenic cycle-specific expression of transcription factor GATA-1 in mouse Sertoli cells.* Development, 1994. **120**(7): p. 1759-66.
177. Silver, L. and J. Palis, *Initiation of murine embryonic erythropoiesis: a spatial analysis.* Blood, 1997. **89**(4): p. 1154-64.
178. Pevny, L., et al., *Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1.* Nature, 1991. **349**(6306): p. 257-60.
179. Weiss, M.J., G. Keller, and S.H. Orkin, *Novel insights into erythroid development revealed through in vitro differentiation of GATA-1 embryonic stem cells.* Genes Dev, 1994. **8**(10): p. 1184-97.
180. Fujiwara, Y., et al., *Arrested development of embryonic red cell precursors in mouse embryos lacking transcription factor GATA-1.* Proc Natl Acad Sci U S A, 1996. **93**(22): p. 12355-8.
181. Whyatt, D., et al., *An intrinsic but cell-nonautonomous defect in GATA-1-overexpressing mouse erythroid cells.* Nature, 2000. **406**(6795): p. 519-24.
182. Shivdasani, R.A., et al., *A lineage-selective knockout establishes the critical role of transcription factor GATA-1 in megakaryocyte growth and platelet development.* Embo J, 1997. **16**(13): p. 3965-73.
183. Yokomizo, T., et al., *Characterization of GATA-1(+) hemangioblastic cells in the mouse embryo.* Embo J, 2007. **26**(1): p. 184-96.
184. Orlic, D., et al., *Pluripotent hematopoietic stem cells contain high levels of mRNA for c-kit, GATA-2, p45 NF-E2, and c-myb and low levels or no mRNA for c-fms and the receptors for granulocyte colony-stimulating factor and interleukins 5 and 7.* Proc Natl Acad Sci U S A, 1995. **92**(10): p. 4601-5.
185. Jippo, T., et al., *Abundant expression of transcription factor GATA-2 in proliferating but not in differentiated mast cells in tissues of mice: demonstration by in situ hybridization.* Blood, 1996. **87**(3): p. 993-8.
186. Lee, M.E., et al., *Cloning of the GATA-binding protein that regulates endothelin-1 gene expression in endothelial cells.* J Biol Chem, 1991. **266**(24): p. 16188-92.
187. Minegishi, N., et al., *The mouse GATA-2 gene is expressed in the para-aortic splanchnopleura and aorta-gonads and mesonephros region.* Blood, 1999. **93**(12): p. 4196-207.
188. Tsai, F.Y., et al., *An early haematopoietic defect in mice lacking the transcription factor GATA-2.* Nature, 1994. **371**(6494): p. 221-6.
189. Tsai, F.Y. and S.H. Orkin, *Transcription factor GATA-2 is required for proliferation/survival of early hematopoietic cells and mast cell formation, but not for erythroid and myeloid terminal differentiation.* Blood, 1997. **89**(10): p. 3636-43.
190. Persons, D.A., et al., *Enforced expression of the GATA-2 transcription factor blocks normal hematopoiesis.* Blood, 1999. **93**(2): p. 488-99.
191. Lohmann, F. and J.J. Bieker, *Activation of Eklf expression during hematopoiesis by Gata2 and Smad5 prior to erythroid commitment.* Development, 2008. **135**(12): p. 2071-82.
192. George, K.M., et al., *Embryonic expression and cloning of the murine GATA-3 gene.* Development, 1994. **120**(9): p. 2673-86.
193. Oosterwegel, M., et al., *Expression of GATA-3 during lymphocyte differentiation and mouse embryogenesis.* Dev Immunol, 1992. **3**(1): p. 1-11.
194. Pandolfi, P.P., et al., *Targeted disruption of the GATA3 gene causes severe abnormalities in the nervous system and in fetal liver haematopoiesis.* Nat Genet, 1995. **11**(1): p. 40-4.
195. Ting, C.N., et al., *Transcription factor GATA-3 is required for development of the T-cell lineage.* Nature, 1996. **384**(6608): p. 474-8.
196. Chen, D. and G. Zhang, *Enforced expression of the GATA-3 transcription factor affects cell fate decisions in hematopoiesis.* Exp Hematol, 2001. **29**(8): p. 971-80.

197. Jan, Y.N. and L.Y. Jan, *HLH proteins, fly neurogenesis, and vertebrate myogenesis.* Cell, 1993. **75**(5): p. 827-30.
198. Murre, C., P.S. McCaw, and D. Baltimore, *A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins.* Cell, 1989. **56**(5): p. 777-83.
199. Begley, C.G., et al., *The gene SCL is expressed during early hematopoiesis and encodes a differentiation-related DNA-binding motif.* Proc Natl Acad Sci U S A, 1989. **86**(24): p. 10128-32.
200. Elefanty, A.G., et al., *SCL expression in the mouse embryo detected with a targeted lacZ reporter gene demonstrates its localization to hematopoietic, vascular, and neural tissues.* Blood, 1999. **94**(11): p. 3754-63.
201. van Eekelen, J.A., et al., *Expression pattern of the stem cell leukaemia gene in the CNS of the embryonic and adult mouse.* Neuroscience, 2003. **122**(2): p. 421-36.
202. Sanchez, M., et al., *An SCL 3' enhancer targets developing endothelium together with embryonic and adult haematopoietic progenitors.* Development, 1999. **126**(17): p. 3891-904.
203. Robb, L., et al., *Absence of yolk sac hematopoiesis from mice with a targeted disruption of the scl gene.* Proc Natl Acad Sci U S A, 1995. **92**(15): p. 7075-9.
204. Ismailoglu, I., et al., *Mesodermal patterning activity of SCL.* Exp Hematol, 2008. **36**(12): p. 1593-603.
205. Mead, P.E., et al., *SCL specifies hematopoietic mesoderm in Xenopus embryos.* Development, 1998. **125**(14): p. 2611-20.
206. Robb, L., et al., *The scl gene product is required for the generation of all hematopoietic lineages in the adult mouse.* Embo J, 1996. **15**(16): p. 4123-9.
207. Elefanty, A.G., et al., *Hematopoietic-specific genes are not induced during in vitro differentiation of scl-null embryonic stem cells.* Blood, 1997. **90**(4): p. 1435-47.
208. Gekas, C., et al., *Mef2C is a lineage-restricted target of Scl/Tal1 and regulates megakaryopoiesis and B cell homeostasis.* Blood, 2009.
209. Kassouf, M.T., et al., *Differential use of SCL/TAL-1 DNA-binding domain in developmental hematopoiesis.* Blood, 2008. **112**(4): p. 1056-67.
210. Kagoshima, H., et al., *The Runt domain identifies a new family of heteromeric transcriptional regulators.* Trends Genet, 1993. **9**(10): p. 338-41.
211. Speck, N.A., *Core binding factor and its role in normal hematopoietic development.* Curr Opin Hematol, 2001. **8**(4): p. 192-6.
212. Wang, Q., et al., *Disruption of the Cbfa2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis.* Proc Natl Acad Sci U S A, 1996. **93**(8): p. 3444-9.
213. Okuda, T., et al., *AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis.* Cell, 1996. **84**(2): p. 321-30.
214. North, T., et al., *Cbfa2 is required for the formation of intra-aortic hematopoietic clusters.* Development, 1999. **126**(11): p. 2563-75.
215. North, T.E., et al., *Runx1 is expressed in adult mouse hematopoietic stem cells and differentiating myeloid and lymphoid cells, but not in maturing erythroid cells.* Stem Cells, 2004. **22**(2): p. 158-68.
216. Ichikawa, M., et al., *AML-1 is required for megakaryocytic maturation and lymphocytic differentiation, but not for maintenance of hematopoietic stem cells in adult hematopoiesis.* Nat Med, 2004. **10**(3): p. 299-304.
217. Lacaud, G., et al., *Runx1 is essential for hematopoietic commitment at the hemangioblast stage of development in vitro.* Blood, 2002. **100**(2): p. 458-66.
218. Yokomizo, T., et al., *Runx1 is involved in primitive erythropoiesis in the mouse.* Blood, 2008. **111**(8): p. 4075-80.
219. Nottingham, W.T., et al., *Runx1-mediated hematopoietic stem-cell emergence is controlled by a Gata/Ets/SCL-regulated enhancer.* Blood, 2007. **110**(13): p. 4188-97.
220. Taoudi, S. and A. Medvinsky, *Functional identification of the hematopoietic stem cell niche in the ventral domain of the embryonic dorsal aorta.* Proc Natl Acad Sci U S A, 2007. **104**(22): p. 9399-403.
221. Dawid, I.B., R. Toyama, and M. Taira, *LIM domain proteins.* C R Acad Sci III, 1995. **318**(3): p. 295-306.
222. Bach, I., *The LIM domain: regulation by association.* Mech Dev, 2000. **91**(1-2): p. 5-17.
223. Taira, M., et al., *Classification of LIM proteins.* Trends Genet, 1995. **11**(11): p. 431-2.
224. Ribeiro, R.C., et al., *Clinical and biologic features of childhood T-cell leukemia with the t(11;14).* Blood, 1991. **78**(2): p. 466-70.

225. Warren, A.J., et al., *The oncogenic cysteine-rich LIM domain protein rbtn2 is essential for erythroid development.* Cell, 1994. **78**(1): p. 45-57.
226. Mead, P.E., et al., *Primitive erythropoiesis in the Xenopus embryo: the synergistic role of LMO-2, SCL and GATA-binding proteins.* Development, 2001. **128**(12): p. 2301-8.
227. Osada, H., et al., *Association of erythroid transcription factors: complexes involving the LIM protein RBTN2 and the zinc-finger protein GATA1.* Proc Natl Acad Sci U S A, 1995. **92**(21): p. 9585-9.
228. Wadman, I.A., et al., *The LIM-only protein Lmo2 is a bridging molecule assembling an erythroid, DNA-binding complex which includes the TAL1, E47, GATA-1 and Ldb1/NLI proteins.* Embo J, 1997. **16**(11): p. 3145-57.
229. Visvader, J.E., et al., *The LIM-domain binding protein Ldb1 and its partner LMO2 act as negative regulators of erythroid differentiation.* Proc Natl Acad Sci U S A, 1997. **94**(25): p. 13707-12.
230. Porter, F.D., et al., *Lhx2, a LIM homeobox gene, is required for eye, forebrain, and definitive erythrocyte development.* Development, 1997. **124**(15): p. 2935-44.
231. Pinto do, O.P., et al., *Multipotent hematopoietic progenitor cells immortalized by Lhx2 self-renew by a cell nonautonomous mechanism.* Exp Hematol, 2001. **29**(8): p. 1019-28.
232. Dahl, L., et al., *Lhx2 expression promotes self-renewal of a distinct multipotential hematopoietic progenitor cell in embryonic stem cell-derived embryoid bodies.* PLoS ONE, 2008. **3**(4): p. e2025.
233. Agulnick, A.D., et al., *Interactions of the LIM-domain-binding factor Ldb1 with LIM homeodomain proteins.* Nature, 1996. **384**(6606): p. 270-2.
234. Matthews, J.M. and J.E. Visvader, *LIM-domain-binding protein 1: a multifunctional cofactor that interacts with diverse proteins.* EMBO Rep, 2003. **4**(12): p. 1132-7.
235. Xu, Z., et al., *Single-stranded DNA-binding proteins regulate the abundance of LIM domain and LIM domain-binding proteins.* Genes Dev, 2007. **21**(8): p. 942-55.
236. Jurata, L.W. and G.N. Gill, *Functional analysis of the nuclear LIM domain interactor NLI.* Mol Cell Biol, 1997. **17**(10): p. 5688-98.
237. Breen, J.J., et al., *Interactions between LIM domains and the LIM domain-binding protein Ldb1.* J Biol Chem, 1998. **273**(8): p. 4712-7.
238. Deane, J.E., et al., *Structural basis for the recognition of ldb1 by the N-terminal LIM domains of LMO2 and LMO4.* Embo J, 2003. **22**(9): p. 2224-33.
239. Tran, Y.H., et al., *Spliced isoforms of LIM-domain-binding protein (CLIM/NLI/Ldb) lacking the LIM-interaction domain.* J Biochem, 2006. **140**(1): p. 105-19.
240. Hiratani, I., et al., *Selective degradation of excess Ldb1 by Rnf12/RLIM confers proper Ldb1 expression levels and Xlim-1/Ldb1 stoichiometry in Xenopus organizer functions.* Development, 2003. **130**(17): p. 4161-75.
241. Kodjabachian, L., et al., *A study of Xlim1 function in the Spemann-Mangold organizer.* Int J Dev Biol, 2001. **45**(1): p. 209-18.
242. Mochizuki, T., et al., *Xlim-1 and LIM domain binding protein 1 cooperate with various transcription factors in the regulation of the goosecoid promoter.* Dev Biol, 2000. **224**(2): p. 470-85.
243. Shawlot, W. and R.R. Behringer, *Requirement for Lim1 in head-organizer function.* Nature, 1995. **374**(6521): p. 425-30.
244. Acampora, D., et al., *Forebrain and midbrain regions are deleted in Otx2-/- mutants due to a defective anterior neuroectoderm specification during gastrulation.* Development, 1995. **121**(10): p. 3279-90.
245. de la Calle-Mustienes, E., et al., *Xenopus Xlmo4 is a GATA cofactor during ventral mesoderm formation and regulates Ldb1 availability at the dorsal mesoderm and the neural plate.* Dev Biol, 2003. **264**(2): p. 564-81.
246. Morcillo, P., et al., *Chip, a widely expressed chromosomal protein required for segmentation and activity of a remote wing margin enhancer in Drosophila.* Genes Dev, 1997. **11**(20): p. 2729-40.
247. van Meyel, D.J., et al., *Chip is an essential cofactor for apterous in the regulation of axon guidance in Drosophila.* Development, 2000. **127**(9): p. 1823-31.
248. van Meyel, D.J., et al., *Chip and apterous physically interact to form a functional complex during Drosophila development.* Mol Cell, 1999. **4**(2): p. 259-65.
249. Mukhopadhyay, M., et al., *Functional ablation of the mouse Ldb1 gene results in severe patterning defects during gastrulation.* Development, 2003. **130**(3): p. 495-505.
250. Morcillo, P., C. Rosen, and D. Dorsett, *Genes regulating the remote wing margin enhancer in the Drosophila cut locus.* Genetics, 1996. **144**(3): p. 1143-54.
251. Valge-Archer, V., A. Forster, and T.H. Rabbitts, *The LMO1 and LDB1 proteins interact in human T cell acute leukaemia with the chromosomal translocation t(11;14)(p15;q11).* Oncogene, 1998. **17**(24): p. 3199-202.

252. Meier, N., et al., *Novel binding partners of Ldb1 are required for haematopoietic development.* Development, 2006. **133**(24): p. 4913-23.
253. Vitelli, L., et al., *A pentamer transcriptional complex including tal-1 and retinoblastoma protein downmodulates c-kit expression in normal erythroblasts.* Mol Cell Biol, 2000. **20**(14): p. 5330-42.
254. Lahlil, R., et al., *SCL assembles a multifactorial complex that determines glycophorin A expression.* Mol Cell Biol, 2004. **24**(4): p. 1439-52.
255. Ostendorff, H.P., et al., *Dynamic expression of LIM cofactors in the developing mouse neural tube.* Dev Dyn, 2006. **235**(3): p. 786-91.
256. Nishioka, N., et al., *Ssdp1 regulates head morphogenesis of mouse embryos by activating the Lim1-Ldb1 complex.* Development, 2005. **132**(11): p. 2535-46.
257. Lundgren, S.E., et al., *Control of neuronal pathway selection by the Drosophila LIM homeodomain gene apterous.* Development, 1995. **121**(6): p. 1769-73.
258. Pfaff, S.L., et al., *Requirement for LIM homeobox gene Isl1 in motor neuron generation reveals a motor neuron-dependent step in interneuron differentiation.* Cell, 1996. **84**(2): p. 309-20.
259. Sheng, H.Z., et al., *Specification of pituitary cell lineages by the LIM homeobox gene Lhx3.* Science, 1996. **272**(5264): p. 1004-7.
260. Tsuchida, T., et al., *Topographic organization of embryonic motor neurons defined by expression of LIM homeobox genes.* Cell, 1994. **79**(6): p. 957-70.
261. Thaler, J.P., et al., *LIM factor Lhx3 contributes to the specification of motor neuron and interneuron identity through cell-type-specific protein-protein interactions.* Cell, 2002. **110**(2): p. 237-49.
262. Jurata, L.W., D.A. Kenny, and G.N. Gill, *Nuclear LIM interactor, a rhombotin and LIM homeodomain interacting protein, is expressed early in neuronal development.* Proc Natl Acad Sci U S A, 1996. **93**(21): p. 11693-8.
263. Lee, S.K. and S.L. Pfaff, *Synchronization of neurogenesis and motor neuron specification by direct coupling of bHLH and homeodomain transcription factors.* Neuron, 2003. **38**(5): p. 731-45.
264. Zhao, Y., et al., *LIM-homeodomain proteins Lhx1 and Lhx5, and their cofactor Ldb1, control Purkinje cell differentiation in the developing cerebellum.* Proc Natl Acad Sci U S A, 2007. **104**(32): p. 13182-6.
265. Kimura, N., et al., *A brain region-specific gene product Lhx6.1 interacts with Ldb1 through tandem LIM-domains.* J Biochem, 1999. **126**(1): p. 180-7.
266. Ramain, P., et al., *Interactions between chip and the achaete/scute-daughterless heterodimers are required for pannier-driven proneural patterning.* Mol Cell, 2000. **6**(4): p. 781-90.
267. Xu, Z., et al., *Identification of a TAL1 target gene reveals a positive role for the LIM domain-binding protein Ldb1 in erythroid gene expression and differentiation.* Mol Cell Biol, 2003. **23**(21): p. 7585-99.
268. Song, S.H., C. Hou, and A. Dean, *A positive role for NLI/Ldb1 in long-range beta-globin locus control region function.* Mol Cell, 2007. **28**(5): p. 810-22.
269. Baron, M.H., *Embryonic origins of mammalian hematopoiesis.* Exp Hematol, 2003. **31**(12): p. 1160-9.
270. Mikkola, H.K., et al., *Placenta as a site for hematopoietic stem cell development.* Exp Hematol, 2005. **33**(9): p. 1048-54.

Chapter 2

Deletion of the mouse *Ldb1* gene leads to severe embryonic developmental defects.

Deletion of the mouse *Ldb1* gene leads to severe embryonic developmental defects.

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Abstract

The Ldb1 (LIM-Domain Binding 1) protein interacts with LIM-Homeodomain and LMO transcription factors via its LID domain and participates in protein complexes that are involved in embryonic development and cell differentiation. Deletion of the *Ldb1* gene leads to a complex phenotype characterized by early embryonic lethality and severe developmental defects, such as anterior truncation, duplication along the posterior axis in 1/3 of the homozygous embryos, absence of heart formation, defective yolk sac morphology and impairement of primitive hematopoiesis. Comparison of gene expression patterns between *Ldb1*+/+ and *Ldb1*-/- embryos revealed that the resulting phenotype is caused by the misregulation of essential developmental pathways and the loss of the expression of many transcription regulators that are involved in cell fate specification.

Introduction

The mouse Ldb1 (LIM- Domain Binding 1) protein, also known as Nli or Clim2, was first identified from a mouse cDNA expression library, via its characteristic ability to bind to LIM domains [1], which are cysteine-rich zinc-finger motifs [2] [3] present in a large number of proteins that can be nuclear, involved in gene transcription or cytoplasmic, associating with the cytoskeleton. These proteins have at least two LIM domains at their N-terminus and they are further categorised according to the number and type of any other additional domains they may contain, which can be nothing else, named LIM-only proteins (LMOs), a homeodomain, named LIM-homeodomains (LIM-HDs), a kinase domain, named LIM-kinases [4]. LIM-HD proteins are able to bind DNA while LMO proteins are not and they require the presence of another DNA binding protein, specifically a bHLH in order to function in a complex. The major role of LIM proteins is to facilitate protein-protein interactions, which are essential for the formation of multi-protein complexes involved in transcription regulation. Through the interaction of its LIM-binding domain with LIM-domains and in particular with the ones present in LIM-HD and LMO proteins, Ldb1, which does bind DNA, can participate in protein complexes and in the regulation of developmental and cell fate determination pathways such as hematopoiesis [5], head development [6, 7], neurogenesis [8], pituitary development [9], kidney development [10]. The stoichiometry within the Ldb1/LIM-HD or Ldb1/LMO complexes is very important for their proper function. It has been recently shown that Ssbp proteins bound on the LCCD domain of Ldb1 are able to maintain this stoichiometry by protecting Ldb1 and the LMO protein bound to it from ubiquitination and subsequent degradation by RLIM [11].

Orthologs of Ldb1 are found in a wide range of species. Caenorhabditis elegans, Drosophila melanogaster, Xenopus laevis, have one copy of the gene known as Ldb. Mammals have two copies of the gene, Ldb1 and Lbd2, and the zebrafish has four copies that are thought to result from gene duplication events. The eleven exon gene that is ubiquitously expressed in embryonic as well as adult tissues, produces two transcripts that result in two peptides of 46.5kDa and 42.7kDa being translated (Ensembl release 52, Dec. 2008). Its most important domain, the LIM-Interaction Domain or LID, is located at the carboxy-terminal of the protein from amino acids 300 to 338. Ldb1 also contains a homodimerization domain at its amino-terminal, from amino acids 1 to 200, while an NLS domain ensures nuclear localization. [12].

The Drosophila ortholog of Ldb1, dLdb or Chip, functions by bringing an enhancer and a promoter in close proximity and thus indirectly facilitating transcription. The homozygous mutant is larval lethal. The dLdb gene has been shown to form a complex with the LIM-HD apterous (ap), which is involved in determining dorsal cell identity and the dorsal-ventral boundary and regulate segmentation and dorsal-ventral patterning in the Drosophila embryo [13] [14]. The dldb/apterous complex has also been shown to regulate neuronal differentiation [15] as well as wing development and for that function the stoichiometry between the two factors is very important, as overexpression of dldb can result in defects

in the wing patterning and the phenotype can be rescued by overexpression of apterous [16] [17]. During neuronal development in Drosophila, dldb forms a complex with Pannier, a GATA transcription factor, for the facilitation of enhancer-promoter communication. The complex activates the expression of ac/sc with the participation of E-box bound ac/sc and Daughterless heterodimers. The complex can also recruit Osa for the repression of ac/sc expression [18].

In Xenopus the Xlim-1 gene encodes a LIM-HD that is expressed in the dorsal lip and dorsal mesoderm of the gastrula embryo in the region of the Spemann-Mangold organizer, which is involved in neural plate, muscle and anterior-posterior axis development [19] [20]. Xldb1 was found to be co-expressed with Xlim1 in the organizer region of the gastrula stage embryos and both proteins were shown to interact in vitro and in vivo. When Xlim1 and Xldb1 mRNA was co-injected in Xenopus embryos, secondary axis formation was observed, as well as ectopic muscle formation along the secondary axis appearing as fused somites. The effect was not seen when each mRNA was injected individually, however the secondary axis phenotype was also observed when a mutated form of Xlim1 mRNA with inactive LIM domains was injected in the ventral equatorial part of the embryos [1].

A protein complex that contains an Ldb1 homodimer, Lmo2, an Scl/E47 heterodimer and Gata-1 was found to regulate the transcription of the protein 4.2 gene (p4.2), a component of the red cell membrane, from two promoter E-box/GATA elements [21]. The bHLH Scl, which binds the E-box sequence CANNTG as a heterodimer with the products of the E2A gene E12/E47 and Lmo2 are essential for hematopoiesis, as knockout mice for the encoding genes are embryonic lethal and lack any hematopoietic activity [22] [5]. The deletion of Gata-1, which is located on the X chromosome, has been shown to affect primitive and definitive hematopoiesis, since Gata-1-/- embryoid bodies, were not able to produce primitive erythroid precursors, while definitive erythroid precursors were able to initially develop but died at the proerythroblast stage [23]. Male Gata-1-/- mice were pale, suffered from anemia and died between 10.5 dpc and 11.5 dpc. However their yolk sac contained vessels and blood islands with hematopoietic cells that were able to produce embryonic globins. Definitive hematopoietic cells, although initially present, failed to mature and died at the proerythroblast stage in accordance with the in vitro studies. Female Gata-1+/- mice were pale after birth because of random X chromosome inactivation, but later recovered [24]. The same complex with the addition of Sp1 was found to regulate the expression of the glycophorin A gene, an erythrocyte membrane glycoprotein, by binding on its promoter [25]. In human erythroblasts TAL-1, E2A, LMO2 and LDB1 form a tetramer that advances to a pentamer through the interaction with protein retinoblastoma (pRb) and down-regulates the expression of c-kit from two inverted E-box type motifs on the promoter [26]. The complex of Ldb1, Tal1, E12/E47, Gata1 and Lmo2 was found to be part of a larger protein complex in non-induced proliferating MEL cells via in vitro biotinylation. In the complex Ldb1 was found to additionally interact with Eto-2, Mtgr1, Cdk9, Lmo4, HEB, Lyl1, E2-2 and both products of the E2A gene, E12 and E47 [27].

Taking into account the participation of Ldb1 in protein complexes that regulate gene expression and the number of developmental pathways that such complexes participate in, it would be interesting to investigate the effect of the disruption of the gene in embryonic development with the generation of mice that lack Ldb1 expression. The Ldb1-/- mice die after 9.5 dpc, with the embryos displaying severe defects, such as anterior truncation, lack of hematopoiesis, heart and gut formation and duplication of the posterior axis in some of the embryos. Gene expression microarrays were used in order to gain information about the effect the Ldb1 deletion has on transcription regulation, cell differentiation and development.

Methods

Generation of an *Ldb1*-/- mouse

The targeting strategy for the generation of a conditional *Ldb1*-/- mouse involved the selective deletion of the first and second exons of the *Ldb1* gene, including the ATG located on exon 1 and the use of a CFP fluorescent protein as a reporter gene. The targeting vector was constructed on a pBluescript SK+ backbone and contained a loxP site followed by the first exon, first intron and second exon, a PMC-Neo cassette flanked by two additional loxP sites, a Mitochondrial Localization Signal (MLS) and a CFP reporter gene. The vector was flanked by two homologous sequences of 2.9 kb on the 5' side and 2.6 kb on the 3' side. The *Ldb1* genomic fragments used to generate the construct were isolated from mouse PAC clones from the UK HGMP Resource Centre. The construct was linearized at a unique NotI site and was used to transfect mouse ES cells via electroporation. The transfected ES cells were put under G418 selection, clones were picked and genomic DNA was isolated for Southern blot analysis, which was used to identify the successfully targeted ES cell clones, i.e. integration of the targeting vector into the SphI site in the second intron of the *Ldb1* locus via homologous recombination. The isolated genomic DNA was digested with either EcoRV or BamHI for the Southern blot analysis. A 0.43 kb PCR generated fragment, located upstream of the 5'UTR and outside of the genes' promoter region, was used as a 5'probe with the EcoRV digestion, to identify the 5 kb fragmented of the *Ldb1* wild type allele and the 6.9 fragment of the targeted allele. A 1kb KpnI-BamHI fragment was used as a 3' probe with the BamHI digestion to identify the 7.2 kb fragment of the wild type allele and the 5.6 kb fragment of the targeted allele. Two successfully targeted ES cell clones, with the correct karyotype, were injected into blastocysts to generate chimeric mice. The male chimeras were mated with wild type female FvB mice and *Ldb1*+/- mice were generated. For the generation of full *Ldb1*-/- mice, the *Ldb1*+/- mice were crossed with CAG-Cre mice that express the Cre recombinase under the control of the cytomegalovirus immediate early enhancer and chicken β-actin hybrid (CAG) promoter, which is a ubiquitously expressed promoter [28]. The complete deletion of the first and second exons of the *Ldb1* gene results in moving the CFP reporter gene at the 5' UTR of the gene. The deletion, when analyzed via Southern blot gives a 5.5 kb fragment for the 5' probe and a 4.2 kb fragment for the 3' probe.

Genotyping

In order to determine the genotype of the bred mice with Polymerase Chain Reaction, the following primers were used: 5' TTTTGATATCGCGTGACCATGTCCGTCCTGAC 3' and 5' TTTTCTGCAGCGAATGGATCTTGGCGCGC 3', which amplify the MLS and will only amplify a fragment of 150 bp, when the mice are heterozygotes for the *Ldb1* targeting construct. For the genotyping of the mouse embryos, the yolk sac was dissected from E8.5 and E9.5 embryos and the genomic DNA isolated. PCR was used to determine the embryo genotype and the primers used were the following: 5' CTAGCAGGCTTCCAGGGGACCTCC 3' and 5' ACTCGGCACGACAAGGTGGGGAGAGACGAG 3'. The reaction amplifies a 400 bp fragment for the wild type allele and a 900 bp fragment for the targeted allele, where the first exon, second intron, second exon and the PMC-Neo cassette are deleted. Western blot analysis was used to verify that no Ldb1 protein was produced in the *Ldb1*-/- embryos. Nuclear extracts were isolated from the embryos and the Ldb1, goat polyclonal IgG, N-18 Santa Cruz antibody was used to identify the Ldb1 protein, which runs as a double band of 42.7 kDa and 46.5 kDa.

Histology

For the histological analysis, E9.5 embryos were dissected, the yolk sac of each embryo was removed for genotyping and the embryo proper was fixed in 2% paraformaldehyde/ PBS for 2 hrs at room temperature. The embryos were equilibrated overnight in 20% sucrose/PBS at 4 ^{0}C, oriented and quick frozen in Tissue-Tek (Sakura Finetek). The embryos were sectioned at 10μm and the sections stained with Hematoxylin. The day that the plug was reported was taken as E0.5.

In situ hybridization

Mouse embryos were dissected at E9.5, fixed in 4% paraformaldehyde overnight at 4^{0}C and dehydrated in 25%, 50%, 75% and 100% methanol/ PBT. The embryos were rehydrated in 75%, 50%, 25% methanol in PBT, treated with 6% hydrogen peroxide, permeabilized with 10μg/ml of proteinase K in PBT, washed with 2mg/ml glycine in PBT, re-fixed in 0.2% glutaraldehyde/4% paraformaldehyde in PBS and hybridized with the digoxygenin-labelled RNA probe of *engrailed 2* 3'UTR at 65^{0}C overnight. Finally the embryos were incubated with an anti-digoxygenin antibody.

CFC assay

The Colony Forming Cell assay was used to examine the hematopoietic potential of the *Ldb1*-/- mice. The yolk sac of *Ldb1*+/+ and *Ldb1*-/- E9.5 embryos was dissected and the cells were disrupted using 2.5% collagenase in PBS. The isolated cells were used for the CFC assay, whereby the cells were grown for 7 days in methycellulose-based media supplemented with 10% FCS, 1% L-glutamine (GIBCO), 0.25μg/ml transferin, 0.25μg/ml ascorbic acid, 2μl/ml monothioglycerol from a 13μl/ml temporary stock, 5% protein free hybridoma medium II, 0.01μg/ml mIL6, 0.001μg/ml IL3, 0.005μg/ml hIL11, 0.003μg/ml GM-CSF, 4U/ml EPO, 0.005μg/ml TPO, 0.1 μg/ml SCF. After 7 days colonies were

identified according to their morphology under an inverted microscope.

Affymetrix Mouse Genome Arrays

Microarray analysis of *Ldb1*+/+ and *Ldb1*-/- E8.5 embryos was performed using the Affymetrix GeneChip® Expression Arrays. *Ldb1*+/+ and *Ldb1*-/- E8.5 embryos were dissected, had their yolk sac removed for genotyping and were frozen in Sigma TRI Reagent®. The day the plug was reported was taken as E0.5. Total RNA was isolated from the embryos using the QIAGEN RNeasy Mini Kit, and the quality was checked using the Agilent Bioanalyzer 2100. 100ng of total RNA from each sample were used. The RNA was reverse transcribed first to single stranded and then double stranded cDNA, which was used as a template for the in vitro transcription (IVT) synthesis of biotin labelled cRNA according to the Affymetrix GeneChip® Eukaryotic Two-Cycle Target Labelling for Expression Analysis protocol, The biotin labelled cRNA was next fragmented and hybridized on the Mouse Genome 430 2.0 Array, which contains 45.000 probe sets of 10 to 20 oligonucleotides. The array was stained with a streptavidin phycoerythrin conjugate and scanned with the Affymetrix GeneChip® Scanner 3000 according to the manufacturer protocol. The signal emitted at 570nm from each probe set, termed the intensity value, was proportional to the hybridization intensity of the biotin labelled cRNA on that location and was used as a measurement for the expression of genes in the original RNA samples.

Microarray data normalization and analysis

The global method for scaling or normalization was applied. The mean (±SD) difference between the scaling or normalization factors for all GeneChips was 0.70±0.26. The intensity values were normalized using the Affymetrix Microarray Suite software, version 5.0 (MAS 5.0). The geometric mean of the hybridization intensities of all the samples was calculated for each probe set. In each sample the level of expression of every probe set was determined relative to the geometric mean and was logarithmically transformed on a base 2 scale, to give equal weight to gene expression levels with similar relative distances from the geometric mean. Consequently deviation from the geometric mean was used as a measure for differential gene expression. Omniviz software version 5.0 was used to perform unsupervised cluster analysis. The clustering of the samples was examined with each probe set that was selected based on the differences observed in the expression level from the geometric mean, in at least one sample. SAM (Significance Analysis of Microarrays) software was used for supervised analysis which correlates gene expression with an external variable. SAM calculates a score for each gene based on the expression changes observed, when compared with the SD of all samples. A q-value of less than 5% was given to each gene to account for the probability that the gene was falsely determined as differentially expressed [29]. The Ingenuity Pathways Analysis software (Ingenuity Systems, http://www.ingenuity.com) was used to group the genes that were found to be differentially expressed between the wild type and the knockout mouse embryos according to function.

Gene expression analysis

RNA was isolated as described above and cDNA was synthesized using SuperScript II Reverse Transcriptase and Oligo(dT)$_{12–18}$ primers according to the manufacturer's instructions (Invitrogen™). Quantitative Real-time PCR was carried out on the synthesized cDNA with 1U of Platinum® Taq polymerase (Invitrogen™), 1x PCR buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl], 1.5mM $MgCl_2$, 10ng/µl of each primer, 250µM dNTPs and SYBR Green (Sigma®) on the Opticon 2® Real-Time PCR Detection System (Bio-Rad). The following cycling conditions were used: 2 minutes at 94^{0}C, 40 cycles of 30 seconds at 94^{0}C, 30 seconds at 55^{0}C, 30 seconds at 72^{0}C and 5 minutes at 72^{0}C. The data was collected and analysed by the MJ Opticon™ analysis software version 3.1 (Bio-Rad). Transcript enrichments were calculated using the comparative C_T method. The C_T threshold was set at 10 cycles to distinguish between non-specific and specific PCR products detected through the incorporation of SYBR Green to amplified DNA. Transcript levels were normalized to the transcript of *Gapdh*, a housekeeping gene, the expression of which was not found to change after the deletion of *Ldb1*.

Results and Discussion

Generation of an *Ldb1*-/- mouse

The genomic structure of the mouse *Ldb1* gene [30] and the strategy for the targeting of one allele for the generation of *Ldb1*-/- mice is shown in Fig. 1A. Using homologous recombination in ES cells, a loxP site was inserted in the 5'UTR of the *Ldb1* gene and a PMC-Neo cassette flanked by two more loxP sites and followed by an MLS and a CFP reporter gene was inserted in the second intron of the gene at an SphI restriction site.

Two positive clones (one is shown) (Fig. 1B) with one wild type and one targeted allele were injected into C57BL/6 mice to generated mouse chimeras. The male chimeras were crossed with FvB wild type females to generate mice that were heterozygous for the targeted allele. The heterozygotes appeared normal and phenotypically similar to the wild type mice and were fertile. The *Ldb1*+/- mice were bred with CAG-Cre mice that ubiquitously express Cre recombinase under the CAG promoter and the first and second exons of the targeted *Ldb1* allele, including the ATG and the PMC-Neo cassette were deleted, bringing the CFP reporter gene in the 5' UTR (Fig. 1B). When these heterozygous mice were crossed, no viable homozygous offspring were born in agreement with the embryonic lethal phenotype also observed by others for the full *Ldb1*-/- mouse [31]. Viable homozygous embryos were found at 7.5 dpc, 8.5 dpc and 9.5 dpc at a Mendelian ratio but not at 12.5 dpc (data not shown). Polymerase chain reaction was used to genotype the embryos from *Ldb1*+/- crosses at 8.5 dpc and 9.5 dpc using genomic DNA isolated from the yolk sac. The reaction amplified a 400 bp fragment from the wild type allele and a 900 bp fragment from the targeted allele (Fig. 1C). Western blot analysis verified that the *Ldb1* -/- embryos did not produce any Ldb1 protein (Fig.1D).

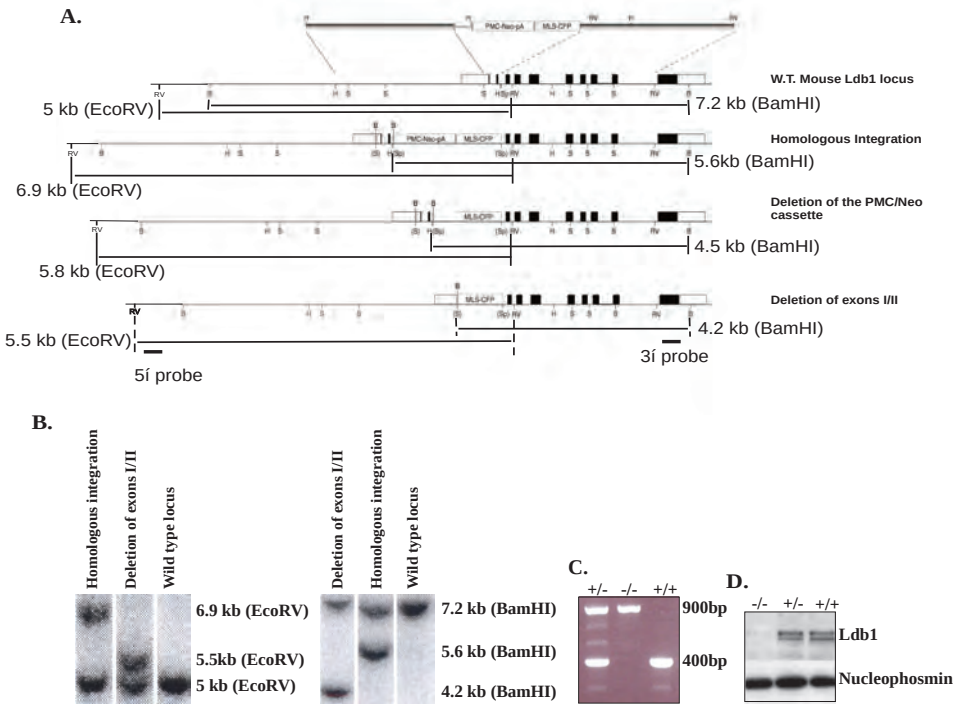


Figure 1. Gene targeting of the mouse *Ldb1* locus. (A) A targeting construct with a PMC-neo cassette and an MLS-CFP was inserted in the second intron of the *Ldb1* allele by homologous recombination. (B) Successful integrations were identified by Southern blot hybridisation (one example is shown). Cre recombinase was used to delete the neo cassette and the first two exons of the gene. ES cell DNA was digested with EcoRV and BamHI for the hybridisation with the 5' and the 3' probes respectively. The EcoRV digest identified a 5 kb wild type fragment, a 6.9 kb homologous integration fragment, a 5.8 kb fragment resulting from the neo deletion and a 5.5 kb fragment resulting from the exon I/II deletion. The BamHI digest resulted in respective fragments of 7.2 kb, 5.6 kb, 4.5 kb and 4.2 kb. (C) PCR was used to genotype the mouse embryos. An amplified fragment of 400 bp indentified the wild type allele and a 900 bp fragment the knockout allele. (D) Western blot on *Ldb1* wild type and knockout mouse embryos showed the presence of Ldb1 protein in the wild type but not in the knockout embryos. Nucleophosmin was used as loading control.

Phenotype of the *Ldb1-/-* mouse

The *Ldb1*-/- mouse is embryonic lethal after 9.5 dpc. The deletion of the gene leads to multiple developmental defects in the embryo. The knockout phenotype begins to be visible at 8.5 dpc, as the knockout embryos are smaller in size in comparison to the wild type embryos (Fig 2A, B). However the developmental defects of the knockout embryos are more obvious at 9.5 dpc. The first striking difference between the wild type and knockout embryos at this stage in development is the complete absence of any blood or vasculature, first from the extra-embryonic yolk sac and secondly from the embryo proper, leading to the conclusion that hematopoiesis and vascular development are completely impaired in the knockouts. Additionally the yolk sac fails to properly surround the knockout embryos (Fig.2C, D, E, F). The *Ldb1*-/- embryos are smaller in size and any heart structure is absent. The head structure is truncated and there is complete absence of any craniofacial features, any foregut structures, forelimbs and hindlimbs, while the somites are malformed (Fig. 2E, F). The knockout embryos appear to have not turned properly and their development seems to have not progressed much beyond 8.5 dpc (Fig. 2E, F).
Wild type and knockout embryos at 9.5 dpc were sectioned and stained with hematoxylin. The head structure anterior of the otic vesicle is absent in the knockouts, while the neural tube and the neural plate appear twisted (Fig 2G, H). Similar phenotypes of anterior truncation have been previously observed in the *Otx2*-/- mice [32] [33], the *Lim1*-/- mice [34] and the *headshrinker* mouse mutant that is characterized by the decreased expression of the *Ssbp1* gene, due to an intronic disruption [35]. Whole embryo in situ hybridizations were done on 9.5 dpc wild type and knockout embryos using *engrailed 2* as a marker [36]. The gene is expressed in the midbrain-hindbrain junction and is absent from the knockout embryos (Fig. 2K).
Duplication along the posterior axis was observed in approximately 1/3 of the knockout embryos (Fig.2I, J). Secondary axis formation has been observed in the *Xenopus* embryo when *Xlim1* mRNA with mutations in the LIM domains or *Xldb1*/*Xlim1* mRNA was injected into the ventral equatorial region. The observed secondary axis in the Xenopus embryos contained neural tissue, somites and cement gland but not notochords [20]. The abovementioned developmental defects of the *Ldb1*-/- embryos, which have been also observed by others [31], show that the *Ldb1* gene is involved in a number of diverse pathways that are fundamental for the development of the mouse embryo, such as hematopoiesis, development of the heart, foregut, somites and central nervous system and the disruption of which can lead to an embryonic lethal phenotype.

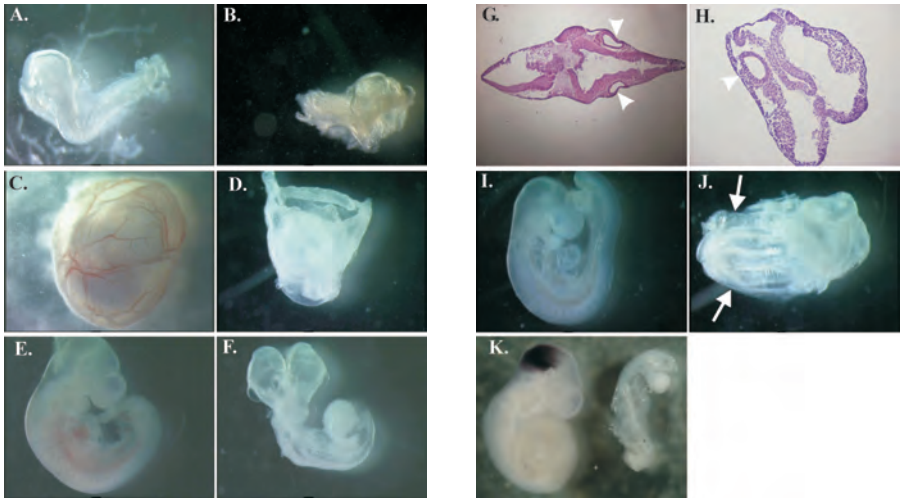


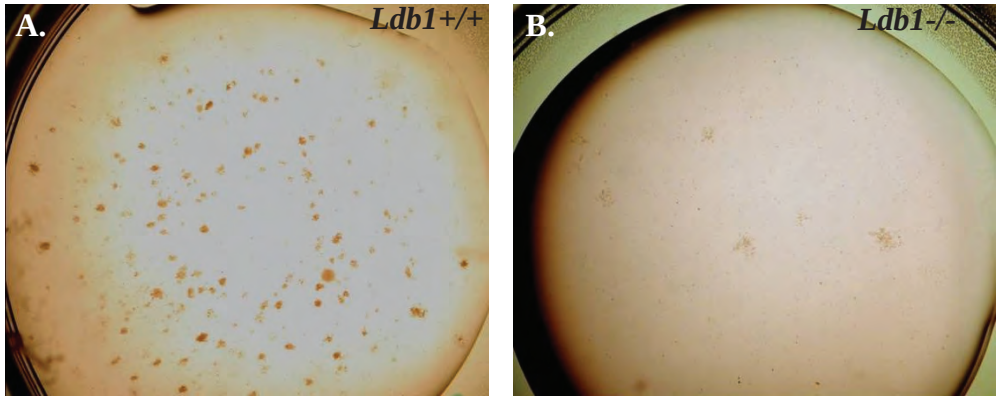
Figure 2. *Ldb1*+/+ embryos, shown on the left and *Ldb1*-/- embryos on the right, were viewed under a light microscope. (A, B) At 8.5 dpc the knockout embryos are smaller in size. (C, D) At 9.5 dpc there is complete absence of blood and a vascular network in the knockout embryo yolk sac, which in addition does not engulf the whole embryo. (E, F) The developmental defects in the knockout embryos at 9.5 dpc include complete absence of hematopoiesis in the embryo proper, absence of heart formation, absence of foregut, lack of craniofacial features and truncation of the anterior, while the somites are smaller and more compact. The embryos have not properly turned and their development seems to have stopped at an earlier stage. (G, H) Hematoxylin stained 10µm sections of 9.5 dpc embryos showed that the knockout embryos suffer from truncation of the head above the otic vesicle (white arrowheads) and have twisted neural tubes. (I, J) Approximately 1/3 of the knockout embryos showed duplication of the posterior axis with four sets of somites (white arrows). (K) Whole embryo in situ hybridizations done on 9.5 dpc embryos show that *engrailed 2*, which is used as a marker for the midbrain-hindbrain junction is expressed in the wild type embryos but is absent from the knockout embryos.

Defects in primitive hematopoiesis in the *Ldb1*-/- mouse

The hematopoietic phenotype of the *Ldb1*-/- embryos was further investigated by examining the hematopoietic potential of 9.5 dpc *Ldb1*-/- yolk sacs. The first wave of hematopoiesis in the mouse embryo, termed primitive hematopoiesis, begins with the appearance of the large primitive erythroblasts, which are nucleated cells that express embryonic globins, in the yolk sac blood islands at 7.5 dpc [37] [38] [39]. These cells enter the blood circulation of the embryo around 8.5 dpc through the yolk sac vasculature [40]. In addition the yolk sac was found to contain macrophages [41]. Primitive hematopoiesis is replaced by definitive hematopoiesis characterized by the smaller, enucleated definitive erythroid cells that produce adult globins [42]. Yolk sacs from wild type and knockout 9.5 dpc embryos were dissected and disrupted with collagenase. The cells were grown for 7

days on methycellulose-based media supplemented with the GM-CSF, EPO, TPO, SCF and the cytokines mIL6, IL3 and hIL11. The resulting colonies were scored according to their morphology and number. The colony forming assay was repeated twice with similar results. A large number of primitive erythroid and macrophage colonies, (red and white colonies) were present in the wild type yolk sac cultures but no primitive erythroid and very few macrophage colonies were present in the knockout yolk sac cultures (Fig 3A, B, C). The above result leads to the conclusion that primitive hematopoiesis is completely impaired in the absence of *Ldb1*. Impaired hematopoiesis is a severe developmental defect that results in embryonic death.

As it was mentioned above, Ldb1 has been shown to act in complex with essential hematopoietic factors such as Scl, Lmo2 and Gata1. When these genes are not expressed primitive hematopoiesis does not initiate. The yolk sacs of the *Scl*-/- and *Lmo2*-/- mice to do not contain blood islands and fail to give rise to any hematopoietic colonies in culture similar to the observed phenotype in the *Ldb1*-/- embryos [22] [5]. It is likely that when the Ldb1 protein is absent, the hematopoietic factors although present, are unable to act in inducing hematopoietic development. This conclusion is supported by the fact that Ldb1 can act as a facilitator in promoter-enhancer interactions as proposed for *Drosophila* [13] and mouse [21].



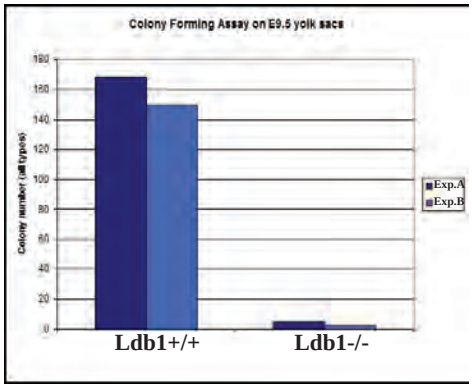


Figure 3. Yolk sacs from 9.5 dpc wild type and knockout embryos were disrupted with collagenase and the cells were grown for 7 days on methycellulose based media supplemented with GM-CSF, EPO, TPO, SCF and the cytokines mIL6, IL3 and hIL11. After 7 days the hematopoietic clones that grew were scored according to their morphology (A, B) and number (C). The assay, which was repeated twice with similar results, shows that the yolk sac of the *Ldb1* knockout embryos, which is the primary site of primitive hematopoiesis, is devoid of hematopoietic potential.

The process of primitive hematopoiesis begins in the yolk sac after the migration of brachyury expressing mesodermal cells through the primitive streak and their differentiation toward hematopoietic or endothelial precursors that collectively form the blood islands. The hematopoietic precursors will give rise to primitive erythroblasts, while the endothelial precursors will form the vasculature. Primitive erythroblasts as well as a vascular network are absent from the *Ldb1*-/- embryo yolk sacs. The mesoderm derived progenitor of hematopoietic and endothelial precursor cells is the hemangioblast that was proposed a century ago (His, 1900 and Sabin, 1920, reviewed by Xiong, 2008). Therefore we can propose that the phenotype of the knockout embryos can be the result of a failure of those mesodermal cells to migrate to the yolk sac, or a failure of those cells to differentiate into hematopoietic or endothelial progenitors. The latter possibility is more likely based on the results obtained by *in vitro* generation of *Scl*-/- embryoid bodies, which has shown that the *Ldb1* co-factor is dispensable for the initial development of the hemangioblast but essential for its further commitment towards the hematopoietic and endothelial lineages [43].

Analysis of *Ldb1*-/- 8.5 dpc embryos using Affymetrix Mouse Genome Arrays

The *Ldb1*-/- embryos suffer from many severe developmental defects, which have probably begun to take place during the initial patterning of the embryo. In order to determine in more detail which genes and developmental pathways are affected due to the absence of *Ldb1*, Affymetrix gene expression arrays were performed on four wild type and four knockout 8.5 dpc embryos. RNA was extracted from the embryo proper and was hybridized on the Affymetrix Mouse Genome 430 2.0 arrays. Taking into account the following parameters that indicate a successful hybridization: the average signal of the hybridization of each sample on the array, the percentage of the present probe sets versus the absent probe sets, the noise and background levels, the ratio of GAPDH 3'to 5' (data not shown) we concluded that the hybridization was successful and that the data from each array can be compared with the others. The microarray data were analysed with the Omniviz software version 5.0 and the SAM (Significance Analysis of Microarrays) software.

The samples' correlation can be seen on Fig. 4A. A negative correlation (blue) indicates that the genes with a high level of expression in one sample will always have a low level of expression in the other sample and the other way around, while a positive correlation (red) indicates that the genes in both samples have similar levels of expression. The clustering of the wild type and knockout samples according to the genes that were found to be differentially expressed is shown on Fig. 4B as a tree diagram at the bottom of the figure. At the left of the figure the tree diagram represents the clustering of the differentially expressed genes. The red colour represents an up-regulation in gene expression of at least 1.5 fold, while the blue colour represents a down-regulation in gene expression of the same level. A total of 330 genes of known function show differential expression between the wild type and the knockout embryos when the fold change threshold was set to 1.5 and the q-value was set at 5%. From those genes, 117 were found to be down-regulated (35%) and 213 were found to be up-regulated (65%). Finally 48 genes (14.5%) that showed differential expression were of unknown function.

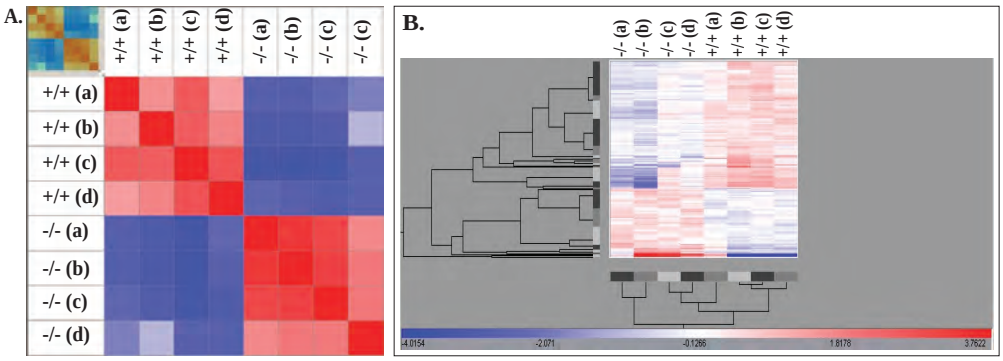


Figure 4. (A) Pairwise correlation of the *Ldb1*+/+ and *Ldb1*-/- embryos. The threshold for the differential expression was set at 1.5 fold and the q-value was set at 1%. The blue and red colour represents the negative and positive Pearson's Correlation Coefficient values respectively. The negative correlation (blue squares) seen between the wild type and the knockout embryos indicates that the genes expressed in the wild type embryos will always be deferentially expressed in the knockout embryos. The positive correlation (red squares) indicates that the genes expressed in all wild type embryos follow the same levels of expression and similarly the genes expressed in all the knockout embryos follow the same levels of expression.
(B) The clustering of the wild type and knockout embryos (the threshold for gene differential expression is set at 1.5 fold and the q-value is set at 5%) based on the genes that show differential expression between the wild type and the knockout embryos according to the above set parameters, is shown as a tree diagram at the bottom of the figure. The clustering of the differentially expressed genes is shown as a tree diagram at the left of the figure. The change in the expression level of each gene is represented by the change of colour from light blue to red. The highest up-regulated genes are represented by red will the most severely down-regulated genes are represented by blue.

Using the Ingenuity Pathways Analysis software the genes found to be differentially expressed in the knockout embryos can be associated with specific biological functions, as shown on Table 1. A differentially expressed gene can be associated with more than one biological function. The significance values of the functional analysis, represented as the negative logarithm of the p-value the threshold of which is set at 5%, are shown on Fig. 5. A low p-value represents the higher probability that the allocation of the genes in a biological function is accurate. Pathways that are involved in cell-to-cell signalling, cell proliferation, growth and death are severely affected in the *Ldb1*-/- embryos, while pathways involved in cell signalling and cell cycle are affected to a lesser extent. Pathways involved in general embryonic development and more specifically in the development of the nervous, cardiovascular, skeletal, muscular, reproductive and hematological systems are affected more than those involved in the development of the respiratory, immune, endocrine and digestive systems.

Table 1. The genes that were found to be differentially expressed in the *Ldb1*-/- embryos were grouped according to the biological function they are most likely to participate in, using the Ingenuity Pathways Analysis software.

Biological Functions	Molecules
Cell to cell signalling and interactions	38
Nervous system development and function	38
Cell death	70
Cellular growth and proliferation	77
Cardiovascular system development and function	26
Embryonic development	24
Hepatic system development and function	5
Reproductive system development and function	23
Skeletal and muscular system development and function	32
Respiratory system development and function	8
Cell signalling	7
Cell cycle	6
Hematological system development and function	19
Immune and lymphatic system development and function	2
Endocrine system development and function	5
Connective tissue development and function	6
Post-translational modification	6
Digestive system development and function	3

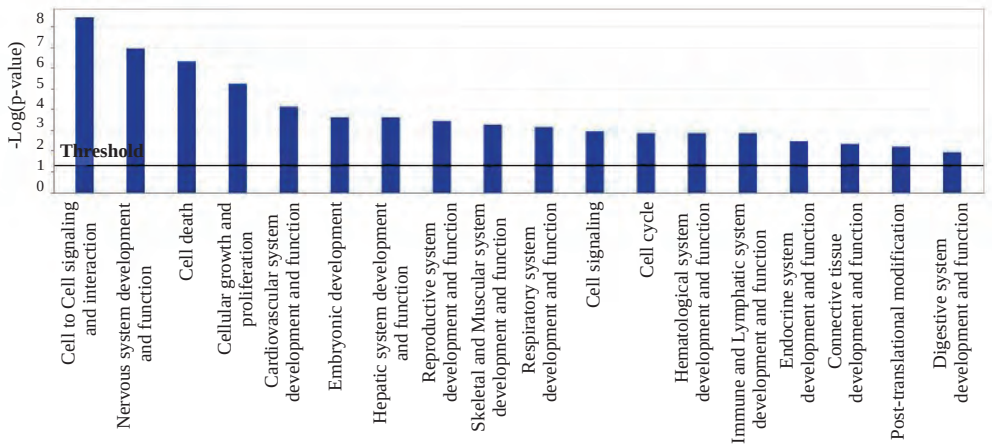


Figure 5. Significance values of the functional analysis done using the Ingenuity Pathways Analysis software. The negative logarithm of the p-value is used in a graphic representation as a measurement of the likelihood that a group of genes was associated with a biological function during the analysis randomly. The threshold for the p-value was set at 5%. The higher the –log (p-value), the lower the possibility that the allocation of a group of genes to a specific biological function is wrong.

Genes that showed differential expression in the *Ldb1*-/- 8.5 dpc embryos, shown in Table 2, were evaluated according to their expression pattern in wild type embryos and the regulatory, cell differentiation and developmental pathways into which they participate in order to determine their biological significance and connection with the phenotype of the *Ldb1*-/- embryos. Among the genes that were found to be down-regulated are many involved in early and essential pathways of embryonic development. The mutants of the majority of these genes are embryonic lethal exhibiting severe developmental and patterning defects a number of which are similar to those observed in the *Ldb1*-/- embryos. Some of the genes that showed differential expression in the *Ldb1*-/- embryos at 8.5 dpc were selected in order to confirm the changes in their expression pattern by quantitative real-time PCR (Fig. 7)

Table 2: A list of genes that show differential expression in the *Ldb1*-/- embryos, compared with the *Ldb1*+/+ embryos. The fold change (up-regulation or down-regulation) of each gene's expression in the knockout embryos is given, as well as the q-value, which determines the significance of the differential expression level.

Gene Symbol	Gene Title	Fold Change	q-value
Hbb-bh1	hemoglobin Z, beta-like embryonic chain	0.068	0.012
Hba-x	hemoglobin X, alpha-like embryonic chain	0.072	0.013
Hbb-y	hemoglobin Y, beta-like embryonic chain	0.077	0.021
Eraf	erythroid associated factor	0.2	0.033
Tbx4	T-box 4	0.234	0.0069
Foxg1	forkhead box G1	0.248	0.0064
Dmrt3	doublesex and mab-3 related transcription factor 3	0.346	0.0062
Otx2	orthodenticle homolog 2 (Drosophila)	0.398	0.01
Asb4	ankyrin repeat and SOCS box-containing protein 4	0.4	0.0007
Dmrta2	doublesex and mab-3 related transcription factor like family A2	0.404	0.0025
Otx1	orthodenticle homolog 1 (Drosophila)	0.41	0.037
Hoxc9	homeo box C9	0.413	1.69E-06
Tcfap2b	transcription factor AP-2 beta	0.425	0.003
Sox10	SRY-box containing gene 10	0.433	0.014
Lhx2	LIM homeobox protein 2	0.465	0.0016
Foxd1	forkhead box D1	0.475	0.0038
Six3	sine oculis-related homeobox 3 homolog (Drosophila)	0.481	0.028
Hhex	hematopoietically expressed homeobox	0.49	0.00013
Vgll2	vestigial like 2 homolog (Drosophila)	0.5	0.034
En2	engrailed 2	0.514	0.0051
Tmem46	transmembrane protein 46	0.524	0.038
Ldb1	LIM domain binding 1	0.525	0.0081
Wnt1	wingless-related MMTV integration site 1	0.539	0.0033
Jun	Jun oncogene	0.54	0.036

Gene Symbol	Gene Title	Fold Change	q-value
Vegfc	vascular endothelial growth factor C	0.543	0.0027
Rprm	reprimo, TP53 dependent G2 arrest mediator candidate	0.544	0.044
Lyl1	lymphoblastomic leukemia	0.545	0.0045
Lmo1	LIM domain only 1	0.554	0.044
Evi1	ecotropic viral integration site 1	0.589	0.038
Dab1	disabled homolog 1 (Drosophila)	0.6	2.54E-05
Fgf9	Fibroblast growth factor 9	0.61	0.0016
E2f1	E2F transcription factor 1	0.612	0.0091
Epha7	Eph receptor A7	0.625	0.019
Cdh6	cadherin 6	0.628	0.011
Neurog2	neurogenin 2	0.631	0.0018
Armc5	armadillo repeat containing 5	0.632	0.011
Ngfr	nerve growth factor receptor	0.636	0.021
Ets1	E26 avian leukemia oncogene 1, 5' domain	0.64	0.036
Twist1	twist gene homolog 1 (Drosophila)	0.642	0.001
Ash1l	ash1 (absent, small, or homeotic)-like (Drosophila)	0.651	0.016
Nr2f2	nuclear receptor subfamily 2, group F, member 2	0.655	0.028
Esam1	endothelial cell-specific adhesion molecule	0.655	0.018
Sox21	SRY-box containing gene 21	0.656	0.043
Raldh2	aldehyde dehydrogenase family 1, subfamily A2	0.66	0.0086
Dsg2	desmoglein 2	1.5	0.017
Fst	follistatin	1.5	0.044
Dkk3	dickkopf homolog 3 (Xenopus laevis)	1.533	0.0011
Emb	embigin	1.547	0.016
Apoa5	apolipoprotein A-V	1.554	0.023
Fgfr4	fibroblast growth factor receptor 4	1.674	0.0092
MGI:1932093	Jun dimerization protein 2	1.696	0.019
Msx3	msh-like 3 homeo box	1.74	0.016
Csf1r/CD115	Colony stimulation factor 1 receptor	1.746	0.011
Apoc3	apolipoprotein C-III	1.762	0.013
Epas1	Endothelial PAS domain protein 1	1.815	0.029
Cbln1	cerebellin 1 precursor protein	1.817	0.0075
Emp2	epithelial membrane protein 2	1.838	0.017
Tac2	tachykinin 2	1.885	0.025
Klf9	Kruppel-like factor 9	1.958	0.022
Apoe	apolipoprotein E	1.998	0.025
Afp	alpha fetoprotein	2	0.044
Klf4	Kruppel-like factor 4	2	0.029
Ttr	transthyretin	2	0.036
Dab2	disabled homolog 2 (Drosophila)	2	0.014
Anxa4	annexin A4	2.2	0.027
Amn	amnionless	2.274	0.047
Adm	adrenomedullin	2.5	0.037

Gene Symbol	Gene Title	Fold Change	q-value
Trf	transferrin	2.656	0.04
Apoa1	apolipoprotein A-I	2.825	0.038
Hnf4a/Tcf4	hepatic nuclear factor 4, alpha	3.053	0.011
Apom	apolipoprotein M	3.27	0.024
Apob	apolipoprotein B	3.322	0.02
Apoa4	Apolipoprotein A-IV	3.482	0.022
Apoc2	apolipoprotein C-II	4.31	0.014
A2m	alpha-2-macroglobulin	4.848	0.049

Genes involved in transcription regulation

The expression of *E2f1*, *Ets1*, *Lyl1*, *Twist* and *c-Jun* is down-regulated in the *Ldb1* -/- embryos. *E2f1* belongs to the E2F family of highly conserved genes that act as transcription activators or repressors. It acts as a regulator of cell proliferation [44] and it can also trigger apoptosis. The knockout mice for *E2f1* are viable, however they exhibit an excess of mature T-cells due to a lack of thymocyte apoptosis and suffer from tumours at a later age [45]. The *Ets1* gene belongs to the Ets family of transcription factors that function at different stages in hematopoiesis. Knockout experiments have shown that *Ets1* functions in lymphoid development and the maturation of T cells [46]. *Lyl1* shares an 82% homology in the bHLH region with the closely related *Scl*, while the encoded protein participates in the same protein complex as Ldb1 in non-induced proliferating MEL cells. Although the *Scl*-/- mice are embryonic lethal, the *Lyl1*-/- mice are viable, but have a much lower B-cell number, which means that the two genes share different roles in the process of hematopoiesis [47]. The bHLH *Twist* is essential for embryonic development in *Drosophila* and *Xenopus.* Its deletion leads to lack of neural tube closure due to defects in the morphology of the head mesenchyme and embryonic lethality at 11.5 dpc [48]. *c-Jun* belongs to the Jun protein family, characterized by a leucine zipper DNA binding domain and can function as a homo- or heterodimer. It is necessary for cellular proliferation and is also involved in triggering mitochondrial induced apoptosis via the JNK pathway [49].

Genes involved in hematopoiesis and vascular development

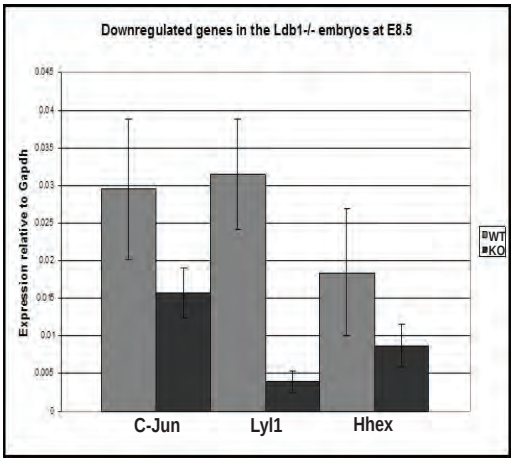
The most severely down-regulated genes in the knockout embryos are *Hbb-y* and *Hbb-bh1* that encode embryonic hemoglobins, which is not surprising since the knockout embryos lack hematopoietic activity. The expression of genes associated with hematopoietic development such as *Evi1* and *Hhex* is decreased in the *Ldb1*-/- embryos. *Evi1* has been shown to participate in the proliferation of hematopoietic stem cells in mice via the regulation of *Gata-2* and the *Evi1*-/- mouse is embryonic lethal [50]. *Hhex* (*Hematopoietically expressed homeobox*) was shown to be involved in murine and zebrafish hematopoiesis and the development of the hemangioblast [51] [52] [53]. Additionally *Hhex* that is expressed in the anterior visceral endoderm and the anterior definitive endoderm participates in the anterior-posterior patterning of the mouse embryo. When its expression is abolished the resulting phenotype includes embryonic lethality and forebrain truncation [54]. The gene is also expressed in the anterior foregut endoderm, which together with the mesodermal septum transversum participates in the formation of the liver [55].

The expression of the orphan nuclear receptor *Nr2f2* or *COUP-TFII* is down-regulated in the *Ldb1*-/- embryos. The gene is regulated by the sonic hedgehog signalling pathway and its deletion leads to embryonic lethality at around 10 dpc, due to extreme hemorrhage and edema in heart and brain as the embryos suffer from defects in angiogenesis and vascular remodelling [56]. *COUP-TFII* is expressed in the endothelium of veins and the smooth muscle cells that surround the arteries and participates in establishing venous-arterial identity by inhibiting the Notch signalling pathway and suppressing the expression of arterial specific genes [57]. Additionally the human ortholog of COUP-*TFII* has been found to function in the repression of apolipoprotein A-IV, apolipoprotein B, apolipoprotein C-II and apolipoprotein C-III [56]. In the *Ldb1*-/- embryos the genes that encode for the apolipoproteins A-IV, B, CII and CIII are up-regulated, possibly due to the absence of a similar repression mechanism involving the action of *COUP-TFII*.

Genes involved in anterior patterning and neural development

Knockout mouse models generated for the genes *Otx2* and *Raldh2*, both of which are down-regulated in the *Ldb1*-/- embryos display anterior patterning defects. The *Otx2* gene is a homolog of the *Drosophila orthodenticle* (*otd*) homeobox gene that is involved in patterning of the anterior head structure [58, 59]. It is initially expressed widely in the epiblast at 5.5 dpc and as gastrulation begins, its expression moves towards the anterior part of the embryo where it becomes restricted [60] but still expressed in all three germ layers. When the gene is absent the resulting phenotype includes gastrulation defects and absence of forebrain, midbrain and anterior hindbrain due to lack of neural plate induction [33]. The *Otx2*-/- mouse also lacks the expression of the *Six3*, *Wnt1* and *En2* genes, which are also down-regulated in the *Ldb1*-/- embryo [61]. The absence of *Six3*, which is expressed in the anterior neural plate leads to forebrain truncation [62], while the absence of *Wnt1* leads to loss of midbrain and a part of the metencephalon [63]. *En2* is expressed in the midbrain-hindbrain junction from where the cerebellum is derived. The deletion of

the gene in mice does not affect embryonic viability, however reduction in the size of the cerebellum and defects in its foliation were observed [64].
The gene *Raldh2* belongs to the RALDH group of proteins whose primary function is to synthesize retinoic acid (RA). Its deletion leads to lack of RA synthesis and embryonic lethality at 10.5 dpc. The defects observed in those embryos include smaller body size when compared with wild type embryos, lack of turning, deformation of the neural tube and lack of closure at the anterior part, lack of an organized network of vessels in the yolk sac, lack of limb buds, small and densely packed somites, heart malformations [65], hypoplastic forebrain and abnormally thin neuroepithelium, possibly due to increased apoptosis between 8.5dpc and 9.5 dpc and lack of cell proliferation in the neuroepithelium and forebrain regions [66]. At this stage of development almost all of the RA present in the mouse embryo is synthesized by *Raldh2,* which is down-regulated in the Ldb1-/- embryos. The absence of RA can be associated with parts of the observed phenotype. Similarities in the knockout phenotypes seen in the *Ldb1*-/- and the *Raldh2*-/- embryos include embryonic lethality at similar stages in development, smaller body size and lack of embryo turning, malformed neural tube, thin neuroepithelium, absence of organized vasculature in the yolk sac, lack of limb buds, small and densely packed somites, heart malformations In an acute lymphoblastic leukemia (T-ALL) human cell line a protein complex of TAL1, LMO and GATA3, was found to regulate the expression of the *RALDH2* gene from a cryptic promoter located in the second intron of the gene that contains a GATA-binding site close to an E-box (CAGGTG) [67]. It is likely that *Ldb1* is involved in the regulation of *Raldh2* expression in the mouse embryo through a similar mechanism. The mouse and human *Raldh2* genes share a 97% homology and a search of the genomic sequence of the mouse gene revealed three E-box sites close to or in the second intron. Also a potential GATA-binding site is located at the beginning of the third exon.
In the *Ldb1*-/- embryos the expression of *neurogenin 2*, *Foxg1* and *Foxd1* is decreased. *Neurogenin 2* is expressed in the neural plate of the mouse embryo and one of its early functions is the initiation of spinal neurogenesis. Later in development the gene participates in the process that determines the different neural cell subtypes. Its expression is regulated by the RA and Shh signalling pathways from an enhancer element that contains two RARE elements and a Gli binding site [68]. The winged-helix transcription factors *Foxg1* and *Foxd1*, also known as *BF-1* and *BF-2*, participate in neurogenesis. *Foxg1* is expressed in the telencephalic neuroepithelium of the developing brain and possibly acts in the patterning of the forebrain. The knockout mice for this gene die shortly after birth and suffer from a severe reduction in the size of the cerebral hemispheres due to a reduction in cell proliferation and changes in the timing of neuronal differentiation in the forebrain neuroepithelium [69]. *Foxd1* is also expressed in the forebrain region of the mouse embryo and specifically in the rostral diencephalon. From overexpression experiments done in *Xenopus* it has been shown that the gene probably acts as a repressor in neural cell migration and mesoderm patterning [70].



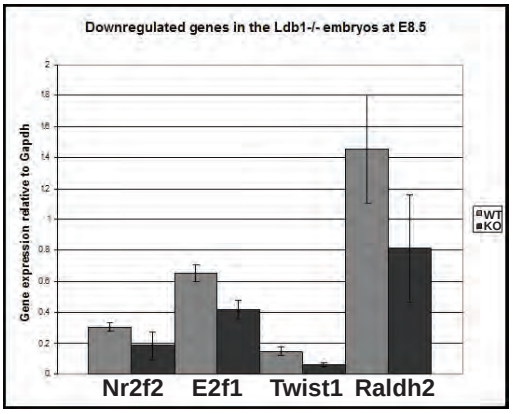


Figure 6. Confirmation of changes in gene expression detected with the microarray analysis of the wild type and *Ldb1* knockout mouse embryos by quantitative real time PCR. The expression of each of the examined genes was normalized to the expression of *Gapdh*, which is not affected from the deletion of *Ldb1*. RNA from three wild type and three *Ldb1* knockout embryos was used.

Conclusion

The severity of the phenotype observed in the embryos that lack *Ldb1* expression is a clear indication of the involvement of the gene in early developmental events that lead to the establishment of the anterior-posterior axis, the development of the nervous system, the formation of the heart and gut, the formation of blood vessels and the initiation of hematopoiesis. There are a number of ways in which *Ldb1* can participate in these vital embryonic development processes. It probably regulates the transcription of developmental factors that begin to act prior to or at the onset of gastrulation, and the lack of which results in the severe pattering defects seen in the knockout embryos. It has been shown by Mukhopadhyay et al 2003 that maternally contributed *Ldb1* mRNA exists in the early mouse zygote. Similarly in *Drosophila* it has been shown that maternally derived *Chip* mRNA is essential for early development, as deficient embryos cannot form segments and are very early embryonic lethal. On the other hand the *Chip*-/- *Drosophila* embryos are able to form segments and die later in the larva stage [71].

Ldb1 participates in a number of distinct regulatory protein complexes, each of which regulates a specific pathway in embryonic development and the collective absence of

those complexes would result in the observed complex phenotype. Our results show that is indeed the case in the mouse. *Ldb1* is ubiquitously expressed in the mouse embryo and it is able to form complexes with a variety of proteins, altering each time the cell type that it functions in and the pathway or process in the regulation of which it participates. Interestingly more genes were up-regulated rather than down-regulated in the knockout embryos, which could either be the result of direct repression by binding on regulatory region(s) of the gene in question or indirect, by regulating the expression of repressor genes that act on the gene in question. It is difficult to establish targets of the *Ldb1* gene by the microarray results alone. It should also be noted that the severity of the phenotype will yield genes whose expression is found to be down-regulated in the microarray due to the absence of the tissues in which the genes are normally expressed and not because their expression is directly affected by the absence of *Ldb1*. However the microarray result can still be an important indicator of the developmental pathways that are affected in the knockout embryos and of the cell types and tissues that are absent. A large number of the genes that are down-regulated have been shown to result to an embryonic lethal phenotype in individual knockout experiments and many are essential developmental factors. Hence the lethal phenotype cannot be attributed to the absence of a specific protein or a specific developmental pathway.

Taking into account the variety of defects observed in the *Ldb1*-/- embryos, it is obvious that all three germ layers are affected. It is possible that *Ldb1* has essential functions prior to or at the onset of gastrulation, which was not completed properly in the knockout embryos leading to the severe patterning defects seen. As mentioned previously the *Xenopus* Xldb1 was found to be expressed together with Xlim1 in the Spemann-Mangold organizer region at the dorsal part of the gastrulating embryos. The organizer, which acts as a signalling centre, has the ability to induce secondary axis formation when transplanted to the ventral part of the embryo [72]. The expression of *Xlim1* in *Xenopus* can be induced by the action of activin A and retinoic acid (RA) [19]. In the mouse embryo equivalent organizer regions have been identified in the node of the late-streak stage embryos, the posterior epiblast, termed the early gastrula organizer (EGO) and the anterior of the primitive streak of the mid-streak stage embryos, termed the mid-gastrula organizer (MGO) [73]. All organizer regions have a secondary axis induction ability [74]. Therefore it is safe to conclude that *Ldb1* is essential as a regulator for the anterior-posterior axis patterning of the mouse embryo.

To summarize *Ldb1* has been identified as a significant regulator of mouse embryonic development. It is involved in the regulation of multiple pathways, although its direct function and targets remain to be discovered.

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References

1. Agulnick, A.D., et al., *Interactions of the LIM-domain-binding factor Ldb1 with LIM homeodomain proteins.* Nature, 1996. **384**(6606): p. 270-2.
2. Breen, J.J., et al., *Interactions between LIM domains and the LIM domain-binding protein Ldb1.* J Biol Chem, 1998. **273**(8): p. 4712-7.
3. Bach, I., *The LIM domain: regulation by association.* Mech Dev, 2000. **91**(1-2): p. 5-17.
4. Retaux, S. and I. Bachy, *A short history of LIM domains (1993-2002): from protein interaction to degradation.* Mol Neurobiol, 2002. **26**(2-3): p. 269-81.
5. Warren, A.J., et al., *The oncogenic cysteine-rich LIM domain protein rbtn2 is essential for erythroid development.* Cell, 1994. **78**(1): p. 45-57.
6. Netchine, I., et al., *Mutations in LHX3 result in a new syndrome revealed by combined pituitary hormone deficiency.* Nat Genet, 2000. **25**(2): p. 182-6.
7. Porter, F.D., et al., *Lhx2, a LIM homeobox gene, is required for eye, forebrain, and definitive erythrocyte development.* Development, 1997. **124**(15): p. 2935-44.
8. Lee, S.K. and S.L. Pfaff, *Synchronization of neurogenesis and motor neuron specification by direct coupling of bHLH and homeodomain transcription factors.* Neuron, 2003. **38**(5): p. 731-45.
9. Sheng, H.Z., et al., *Specification of pituitary cell lineages by the LIM homeobox gene Lhx3.* Science, 1996. **272**(5264): p. 1004-7.
10. Chen, H., et al., *Limb and kidney defects in Lmx1b mutant mice suggest an involvement of LMX1B in human nail patella syndrome.* Nat Genet, 1998. **19**(1): p. 51-5.
11. Xu, Z., et al., *Single-stranded DNA-binding proteins regulate the abundance of LIM domain and LIM domain-binding proteins.* Genes Dev, 2007. **21**(8): p. 942-55.
12. Matthews, J.M. and J.E. Visvader, *LIM-domain-binding protein 1: a multifunctional cofactor that interacts with diverse proteins.* EMBO Rep, 2003. **4**(12): p. 1132-7.
13. Morcillo, P., et al., *Chip, a widely expressed chromosomal protein required for segmentation and activity of a remote wing margin enhancer in Drosophila.* Genes Dev, 1997. **11**(20): p. 2729-40.
14. Fernandez-Funez, P., et al., *The relative expression amounts of apterous and its co-factor dLdb/Chip are critical for dorso-ventral compartmentalization in the Drosophila wing.* Embo J, 1998. **17**(23): p. 6846-53.
15. van Meyel, D.J., et al., *Chip is an essential cofactor for apterous in the regulation of axon guidance in Drosophila.* Development, 2000. **127**(9): p. 1823-31.
16. Milan, M. and S.M. Cohen, *Regulation of LIM homeodomain activity in vivo: a tetramer of dLDB and apterous confers activity and capacity for regulation by dLMO.* Mol Cell, 1999. **4**(2): p. 267-73.
17. van Meyel, D.J., et al., *Chip and apterous physically interact to form a functional complex during Drosophila development.* Mol Cell, 1999. **4**(2): p. 259-65.
18. Heitzler, P., et al., *Enhancer-promoter communication mediated by Chip during Pannier-driven proneural patterning is regulated by Osa.* Genes Dev, 2003. **17**(5): p. 591-6.
19. Taira, M., et al., *The LIM domain-containing homeo box gene Xlim-1 is expressed specifically in the organizer region of Xenopus gastrula embryos.* Genes Dev, 1992. **6**(3): p. 356-66.
20. Taira, M., et al., *Role of the LIM class homeodomain protein Xlim-1 in neural and muscle induction by the Spemann organizer in Xenopus.* Nature, 1994. **372**(6507): p. 677-9.
21. Xu, Z., et al., *Identification of a TAL1 target gene reveals a positive role for the LIM domain-binding protein Ldb1 in erythroid gene expression and differentiation.* Mol Cell Biol, 2003. **23**(21): p. 7585-99.
22. Robb, L., et al., *Absence of yolk sac hematopoiesis from mice with a targeted disruption of the scl gene.* Proc Natl Acad Sci U S A, 1995. **92**(15): p. 7075-9.
23. Weiss, M.J., G. Keller, and S.H. Orkin, *Novel insights into erythroid development revealed through in vitro differentiation of GATA-1 embryonic stem cells.* Genes Dev, 1994. **8**(10): p. 1184-97.
24. Fujiwara, Y., et al., *Arrested development of embryonic red cell precursors in mouse embryos lacking transcription factor GATA-1.* Proc Natl Acad Sci U S A, 1996. **93**(22): p. 12355-8.
25. Lahlil, R., et al., *SCL assembles a multifactorial complex that determines glycophorin A expression.* Mol Cell Biol, 2004. **24**(4): p. 1439-52.
26. Vitelli, L., et al., *A pentamer transcriptional complex including tal-1 and retinoblastoma protein downmodulates c-kit expression in normal erythroblasts.* Mol Cell Biol, 2000. **20**(14): p. 5330-42.
27. Meier, N., et al., *Novel binding partners of Ldb1 are required for haematopoietic development.* Development, 2006. **133**(24): p. 4913-23.

28. Sakai, K. and J. Miyazaki, *A transgenic mouse line that retains Cre recombinase activity in mature oocytes irrespective of the cre transgene transmission.* Biochem Biophys Res Commun, 1997. **237**(2): p. 318-24.
29. Valk, P.J., et al., *Prognostically useful gene-expression profiles in acute myeloid leukemia.* N Engl J Med, 2004. **350**(16): p. 1617-28.
30. Yamashita, T., et al., *Genomic structure and chromosomal localization of the mouse LIM domain-binding protein 1 gene, Ldb1.* Genomics, 1998. **48**(1): p. 87-92.
31. Mukhopadhyay, M., et al., *Functional ablation of the mouse Ldb1 gene results in severe patterning defects during gastrulation.* Development, 2003. **130**(3): p. 495-505.
32. Acampora, D., et al., *Forebrain and midbrain regions are deleted in Otx2-/- mutants due to a defective anterior neuroectoderm specification during gastrulation.* Development, 1995. **121**(10): p. 3279-90.
33. Ang, S.L., et al., *A targeted mouse Otx2 mutation leads to severe defects in gastrulation and formation of axial mesoderm and to deletion of rostral brain.* Development, 1996. **122**(1): p. 243-52.
34. Shawlot, W. and R.R. Behringer, *Requirement for Lim1 in head-organizer function.* Nature, 1995. **374**(6521): p. 425-30.
35. Nishioka, N., et al., *Ssdp1 regulates head morphogenesis of mouse embryos by activating the Lim1-Ldb1 complex.* Development, 2005. **132**(11): p. 2535-46.
36. Davidson, D., et al., *A gene with sequence similarity to Drosophila engrailed is expressed during the development of the neural tube and vertebrae in the mouse.* Development, 1988. **104**(2): p. 305-16.
37. Moore, M.A. and D. Metcalf, *Ontogeny of the haemopoietic system: yolk sac origin of in vivo and in vitro colony forming cells in the developing mouse embryo.* Br J Haematol, 1970. **18**(3): p. 279-96.
38. Barker, J.E., *Development of the mouse hematopoietic system. I. Types of hemoglobin produced in embryonic yolk sac and liver.* Dev Biol, 1968. **18**(1): p. 14-29.
39. Steiner, R. and H. Vogel, *On the kinetics of erythroid cell differentiation in fetal mice. I. Microspectrophotometric determination of the hemoglobin content in erythroid cells during gestation.* J Cell Physiol, 1973. **81**(3): p. 323-38.
40. Dzierzak, E. and A. Medvinsky, *Mouse embryonic hematopoiesis.* Trends Genet, 1995. **11**(9): p. 359-66.
41. Lacaud, G., et al., *Regulation of hemangioblast development.* Ann N Y Acad Sci, 2001. **938**: p. 96-107; discussion 108.
42. Baron, M.H., *Embryonic origins of mammalian hematopoiesis.* Exp Hematol, 2003. **31**(12): p. 1160-9.
43. D'Souza, S.L., A.G. Elefanty, and G. Keller, *SCL/Tal-1 is essential for hematopoietic commitment of the hemangioblast but not for its development.* Blood, 2005. **105**(10): p. 3862-70.
44. Wu, C.L., et al., *Expression of dominant-negative mutant DP-1 blocks cell cycle progression in G1.* Mol Cell Biol, 1996. **16**(7): p. 3698-706.
45. Field, S.J., et al., *E2F-1 functions in mice to promote apoptosis and suppress proliferation.* Cell, 1996. **85**(4): p. 549-61.
46. Bartel, F.O., T. Higuchi, and D.D. Spyropoulos, *Mouse models in the study of the Ets family of transcription factors.* Oncogene, 2000. **19**(55): p. 6443-54.
47. Capron, C., et al., *The SCL relative LYL-1 is required for fetal and adult hematopoietic stem cell function and B-cell differentiation.* Blood, 2006. **107**(12): p. 4678-86.
48. Chen, Z.F. and R.R. Behringer, *twist is required in head mesenchyme for cranial neural tube morphogenesis.* Genes Dev, 1995. **9**(6): p. 686-99.
49. Hess, J., P. Angel, and M. Schorpp-Kistner, *AP-1 subunits: quarrel and harmony among siblings.* J Cell Sci, 2004. **117**(Pt 25): p. 5965-73.
50. Yuasa, H., et al., *Oncogenic transcription factor Evi1 regulates hematopoietic stem cell proliferation through GATA-2 expression.* Embo J, 2005. **24**(11): p. 1976-87.
51. Chan, R.J., R. Hromas, and M.C. Yoder, *The role of Hex in hemangioblast and hematopoietic development.* Methods Mol Biol, 2006. **330**: p. 123-33.
52. Kubo, A., et al., *The homeobox gene HEX regulates proliferation and differentiation of hemangioblasts and endothelial cells during ES cell differentiation.* Blood, 2005. **105**(12): p. 4590-7.
53. Liao, W., et al., *Hhex and scl function in parallel to regulate early endothelial and blood differentiation in zebrafish.* Development, 2000. **127**(20): p. 4303-13.

54. Martinez Barbera, J.P., et al., *The homeobox gene Hex is required in definitive endodermal tissues for normal forebrain, liver and thyroid formation.* Development, 2000. **127**(11): p. 2433-45.
55. Bogue, C.W., et al., *Hex expression suggests a role in the development and function of organs derived from foregut endoderm.* Dev Dyn, 2000. **219**(1): p. 84-9.
56. Pereira, F.A., et al., *The orphan nuclear receptor COUP-TFII is required for angiogenesis and heart development.* Genes Dev, 1999. **13**(8): p. 1037-49.
57. You, L.R., et al., *Suppression of Notch signalling by the COUP-TFII transcription factor regulates vein identity.* Nature, 2005. **435**(7038): p. 98-104.
58. Finkelstein, R., et al., *The orthodenticle gene encodes a novel homeo domain protein involved in the development of the Drosophila nervous system and ocellar visual structures.* Genes Dev, 1990. **4**(9): p. 1516-27.
59. Simeone, A., et al., *A vertebrate gene related to orthodenticle contains a homeodomain of the bicoid class and demarcates anterior neuroectoderm in the gastrulating mouse embryo.* Embo J, 1993. **12**(7): p. 2735-47.
60. Ang, S.L., et al., *Positive and negative signals from mesoderm regulate the expression of mouse Otx2 in ectoderm explants.* Development, 1994. **120**(10): p. 2979-89.
61. Rhinn, M., et al., *Sequential roles for Otx2 in visceral endoderm and neuroectoderm for forebrain and midbrain induction and specification.* Development, 1998. **125**(5): p. 845-56.
62. Lagutin, O.V., et al., *Six3 repression of Wnt signaling in the anterior neuroectoderm is essential for vertebrate forebrain development.* Genes Dev, 2003. **17**(3): p. 368-79.
63. McMahon, A.P., et al., *The midbrain-hindbrain phenotype of Wnt-1-/Wnt-1- mice results from stepwise deletion of engrailed-expressing cells by 9.5 days postcoitum.* Cell, 1992. **69**(4): p. 581-95.
64. Millen, K.J., et al., *Abnormal embryonic cerebellar development and patterning of postnatal foliation in two mouse Engrailed-2 mutants.* Development, 1994. **120**(3): p. 695-706.
65. Niederreither, K., et al., *Embryonic retinoic acid synthesis is essential for early mouse post-implantation development.* Nat Genet, 1999. **21**(4): p. 444-8.
66. Ribes, V., et al., *Retinaldehyde dehydrogenase 2 (RALDH2)-mediated retinoic acid synthesis regulates early mouse embryonic forebrain development by controlling FGF and sonic hedgehog signaling.* Development, 2006. **133**(2): p. 351-61.
67. Ono, Y., N. Fukuhara, and O. Yoshie, *TAL1 and LIM-only proteins synergistically induce retinaldehyde dehydrogenase 2 expression in T-cell acute lymphoblastic leukemia by acting as cofactors for GATA3.* Mol Cell Biol, 1998. **18**(12): p. 6939-50.
68. Ribes, V., et al., *Combinatorial signalling controls Neurogenin2 expression at the onset of spinal neurogenesis.* Dev Biol, 2008. **321**(2): p. 470-81.
69. Xuan, S., et al., *Winged helix transcription factor BF-1 is essential for the development of the cerebral hemispheres.* Neuron, 1995. **14**(6): p. 1141-52.
70. Gomez-Skarmeta, J.L., et al., *Xenopus brain factor-2 controls mesoderm, forebrain and neural crest development.* Mech Dev, 1999. **80**(1): p. 15-27.
71. Morcillo, P., C. Rosen, and D. Dorsett, *Genes regulating the remote wing margin enhancer in the Drosophila cut locus.* Genetics, 1996. **144**(3): p. 1143-54.
72. Bouwmeester, T., *The Spemann-Mangold organizer: the control of fate specification and morphogenetic rearrangements during gastrulation in Xenopus.* Int J Dev Biol, 2001. **45**(1): p. 251-8.
73. Robb, L. and P.P. Tam, *Gastrula organiser and embryonic patterning in the mouse.* Semin Cell Dev Biol, 2004. **15**(5): p. 543-54.
74. Beddington, R.S., *Induction of a second neural axis by the mouse node.* Development, 1994. **120**(3): p. 613-20.

Chapter 3

Ldb1 is essential for primitive hematopoiesis and the development of the hemangioblast

Ldb1 is essential for primitive hematopoiesis and the development of the hemangioblast

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Abstract

The Ldb1 (LIM-Domain Binding 1) protein interacts with LIM-Homeodomain and LMO factors and participates in multiprotein complexes, which can additionally include bHLH and GATA factors. Such complexes are involved in transcription regulation and cell differentiation. The deletion of *Ldb1* is characterized by a severe phenotype and leads to embryonic lethality after 9.5 dpc. Among the many defects identified in the *Ldb1*-/- embryo is an impairement of yolk sac hematopoiesis and vascular development. We investigated the role of *Ldb1* in primitive hematopoiesis with the use of knockout ES cells differentiated into embryoid bodies. Our data did show that *Ldb1* is essential both for the emergence and differentiation of the hemangioblast, as *Ldb1*-/- BL-CFCs are both reduced in number and unable to differentiate into blast colonies *in vitro*. In addition *Ldb1* was found to act upstream of many essential transcription factors that govern many aspects of hematopoietic development.

Manuscript in preparation

Introduction

During gastrulation *brachyury* expressing mesodermal cells migrate from the primitive streak, located at the anterior of the mouse embryo to the extra-embryonic visceral yolk sac where they contribute to the formation of blood islands [1], first studied in 1909 [2]. These are the sites where both primitive erythropoiesis and vascular development are initiated as the outer cells differentiate towards the endothelial lineage that will give rise to the vascular network and the inner cells form the large nucleated primitive erythroblasts that produce embryonic globins [3] and will enter the embryo circulation at approximately 8.5 dpc [4]. In addition definitive erythroid and macrophage precursors are believed to arise in the yolk sac from 8.25 dpc [5, 6]. At approximately 9.5 dpc, definitive hematopoiesis is initiated in the aorta-gonad-mesonephros, also known as the AGM region, which emerges from the para-aortic splanchnopleura (PSp) and is the site where the first hematopoietic stem cells (HSCs) emerge [7] [8]. HSCs migrate to the fetal liver, which becomes the primary hematopoietic site of the mouse embryo from 12 dpc until birth. Fetal liver definitive hematopoiesis is a multineage process; which includes erythropoiesis, myelopoiesis and lymphopoiesis [9]. Hematopoiesis shifts to the bone marrow shortly before birth and continues throughout adult life.

The emergence of both primitive erythroid and endothelial progenitors occurs almost simultaneously in the yolk sac blood islands and begs the question whether these cells originate from a common progenitor identified as the hemangioblast [10]. Its existence has been studied in mouse, chick and zebrafish embryos (reviewed by [11]) and is supported by the observation that hematopoietic and endothelial progenitors share the expression of many common genes, such as *Flk1* [12], *Tie*, *Tie-2* [13], *EpoR* [14], *VE-cadherin* [15], *CD34* [16], *Pecam-1* [17], *Scl* [18]. The hemangioblast has also been studied *in vitro* through the differentiation of embryonic stem cells into embryoid bodies, which are spherical clusters of differentiated cells [19] and are used as the *in vitro* equivalent of embryonic development. Embryoid bodies were found to contain a type of cell identified as the *in vitro* equivalent of the hemangioblast. This is the blast colony forming cell (BL-CFC) that can generate blast colonies able to differentiate into both hematopoietic and endothelial progenitors in culture [20].

A number of genes have been identified as essential for primitive hematopoiesis in mice, mainly from loss of function studies. The deletion of *Flk1*, the most common marker used to identify the hemangioblast, leads to embryonic lethality between 8.5 dpc and 9.5 dpc possibly due to the complete absence of blood islands and vessels from the yolk sac [21]. *Scl* was initially identified from a translocation in a human leukemic cell line [22] and its absence leads to lack of hematopoiesis and defects in vascular development in the yolk sac with subsequent embryonic lethality after 10.5 dpc [23] [24]. The deletion of *Scl* in embryoid bodies showed that the factor is not required for the emergence of the BL-CFCs, but functions downstream towards their differentiation into progenitors of the hematopoietic and endothelial lineages [25]. Scl forms heterodimers with the products of

the *E2A* gene (E12/E47) which bind on the DNA regulatory sequence CANNTG, termed the E-box. A similar hematopoietic phenotype with the *Scl*-/- embryos was observed in *Lmo2*-/- embryos [26]. Two members of the GATA family of transcription factors, *Gata-1*, located on the X chromosome and *Gata-2*, have been identified as essential regulators of mouse hematopoiesis. The deletion of *Gata-2* leads to embryonic lethality between 10 dpc and 11 dpc, as the mutants suffer from severe anaemia due to a marked reduction in primitive hematopoiesis in the yolk sac. *Gata-2*-/- embryoid bodies were characterized by a reduction in primitive and definitive erythroid cells [27]. *Gata-1*-/- embryoid bodies did not give rise to primitive erythroid precursors, while definitive erythroid precursors were able to initially develop but died at the proerythroblast stage [28]. Male *Gata-1*-/- mice were pale, suffering from severe anemia and died between 10.5 dpc and 11.5 dpc. However their yolk sacs showed normal vascular organization and contained blood islands with hematopoietic cells that were able to produce embryonic globins. Definitive hematopoietic cells, although initially present, failed to mature and died at the proerythroblast stage similar to the *in vitro* studies. Female *Gata-1*+/- mice were also pale after birth because of random inactivation of the X chromosome, but later were able to recover [29].

The Scl/E47 heterodimer, Lmo2 and Gata-1 form a complex that is binds on an E-box/GATA motif, with the additional participation of the non-DNA binding Ldb1 in erythroid cells and regulate transcription. For the formation of the complex, Lmo2 acts as a bridging molecule between the DNA-binding elements Scl/E47/Gata-1 and Ldb1 [30]. Examples of how such a protein complex can function in transcription regulation were demonstrated on the promoters of the *protein 4.2* gene (*p4.2*) a component of the red cell membrane [31] and the erythrocyte membrane glycoprotein *glycophorin A* [32]. In addition the complex was found to be part of a larger one in non-induced proliferating MEL cells via *in vitro* biotinylation. Apart from Scl/E47, Lmo2 and Gata-1, Ldb1 was found to further interact with E12, Eto-2, Mtgr1, Cdk9, Lmo4, HEB, Lyl1, E2-2 [33].

The *Ldb1* gene was identified from a mouse cDNA expression library through its ability to bind LIM domains [34], present in a large number of regulatory proteins essential for differentiation and development. The highly conserved eleven exon gene is ubiquitously expressed in embryonic and adult tissues. Its function is mainly achieved through the LID (LIM-Interaction-Domain) domain; which binds on the LIM domains of LIM-HD and LMO proteins. Furthermore Ldb1 contains a nuclear localization signal, a homodimerization domain and the LCCD domain that facilitates the interaction with single strand DNA binding proteins (Ssbp proteins) [35, 36]. The *Drosophila* ortholog of *Ldb1*, *dLdb* or *Chip*, functions by enabling long range interactions between promoters and enhancers for transcription regulation [37].

The *Ldb1*-/- mouse is embryonic lethal after 9.5 dpc and suffers from severe developmental defects that include small size, anterior truncation, absence of heart and foregut structures, impairment of both yolk sac hematopoiesis and vascular development. Approximately 1/3 of the *Ldb1*-/- embryos show duplication of the posterior axis with four rows of somites (Chapter 2, [38]).

The embryonic lethality of the *Ldb1*-/- mouse poses limitations in the study of primitive hematopoiesis. Here the generation of an *Ldb1*-/- ES cell line is described. *Ldb1*-/- embryoid bodies were found to be devoid of hematopoietic potential and contained a reduced number of Flk1+ BL-CFCs that failed to give rise to any blast colonies in culture. Changes in the pattern of gene expression in the *Ldb1*-/- Flk1+ cells were examined with the use of expression microarrays, in order to determine the genes that function downstream of *Ldb1* in the regulation of the hemangioblast development and to gain further insight into its role during primitive hematopoiesis.

Methods

Generation of an *Ldb1*-/- ES cell line

In order to generate an *Ldb1* null ES cell line both alleles of the *Ldb1* locus were targeted with the same construct that was built on a pBluescript SK+ backbone and contained a loxP site followed by the *Ldb1* first exon, first intron and second exon, a PMC-neo cassette flanked by two additional loxP sites, a Mitochondrial Localization Signal (MLS) and a CFP reporter gene, all of which was flanked by two homologous sequences of 2.9 kb on the 5' side and 2.6 kb on the 3' side. The *Ldb1* genomic fragments used to generate the construct were isolated from mouse PAC clones from the UK HGMP Resource Centre. The construct was linearized at a unique NotI site and was used to transfect mouse ES cells via electroporation. The transfected ES cells were put under G418 selection, clones were picked and genomic DNA was isolated for Southern blot analysis, in order to identify the successfully targeted ES cell clones, where the targeting vector was incorporated into the SphI site in the second intron of the *Ldb1* locus via homologous recombination. Genomic DNA was digested with EcoRV for the Southern blot analysis. A 0.43 kb PCR generated fragment, located upstream of the 5'UTR and outside of the *Ldb1* promoter region, was used as a 5'probe with the EcoRV digestion, to identify the 5 kb fragmented of the *Ldb1* wild type allele and the 6.9 fragment of the targeted allele. The clone was transiently transfected with a vector expressing Cre recombinase under puromycin selection for the deletion of the *Ldb1* first and second exons, including the ATG, and the PMC-neo cassette. Clones were picked; the genomic DNA was isolated and digested with EcoRV for Southern blot analysis using the same 5' probe as before to identify the 5.5 kb fragment of the targeted allele that resulted from the deletion. The targeting was repeated one more time with the same construct for the other *Ldb1* allele. Southern blot analysis was used as before with the same PCR generated 5' probe that identified the 5.5 kb fragment of the first targeted allele with the neo cassette and exon I/II deletion and the 6.9 kb fragment of the second targeted allele. The karyotype of the ES cells was checked after each transfection step and found to be correct. Nuclear extracts were isolated from wild type ES cells and the *Ldb1* null ES cell clone and the goat polyclonal IgG anti-Ldb1 antibody, N-18 (Santa Cruz®) was used to identify the Ldb1 protein, which runs as a double band of 42.7 kDa and 46.5 kDa.

Immunofluoresence

Wild type and *Ldb1* null ES cells were cultured on glass cover-slips. The cells were fixed in 4% Paraformaldehyde/PBS for 15 minutes at room temperature, permeabilized with 0.1% Triton®X-100/PBS, blocked with PBS/0.5% BSA/0.15% Glycine, incubated overnight at 4^0C with the anti-Ldb1, goat polyclonal IgG, N-18 (Santa Cruz®) antibody and for 2 hours at room temperature with a rabbit anti-goat IgG FITC secondary antibody. The cover-slips were mounted on glass slides with DAPI/Vectashield for nuclei staining.

Differentiation of ES cells into embryoid bodies

Wild type and *Ldb1* null ES cells were grown in suspension at a density of 10.000 cells/ml for the generation of day 4, day 6 and day 8 embryoid bodies, in IMDM based medium supplemented with 15% FCS, 1% penicillin-streptomycin, 1% L-glutamine from a 100x stock (GIBCO), 0.05μg/ml transferin, 0.05μg/ml ascorbic acid, 3μl/ml monothioglycerol (Sigma) for the day 4 embryoid bodies and 1.8μl/ml for the day 6 and day 8 embryoid bodies from a 13μl/ml freshly made stock. Also for the day 6 and day 8 embryoid bodies 5% protein free hybridoma medium II (GIBCO) was added.

BL-CFC assay

For the Blast Colony Forming Cell assay day 4 embryoid bodies were disrupted with trypsin-EDTA and the cells were left to grow for 3 days in methycellulose-based media supplemented with 10% FCS, 1% penicillin-streptomycin, 1% L-glutamine from a 100x stock (GIBCO), 0.25μg/ml transferin, 0.25μg/ml ascorbic acid, 2μl/ml monothioglycerol (Sigma) from a 13μl/ml freshly made stock, 0.01μg/ml mIL6 (R&D Systems), 0.005μg/ml hVEGF (R&D Systems). The colonies were scored according to their morphology and number under an inverted microscope.

CFC assay

For the Colony Forming Cell assay day 6 embryoid bodies were disrupted with 2.5% collagenase in 10%FCS/PBS and the cells were left to grow for 6 days in methycellulose-based media supplemented with 10% FCS, 1% penicillin-streptomycin, 1% L-glutamine from a 100x stock (GIBCO), 0.25μg/ml transferin, 0.25μg/ml ascorbic acid, 2μl/ml monothioglycerol (Sigma) from a 13μl/ml temporary stock, 5% protein free hybridoma medium II (GIBCO), 0.01μg/ml mIL6 (R&D Systems), 0.001μg/ml IL3 (R&D Systems), 0.005μg/ml hIL11 (R&D Systems), 0.003μg/ml GM-CSF (R&D Systems), 4U/ml EPO (R&D Systems), 0.005μg/ml TPO (R&D Systems), 0.1 μg/ml SCF (R&D Systems). After 6 days colonies were identified according to their morphology under an inverted microscope.

FACS analysis

For FACS analysis day 4, day 6 and day 8 embryoid bodies were disrupted with trypsin-EDTA (for day 4) or 2.5% collagenase in 10%FCS/ PBS (for day 6 and day 8). Single cell suspensions were stained in 1% BSA/PBS on ice for 30 minutes with the following phycoerythrin (PE) or FITC conjugated antibodies: Flk1-PE Rat IgG2a, k , clone Avas 12α1 (BD Pharmigen™), CD41 (Integrin aIIb)-PE rat IgG1, clone MWReg30 (Santa

Cruz®), CD31 (PECAM-1)-FITC Rat IgG2a, k, clone MEC 13.3 (BD Pharmigen™). Dead cells were visualized and excluded from the analysis by 7-aminoactinomycin D (7-AAD) (Invitrogen™) (1µg/ml) staining. The flow cytometry was performed on FACScan (Becton Dickinson) and was analyzed with the Cell Quest software. Flk1+ cells were sorted from the day 4 embryoid bodies by flow cytometry on FACSAria (BD). Single cell suspensions were stained with the previous Flk1-PE conjugated antibody (BD Pharmigen™) in 1% BSA/PBS. The dead cells were excluded by Hoechst 33258 (1µg/ml) (Molecular Probes) staining. For the analysis the software Diva version 5.1 (BD) was used.

Affymetrix Mouse Genome Arrays

Microarray analysis of *Ldb1*+/+ and *Ldb1*-/- Flk1+ cells was performed as described in Chapter 2.

Microarray data normalization and analysis

Described in Chapter 2.

Gene expression analysis

RNA was isolated as described above and cDNA was synthesized using SuperScript II Reverse Transcriptase and Oligo(dT)$_{12–18}$ primers according to the manufacturer's instructions (Invitrogen™). Quantitative Real-time PCR was carried out on the synthesized cDNA with 1U of Platinum® Taq polymerase (Invitrogen™), 1x PCR buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl], 1.5mM $MgCl_2$, 10ng/µl of each primer, 250µM dNTPs and SYBR Green (Sigma®) on the Opticon 2® Real-Time PCR Detection System (Bio-Rad). The following cycling conditions were used: 2 minutes at 94^{0}C, 40 cycles of 30 seconds at 94^{0}C, 30 seconds at 55^{0}C, 30 seconds at 72^{0}C and 5 minutes at 72^{0}C. The data was collected and analysed by the MJ Opticon™ analysis software version 3.1 (Bio-Rad). Transcript enrichments were calculated using the comparative C_T method. The C_T threshold was set at 10 cycles to distinguish between non-specific and specific PCR products detected through the incorporation of SYBR Green to amplified DNA. Transcript levels were normalized to the transcript of *Gapdh*, a housekeeping gene, the expression of which was not found to change after the deletion of *Ldb1* in the Flk1+ cells.

Results and Discussion

Generation of an *Ldb1-/-* ES cell line

In order to investigate the involvement of *Ldb1* in primitive hematopoiesis and hemangioblast differentiation an *Ldb1* knockout ES cell line was generated. Both alleles of the *Ldb1* locus were sequentially targeted with the same construct. The integration of the construct in the *Ldb1* locus via homologous recombination results in the insertion of a loxP site at the 5' UTR and a PMC-neo cassette flanked by two more loxP sites and followed by an MLS and a CFP reporter gene at an SphI restriction site in the second intron. The genomic structure of the mouse *Ldb1* gene [39] and the strategy for the targeting of the *Ldb1* locus for the generation of the cell line is shown in Fig. 1A. A positive clone (Fig. 1B) with one wild type

and one targeted allele was selected and transiently transfected with a Cre recombinase expressing vector for the deletion of the PMC-neo cassette and the first two exons of the gene, including the ATG. Due to the deletion the CFP reporter gene moves to the 5' UTR of *Ldb1* (Fig. 1B). The same targeting step was repeated once more and a positive clone was selected where the targeting construct was inserted by homologous recombination in the remaining wild type allele (Fig. 1B). Due to an unexpected design characteristic of the targeting construct, as observed previously in the attempt to generate conditional *Ldb1*-/- mice (unpublished data), the integration of the targeting construct via homologous recombination results in a full knocked out allele, without the need to delete the first two gene exons, possibly due to an interference of the CFP reporter gene with the transcription or translation machinery. Therefore the first two exons and the PMC-neo cassette were not deleted in the second targeted *Ldb1* allele (Fig. 1B). In order to confirm that no Ldb1 protein was present in the *Ldb1* null ES cells after the successful targeting of the locus two independent techniques were used. Nuclear extracts were isolated from wild type and *Ldb1* null ES cells for western blot analysis with an antibody against Ldb1, which appears as a double band, because of the two *Ldb1* isoforms being translated (Fig. 1C). Additionally wild type and *Ldb1* null ES cells were grown on glass cover-slips for immunofluoresence analysis with the use of the same antibody, to confirm the complete absence of Ldb1 from the cell nucleus (Fig. 1D). As shown on these figures no detectable Ldb1 protein is present in the *Ldb1* null ES cell line. The deletion of *Ldb1* did not have any obvious effects on the ES cells, which appear similar to the wild type cells under a light microscope and grow with a similar rate (Fig. 1E)Next the differentiation potential of the *Ldb1* null ES cells was investigated with the generation of embryoid bodies. When ES cells are left to grow in suspension in non-adherent culture dishes and LIF is withdrawn from the medium, they form spherical cell clusters that mimic the early stages of embryonic development. Wild type and *Ldb1* null ES cells were cultured in suspension and in hematopoiesis inducing media. When viewed under a light microscope the embryoid bodies did not show any phenotypical differences at days 3 and 4 as they appeared similar in size and structure. However when they were left to grow for 8 days, a striking difference was immediately obvious from the absence of erythroid clusters in the *Ldb1* null embryoid bodies, which additionally appeared darker and more compact in comparison to wild type ones (Fig. 1F). *Ldb1* null embryoid bodies were still able to survive at least until day 10 and they did not appear to suffer from early induced apoptosis; however they were more adherent than the wild type ones and after 10 days in culture the majority of them were found to attach on the bottom of the culture dish (data not shown).

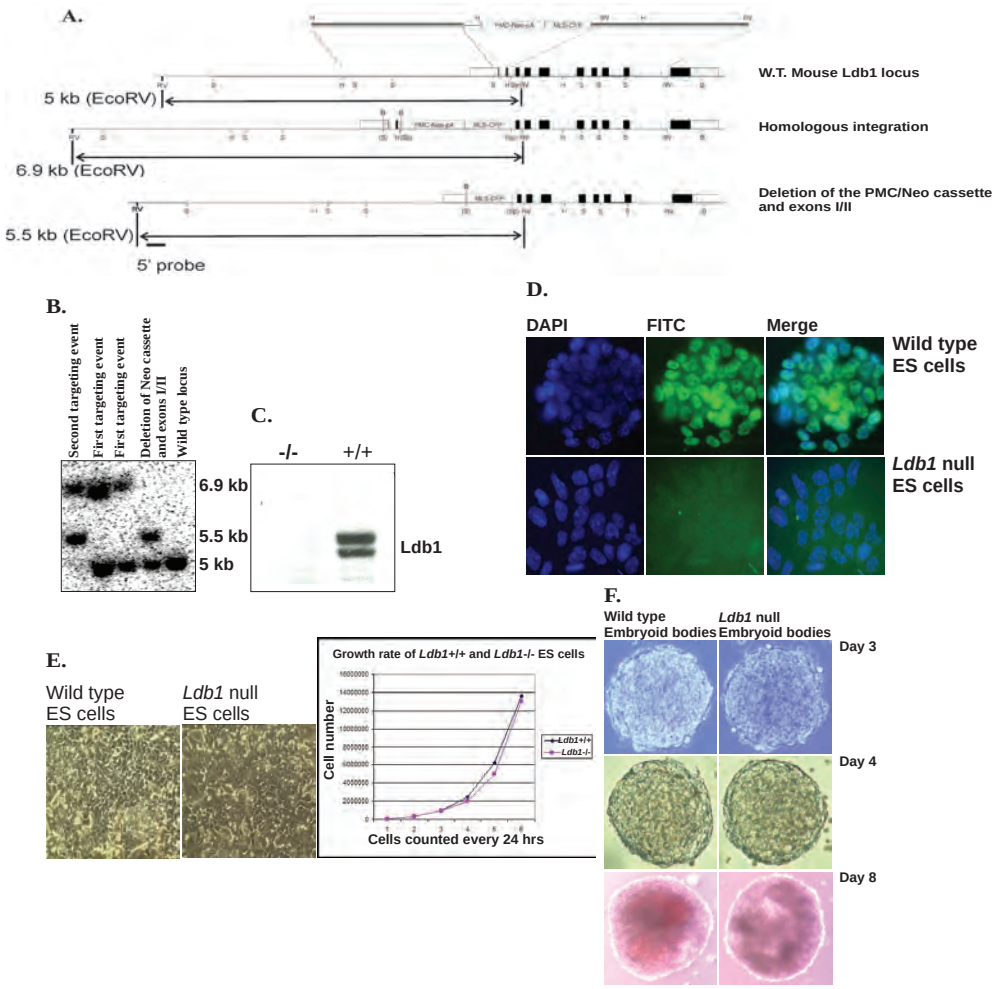


Figure 1. Scheme for the generation of the *Ldb1* null ES cell line. (A) A targeting construct with a PMC-neo cassette and an MLS-CFP was inserted in the second intron of both alleles in the *Ldb1* locus by homologous recombination through sequential targeting. Transfection with Cre recombinase was used to delete the neo cassette and the two first exons in one allele before the targeting of the locus with the same construct. (B) ES cell DNA was digested with EcoRV for the hybridisation with the 5' probe in order to identify the 5 kb fragmented of the *Ldb1* wild type allele, the 6.9 fragment of the targeted allele and the 5.5 kb fragment of the targeted allele that resulted from the deletion of the neo cassette and the first two exons. (C) Western blot analysis of wild type and *Ldb1* null ES cells showed the presence of Ldb1 protein in the wild type but not in the knockout cells. (D) Immunofluoresence analysis of wild type and *Ldb1* null ES cells revealed that there is no Ldb1 protein present in the nucleus of the knockout ES cells. (E) The *Ldb1* null ES cells look similar to the wild type ones and grow at similar rates. (F) Wild type and *Ldb1* null ES cells were differentiated into embryoid bodies. After 3 and 4 days of

differentiation the knockout embryoid bodies look similar to the wild type ones. After 8 days of differentiation the knockout embryoid bodies lack erythroid clusters, while they appear darker and more compact.

Primitive erythroid cells do not develop in *Ldb1* null embryoid bodies

Ldb1-/- mouse embryos show a complete impairment in primitive hematopoiesis, vascular development and subsequent embryonic death after 9.5 dpc [38] (Mylona et al. unpublished data). When yolk sacs were dissected from *Ldb1*+/+ and *Ldb1*-/- embryos, and the cells disrupted with collagenase and cultured on methycellulose-based media supplemented with the GM-CSF, EPO, TPO, SCF and the cytokines mIL6, IL3 and hIL11 for 7 days, a large number of primitive erythroid and macrophage colonies, was observed in the wild type yolk sac cultures but very few macrophage colonies were present in the knockout yolk sac cultures (Chapter 2: Fig 3A, B, C).

Similarly when the *Ldb1* null ES cells are cultured in hematopoiesis inducing media and under specific conditions to generate embryoid bodies, it was clear that the wild type embryoid bodies contained clusters of erythroid cells, while the *Ldb1* null ones did not. Thus the hematopoietic defect of the *Ldb1*-/- mice is replicated *in vitro*. In order to investigate the extent of the defect in more depth, colony forming assays were performed with cell suspensions of day 6 embryoid bodies cultured in hematopoiesis inducing media. The embryoid bodies were disrupted with collagenase and the isolated cells were cultured for 6 days on methycellulose-based media supplemented with the GM-CSF, EPO, TPO, SCF and the cytokines mIL6, IL3 and hIL11. The colonies that formed were scored according to their morphology and number (Fig. 2A, B, C). Wild type day 6 embryoid bodies give rise to bright red primitive erythroid colonies and macrophage colonies, which are white and composed of round cells that grow in clumps. Both types of colonies are completely absent in the *Ldb1* null cultures (Fig. 2D). The colony forming assay was repeated three times with similar results.

Wild type and *Ldb1* null embryoid bodies grown for 4, 6 and 8 days were investigated with quantitative real time PCR, for the presence of the mouse embryonic hemoglobins Hbb-y and Hbb-bh1 and of the mouse adult hemoglobin Hbb-bmajor. Hbb-y and Hbb-bh1 are absent from day 4 embryoid bodies, but their expression begins soon after, as they are present in day 6 and day 8 embryoid bodies. Also present at these time points in embryoid bodies is Hbb-bmajor, albeit in very small amounts. No embryonic or adult hemoglobins are produced in the *Ldb1* null embryoid bodies (Fig. 2E).

In order to determine whether any erythroid progenitors were present in the *Ldb1* null embryoid bodies, which perhaps failed to further develop into primitive erythroid colonies in the *in vitro* cultures, day 6 and day 8 embryoid bodies from wild type and *Ldb1* null ES cells were examined by flow cytometry for the presence of such cells. CD41 (or integrin alpha-IIb) was initially identified as part of a platelet specific receptor complex,

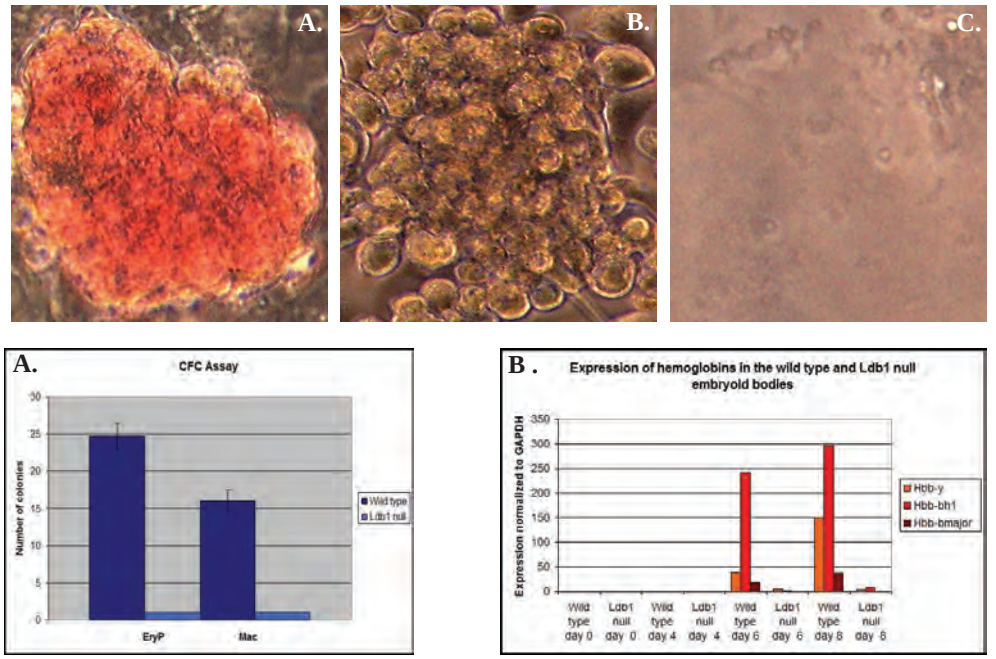


Figure 2. Cell suspensions from wild type and *Ldb1* null embryoid bodies, grown for 6 days, were cultured for a further 6 days on methycellulose based media supplemented with GM-CSF, EPO, TPO, SCF and the cytokines mIL6, IL3 and hIL11. Primitive erythroid and macrophage colonies were able to grow on the wild type cultures (A, B) but not on the knockout cultures (C). (D) The graph summarizes the total absence of primitive erythroid and macrophage colonies in the knockout cultures from three different CFC assays. (E) *Ldb1* null embryoid bodies do not produce any embryonic or adult globins.

but it has recently been identified as a marker for primitive erythroid progenitor cells in the early embryo yolk sac [40]. The wild type embryoid bodies contained 2.6% and 12% of CD41+ cells at day 6 and day 8 respectively, however the *Ldb1* null embryoid bodies did not contain any CD41+ erythroid progenitor cells, in accordance with the total absence of primitive erythroid colonies in the *Ldb1* null CFC cultures (Fig. 3). Flow cytometry was also used to investigate the presence of endothelial progenitors in day 6 and day 8 embryoid bodies, taking into account that the *Ldb1*-/- embryos suffer from a severe defect in vascular development. CD31 (or PECAM-1) is a cell adhesion molecule that is expressed on platelets and at the intercellular junctions of endothelial cells and has been extensively used as a marker for endothelial cells within the embryo and the yolk sac [41]. At day 6 and day 8 the wild type embryoid bodies contained 21% and 16% CD31+ cell respectively. Endothelial CD31+ cells were also found to be present in the *Ldb1* null embryoid bodies at the same time points but their population was reduced to less than half when compared to wild type embryoid bodies. The knockout embryoid bodies contain

5.6% of CD31+ cells at day 6 and 7% at day 8 (Fig. 3). Therefore we can conclude that the hematopoietic defect seen in the knockout embryoid bodies and additionally in the knockout embryos is the result of the absence of primitive hematopoietic progenitor cells, which do not emerge in the absence of *Ldb1*. There is an additional defect in endothelial development, which has also been observed in the *Ldb1*-/- mouse embryos. Although there are still some CD31+ endothelial cells in the *Ldb1* null embryoid bodies, their number is severely reduced. Repeat experiments gave the same results. We therefore conclude that the defect in primitive hematopoiesis in the absence of *Ldb1* must occur at an earlier stage in development.

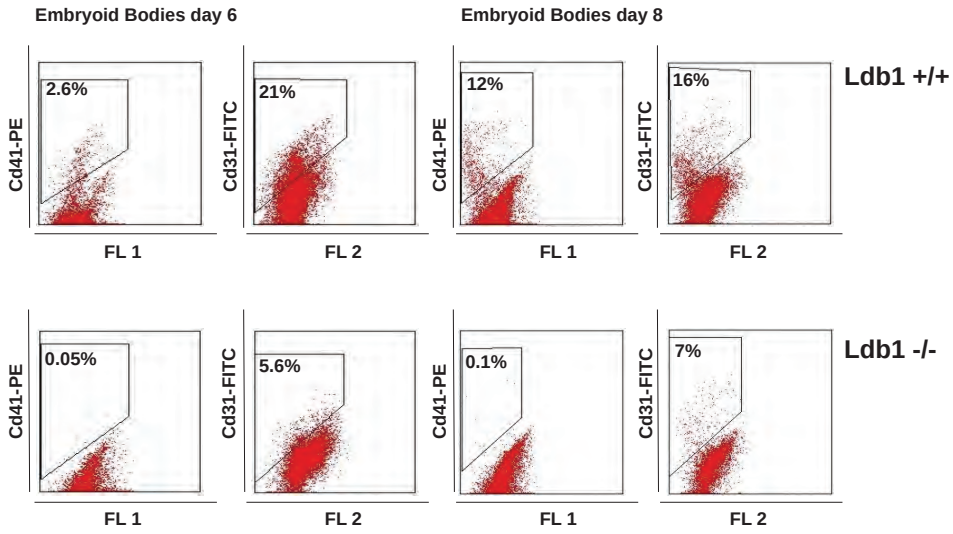


Figure 3. Analysis of day 6 and day 8 wild type and *Ldb1* null embryoid bodies by flow cytometry. CD41+ primitive erythroid progenitors are present in the wild type but not in the *Ldb1* null embryoid bodies. CD31+ endothelial cells are present in both the wild type and the *Ldb1* null embryoid bodies, but their overall number in the *Ldb1* null embryoid bodies is less than half of the endothelial cells present in the wild type embryoid bodies. The cells were stained with either CD41-PE or CD31-FITC. The CD41-PE+ cells were detected through the FL2 channel and plotted against the FL1 channel that should detect no stained cells. The CD31-FITC+ cells were detected through the FL1 channel and plotted against the FL2 channel that should detect no stained cells.

Ldb1 null embryoid bodies do not give rise to blast colonies

The first step in hematopoiesis and vascular development of the mouse embryo is the emergence of the hemangioblast and its subsequent differentiation into hematopoietic and endothelial progenitors. Studies done with embryoid bodies derived from differentiated mouse ES cells have identified the blast colony-forming cells (BL-CFCs) that can form blast colonies with both hematopoietic and endothelial potential as the *in vitro* equivalent of the hemangioblast [42, 43]. We therefore investigated whether BL-CFCs were present in the *Ldb1* null embryoid bodies. The most common marker used to identify these cells is Flk1. Flow cytometric analysis was performed on day 4 wild type and *Ldb1* null embryoid bodies in order to determine the presence of Flk1+ BL-CFCs (Fig. 4A). In both wild type and *Ldb1* null embryoid bodies Flk1+ cells were present, however a striking difference was observed in the number of those cells. The *Ldb1* null embryoid bodies contain on average half the number of Flk1+ cells compared to those present in the wild type embryoid bodies. Repeat experiments gave the same result.

In addition to the hemangioblast, Flk1 is also expressed in endothelial progenitors and fully differentiated endothelial cells. It is therefore possible that the Flk1+ cells present in the *Ldb1* null embryoid bodies were only endothelial progenitors, which could explain the reduced numbers. In order to clarify this question, cell suspensions of wild type and *Ldb1* null day 4 embryoid bodies were analyzed by flow cytometry for the presence of Flk1+CD31+ double positive cells. As shown in Fig. 4A, the Flk1+ cells present in both wild type and *Ldb1* null day 4 embryoid bodies are not CD31+, which shows that the Flk1+ cells are BL-CFCs and not endothelial progenitor cells.

Since Flk1+ BL-CFCs are present in the *Ldb1* null embryoid bodies, the next question is whether they are able to form blast colonies. For that purpose blast colony forming assays were performed and repeated three times, using cell suspensions from wild type and *Ldb1* null day 4 embryoid bodies that were subsequently cultured for 3 days on methycellulose based media with the addition of mouse IL6 and human VEGF. Blast colonies did form in the wild type cultures; however no colonies were formed in the knockout cultures (Fig. 4B). Thus BL-CFCs are present in the *Ldb1* null embryoid bodies, but they are unable to develop into fully grown blast colonies. The Flk1+ cells of the *Ldb1* null day 4 embryoid bodies eventually die (data not shown). The fact that these cells represent the common precursor of both hematopoietic and endothelial progenitors indicates that the hematopoietic defect seen in knockout embryoid bodies and knockout mouse embryos is an early one.

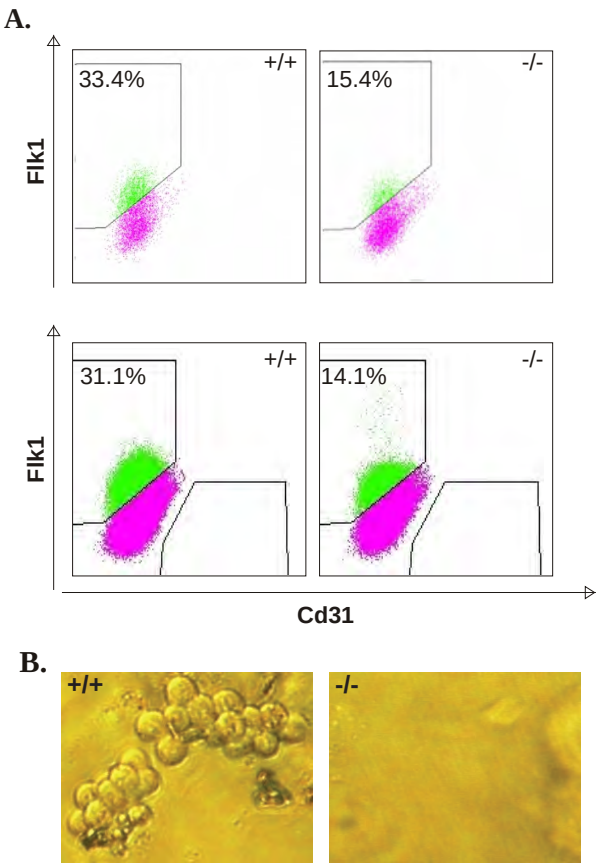


Figure 4. (A) Flow cytometric analysis of day 4 wild type and *Ldb1* null embryoid bodies. The knockout embryoid bodies contain approximately 50% less Flk1+CD31- cells than the wild type ones. (B) Cell suspensions from wild type and *Ldb1* null embryoid bodies, grown for 4 days, were cultured for a further 3 days on methycellulose based media supplemented with mouse IL6 and human VEGF. The wild type Flk1+ cells are able to give fully grown blast colonies, whereas the *Ldb1* null Flk1+ cells do not give any blast colonies.

Changes in gene expression pattern between wild type and *Ldb1* null Flk1+ cells

In order to gain an insight into why the Flk1+ BL-CFCs present in the *Ldb1* null embryoid bodies fail to develop into blast colonies, changes in gene expression were investigated. Flk1+ BL-CFCs were isolated from day 4 wild type and *Ldb1* null embryoid bodies by FACS sort. RNA was isolated from equal numbers of wild type and *Ldb1* null Flk1+ cells and 100ng of each was used for hybridization on the Affymetrix Mouse Genome 430 2.0 arrays. The following parameters were fulfilled as a measure of a successful hybridization: the average signal of the hybridization of each sample on the array, the percentage of the present probe sets versus the absent probe sets, the noise and background levels, the ratio of GAPDH 3'to 5' (data not shown). The microarray data were analysed with the Omniviz software version 5.0 and the SAM (Significance Analysis of Microarrays) software. The correlation of the samples is shown in Fig. 5A. The blue and red colours represent the Pearson's Correlation Coefficient values, where the red colour represents positive and the blue colour negative correlations. A negative correlation (represented in blue) indicates that the genes with a high level of expression in one sample will always have

a low level of expression in the other sample and the other way around, while a positive correlation (represented in red) indicates that the genes in both samples have similar levels of expression. The clustering of the wild type and knockout samples according to the most significant differentially expressed genes based on the q-value threshold of 5% is shown on Fig. 5B as a tree diagram at the top of the figure. At the left of the figure the tree diagram represents the clustering of differentially expressed genes. The red colour represents an up-regulation in gene expression of at least 1.5 fold, while the green colour represents a down-regulation in gene expression of the same level. A total number of 2840 genes showed a higher than 1.5 fold differential expression between the wild type and the *Ldb1* null Flk1+ cells, from which 1730 genes were of known function. From those genes, 1290 genes were found to be down-regulated (74.56%) and 440 were found to be up-regulated (25.43%). Finally 1099 from the total number of differentially expressed genes (38.69%) were of unknown function. From those 640 (58.23%) were down-regulated and 459 (41.76%) were up-regulated. A list of genes that are differentially expressed in the *Ldb1* null Flk1+ cells is shown in Table 1. The higher p-values associated with the differential expression of some of the genes are probably the result of the small amounts of RNA used for the analysis and possible variations in the hybridization efficiency of the samples. Additionally any observed up- or down-regulation of genes expressed at low levels is associated with elevated p-values. A number of the genes that showed differential expression in the *Ldb1* null Flk1+ cells were selected from the above list in order to confirm the changes in their expression pattern by quantitative real-time PCR (Fig. 7)

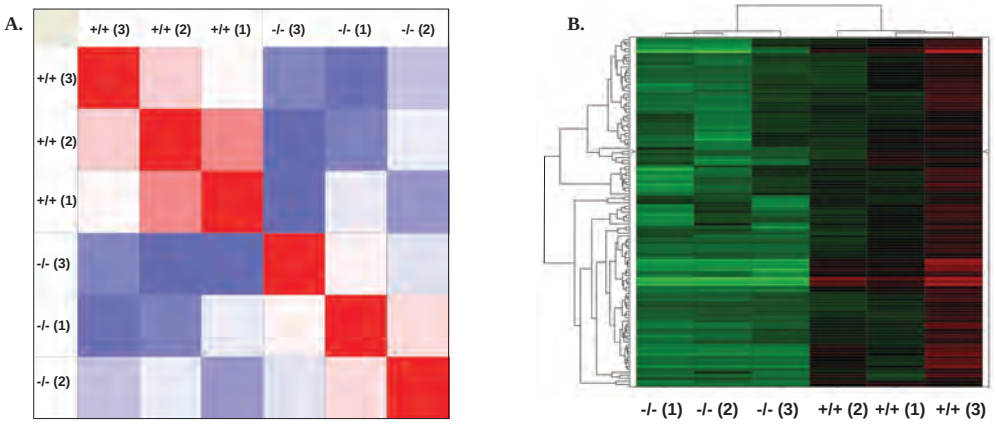


Figure 5. (A) Pairwise correlation of the wild type and *Ldb1* null Flk1+ cells. The threshold for the differential expression was set at 1.5 fold and the q-value was set at 5%. The blue and red colour represents the negative and positive Pearson's Correlation Coefficient values respectively. The negative correlation (blue squares) seen between the wild type and the *Ldb1* null Flk1+ cells indicates that the genes expressed in the wild type cells will always be differentially expressed

in the knockout cells. The positive correlation (red squares) indicates that the genes expressed in all wild type cells follow the same levels of expression and similarly the genes expressed in all the knockout cells follow the same levels of expression. (B) The clustering of the wild type and *Ldb1* null Flk1+ cells (the threshold for gene differential expression is set at 1.5 fold and the q-value is set at 5%) based on the genes that show differential expression between the wild type and the knockout Flk1+ cells according to the above set parameters is shown as a tree diagram at the top of the figure. The change in the expression level of each gene is represented by the change of colour from light green to red. The highest up-regulated genes are represented by red will the most severely down-regulated genes are represented by light green.

Table 1. A list of genes that show differential expression in the *Ldb1* null Flk1+ cells, compared with the wild type Flk1+ cells. The fold change (up-regulation or down-regulation) of each gene's expression in the double knockout cells is given. Bold characters indicate the genes for which the change in expression is associated with a q-value below the threshold of 5%.

Gene Symbol	Gene Title	Fold Change	q-value
Gata1	**GATA binding protein 1**	**0.222**	**0.034**
Fli1	friend leukemia integration 1	0.423	0.319
Gfi1b	**growth factor independent 1B**	**0.36**	**0.021**
Hbb-bh1	**hemoglobin Z, beta-like embryonic chain**	**0.072**	**0.0075**
Hbb-y	hemoglobin Y, beta-like embryonic chain	0.633	0.182
CD117/c-kit	kit ligand	0.434	0.153
Eklf	Kruppel-like factor 1 (erythroid)	0.6	0.509
Lyl1	lymphoblastomic leukemia	0.33	0.151
Runx1t1/Eto	runt-related transcription factor 1	0.478	0.156
Eto2	**core-binding factor alpha subunit 2, translocated to, 3**	**0.49**	**0.073**
Nfe2	nuclear factor, erythroid derived 2	0.536	0.348
Runx1	**runt related transcription factor 1**	**0.552**	**0.036**
Cd24a	CD24a antigen	1.59	0.401
Stat5a	signal transducer and activator of transcription 5A	0.647	0.462
Stat5b	signal transducer and activator of transcription 5B	0.584	0.186
Tcf12	transcription factor 12	1.534	0.713
Gypc	glycophorin C	0.32	0.173
Mef2c	**myocyte enhancer factor 2C**	**0.428**	**0.055**
Ikzf1	**IKAROS family zinc finger 1**	**0.215**	**0.0048**
Ikzf2	IKAROS family zinc finger 2	0.475	0.194

Gene Symbol	Gene Title	Fold Change	q-value
Id2	inhibitor of DNA binding 2	2.17	0.113
Id4	inhibitor of DNA binding 4	3	0.144
Lmo1	LIM domain only 1	1.89	0.446
Ldb2	LIM domain binding 2	0.633	0.320
Rnf12	ring finger protein 12	0.657	0.73
Ssbp2	single-stranded DNA binding protein 2	2.168	0.439
Ssbp3	Single-stranded DNA binding protein 3	1.69	0.648
Bcl11a	B-cell CLL/lymphoma 11A (zinc finger protein)	1.984	0.357
Bclaf1	BCL2-associated transcription factor 1	1.658	0.84
Bnip3	BCL2/adenovirus E1B interacting protein 1, NIP3	0.643	0.379
Myb	myeloblastosis oncogene	0.21	0.006
Mycn	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived	1.56	0.373
Bmp2	bone morphogenetic protein 2	0.523	0.144
Bmp5	bone morphogenetic protein 5	0.543	0.168
Bmp7	bone morphogenetic protein 7	0.643	0.383
Smad3	MAD homolog 3 (Drosophila)	0.664	0.407
Wnt5a	wingless-related MMTV integration site 5A	1.584	0.812
Wnt6	wingless-related MMTV integration site 6	0.546	0.622
Dkk1	dickkopf homolog 1 (Xenopus laevis)	1.517	0.461
Dkk3	dickkopf homolog 3 (Xenopus laevis)	0.61	0.513
Sfrp1	secreted frizzled-related protein 1	0.3	0.051
Sfrp2	secreted frizzled-related protein 2	0.549	0.238
Frzb/Sfrp3	frizzled-related protein	1.79	0.942
Sfrp5	secreted frizzled-related sequence protein 5	0.641	0.468
Fzd1	frizzled homolog 1 (Drosophila)	0.658	0.372
Fzd3	frizzled homolog 3 (Drosophila)	1.546	0.667
Fzd4	frizzled homolog 4 (Drosophila)	0.431	0.347
Fzd9	frizzled homolog 9 (Drosophila)	0.643	0.373

Gene Symbol	Gene Title	Fold Change	q-value
Dll3	delta-like 3 (Drosophila)	0.612	0.354
Dll4	delta-like 4 (Drosophila)	0.377	0.373
Fgf15	fibroblast growth factor 15	0.409	0.119
Fgf3	**fibroblast growth factor 3**	**0.406**	**0.021**
Fgf4	fibroblast growth factor 4	0.484	0.178
Fgf5	fibroblast growth factor 5	2.24	0.232
Fgf8	fibroblast growth factor 8	1.523	0.762
Fgfr2	**fibroblast growth factor receptor 2**	**0.431**	**0.088**
Fgfr3	fibroblast growth factor receptor 3	0.49	0.343
Fgfr4	fibroblast growth factor receptor 4	0.492	0.373
Hoxa5	homeo box A5	0.47	0.72
Hoxb1	homeo box B1	0.6	0.29
Hoxb2	homeo box B2	0.512	0.385
Hoxb3	**homeo box B3**	**0.394**	**0.084**
Hoxb4	**homeo box B4**	**0.548**	**0.082**
Hoxb5	**homeo box B5**	**0.553**	**0.069**
Hoxb6	homeo box B6	0.343	0.273
Hoxc6	homeo box C6	0.513	0.373
Hoxd1	homeo box D1	0.435	0.197
Hoxd8	homeo box D8	0.42	0.117
Hoxd9	homeo box D9	0.498	0.3
Cx3cl1	chemokine (C-X3-C motif) ligand 1	1.527	0.68
Cxcl14	chemokine (C-X-C motif) ligand 14	0.618	0.373
Cxcl17	chemokine (C-X-C motif) ligand 17	0.535	0.581
Cxcr3	chemokine (C-X-C motif) receptor 3	0.614	0.513
Cxcr4	chemokine (C-X-C motif) receptor 4	0.662	0.539
Cxcr7	chemokine (C-X-C motif) receptor 7	0.539	0.466
Efna1	ephrin A1	0.622	0.482
Efnb2	**ephrin B2**	**0.522**	**0.085**
Epha1	eph receptor A1	1.941	0.251
Epha2	eph receptor A2	1.612	0.304
Epha4	eph receptor A4	0.542	0.186

Gene Symbol	Gene Title	Fold Change	q-value
Sox17	**SRY-box containing gene 17**	**0.473**	**0.037**
Sox2	SRY-box containing gene 2	1.648	0.42
Sox21	SRY-box containing gene 21	1.679	0.329
Lhx2	LIM homeobox protein 2	0.65	0.373
Lhx3	LIM homeobox protein 3	0.548	0.45
Pdlim3	PDZ and LIM domain 3	2	0.17
Pdlim4	**PDZ and LIM domain 4**	**0.387**	**0.05**
Esrrb	estrogen related receptor, beta	0.655	0.498
Esrrg	estrogen-related receptor gamma	0.594	0.45
Meis1	**Meis homeobox 1**	**0.431**	**0.049**
Mesp2	mesoderm posterior 2	0.433	0.162
Mest	**mesoderm specific transcript**	**0.046**	**0.01**
Mixl1	Mix1 homeobox-like 1 (Xenopus laevis)	2	0.418
T	brachyury	1.93	0.319
Twist1	twist gene homolog 1 (Drosophila)	1.588	0.5
Hes6	hairy and enhancer of split 6 (Drosophila)	1.776	0.373
Hey2	hairy/enhancer-of-split related with YRPW motif 2	0.6	0.5
Dhh	desert hedgehog	0.631	0.412
Cdh1	cadherin 1	1.52	0.821
Nodal	nodal	2.28	0.325
Runx2	runt related transcription factor 2	0.544	0.373
Klf4	Klf4	0.6	0.537
Etv2	ets variant gene 2	1.532	0.592
Hand1	**heart & neural crest derivatives expressed transcript 1**	**0.469**	**0.073**
Raldh2	**aldehyde dehydrogenase family 1, subfamily A2**	**0.297**	**0.083**
Gata6	GATA binding protein 6	0.622	0.464
Sae1	SUMO1 activating enzyme subunit 1	0.382	0.511
Smarca2	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2	0.668	0.568
Smarcd3	**SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 3**	**0.324**	**0.072**
Nxf3	**nuclear RNA export factor 3**	**0.188**	**0.01**

Gene Symbol	Gene Title	Fold Change	q-value
Ablim1	actin-binding LIM protein 1	0.569	0.219
Abtb2	**ankyrin repeat and BTB (POZ) domain containing 2**	**0.412**	**0.055**
Ank2	**ankyrin 2, brain**	**0.35**	**0.056**
E2f5	E2F transcription factor 5	0.439	0.58
Egfr	epidermal growth factor receptor	0.644	0.351
Igf1	insulin-like growth factor 1	0.58	0.373
Inpp5d	inositol polyphosphate-5-phosphatase D	0.432	0.47
Nfib	nuclear factor I/B	2	0.109

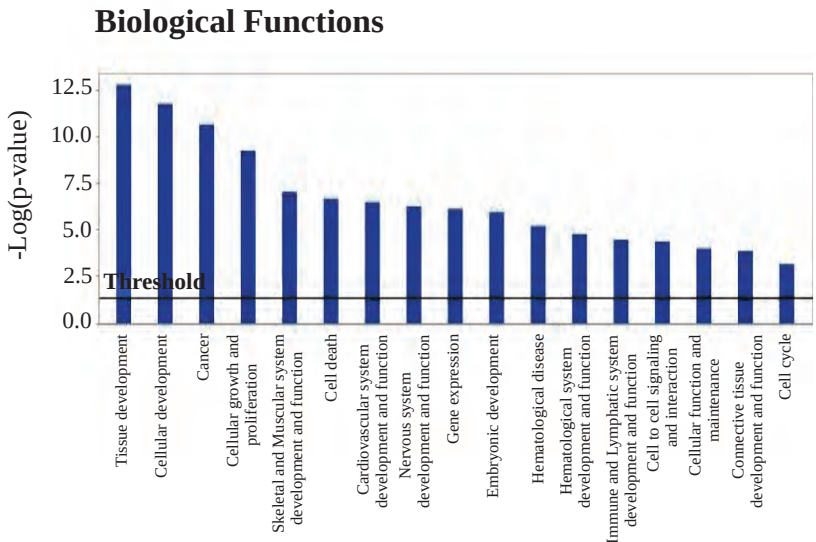
Biological functions and signalling pathways affected due to the deletion of *Ldb1* in Flk1+ cells

Gene expression analysis of wild type and *Ldb1* null Flk1+ cells identified a large number of genes that showed misregulation in their expression pattern. With the use of the Ingenuity Pathways Analysis software the genes found to be differentially expressed in the wild type and the *Ldb1* null Flk1+ cells were associated with specific biological functions (Table 2). A differentially expressed gene can be associated with more than one biological function. The graph in Fig. 6A shows the significance values of the biological function analysis, which are a measurement of the probability that the association of a group of genes with a specific biological function was accurate. The negative logarithm of the p-value with a threshold of 5% was used as the measurement of the significance. A higher p-value represents the higher probability that the allocation of the genes in a biological function is not correct. Genes that are involved in tissue development, cellular development, growth and proliferation, embryonic development, gene expression, cell death and cancer show differential expression in the *Ldb1*-/- Flk1+ cells. Cell cycle and cell signalling genes are affected much less, since very few genes that are involved in these process show changes in their expression pattern due too the absence of *Ldb1*. A number of genes that show differential expression in the *Ldb1* null Flk1+ cells participate in the development of the hematopoietic and immune system, but also in the development of the nervous and cardiovascular systems. Additionally the Ingenuity Pathways Analysis software was used to group the differentially expressed genes according to the biological pathway that they are more likely to participate in, as shown in Fig, 6B. The ratio of the number of genes allocated to a biological pathway over all the differentially expressed genes is represented on the y-axis, while the yellow line represents the logarithm of the p-value, the threshold for which was set at 5% and is used as a measurement of the probability that the allocation of genes to a specific biological pathway was random. A low p-value indicates that the allocation of a group of genes to a specific biological pathway is correct. As was mentioned in the biological function analysis a gene may be allocated to more than one pathway. Essential development pathways for the regulation of hematopoiesis such as, the Shh signalling pathway [44], the BMP pathway [45-47], the WNT pathway [48, 49], the Notch signalling pathway [50, 51], the FGF signalling pathway [52, 53], the MAPK signalling pathway [54, 55], erythropoietin signalling [56-58], the TGF-beta signalling pathway [59-62] are affected in the *Ldb1* null Flk1+ cells while the p53 and VEGF signalling pathways are not affected by changes in gene expression in these cells.

Table 2. The genes that were found to be differentially expressed in the *Ldb1* null Flk1+ cells were grouped according to the biological function they are most likely to participate in, using the Ingenuity Pathways Analysis software.

Biological Function	Molecules
Tissue Development	262
Cellular Development	321
Cancer	489
Cellular Growth and Proliferation	408
Skeletal and Muscular System Development and Function	151
Cell Death	351
Cardiovascular System Development and Function	126
Nervous System Development and Function	222
Gene Expression	268
Embryonic Development	136
Hematological Disease	126
Hematological System Development and Function	148
Immune and Lymphatic System Development and Function	89
Cell-To-Cell Signalling and Interaction	195
Cellular Function and Maintenance	19
Connective Tissue Development and Function	34
Cell Cycle	145

A.



B.

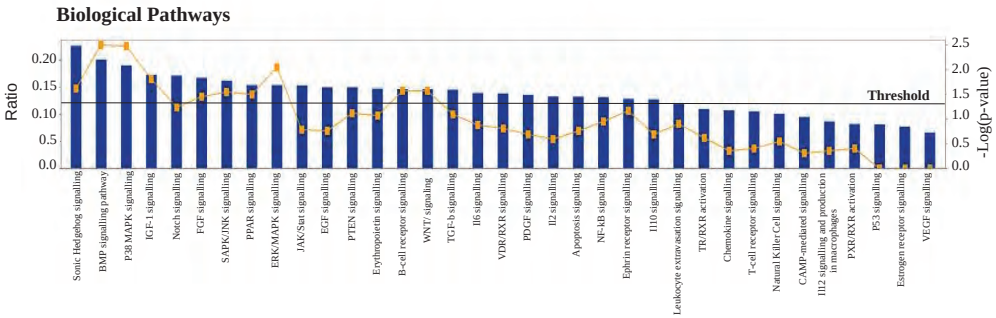


Figure 6. (A) Significance values of the functional analysis done using the Ingenuity Pathways Analysis software. The negative logarithm of the p-value (y-axis) is used in a graphic representation as a measurement of the likelihood that a group of genes was associated with a biological function during the analysis accurately. The threshold for the p-value was set at 5%. The higher the –log (p-value), the lower the probability that the allocation of a group of genes to a specific biological function is wrong. (B) Grouping of the genes found to be differentially expressed in the *Ldb1* null Flk1+ cells into the pathways they are most likely to participate, using the Ingenuity Pathways Analysis software. The y-axis represents the ratio of the genes allocated to a specific pathway over the total number of the differentially expressed genes. The yellow line represents the probability that the allocation of genes to a specific biological pathway was accurate.

Identification of genes involved in hematopoietic regulation that show altered expression in *Ldb1* null Flk1+ cells

The analysis described above showed changes in the expression pattern of genes that are involved in the regulation of mouse embryonic development and more specifically in the regulation of hematopoiesis. A group of these genes was selected based on their possible involvement in hematopoiesis and hemangioblast development (Fig. 8) and will be discussed below.

As mentioned above, *Gata-1*-/- embryoid bodies suffer from a defect in primitive hematopoiesis. Gene expression analysis of blast colonies at different time points in their development has shown that the expression of *Gata-1* starts and is steadily up-regulated after 24 hours of blast colony formation [63].

The involvement of *Runx1* in hematopoiesis has been investigated with the use of *Runx1* -/- mice and embryoid bodies. In accordance with the mouse phenotype *Runx1*-/- embryoid bodies lacked definitive hematopoietic progenitors, however they also gave rise to fewer blast colony forming cells, which indicates that contrary to previous observations *Runx1* may also be involved in the regulation of hemangioblast development [9, 64]. A defect in primitive hematopoiesis and yolk sac erythroblast development was observed in mice heterozygous for the gene fusion CBFβ-MYH11 caused by an inversion in chromosome 16 [65] due to a possible interference with the correct function of Runx1 [66]. During blast colony formation *Runx1* was found to be expressed at low levels after the first 24 hours, with the expression increasing after 48 hours as the first hematopoietic progenitors of the erythroid and macrophage lineages begin to emerge [63].

The involvement of *Fli1* in hematopoiesis and hemangioblast development has been examined in detail in zebrafish and *Xenopus* embryos. A wide range of morpholino experiments has shown that in the absence of *Fli1*, hemangioblast cells do not differentiate and undergo apoptosis due to a block in their development, leading to the conclusion that the gene is essential for both hematopoietic and endothelial development. *Fli1*, probably regulated by the BMP pathway, was found to act upstream of the essential hematopoietic regulators *Gata2*, *Scl*, and *Lmo2* [67].

Scl was not identified as differentially expressed in the microarray analysis of the *Ldb1* null Flk1+ cells; however the use of quantitative real time PCR did show that the expression is down-regulated in these cells (Fig 6).

Lyl1 shares an 82% homology with Scl in the DNA binding region. Additionally the bHLH protein participates in the same complex with Ldb1 in non-induced proliferating MEL cells. Mouse knockout experiments have shown that despite the above observations, its involvement in hematopoiesis is different from *Scl* since its deletion does not lead to embryonic lethality and the only hematopoietic defect the mice suffer from is lower B-cell count [68].

The involvement of *Id2* and *Id4* in the regulation of hemangioblast development has been investigated with *in vitro* BL-CFC assays. Id2 and Id4 belong to the Id family of HLH

proteins; they do not bind DNA and mainly function as repressors by forming heterodimers with bHLH proteins that render them unable to bind DNA thereby eliminating their function. Both genes are expressed in ES cells, while in blast colonies *Id4* is not expressed and *Id2* is expressed at a constant level [69]. In the *Ldb1* null Flk1+ cells the observed up-regulation could inhibit the function of bHLH factors such as *Scl1* or *Lyl1*.

The KLF family member *Klf4* was also down-regulated in the *Ldb1* null Flk1+ cells. *Klf4* was shown to participate in the regulation of embryonic globin expression and primitive but not definitive hematopoiesis in zebrafish knockdown experiments [70].

The *Ldb1* null Flk1+ cells express higher levels of the mesodermal marker *brachyury* compared to the wild type Flk1+ cells. *Brachyury* is initially expressed in Flk1+ BL-CFCs but the expression is lost after the first 24 hours of blast colony formation [63].

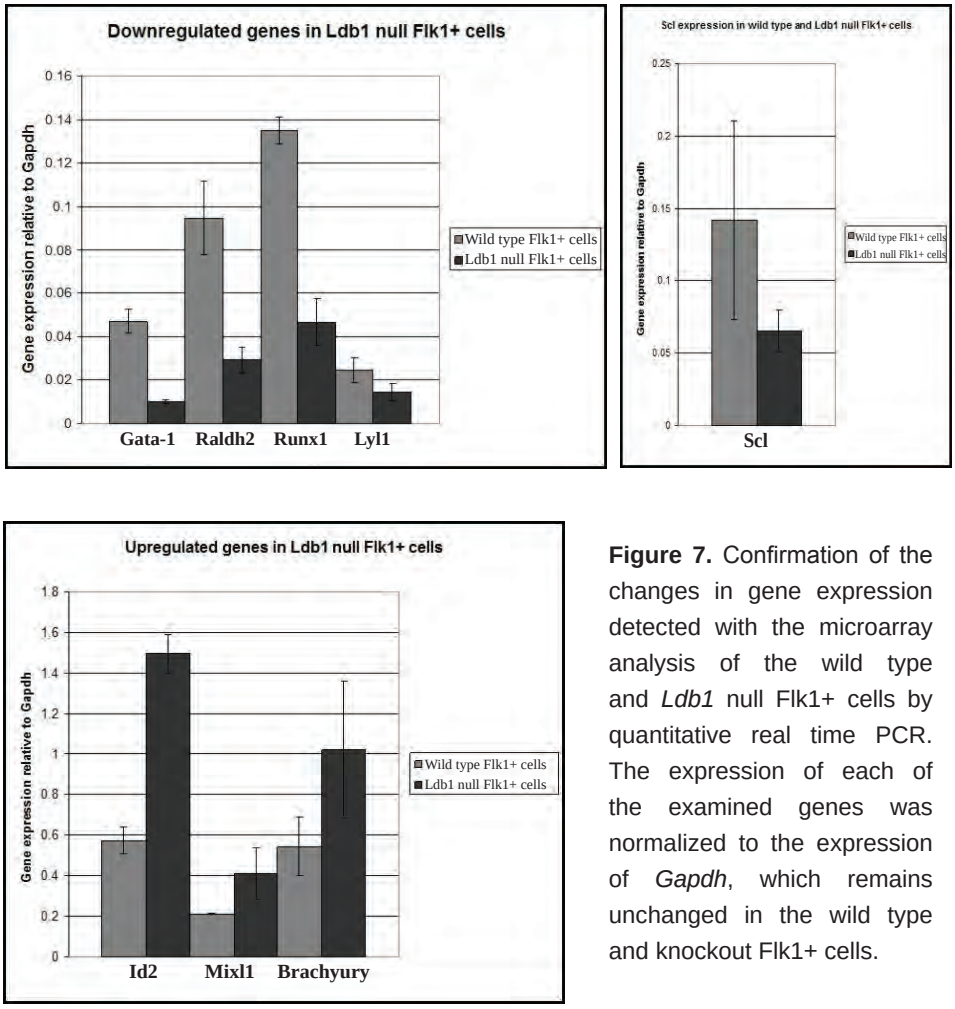


Figure 7. Confirmation of the changes in gene expression detected with the microarray analysis of the wild type and *Ldb1* null Flk1+ cells by quantitative real time PCR. The expression of each of the examined genes was normalized to the expression of *Gapdh*, which remains unchanged in the wild type and knockout Flk1+ cells.

The WNT signalling pathway has a positive role in the regulation of primitive but not definitive hematopoietic development [71]. When the pathway is blocked with the addition of the inhibitor Dkk1, the potential of Flk1+ hemangioblasts to generate blast colonies with a primitive hematopoietic potential is severely reduced, while the effect is rescued by the addition of β-catenin. The action of Dkk1 in the WNT pathway regulation of hematopoiesis is highly time dependent. The addition of Dkk1 was found to have no effect in definitive hematopoietic development. The expression of the WNT pathway inhibitor *Sfrp5* begins after 12 hours of blast colony growth and is gradually reduced in time while *Sfrp1* and *Sfrp2* expression begins after 48 hours of blast colony formation [63]. All three inhibitor genes are down-regulated in the *Ldb1* null Flk1+ cells. A severe reduction in the expression of *Sfrp1* and has also been previously observed in the *Ldb1*-/- mouse embryo [38].
Gfi1b is a zinc finger proto-oncogene with repressor activity. The deletion of the gene leads to hemorrhages in the embryos and lethality after 15 dpc. In the *Gfi1b*-/- mouse embryos primitive erythrocytes are present but their morphology is abnormal and their maturation is delayed. Fetal liver definitive erythropoiesis in the knockout embryos is impaired, while megakaryocyte development shows a maturation defect [72]. *Gfi1b* is probably not involved in the initial emergence of hematopoietic progenitor cells but is involved in the subsequent differentiation and maturation steps.
Eto-2, as mentioned previously, participates in the Ldb1/Scl regulatory complexes in erythroid cells. The stoichiometry of Eto-2 to Scl is very important within such a complex, since Eto-2 functions mainly as a repressor to the activator function of Scl. During hematopoiesis the self-renewal versus differentiation of hematopoietic progenitors is a tightly controlled. It is proposed that the Eto-2 to Scl ratio is essential for the fine tuning of this process as Eto-2 is required for the proliferation of erythroid progenitors but not for their differentiation [73].
The *c-myb* proto-oncogene is present in erythroid, myeloid and lymphoid progenitors and its expression is reduced as the cells differentiate. The deletion of *c-myb* in mice leads to severe anemia at 15 dpc and death soon after. The number of nucleated erythrocytes from the wave of primitive erythropoiesis is similar in the wild type and knockout embryos. In contrast the number of enucleated erythrocytes is severely reduced in the *c-myb*-/- mice. These observations led to the conclusion that the gene is essential for definitive erythropoiesis only [74]. Thus although the expression of *c-myb* is down-regulated in the *Ldb1*-/- Flk1+ cells, its involvement in the process of hemangioblast differentiation is probably dispensable. This does not exclude the possibility that *c-myb* may be involved in the regulatory pathway that leads to the emergence of definitive hematopoietic progenitors in the yolk sac, or in the regulation of blast colony formation.
Eklf was also down-regulated in the *Ldb1* null Flk1+ cells. *Eklf* deficient embryos die from anemia at 14 dpc and suffer from a reduction in β-globin expression. The effect of the deletion in primitive hematopoiesis is much more subtle, since primitive erythroid cells in the knockout mouse embryos are functional even though they demonstrate some morphological abnormalities [75-77].

Definitive hematopoiesis and megakaryocyte development are also defective in mice with a homozygous deletion of *Meis1* [78], another gene expressed at lower levels in the *Ldb1* null Flk1+ cells.

Raldh2 is involved in the synthesis of retinoic acid from vitamin A (retinol) in the mouse embryo. Its deletion leads to embryonic lethality at 10.5 dpc due to the lack of retinoic acid synthesis [79, 80]. It is expressed in the visceral endoderm of the mouse embryo yolk sac between 7.5 dpc and 8.5 dpc, which is the time when vascular development commences in the adjacent mesoderm. In addition yolk sac endothelial cells express retinoic acid receptors. In the absence of *Raldh2* and consequently RA yolk sac vascular development is defective. *Raldh2* is not an important factor for the regulation for endothelial cell differentiation. Instead it is involved in endothelial cell maturation, due to its function as an inhibitor of cell growth and proliferation, via the up-regulation of *p21* and *p27*, which act as inhibitors to the Cdk4 mediated transition from G1 to S during the cell cycle [81, 82]. *Raldh2* has not been shown to be involved in primitive hematopoiesis. However a novel role for the gene as an inducer of differentiation of the hemogenic endothelium in definitive hematopoietic development has been identified [83].

Studies performed in chick embryos have suggested that the FGF signalling pathway is involved in maintaining hematopoietic progenitor cells in an immature state by exerting a block in their final differentiation. The main regulator of this pathway *Fgfr2* [52] is down-regulated in the *Ldb1* null Flk1+ cells, which should lead to an increased level of differentiation, since the block can no longer be maintained. However the opposite effect is obvious in the *Ldb1* null Flk1+ cells, as they lack any differentiation potential, which in light of the severe down-regulation in the expression of many essential hematopoietic regulatory factors is to be expected.

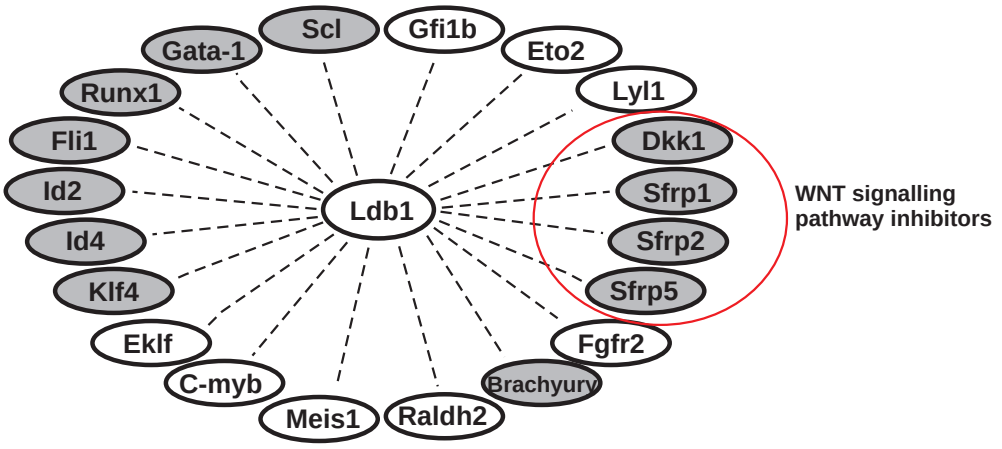


Figure 8. Possible downstream targets of *Ldb1* in Flk1+ BL-CFCs identified through gene expression arrays. The grey circles represent genes that may be involved in primitive hematopoiesis regulation and hemangioblast development. The white circles represent genes for which an involvement in definitive hematopoiesis only has been demonstrated so far.

Conclusion

The deletion of *Ldb1* in ES cells did not affect their capacity to self-renew or generate embryoid bodies. Therefore we can conclude that *Ldb1* is not involved in the regulation of essential ES cell genes and is not necessary for their survival, proliferation and maintenance their ability to differentiate. It has been shown in *Drosophila* primarily but also in the mouse that the involvement of *Ldb1* in the regulation of development, centres around its function as a bridging molecule in complexes of transcription factors bound on distant DNA regulatory sequences. It is possible that long range interactions between promoters and other regulatory elements positioned up- or downstream of a gene and requiring *Ldb1* do not play an important role in the transcription regulation of essential genes in ES cells. Probably such interactions are also not required during the early steps of ES cell differentiation. ES cells are derived from the inner cell mass (ICM) of the blastocyst at 3.5 dpc. They can be maintained in adherent cultures for an unlimited period of time, due to their ability to self-renew. It is likely that the requirement for the function of gene regulatory elements located within a long distance of a promoter and the transcription initiation site takes place during more distinct differentiation pathways that will give rise to one specific progenitor cell population. At that point a bridging molecule that will act in order to bring together DNA bound regulatory factors for the formation of transcription regulatory complex may be needed.

Our results show that *Ldb1* is involved in the proliferation and differentiation of BL-CFCs, the *in vitro* equivalent of the hemangioblast. *Ldb1* null embryoid bodies grown for 4 days contain half the number of BL-CFCs compared to the wild type ones, while the cells do not have the potential to develop into fully grown blast colonies and eventually die in culture. These observations also explain the hematopoietic and vascular phenotype of the *Ldb1*-/- embryos and show that *Ldb1* is crucial for the regulation of the earliest step of hematopoiesis.

It is still not clear how the hemangioblast emerges from the mouse embryonic mesoderm and how exactly the subsequent differentiation into hematopoietic and endothelial progenitors is regulated, although loss of function studies in mice and ES cells have provided valuable information towards our understanding of these processes. Here we show that the *Ldb1* gene acts upstream of a number of essential hematopoietic factors, such as *Fli1*, *Scl*, *Gata1*, *Runx1*, *Gfi1b*, *Eto2*, *c-myb* and others, while it has previously been shown to form complexes with a number of these factors for transcription regulation in erythroid cells. The absence of these hematopoietic regulators from the Flk1+ cells explains their failure

to develop into blast colonies. In more detail, the deletion of *Runx1* has been previously shown to result in the emergence of fewer blast colonies from embryoid bodies. The down-regulation of *Runx1* in the *Ldb1* null embryoid bodies could be the cause of the decrease seen in the number of Flk1+ cells. However while *Runx1* is involved in definitive hematopoiesis development, its role in primitive hematopoiesis is not as essential, since primitive hematopoietic colonies and blast colonies are still able to grow in its absence. The inability of the Flk1+ cells that are present in the *Ldb1* null embryoid bodies to grow into blast colonies can be attributed to the loss of *Fli1*, since a similar phenotype has been shown in *Xenopus* morpholino experiments. Lack of *Fli1* expression was shown to lead to an end of the hemangioblast differentiation program and subsequent apoptosis, similar to the phenotype seen in the *Ldb1* null Flk1+ cells.
Furthermore the up-regulation of the WNT pathway inhibitor *Dkk1* and the down-regulation of the same pathway inhibitors *Sfrp1*, *Sfrp2* and *Sfrp5* is an indicator that the pathway, which participates in the control of primitive hematopoiesis, is severely misregulated. It is interesting to note that the expression of *Brachyury* is up-regulated in the *Ldb1* null Flk1+ cells. The gene is expressed in the mesoderm and was found to be expressed only during the first 12 hours of blast colony formation. The up-regulation of *Brachyury* can indicate that the *Ldb1* null Flk1+ cells have not lost their mesodermal identity/properties and hence are unable to differentiate into blast colonies.
In summary *Ldb1* was identified as an essential regulator of the proliferation and differentiation of the mouse hemangioblast. The severity of the hematopoietic phenotype is the result of a decreased number of hemangioblast cells and their inability to differentiate further down the hematopoietic and endothelial lineages. The control that *Ldb1* exerts in hematopoietic development is two-fold. First protein complexes that act as transcription regulators in hematopoietic development are obviously not able to form in the absence of *Ldb1* and secondly the expression of essential transcription factors for hematopoietic development is misregulated, placing *Ldb1* upstream of the regulatory pathways that control hematopoietic development. How exactly *Ldb1* controls the expression of such a large number of transcription factors remains to be investigated. Identifying gene targets of *Ldb1* in Flk1+ cells and embryoid bodies grown for 2-4 days will provide valuable information for this question.

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References

1. Baron, M.H., *Early patterning of the mouse embryo: implications for hematopoietic commitment and differentiation.* Exp Hematol, 2005. **33**(9): p. 1015-20.
2. Maximow, *Untersuchungen uber blut und bindegewebe 1. Die fruhesten entwicklungsstadien der blut und bindegewebszellan bein saugeberembryo, bis zum anfang der blutbilding unden leber.* Arch Mikroskop Anat, 1909. **73**.
3. Barker, J.E., *Development of the mouse hematopoietic system. I. Types of hemoglobin produced in embryonic yolk sac and liver.* Dev Biol, 1968. **18**(1): p. 14-29.
4. Haar, J.L. and G.A. Ackerman, *A phase and electron microscopic study of vasculogenesis and erythropoiesis in the yolk sac of the mouse.* Anat Rec, 1971. **170**(2): p. 199-223.
5. Moore, M.A. and D. Metcalf, *Ontogeny of the haemopoietic system: yolk sac origin of in vivo and in vitro colony forming cells in the developing mouse embryo.* Br J Haematol, 1970. **18**(3): p. 279-96.
6. Palis, J., et al., *Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse.* Development, 1999. **126**(22): p. 5073-84.
7. Medvinsky, A. and E. Dzierzak, *Definitive hematopoiesis is autonomously initiated by the AGM region.* Cell, 1996. **86**(6): p. 897-906.
8. Dzierzak, E., *Ontogenic emergence of definitive hematopoietic stem cells.* Curr Opin Hematol, 2003. **10**(3): p. 229-34.
9. Lacaud, G., et al., *Regulation of hemangioblast development.* Ann N Y Acad Sci, 2001. **938**: p. 96-107; discussion 108.
10. Sabin, *Studies on the origin of blood vessels and of red corpuscles as seen in the living blastoderm of the chick during the second day of incubation.* Contrib Embryol, 1920. **9**: p. 213–262.
11. Xiong, J.W., *Molecular and developmental biology of the hemangioblast.* Dev Dyn, 2008. **237**(5): p. 1218-31.
12. Huber, T.L., et al., *Haemangioblast commitment is initiated in the primitive streak of the mouse embryo.* Nature, 2004. **432**(7017): p. 625-30.
13. Yano, M., et al., *Expression and function of murine receptor tyrosine kinases, TIE and TEK, in hematopoietic stem cells.* Blood, 1997. **89**(12): p. 4317-26.
14. Anagnostou, A., et al., *Erythropoietin receptor mRNA expression in human endothelial cells.* Proc Natl Acad Sci U S A, 1994. **91**(9): p. 3974-8.
15. Nishikawa, S.I., et al., *Progressive lineage analysis by cell sorting and culture identifies FLK1+VE-cadherin+ cells at a diverging point of endothelial and hemopoietic lineages.* Development, 1998. **125**(9): p. 1747-57.
16. Fina, L., et al., *Expression of the CD34 gene in vascular endothelial cells.* Blood, 1990. **75**(12): p. 2417-26.
17. Watt, S.M., S.E. Gschmeissner, and P.A. Bates, *PECAM-1: its expression and function as a cell adhesion molecule on hemopoietic and endothelial cells.* Leuk Lymphoma, 1995. **17**(3-4): p. 229-44.
18. Kallianpur, A.R., J.E. Jordan, and S.J. Brandt, *The SCL/TAL-1 gene is expressed in progenitors of both the hematopoietic and vascular systems during embryogenesis.* Blood, 1994. **83**(5): p. 1200-8.
19. Keller, G.M., *In vitro differentiation of embryonic stem cells.* Curr Opin Cell Biol, 1995. **7**(6): p. 862-9.
20. Choi, K., *Hemangioblast development and regulation.* Biochem Cell Biol, 1998. **76**(6): p. 947-56.
21. Shalaby, F., et al., *Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice.* Nature, 1995. **376**(6535): p. 62-6.
22. Begley, C.G., et al., *Chromosomal translocation in a human leukemic stem-cell line disrupts the T-cell antigen receptor delta-chain diversity region and results in a previously unreported fusion transcript.* Proc Natl Acad Sci U S A, 1989. **86**(6): p. 2031-5.
23. Robb, L., et al., *Absence of yolk sac hematopoiesis from mice with a targeted disruption of the scl gene.* Proc Natl Acad Sci U S A, 1995. **92**(15): p. 7075-9.
24. Visvader, J.E., Y. Fujiwara, and S.H. Orkin, *Unsuspected role for the T-cell leukemia protein SCL/tal-1 in vascular development.* Genes Dev, 1998. **12**(4): p. 473-9.
25. D'Souza, S.L., A.G. Elefanty, and G. Keller, *SCL/Tal-1 is essential for hematopoietic commitment of the hemangioblast but not for its development.* Blood, 2005. **105**(10): p. 3862-70.
26. Warren, A.J., et al., *The oncogenic cysteine-rich LIM domain protein rbtn2 is essential for erythroid development.* Cell, 1994. **78**(1): p. 45-57.
27. Tsai, F.Y., et al., *An early haematopoietic defect in mice lacking the transcription factor GATA-2.* Nature, 1994. **371**(6494): p. 221-6.

28. Weiss, M.J., G. Keller, and S.H. Orkin, *Novel insights into erythroid development revealed through in vitro differentiation of GATA-1 embryonic stem cells.* Genes Dev, 1994. **8**(10): p. 1184-97.
29. Fujiwara, Y., et al., *Arrested development of embryonic red cell precursors in mouse embryos lacking transcription factor GATA-1.* Proc Natl Acad Sci U S A, 1996. **93**(22): p. 12355-8.
30. Wadman, I.A., et al., *The LIM-only protein Lmo2 is a bridging molecule assembling an erythroid, DNA-binding complex which includes the TAL1, E47, GATA-1 and Ldb1/NLI proteins.* Embo J, 1997. **16**(11): p. 3145-57.
31. Xu, Z., et al., *Identification of a TAL1 target gene reveals a positive role for the LIM domain-binding protein Ldb1 in erythroid gene expression and differentiation.* Mol Cell Biol, 2003. **23**(21): p. 7585-99.
32. Lahlil, R., et al., *SCL assembles a multifactorial complex that determines glycophorin A expression.* Mol Cell Biol, 2004. **24**(4): p. 1439-52.
33. Meier, N., et al., *Novel binding partners of Ldb1 are required for haematopoietic development.* Development, 2006. **133**(24): p. 4913-23.
34. Agulnick, A.D., et al., *Interactions of the LIM-domain-binding factor Ldb1 with LIM homeodomain proteins.* Nature, 1996. **384**(6606): p. 270-2.
35. Xu, Z., et al., *Single-stranded DNA-binding proteins regulate the abundance of LIM domain and LIM domain-binding proteins.* Genes Dev, 2007. **21**(8): p. 942-55.
36. Matthews, J.M. and J.E. Visvader, *LIM-domain-binding protein 1: a multifunctional cofactor that interacts with diverse proteins.* EMBO Rep, 2003. **4**(12): p. 1132-7.
37. Morcillo, P., et al., *Chip, a widely expressed chromosomal protein required for segmentation and activity of a remote wing margin enhancer in Drosophila.* Genes Dev, 1997. **11**(20): p. 2729-40.
38. Mukhopadhyay, M., et al., *Functional ablation of the mouse Ldb1 gene results in severe patterning defects during gastrulation.* Development, 2003. **130**(3): p. 495-505.
39. Yamashita, T., et al., *Genomic structure and chromosomal localization of the mouse LIM domain-binding protein 1 gene, Ldb1.* Genomics, 1998. **48**(1): p. 87-92.
40. Ferkowicz, M.J., et al., *CD41 expression defines the onset of primitive and definitive hematopoiesis in the murine embryo.* Development, 2003. **130**(18): p. 4393-403.
41. Baldwin, H.S., et al., *Platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31): alternatively spliced, functionally distinct isoforms expressed during mammalian cardiovascular development.* Development, 1994. **120**(9): p. 2539-53.
42. Choi, K., et al., *A common precursor for hematopoietic and endothelial cells.* Development, 1998. **125**(4): p. 725-32.
43. Kennedy, M., et al., *A common precursor for primitive erythropoiesis and definitive haematopoiesis.* Nature, 1997. **386**(6624): p. 488-93.
44. Bhardwaj, G., et al., *Sonic hedgehog induces the proliferation of primitive human hematopoietic cells via BMP regulation.* Nat Immunol, 2001. **2**(2): p. 172-80.
45. Schmerer, M. and T. Evans, *Primitive erythropoiesis is regulated by Smad-dependent signaling in postgastrulation mesoderm.* Blood, 2003. **102**(9): p. 3196-205.
46. Snyder, A., S.T. Fraser, and M.H. Baron, *Bone morphogenetic proteins in vertebrate hematopoietic development.* J Cell Biochem, 2004. **93**(2): p. 224-32.
47. Walmsley, M., A. Ciau-Uitz, and R. Patient, *Adult and embryonic blood and endothelium derive from distinct precursor populations which are differentially programmed by BMP in Xenopus.* Development, 2002. **129**(24): p. 5683-95.
48. Austin, T.W., et al., *A role for the Wnt gene family in hematopoiesis: expansion of multilineage progenitor cells.* Blood, 1997. **89**(10): p. 3624-35.
49. Van Den Berg, D.J., et al., *Role of members of the Wnt gene family in human hematopoiesis.* Blood, 1998. **92**(9): p. 3189-202.
50. Maillard, I., Y. He, and W.S. Pear, *From the yolk sac to the spleen: New roles for Notch in regulating hematopoiesis.* Immunity, 2003. **18**(5): p. 587-9.
51. Robert-Moreno, A., et al., *Impaired embryonic haematopoiesis yet normal arterial development in the absence of the Notch ligand Jagged1.* Embo J, 2008. **27**(13): p. 1886-95.
52. Nakazawa, F., et al., *Negative regulation of primitive hematopoiesis by the FGF signaling pathway.* Blood, 2006. **108**(10): p. 3335-43.
53. Ratajczak, M.Z., et al., *Effect of basic (FGF-2) and acidic (FGF-1) fibroblast growth factors on early haemopoietic cell development.* Br J Haematol, 1996. **93**(4): p. 772-82.
54. Elorza, A., et al., *UCP2 modulates cell proliferation through the MAPK/ERK pathway during erythropoiesis and has no effect on heme biosynthesis.* J Biol Chem, 2008. **283**(45): p. 30461-70.
55. Filippi, M.D., et al., *Requirement for mitogen-activated protein kinase activation in the response*

of embryonic stem cell-derived hematopoietic cells to thrombopoietin in vitro. Blood, 2002. **99**(4): p. 1174-82.
56. Lee, R., et al., *Erythropoietin (Epo) and EpoR expression and 2 waves of erythropoiesis.* Blood, 2001. **98**(5): p. 1408-15.
57. Munugalavadla, V. and R. Kapur, *Role of c-Kit and erythropoietin receptor in erythropoiesis.* Crit Rev Oncol Hematol, 2005. **54**(1): p. 63-75.
58. Richmond, T.D., M. Chohan, and D.L. Barber, *Turning cells red: signal transduction mediated by erythropoietin.* Trends Cell Biol, 2005. **15**(3): p. 146-55.
59. Dickson, M.C., et al., *Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice.* Development, 1995. **121**(6): p. 1845-54.
60. Kim, S.J. and J. Letterio, *Transforming growth factor-beta signaling in normal and malignant hematopoiesis.* Leukemia, 2003. **17**(9): p. 1731-7.
61. Oshima, M., H. Oshima, and M.M. Taketo, *TGF-beta receptor type II deficiency results in defects of yolk sac hematopoiesis and vasculogenesis.* Dev Biol, 1996. **179**(1): p. 297-302.
62. Perlingeiro, R.C., *Endoglin is required for hemangioblast and early hematopoietic development.* Development, 2007. **134**(16): p. 3041-8.
63. Cheng, X., et al., *Numb mediates the interaction between Wnt and Notch to modulate primitive erythropoietic specification from the hemangioblast.* Development, 2008. **135**(20): p. 3447-58.
64. Lacaud, G., et al., *Runx1 is essential for hematopoietic commitment at the hemangioblast stage of development in vitro.* Blood, 2002. **100**(2): p. 458-66.
65. Le Beau, M.M., et al., *Association of an inversion of chromosome 16 with abnormal marrow eosinophils in acute myelomonocytic leukemia. A unique cytogenetic-clinicopathological association.* N Engl J Med, 1983. **309**(11): p. 630-6.
66. Castilla, L.H., et al., *Failure of embryonic hematopoiesis and lethal hemorrhages in mouse embryos heterozygous for a knocked-in leukemia gene CBFB-MYH11.* Cell, 1996. **87**(4): p. 687-96.
67. Liu, F., et al., *Fli1 acts at the top of the transcriptional network driving blood and endothelial development.* Curr Biol, 2008. **18**(16): p. 1234-40.
68. Capron, C., et al., *The SCL relative LYL-1 is required for fetal and adult hematopoietic stem cell function and B-cell differentiation.* Blood, 2006. **107**(12): p. 4678-86.
69. Nogueira, M.M., et al., *Regulation of Id gene expression during embryonic stem cell-derived hematopoietic differentiation.* Biochem Biophys Res Commun, 2000. **276**(2): p. 803-12.
70. Gardiner, M.R., et al., *A global role for zebrafish klf4 in embryonic erythropoiesis.* Mech Dev, 2007. **124**(9-10): p. 762-74.
71. Nostro, M.C., et al., *Wnt, activin, and BMP signaling regulate distinct stages in the developmental pathway from embryonic stem cells to blood.* Cell Stem Cell, 2008. **2**(1): p. 60-71.
72. Saleque, S., S. Cameron, and S.H. Orkin, *The zinc-finger proto-oncogene Gfi-1b is essential for development of the erythroid and megakaryocytic lineages.* Genes Dev, 2002. **16**(3): p. 301-6.
73. Goardon, N., et al., *ETO2 coordinates cellular proliferation and differentiation during erythropoiesis.* Embo J, 2006. **25**(2): p. 357-66.
74. Mucenski, M.L., et al., *A functional c-myb gene is required for normal murine fetal hepatic hematopoiesis.* Cell, 1991. **65**(4): p. 677-89.
75. Perkins, A.C., A.H. Sharpe, and S.H. Orkin, *Lethal beta-thalassaemia in mice lacking the erythroid CACCC-transcription factor EKLF.* Nature, 1995. **375**(6529): p. 318-22.
76. Nuez, B., et al., *Defective haematopoiesis in fetal liver resulting from inactivation of the EKLF gene.* Nature, 1995. **375**(6529): p. 316-8.
77. Drissen, R., et al., *The erythroid phenotype of EKLF-null mice: defects in hemoglobin metabolism and membrane stability.* Mol Cell Biol, 2005. **25**(12): p. 5205-14.
78. Azcoitia, V., et al., *The homeodomain protein Meis1 is essential for definitive hematopoiesis and vascular patterning in the mouse embryo.* Dev Biol, 2005. **280**(2): p. 307-20.
79. Niederreither, K., et al., *Embryonic retinoic acid synthesis is essential for early mouse post-implantation development.* Nat Genet, 1999. **21**(4): p. 444-8.
80. Zhao, D., et al., *Molecular identification of a major retinoic-acid-synthesizing enzyme, a retinaldehyde-specific dehydrogenase.* Eur J Biochem, 1996. **240**(1): p. 15-22.
81. Bohnsack, B.L., et al., *Signaling hierarchy downstream of retinoic acid that independently regulates vascular remodeling and endothelial cell proliferation.* Genes Dev, 2004. **18**(11): p. 1345-58.
82. Lai, L., et al., *Retinoic acid regulates endothelial cell proliferation during vasculogenesis.* Development, 2003. **130**(26): p. 6465-74.
83. Goldie, L.C., et al., *Cell signaling directing the formation and function of hemogenic endothelium during murine embryogenesis.* Blood, 2008. **112**(8): p. 3194-204.

Chapter 4

Ldb1 is involved in the regulation of neural stem cell self-renewal

Ldb1 is involved in the regulation of neural stem cell self-renewal

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Abstract

The Ldb1 (LIM-Domain Binding 1) protein functions as a regulator of gene expression during cell differentiation and embryonic development. Due to its ability to bind on the LIM domains of LIM-Homeodomain and LMO proteins it participates together with bHLH and GATA factors in protein complexes that control transcription initiation. The involvement of *Ldb1* in the regulation of hematopoiesis has been established and studied in detail, while its role in other developmental processes such as neural development has been investigated to a lesser extent. The deletion of *Ldb1* in the mouse embryo leads to embryonic lethality after 9.5 dpc and the resulting phenotype is highly complex. Among the observed defects is a truncation of the anterior above the otic vesicle, which results in the absence of forebrain, midbrain and anterior hindbrain and abnormal neural tube morphology. In order to investigate the role of *Ldb1* in neural development, neural stem cell lines were generated from *Ldb1*+/+ and *Ldb1*-/- ES cells. The deletion of *Ldb1* did not affect the ability of mouse ES cells to differentiate towards the neural lineage. However it did have a profound effect on the self-renewal capacity of the neural stem cells, which was identified as a severe delay in their proliferation, believed to be the result of prolonged cell cycle progression.

Work in progress

Introduction

Gastrulation begins after the implantation of the mouse embryo in the uterus at 6.5 dpc and results in the formation of the body plan, which acts as a guidance for the subsequent morphogenesis. During gastrulation mesoderm induction from the primitive streak is initiated. Afterwards the mouse embryo consists of three definitive germ layers, which are ectoderm and endoderm in addition to mesoderm. These germ layers will give rise to the different tissues and organs of the embryo [1]. Ectodermal cells receiving signals from the notochord acquire neural specification giving rise to the neuroectoderm and subsequently the neural plate, which folds and forms the neural groove consisting of the left and right neural folds. The two unite and generate the neural tube, which will give rise to the brain and spinal cord of the embryo [2]. The neural crest forms at the edge of the neural plate and the adjacent ectoderm and consists of pluripotent cells that are able to migrate into different parts of the embryo body and give rise to a variety of neural and non-neural cells types. The differentiation fate of the neural crest cells, which are generated along most of the vertebrae axis, depends on their original location along the body axis prior to their migration from the neural tube [3]. Neural crest cells differentiate towards the neurons and glial cells of the peripheral nervous system but they also give rise to melanocytes and endocrine cells, while they are also essential for craniofacial development as they give rise to mesenchymal derivatives such as cartilage, bone, muscle and connective tissue cells [4]. Since neural crest cells have both the ability to self-renew and to differentiate towards various cell types they are characterized as a stem cell population.

Mouse embryonic stem cells are derived from the inner cell mass (ICM) of the blastocyst at 3.5 dpc. They are characterized by self-renewal and pluripotency, as they can give rise to every cell type in the mouse embryo [5]. ES cells can be maintained in adherent cultures for an unlimited period of time, while being able to readily differentiate into committed cell types under specific culture conditions. Progenitor cells have also the ability to self-renew albeit for a limited number of cell divisions before committing to a fully differentiated cell type. Often progenitor cells appear as an intermediate derivative during the differentiation process of ES cells [6]. Stem cells in the mouse embryo exist in specific micro-environments termed niches and are responsible for efficient tissue regeneration. Examples of these include the hematopoietic stem cells (HSCs), the neural stem cells (NSCs) and the epidermal stem cells. Their role is achieved through a tightly controlled balance between self-renewal of the stem cells and differentiation toward the specific tissue cell lineage [7].

The development of the central nervous system (CNS) relies on the presence of neural stem cells, known as neuroepithelial cells, in the neural tube. These cells demonstrate the two essential characteristics of stem cells as they are able to self-renew and also to generate neuronal and glial progenitor cells that will further differentiate to mature neurons and glial cells. Primary neural stem cells have been successfully isolated from the fetal forebrain and the adult sub-ventricular zone (SVZ) and maintained in adherent cultures

[8]. Neural stem cells have also been isolated from the neural tube [9]. The maintenance of these stem cells in culture requires the formation of neurospheres in suspension [10]. These are spherical cell clusters, which contain progenitor and differentiated cells of neural lineages together with neural stem cells [11]. However the actual number of true neural stem cells within a neurosphere is low [12] and these cells are difficult to maintain in culture due to the frequent occurrence of differentiation and cell death [13].

Ldb1 (LIM-Domain Binding 1) has been shown to participate in multiprotein complexes and to function as a bridging molecule, bringing together promoters and enhancer elements for transcription regulation [14, 15] [16-18]. The *Ldb1*-/- mouse is embryonic lethal after 9.5 dpc and suffers from severe developmental defects among which is a truncation of the head structure anterior of the otic vesicle. The anterior neural plate and the neural tube display a twisted shape, which is probably the result of a defect in the growth and expansion of the neuroepithelial cells. The expression of *engrailed 2*, which marks the boundary between the midbrain and the hindbrain is absent from the knockout embryos [19], while the expression of *Otx2*, which is involved in patterning of the anterior head structure [20, 21] is severely down-regulated (Mylona et al unpublished data). The deletion of *Otx2* in mice results in gastrulation defects and absence of a forebrain, midbrain and anterior hindbrain due to lack of neural plate induction [22].

The function of *Ldb1* has been mainly studied in hematopoiesis, in which the gene plays an important regulatory role, since in its absence primitive erythropoiesis and vascular development are impaired [19]; (Mylona et al unpublished data, Chapter 3 this thesis). However *Ldb1* has also been implicated in neural development initially from studies in *Drosophila* and also in mice. In *Drosophila* the deletion of the *Ldb1* ortholog, *dLdb* or *Chip*, causes a larval lethal phenotype. Chip is involved in neural development through the formation of a complex with apterous [23]. It also acts as a bridging molecule for the formation of a complex with the GATA factor Pannier, and the E-box bound ac/sc and Daughterless heterodimers. The complex regulates the expression of the neural development regulatory factor *ac/sc*. Additionally the dLdb/Pannier complex can recruit Osa, a member of the chromatin remodelling complex Brahma [24], for the repression of *ac/sc* expression [25].

A similar phenotype of anterior truncation with the *Ldb1*-/- mouse has been observed in the *Lim1*-/- mouse, due to misregulation of the head organizer [26] and the mouse mutant *headshrinker* (*hsk*), which suffers from a disruption in the expression of the gene *Ssbp1* [27]. The Ldb1 protein is able to form complexes with LIM-Only proteins (LMOs) and LIM-Homeodomain (LIM-HDs) proteins. The stoichiometry in the Ldb1/LIM-HD or Ldb1/LMO complexes is essential for their function as transcription regulators [28]. Ssbp proteins can protect the stoichiometry by binding on the LCCD domain of Ldb1 and prevent the ubiquitination and subsequent degradation of the formed complex by RLIM [29].

Ldb1 and RLIM were shown to co-express in the dorsal part of the developing neural tube of mouse embryos, between 8 dpc and 10 dpc, in the same regions that also express LIM-HD proteins [30], which play an important role in neural development [31] and the

differentiation of neurons [32, 33]. Ldb1 together with the LIM-HD proteins Lhx1 and Lhx5 are involved in the differentiation and proliferation of Purkinje cells, one of the two neuron types of the cerebellum [34]. Furthermore the ability of Ldb1 to act as a bridging molecule bringing together DNA bound factors has been demonstrated in motor neuron differentiation. In this case the dimerization of Ldb1 facilitates the formation of a complex with the LIM-HD regulatory factors Lhx3 and Isl1 and the E-box bound heterodimer NeuroM/E47 [35].

ES cells can be efficiently differentiated in culture into neural stem cells [36], which can be used as tool in order to investigate neural development. The participation of *Ldb1* in neural development has been demonstrated by its ability to form complexes with other regulatory factors that are important for that process and by the phenotype of the mouse knockout. However questions still remain about the direct functions of *Ldb1* and the identity of the genes, whose expression is regulated by Ldb1 containing complexes. In order to gain further insight into the role of *Ldb1* in neural development, a knockout ES cell line was created, which was then differentiated into a neural stem cell line. Wild type and *Ldb1* knockout neural stem cell lines were examined for the expression of markers that distinguish neural stem cells from committed progenitors. The deletion of *Ldb1* did not affect the ability of mouse ES cells to differentiate into a homogenous population of neural stem cells or the ability of those cells to further differentiate into committed astrocyte progenitors. However the absence of *Ldb1* had a profound effect on the proliferation capacity of the differentiated neural stem cells. Gene expression arrays were carried out using RNA isolated from wild type and the knockout neural stem cells in order to determine the genes that function downstream of *Ldb1* in neural development and in particular in the proliferation of neural stem cells.

Methods

Generation of an *Ldb1-/-* mouse ES cell line

The generation of the *Ldb1* null ES cell line is described in Chapter 3. The karyotype of the ES cells was checked and found to be correct. Nuclear extracts were isolated from wild type and *Ldb1* null ES cells and the anti-Ldb1, goat polyclonal IgG, N-18 (Santa Cruz®) antibody was used to identify the Ldb1 protein, which runs as a double band of 42.7 kDa and 46.5 kDa.

Generation of Neural Stem Cell lines

Wild type and *Ldb1* null ES cells were differentiated towards neural stem cells with the use of NDiff™ N2B27™ Neural Differentiation Medium (Stem Cell Sciences). $1x10^6$ ES cells were cultured on 0.1% gelatin coated dishes for 7 days, trypsinized and $3x10^6$ cells were cultured for a further 3 days in suspension and in the same medium with the addition of 10ng/ml Recombinant Murine EGF (PeproTech) and Recombinant Human FGF-basic (FGF-2) (PeproTech) (expansion medium), for the formation of cell aggregates. The aggregates were replated on 0.1% gelatin coated dishes and cultured in the expansion

medium for 7 days, during which they attached at the bottom of the dish and soon after cells started to grow out with the characteristic bipolar neural stem cell morphology. After extensive outgrowth all the cells were trypsinized and single cells were plated on 0.1% gelatin coated dishes and from then on cultured exclusively in the expansion medium. After a few passages a uniform neural stem cell population was achieved. The differentiation protocol was repeated 4 times for wild type and *Ldb1* null ES cells.

Immunofluoresence

Wild type and *Ldb1* null ES cells and NSCs were cultured on glass cover-slips. The cells were fixed in 4% paraformaldehyde/PBS for 10 minutes at room temperature, permeabilized with 0.4% Triton®X-100/PBS, blocked with 10% FCS in PBS/0.05% TWEEN®20, incubated overnight at 4^{0}C with the following antibodies in blocking solution: anti-Ldb1 goat polyclonal IgG, N-18 (Santa Cruz®), anti-RC2 (Radial glial cell marker) mouse IgM (DSHB Hybridoma Bank), anti-nestin mouse IgG_1, clone Rat 401 (BD Pharmigen™), anti-Tuj1 (Neuronal Class III β-Tubulin) mouse IgG2a (Covance®), anti-Oct3/4 goat IgG, clone N-19 (Santa Cruz®), anti-GFAP (Glial Fibrillary Acidic Protein) rabbit IgG (Sigma-Aldrich®) and for 2 hours at room temperature with the following secondary antibodies: anti-goat IgG FITC, anti-mouse IgM $Alexa_{594}$, anti-mouse IgG $Alexa_{594}$, anti-rabbit IgG $Alexa_{594}$. The cover-slips were mounted on glass slides with DAPI/Vectashield for nuclei staining.

Ldb1+/+ and *Ldb1*-/- 9.5 dpc embryos were dissected, the yolk sac of each embryo was removed for genotyping and the embryo proper was fixed in 2% paraformaldehyde/PBS for 2 hrs at room temperature. The embryos were equilibrated overnight in 20% sucrose/PBS at 4^{0}C, oriented and quick frozen in Tissue-Tek (Sakura Finetek). The embryos were sectioned at 10μm. Before staining with the antibody the sections were fixed in cold acetone for 10 min. The cells were permeabilized with 0.05% Tween®20/PBS, blocked with 1%BSA, 0.05% Tween®20/PBS and incubated with the primary antibody anti-nestin mouse IgG_1, clone Rat 401 (BD Pharmigen™) in blocking solution overnight at 4^{0}C and an anti-mouse IgG $Alexa_{594}$ secondary antibody in blocking solution for 2 hours at room temperature. The sections were mounted with DAPI/Vectashield for nuclei staining. The day that the plug was reported was taken as 0.5 dpc.

Synchronization and BrdU labelling

Wild type and *Ldb1* null NSCs were incubated for 24 hours with 0.5mM of mimosine (Sigma-Aldrich®) at 37^{0}C. The cells were washed twice in PBS and the medium was refreshed to release them from the mimosine block. The cells were incubated with 1μM BrdU for 10 minutes at 37^{0}C and harvested by trypsinization 4, 12 and 24 hours after the release. The cells were fixed in 70% EtOH at 4^{0}C overnight, treated with 0.1N HCl containing 0.5mg/ml pepsin (Merck) for 20 minutes at room temperature with rotation, washed in 0.5% Tween®20/0.1%BSA/PBS, treated with 2N HCl for 12 minutes at 37^{0}C, washed with borate buffer pH 8.5 at 4^{0}C, washed in 0.5% Tween®20/0.1%BSA/PBS and incubated with a FITC conjugated anti-BrdU antibody (Becton & Dickinson) in 0.5% Tween®20/0.1%BSA/PBS for 1 hour at 4^{0}C. The cells were treated with RNase (10μg/ml) and counterstained with PI

(propidium iodine) (10µg/ml) for 30 minutes at 37^{0}C. The flow cytometry was performed on the FACScan (Becton Dickinson) and was analyzed with the Cell Quest software.

Affymetrix Mouse Genome Arrays

Microarray analysis of *Ldb1*+/+ and *Ldb1*-/- NSCs was performed as described in Chapter 2.

Microarray data normalization and analysis

Described in Chapter 2.

Gene expression analysis

RNA was isolated as described above and cDNA was synthesized using SuperScript II Reverse Transcriptase and Oligo(dT)$_{12–18}$ primers according to the manufacturer's instructions (Invitrogen™). Quantitative Real-time PCR was carried out on the synthesized cDNA with 1U of Platinum® Taq polymerase (Invitrogen™), 1x PCR buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl], 1.5mM $MgCl_2$, 10ng/µl of each primer, 250µM dNTPs and SYBR Green (Sigma®) on the Opticon 2® Real-Time PCR Detection System (Bio-Rad). The following cycling conditions were used: 2 minutes at 94^{0}C, 40 cycles of 30 seconds at 94^{0}C, 30 seconds at 55^{0}C, 30 seconds at 72^{0}C and 5 minutes at 72^{0}C. The data was collected and analysed by the MJ Opticon™ analysis software version 3.1 (Bio-Rad). Transcript enrichments were calculated using the comparative C_T method. The C_T threshold was set at 10 cycles to distinguish between non-specific and specific PCR products detected through the incorporation of SYBR Green to amplified DNA. Transcript levels were normalized to the transcript of *Gapdh*, a housekeeping gene, the expression of which was not found to change after the deletion of *Ldb1* in NSCs.

Results and discussion

Generation of an *Ldb1*-/- mouse ES cell line

In order to investigate the involvement of *Ldb1* in neural development a knockout ES cell line was created. The *Ldb1* locus was targeted twice with the same construct. The integration of the construct via homologous recombination in the locus leads to the insertion of a loxP site at the 5' UTR and a PMC-neo cassette flanked by two more loxP sites and followed by an MLS and a CFP reporter gene in the second intron. The genomic structure of the mouse *Ldb1* gene [37] and the strategy for the targeting of the *Ldb1* locus for the generation of the cell line is shown in Fig. 1A. A positive clone (Fig. 1B) with one wild type and one targeted allele was selected and transiently transfected with a Cre recombinase expressing vector for the deletion of the PMC-neo cassette and the first two exons including the ATG. Due to the deletion the CFP reporter gene moves at the 5' UTR of *Ldb1* (Fig. 1B). The same targeting step was repeated once more and a positive clone was selected, where the targeting construct was incorporated in the remaining wild type allele (Fig. 1B). Because of an unexpected design characteristic of the targeting construct, as observed previously in the attempt to generate conditional *Ldb1*-/- mice (unpublished

data), the integration results in a full knocked out allele without the need to delete the first two exons, possibly due to an interference of the CFP reporter gene with the transcription or translation machinery. Therefore exons I/II and the PMC-neo cassette were not deleted in the second targeted allele (Fig. 1B). In order to confirm that no Ldb1 protein was present in the *Ldb1* null ES cells after the successful targeting of the locus two independent techniques were used. Nuclear extracts were isolated from wild type and *Ldb1* null ES cells for western blot analysis with an antibody against Ldb1, which appears as a double band (Fig. 1C). Additionally wild type and *Ldb1* null ES cells were grown on glass coverslips for immunofluoresence analysis with the use of the same antibody, to confirm the complete absence of Ldb1 from the cell nucleus (Fig. 1D). As shown on these figures no Ldb1 protein is present in the *Ldb1* null ES cell line.

The deletion of *Ldb1* did not have any obvious effects on the ES cells, which appear similar to the wild type cells under an inverted microscope and grow with a similar rate (Fig. 1E). We conclude that *Ldb1* is not essential for the survival, maintenance or self-renewal of ES cells.

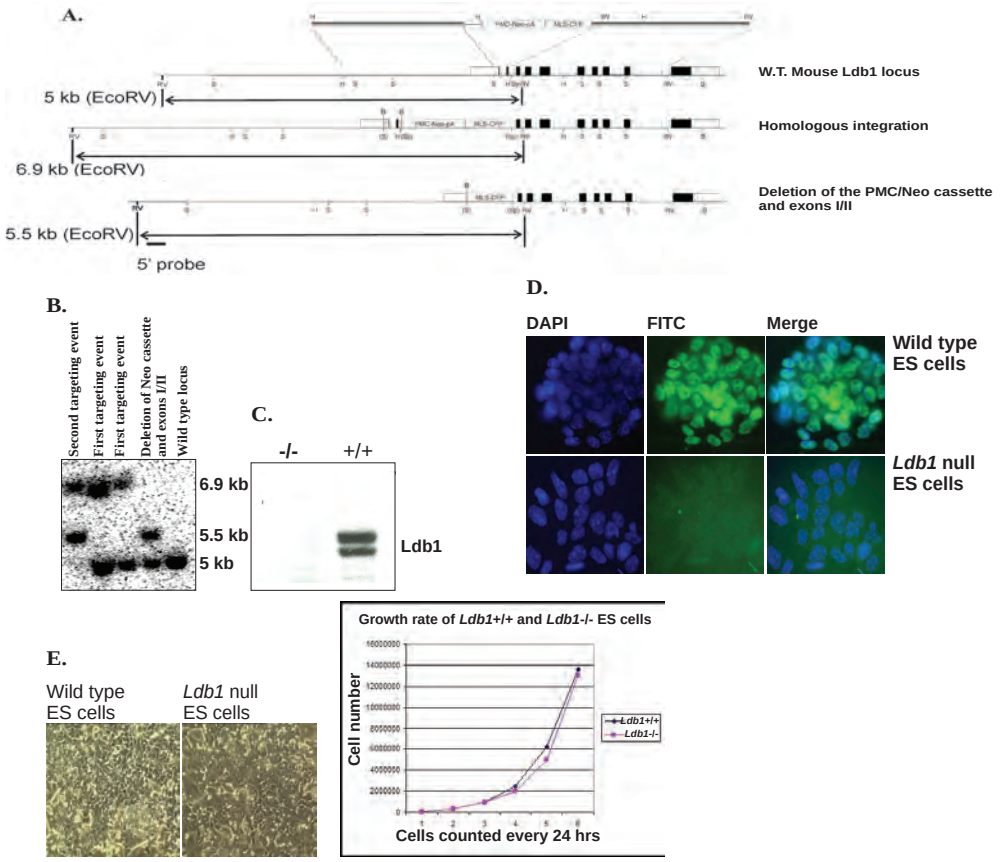


Figure 1. Scheme for the generation of an *Ldb1* null ES cell line. (A) A targeting construct with a PMC-neo cassette and an MLS-CFP was inserted in the second intron of both alleles in the *Ldb1* locus by homologous recombination through sequential targeting. Transfection with Cre recombinase was used to delete the neo cassette and the two first exons in one allele before the targeting of the locus with the same construct. (B) ES cell DNA was digested with EcoRV for the hybridisation with the 5' probe in order to identify the 5 kb fragmented of the *Ldb1* wild type allele, the 6.9 fragment of the targeted allele and the 5.5 kb fragment of the targeted allele that resulted from the deletion of the neo cassette and exons I/II. (C) Western blot analysis of wild type and *Ldb1* null ES cells showed the presence of Ldb1 protein in the wild type but not in the knockout cells. (D) Immunofluoresence analysis of wild type and *Ldb1* null ES cells revealed that there is no Ldb1 protein present in the nucleus of the knockout ES cells. (E) The *Ldb1* null ES cells look similar to the wild type ones and grow with similar rates.

Wild type and *Ldb1-/-* null mouse ES cells can successfully differentiate into Neural Stem Cells (NSCs)

Mouse ES cells can successfully differentiate into a pure population of adherent NSCs, when LIF is removed and the cells are cultured in the serum free basal medium NS-A supplemented with 2mM L-glutamine, modified N2 supplement, epidermal growth factor (EGF) and FGF-2 [8, 36, 38]. For that purpose wild type and *Ldb1* null ES cells were cultured in the basal medium with the N2 supplement for 7 days in order to initiate neural lineage specification. The medium used does not support the growth and proliferation of ES cells, which as a result disappear from the differentiating cultures. Next the cells were cultured for a further 3 days in suspension and with the addition of EGF and FGF-2 for the formation of floating aggregates that contain NSCs. The aggregates were collected and re-plated on gelatine coated dishes, whereby they adhered to the bottom of the culture dish and the NSCs with the characteristic bipolar morphology began to grow outwards. Prolonged culturing of these adherent NSCs eventually resulted in a pure population.

The first step in order to confirm that a homogenous NSC population was obtained was to examine whether any multipotent ES cells still remained in the differentiated populations. For that purpose wild type and *Ldb1* null NSCs were grown on glass coverslips and incubated with an antibody against Oct4, which is a marker for undifferentiated ES cells [39]. As shown on Fig. 2A wild type ES cells express Oct4 in the nucleus while wild type (Fig. 2B) and *Ldb1* null NSCs (Fig. 2C) do not. We conclude that the differentiated cell populations follow a neural lineage.

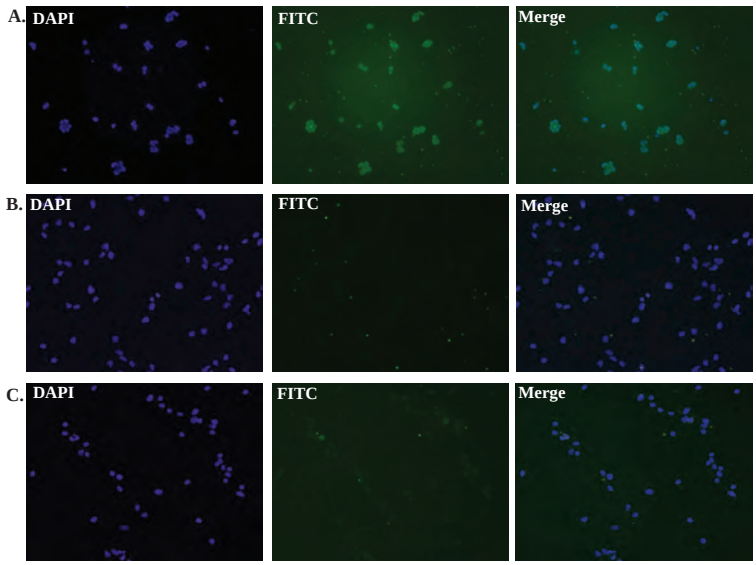


Figure 2. Wild type mouse ES cells (A), differentiated wild type NSCs (B) and differentiated *Ldb1* null NSCs (C) were incubated with an antibody against Oct4 in order to confirm that the NSC populations obtained after the differentiation process were pure and did not contain any undifferentiated ES cells (x10 magnification).

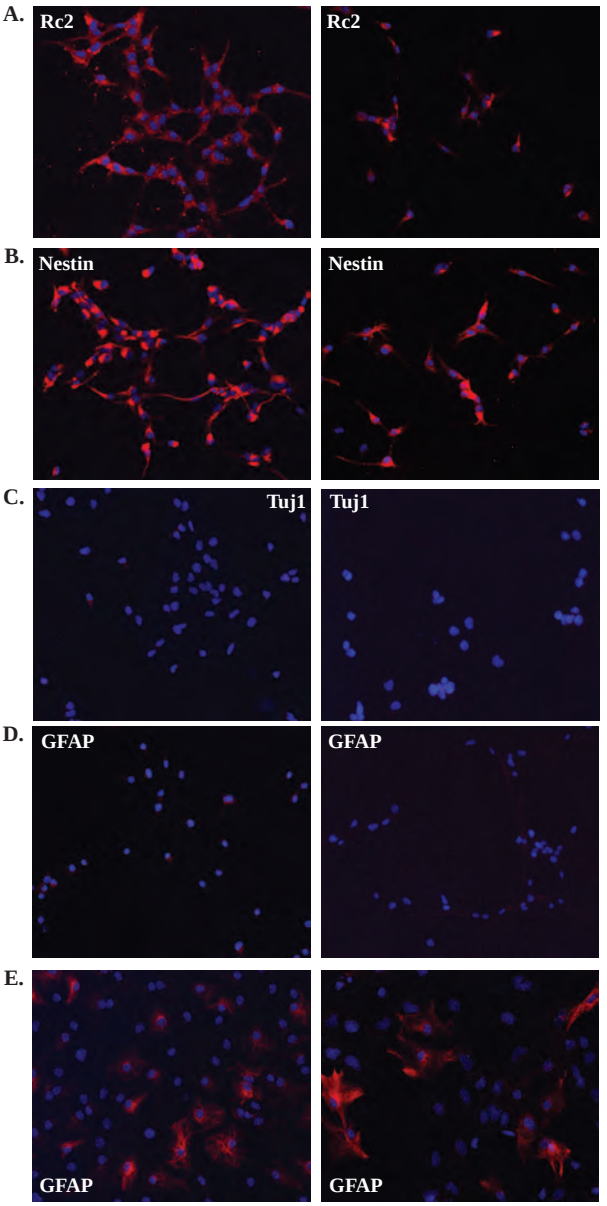


Figure 3. Wild type differentiated cells, appearing on the left and *Ldb1* null differentiated cells, appearing on the right, express the NSC markers RC2 (A) and nestin (B), while they do not express the neuron marker type III β-tubulin or Tuj1 (C) and the astrocyte marker glial fibrillary acidic protein (GFAP) (D). (E) Wild type NSCs on the left and *Ldb1* null NSCs on the right are able to generate GFAP expressing astrocytes (x10 magnification).

The second step was to examine whether the differentiated cell populations expressed markers specific for NSCs. Two such markers were selected for that purpose, RC2 and nestin [36]. Wild type and *Ldb1* null NSCs were grown on glass coverslips and incubated with an antibody against each marker. In Fig. 3 the wild type NSCs appear on the left and the *Ldb1* null NSCs appear on the right. The figure shows that the wild type and the knockout cells express both neural stem cell markers (Fig. 3A, 3B).

Finally it is important to confirm that the differentiated NSC populations have not differentiated further towards neuronal or astrocyte lineages. For that purpose an antibody against the type III β-tubulin (Tuj1) was used in order to identify differentiated neurons and an antibody against the glial fibrillary acidic protein (GFAP) was used in order to identify differentiated astrocytes [38]. Fig. 3C and 3D show that both the wild type, on the left, and the *Ldb1* null differentiated cells, on the right, do not express the two markers selected for terminally differentiated cells.

NSCs are able to give rise to both neurons and astrocytes *in vitro* under specific culture conditions for each cell type. The further differentiating capacity of the *Ldb1* null NSCs was investigated and the astrocyte generation pathway was chosen for that purpose. Wild type and *Ldb1* null NSCs were cultured on adherent dishes and in the basal NS-A medium supplemented with N2 and 1% Fetal Calf Serum, as a source of BMP4 necessary for the generation of astrocytes. We can see on Fig. 3E that both wild type and *Ldb1* null NSCs are capable of efficiently further differentiating towards astrocytes, which were identified from their characteristic morphology and strong expression of GFAP.

From the above observations we conclude that mouse ES cells were successfully differentiated into a pure and homogenous population of NSCs and that the deletion of *Ldb1* did not have an effect on the differentiation process. Moreover the emerging knockout NSCs have not lost the capacity to differentiate further as they can successfully generate astrocytes.

Ldb1-/- null mouse NSCs display a defect in proliferation

The morphology of the of the *Ldb1* null NSCs was examined in comparison to the wild type cells under an inverted microscope in order to examine the effect of the *Ldb1* deletion. On Fig. 4A and 4B the morphology of the wild type NSCs is shown at two different magnifications, with which the knockout NSCs were compared. The differentiation of the *Ldb1* null ES cells towards the neural lineage was repeated four times and the resulting knockout NSC cultures can be seen on Fig. 4C to 4J. The knockout NSCs appear smaller than the wild type cells and they do not form networks with the same efficiency, possibly because the cells have smaller branches that do not extend to the neighbouring cells. The phenotype of the resulting knockout NSCs could not be replicated completely with differences in morphology between the populations that resulted from each differentiation, which indicates that the absence of *Ldb1* in these cells results in a semi-prevalent phenotype.

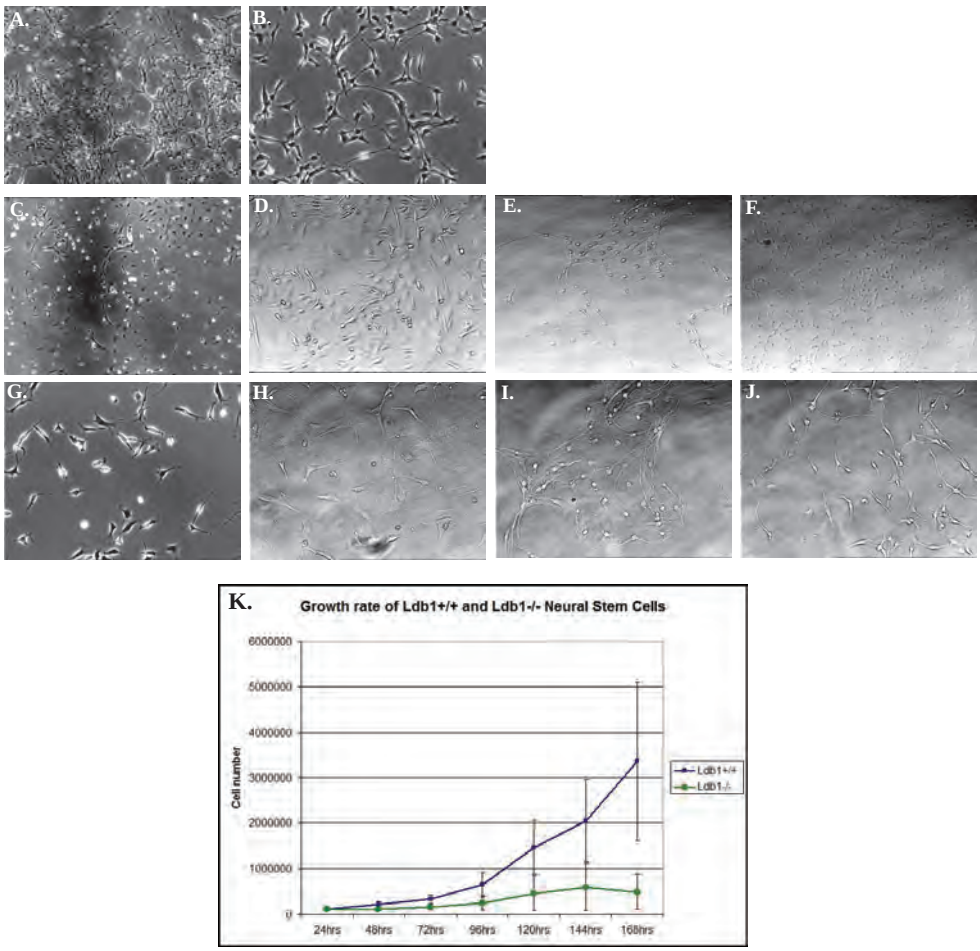


Figure 4. Wild type and *Ldb1* null NSCs viewed under an inverted microscope. (A) x10 magnification and (B) x20 magnification of wild type NSCs. (C, D, E, F) x10 magnification of four separate differentiated populations of *Ldb1* null NSCs, (G, H, I, J) x20 magnification of the same four differentiated populations of *Ldb1* null NSCs. The *Ldb1* null NSCs are smaller in size and they do not form networks with the same efficiency as the wild type cells, probably due to shorter branches. (K) The *Ldb1* null NSCs grow at a much slower rate in comparison with the wild type cells, which double approximately every 24 hours.

The common characteristic observed between the four different populations of *Ldb1* null NSCs was a much slower growth rate compared to the wild type cells. Equal numbers of four separate differentiated populations of wild type and *Ldb1* null NSCs were put in culture and grown for 7 days. The cell populations were counted every 24 hours. We can see from Fig. 4K that while the wild type NSCs grow with the expected rate, on average doubling every 24 hours, the *Ldb1* null cells grow at a much slower rate, their numbers do

not double every 24 hours and they never reach the total number of wild type cells after 7 days in culture. The same observation was reached when the cells were put in culture for longer periods of time (data not shown). The *Ldb1* null NSCs did not show increased apoptosis, therefore the lower cell numbers counted were not the result of extensive cell death.

We conclude that the lack of *Ldb1* expression in NSCs affects their ability to form networks with other cells, possibly due a defect in their morphology and more importantly it severely affects their growth rate as the cells are unable to proliferate efficiently.

Nestin expressing NSCs are present in *Ldb1*+/+ and *Ldb1*-/- mouse embryos at 9.5 dpc

Nestin encodes an intermediate filament that is strongly expressed in the proliferating pluripotent neuroepithelial cells of the neural tube. Its expression decreases as the cells differentiate towards the neuronal or glial lineages [40, 41]. The deletion of *Ldb1* was shown to affect the proliferation efficiency of NSCs *in vitro*, while the deletion in the mouse embryo was associated with defects in the morphology of the anterior neural plate, the neural tube and the expansion of the neuroepithelium. Sections of the anterior neural tube from *Ldb1*+/+ and *Ldb1*-/- 9.5 dpc embryos were incubated with an antibody against nestin in order see whether the defects seen in the *Ldb1* null NSCs *in vitro* correspond to the *in vivo* phenotype of the *Ldb1*-/- mouse embryo. As it is shown on Fig. 5A sections of the anterior of *Ldb1*+/+ (top panel) and *Ldb1*-/- (bottom panel) mouse embryos at the level of the optic vesicle, reveal the truncation of the forebrain structure and the lack of neural tube closure due to the deletion of *Ldb1*. The neural tube of the *Ldb1*-/- embryo has a twisted shape and the expansion of nestin positive neuroepithelial cells is defective, possibly due to a reduction in their population. In addition stem cells of the neural crest are absent (white arrows). The same phenotype can be seen in the neural tube sections of *Ldb1*+/+ (top panel) and *Ldb1*-/- (bottom panel) mouse embryos at the level of the otic vesicle (Fig. 5B). As described before, the *Ldb1*-/- neural tube has a compact and twisted shape in comparison with the *Ldb1*+/+ embryo. Nestin expressing neuroepithelial cells are present in the *Ldb1*+/+ and the *Ldb1*-/- embryos as expected, since the *Ldb1* null ES cells are still able to differentiate into nestin expressing NSCs. However there is stronger nestin expression in the *Ldb1*+/+ neural tube and dorsal neural plate (white arrowheads) when compared with the expression pattern in the *Ldb1*-/- embryo. This appears to be due (at least in part) to the presence of a larger number of neuroepithelial cells in the *Ldb1*+/+ neural tube and specifically in the neural plate, than the ones present in the *Ldb1*-/- mouse embryo.

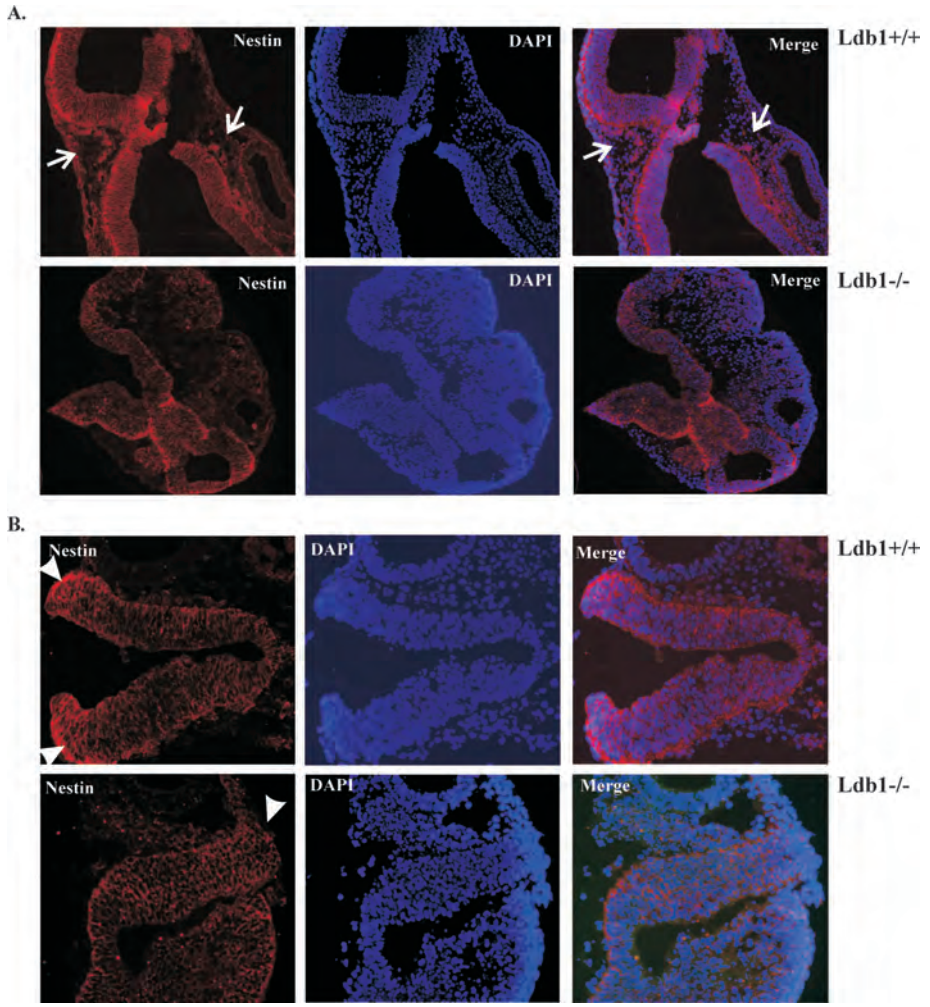


Figure 5. Neural tube sections (10µm) of 9.5 dpc *Ldb1*+/+ and *Ldb1*-/- embryos were stained with an antibody against nestin and DAPI for the visualization of cell nuclei. The top panel shows the *Ldb1*+/+ neural tube, while the bottom panel shows the *Ldb1*-/- neural tube. (A) Sections show the forebrain vesicle, next to the optic vesicle and the fourth ventricle, which ends at the roof of the hindbrain. Note the lack of expansion of nestin positive neuroepithelial cells, the absence of neural crest stem cells (white arrows), the forebrain truncation and the lack of neural tube closure in the *Ldb1*-/- sections. (B) Sections of the neural tube at the level of the otic vesicle. Note the twisted shape of the *Ldb1*-/- neural tubes and the lower expression of nestin possibly due to a reduction in the population of neuroepithelial cells. The expression of nestin is stronger in the *Ldb1*+/+ neural plate (dorsal neural tube) (white arrowheads) and is absent from the *Ldb1*-/- (x10 magnification).

Changes in gene expression pattern between wild type and *Ldb1* null NSCs

In order to gain more insight into the proliferation defect and the morphological differences observed in the *Ldb1* NSCs when compared with the wild type NSCs, RNA was collected from three separate differentiated populations of wild type and *Ldb1* null NSCs. Equal amounts of RNA from each population were hybridized on the Affymetrix Mouse Genome 430 2.0 arrays. Taking into account the following parameters that indicate a successful hybridization: the average signal of the hybridization of each sample on the array, the percentage of the present probe sets versus the absent probe sets, the noise and background levels, the ratio of GAPDH 3'to 5' (data not shown) we concluded that the hybridization was successful and that the data from each array can be compared with the others. The microarray data were analysed with the Omniviz software version 5.0 and the SAM (Significance Analysis of Microarrays) software. The samples' correlation is shown in Fig. 6A. The blue and red colours represent the Pearson's Correlation Coefficient values, where the red colour represents positive and the blue colour negative correlations. A negative correlation (shown in blue) is an indicator of differences in gene expression between the compared samples, while a positive correlation (shown in red) indicates that the genes in both samples have similar levels of expression. The clustering of the wild type and knockout samples according to the genes that were found to be differentially expressed is shown on Fig. 6B as a tree diagram at the top of the figure. At the left of the figure the tree diagram represents the clustering of the differentially expressed genes. The red colour represents an up-regulation in gene expression of at least 1.5 fold, while the green colour represents a down-regulation in gene expression of the same level. A total of 499 genes showed differential expression between the wild type and the *Ldb1* null NSCs, when the fold change threshold was set to 1.5, 396 of which were of known function. From those genes 173 were found to be down-regulated (43.68%) and 223 were found to be up-regulated (56.31%). Finally 103 genes from the total number of differentially expressed genes (20.6%) were of unknown function.

A list of genes that are differentially expressed in the *Ldb1* null NSCs can be seen in Table 1. Some of these genes that showed differential expression were selected from the above list in order to confirm the changes in their expression pattern by quantitative real-time PCR (Fig. 8).

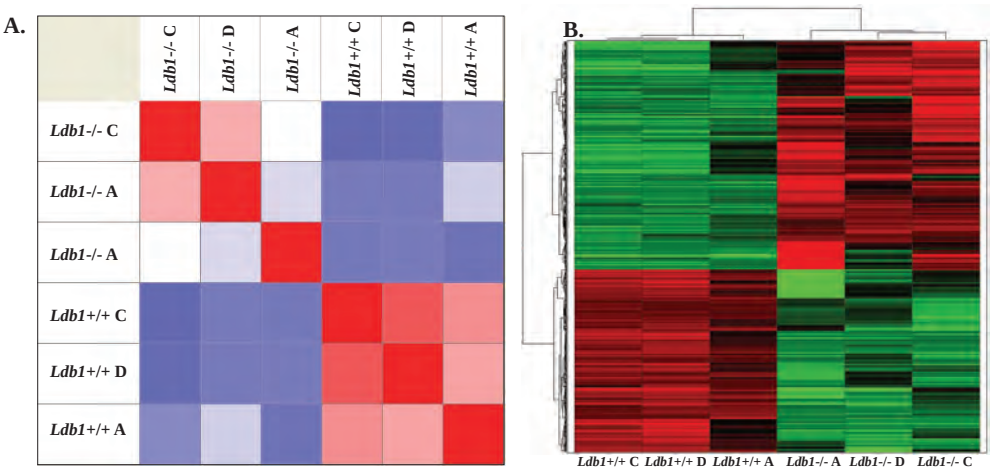


Figure 6. (A) Pairwise correlation of the wild type and *Ldb1* null NSCs. The threshold for the differential expression was set at 1.5 fold and the q-value was set at 5%. The blue and red colour represents the negative and positive Pearson's Correlation Coefficient values respectively. The negative correlation (blue squares) seen between the wild type and the knockout NSC samples is an indicator of the differences in gene expression. The positive correlation (red squares) indicates that the genes expressed in all wild type NSC samples follow the same levels of expression and similarly the genes expressed in all the knockout NSC samples follow the same levels of expression.

(B) The clustering of the wild type and *Ldb1* NSCs (the threshold for gene differential expression is set at 1.5 fold and the q-value is set at 5%) based on the genes that show differential expression between the wild type and the knockout NSCs according to the above set parameters, is shown as a tree diagram at the top of the figure. The clustering of the differentially expressed genes is shown as a tree diagram at the left of the figure. The change in the expression level of each gene is represented by the change of colour from light green to red. The highest up-regulated genes are represented by red will the most severely down-regulated genes are represented by light green.

Table 1. A list of genes that show differential expression in the *Ldb1* NSCs, compared with the wild type cells. The fold change (up-regulation or down-regulation) of each gene's expression in the knockout cells is given. Bold characters indicate the genes for which the change in expression is associated with a q-value below the threshold of 5%.

Gene Symbol	Gene Title	Fold Change
Ednrb	**endothelin receptor type B**	**0.071**
Gpr56	**G protein-coupled receptor 56**	**0.0718**
Ttyh1	**tweety homolog 1 (Drosophila)**	**0.077**
Wnt7a	**wingless-related MMTV integration site 7A**	**0.0799**
PACAP-R1/ PAC1	**pituitary adenylate cyclase-activating polypeptide type I receptor**	**0.09**
Fbln2	**fibulin 2**	**0.1**
Atp1a2	**ATPase, Na+/K+ transporting, alpha 2 polypeptide**	**0.111**
Lfng	**LFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase**	**0.113**
Mash1	**achaete-scute complex homolog 1 (Drosophila)**	**0.123**
Lmo1	**LIM domain only 1**	**0.127**
Ptprz1	**protein tyrosine phosphatase, receptor type Z, polypeptide 1**	**0.134**
Sox3	**SRY-box containing gene 3**	**0.136**
Sox8	**SRY-box containing gene 8**	**0.142**
Fzd9	**frizzled homolog 9 (Drosophila)**	**0.142**
Hey1	**hairy/enhancer-of-split related with YRPW motif 1**	**0.147**
Cxcr4	**chemokine (C-X-C motif) receptor 4**	**0.149**
Olig2	**oligodendrocyte transcription factor 2**	**0.152**
Rnd2	**Rho family GTPase 2**	**0.153**
F-spondin	**spondin 1, (f-spondin) extracellular matrix protein**	**0.155**
Slc1a3	**solute carrier family 1 (glial high affinity glutamate transporter), member 3**	**0.16**
Nrarp	**notch-regulated ankyrin repeat protein**	**0.161**
Dab1	**disabled homolog 1 (Drosophila)**	**0.172**
Fabp7	**fatty acid binding protein 7, brain**	**0.173**
Tnc	**tenascin C**	**0.176**
Dll1	delta-like 1 (Drosophila)	0.178
Grm5	**glutamate receptor, metabotropic 5**	**0.181**
Pttg1	**pituitary tumor-transforming 1**	**0.182**
Sox6	**SRY-box containing gene 6**	**0.186**
Ncan	Neurocan	0.195
Gbx2	gastrulation brain homeobox 2	0.2
Qk	**quaking**	**0.2**

Gene Symbol	Gene Title	Fold Change
Olig1	**oligodendrocyte transcription factor 1**	**0.211**
Prdm1	**PR domain containing 1, with ZNF domain**	**0.212**
Vangl2	vang-like 2 (van gogh, Drosophila)	0.212
Satb1	special AT-rich sequence binding protein 1	0.213
Bmpr1b	bone morphogenetic protein receptor, type 1B	0.226
Cdc25c	**cell division cycle 25 homolog C (S. pombe)**	**0.227**
Hoxd8	homeo box D8	0.228
Hes6	hairy and enhancer of split 6 (Drosophila)	0.235
Ccnb1	cyclin B1	0.236
Sox21	SRY-box containing gene 21	0.236
Epha5	Eph receptor A5	0.238
Cdh20	cadherin 20	0.239
Igsf8	immunoglobulin superfamily, member 8	0.245
Neud4	neuronal d4 domain family member	0.245
Sox5	SRY-box containing gene 5	0.249
Sema6a	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6A	0.249
Skp2	S-phase kinase-associated protein 2 (p45)	0.274
Ccnb1	cyclin B1	0.275
Ttk	Ttk protein kinase	0.278
Aspm	asp (abnormal spindle)-like, microcephaly associated (Drosophila)	0.293
Slc6a1	solute carrier family 6 (neurotransmitter transporter, GABA), member 1	0.319
Mlc1	**megalencephalic leukoencephalopathy with subcortical cysts 1 homolog (human)**	**0.393**
Smad3	MAD homolog 3 (Drosophila)	3.34
Mmp14	matrix metallopeptidase 14 (membrane-inserted)	3.518
Gfra2	glial cell line derived neurotrophic factor family receptor alpha 2	3.624
Ghr	growth hormone receptor	3.688
Gadd45a	growth arrest and DNA-damage-inducible 45 alpha	4
Cd9	CD9 antigen	4.153
F3	coagulation factor III	4.56
Crim1	cysteine rich transmembrane BMP regulator 1 (chordin like)	4.621
Actn1	actinin, alpha 1	4.635
Ndrg1	N-myc downstream regulated gene 1	4.722
Plxnd1	plexin D1	4.73
Reck	reversion-inducing-cysteine-rich protein with kazal motifs	4.748
Nrp1	neuropilin 1	4.8
Adam23	a disintegrin and metallopeptidase domain 23	4.965

Gene Symbol	Gene Title	Fold Change
Sdc2	syndecan 2	4.969
Prrx1	paired related homeobox 1	5.233
Bmp2	bone morphogenetic protein 2	5.239
Cited2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	5.244
Epha2	eph receptor A2	5.322
Apoe	apolipoprotein E	5.37
Col4a1	**collagen, type IV, alpha 1**	**5.375**
Lrrk2	**leucine-rich repeat kinase 2**	**5.678**
Dlc1	**deleted in liver cancer 1**	**5.947**
Tgfbr3	**transforming growth factor, beta receptor III**	**6**
Ptgs2	prostaglandin-endoperoxide synthase 2	6.155
Unc5b	unc-5 homolog B (C. elegans)	6.275
Socs3	suppressor of cytokine signalling 3	6.764
Cd38	**CD38 antigen**	**7.113**
Dab2	disabled homolog 2 (Drosophila)	7.324
Efnb2	**ephrin B2**	**7.586**
Tgfb1	transforming growth factor, beta 1	8.122
Col6a1	**collagen, type VI, alpha 1**	**8.393**
Tgfbi	transforming growth factor, beta induced	9.623
Tgfb1i1	**transforming growth factor beta 1 induced transcript 1**	**10.538**
Adm	**adrenomedullin**	**13.65**
Vgf	**VGF nerve growth factor inducible**	**17.455**
Ret	**ret proto-oncogene**	**17.542**
Ccl2	**chemokine (C-C motif) ligand 2**	**22.161**
S100a4	**S100 calcium binding protein A4**	**23.3**
Igf2	insulin-like growth factor 2	54.239

Biological functions and signalling pathways affected due to the deletion of *Ldb1* in NSCs

Gene expression analysis of wild type and *Ldb1* null NSCs identified many genes that showed misregulation in their expression pattern in the absence of *Ldb1*. With the use of the Ingenuity Pathways Analysis software the genes found to be differentially expressed in the wild type and the *Ldb1* null NSCs were associated with specific biological functions, which can be seen in Table 2. A differentially expressed gene can be associated with more than one biological function. The graph in Fig. 7A shows the significance values of the biological function analysis. The significance is represented as a negative logarithm of the p-value, the threshold of which was set at 5% and is a measurement of the probability that a group of genes was associated with a specific biological function accurately. A higher p-value represents the higher probability that the allocation of the genes in a biological function is random. As it is shown on the graph genes that are involved in cellular growth, proliferation, development and morphology, cell cycle, cancer, cell-to-cell signalling show differential expression in the *Ldb1* null NSCs. A number of genes that show differential expression in the *Ldb1* null NSCs are indentified by the analysis as involved in the development of the cardiovascular, nervous and hematological systems and in general embryonic and organ development processes. It has been previously shown that neural and hematopoietic stem cells express common genes [42, 43], while NSCs were shown to give rise to hematopoietic and erythroid cells [44, 45]. Additionally the Ingenuity Pathways Analysis software was used to group the differentially expressed genes according to the biological pathway that they are more likely to participate in, as shown in Fig, 7B. The ratio of the number of genes allocated to a biological pathway over all the differentially expressed genes is represented on the y-axis, while the yellow line represents the negative logarithm of the p-value, the threshold for which was set at 5% and is used as a measurement of the probability that the allocation of genes to a specific biological pathway was random. A low p-value indicates that the allocation of a group of genes to a specific biological pathway is correct. Similarly to the biological function analysis a gene may be allocated as a member of more than one pathway. As it is shown the graph the two DNA damage induced cell cycle checkpoints, G1/S and G2/M are affected in NSCs when *Ldb1* is absent. Essential development pathways such as WNT [46-48], TGF-β [49-51] and Notch [52, 53] signalling pathways are affected to some extent but not substantially in the *Ldb1* null NSCs when the comparison of the ratio (y-axis) and the p-value (yellow line) is taken into account, while the p53 and apoptosis signalling pathways are not affected at all by changes in gene expression in these cells, which is expected since the cells did not show excessive cell death.

Table 2. The genes that were found to be differentially expressed in the *Ldb1* null NSCs were grouped according to the biological function they are most likely to participate in, using the Ingenuity Pathways Analysis software.

Biological Function	Molecules
Cancer	182
Cell Death	133
Cellular Growth and Proliferation	156
Cardiovascular System Development and Function	55
Organismal Development	66
Cellular Movement	96
Cellular Development	112
Cell Morphology	86
Cell-To-Cell Signalling and Interaction	80
Tissue Development	86
Cell Cycle	64
Tissue Morphology	63
Nervous System Development and Function	77
Cellular Function and Maintenance	22
Hematological System Development and Function	59
DNA Replication, Recombination, and Repair	31
Gene Expression	71
Organ Development	48
Embryonic Development	31

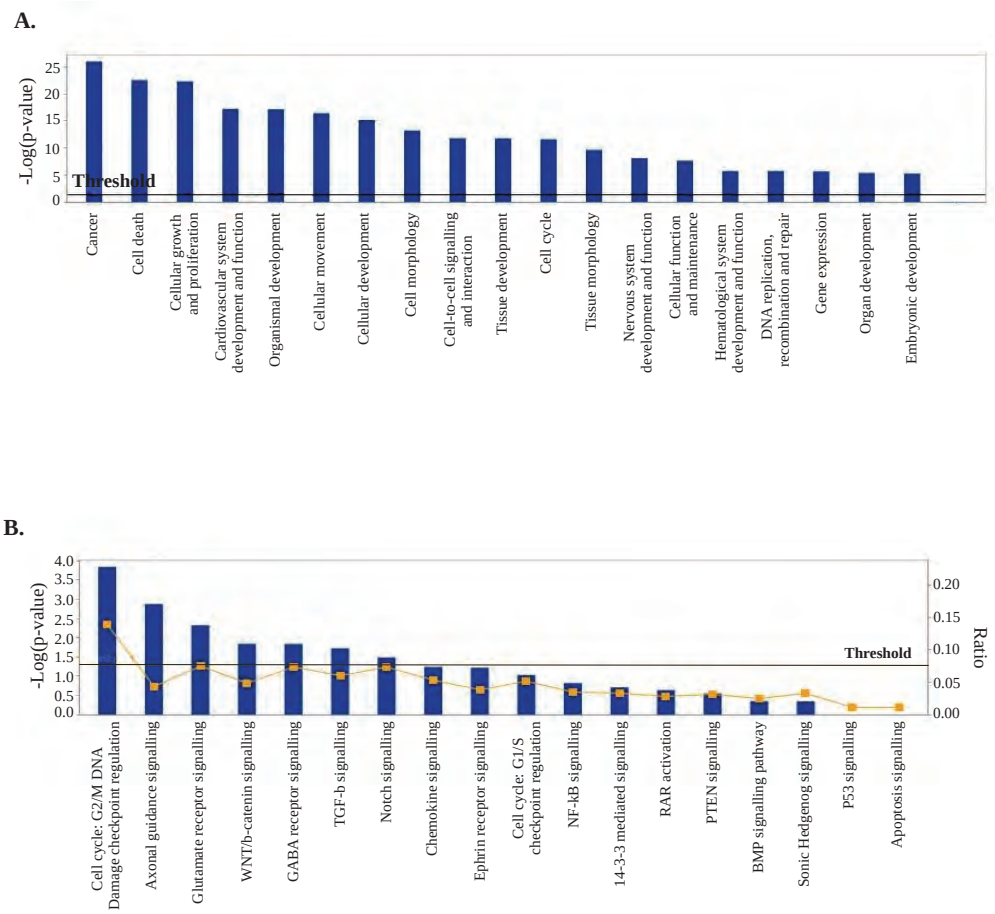


Figure 7. (A) Significance values of the functional analysis done using the Ingenuity Pathways Analysis software. The negative logarithm of the p-value is used in a graphic representation as a measurement of the likelihood that a group of genes was associated with a biological function during the analysis randomly. The threshold for the p-value was set at 5%. The higher the –log (p-value), the lower the possibility that the allocation of a group of genes to a specific biological function is wrong. (B) Grouping of the genes found to be differentially expressed in the *Ldb1* NSCs into the pathways they are most likely to participate, using the Ingenuity Pathways Analysis software. The y-axis represents the ratio of the genes allocated to a specific pathway over the total number of the differentially expressed genes. The yellow line {–log (p-value)} represents the probability that the allocation of genes to a specific biological pathway was accurate.

The deletion of *Ldb1* affects the expression of genes that are involved in the proliferation of neural stem and progenitor cells

The gene expression analysis done on wild type and *Ldb1* null NSCs revealed the misregulation of many genes. Since the lack of *Ldb1* expression affects the proliferation capacity of the cells at a great extent, the focus was put on identifying genes that are *Ldb1* dependent and are known to regulate cell proliferation during neural development.

For example one of the down-regulated genes encodes Tenascin C, a conserved glycoprotein of the extracellular matrix [54]. Due to its complex structure a large number of isoforms have been identified that are involved in neural development. More specifically 27 isoforms of *tenascin C* were shown to be present in the developing CNS of the mouse embryo [55, 56]. The gene is expressed in the forebrain ventricular and sub-ventricular zones and specifically in radial glia cells [57, 58]. In addition *tenascin C* is also expressed strongly in NSCs that exist within neurospheres [55]. In the forebrain ventricular zone of 13 dpc mouse embryos *tenascin C* is expressed in a highly proliferative cell population [59]. The number of neural stem and progenitor cells that can successfully go through cell cycle is reduced in the forebrain of *tenascin C*-/- embryos, leading to the conclusion that the gene is important for their proliferation [60]. It is believed that *tenascin C* regulates the responsiveness of NSCs grown in culture to the effects of the added FGF and EGF, which is essential for their growth and maintenance [11]. In particular EGF is responsible for maintaining the proliferative capacity of the NSCs *in vitro* [61]. The expression of *tenascin C* is lost in the *Ldb1* null NSCs.

As mentioned above the *Drosophila* ortholog of *Ldb1* participates in a complex that regulates the expression of the bHLH proneural regulatory genes *achaete-scute*, expressed in the ectoderm. They control the emergence and specification of the neural precursor cells from the proneural clusters that exist in the ectoderm [62, 63]. Loss of *achaete-scute* expression leads to fewer neural precursors emerging due to absence of neural specification signals [64]. The expression of the mouse ortholog *Mash1* is severely down-regulated in the *Ldb1* null NSCs. The deletion of *Mash1* in mice leads to lethality just after birth and a reduction in the numbers of sympathetic, parasympathetic, enteric and sensory neurons of the olfactory epithelium [65]. *Mash1* is expressed in the ventricular and sub-ventricular zones of the ventral forebrain and a role for it in the regulation of cell proliferation in these regions has been described, as the expression of *Mash1* is linked to the balance of cell proliferation versus differentiation through a lateral inhibition pathway [65].

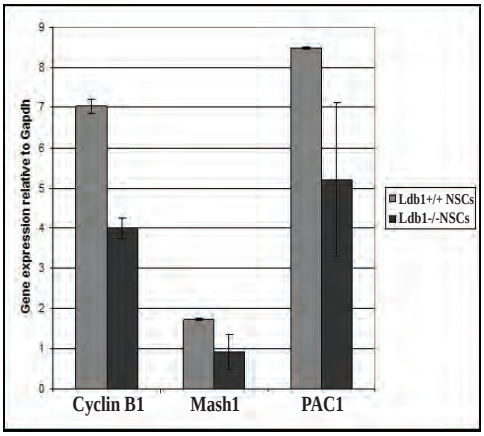
The *pituitary adenylate cyclase-activating polypeptide type I receptor* (*PAC1*) gene is not expressed in NSCs in the absence of *Ldb1*. PAC1 is a receptor for the pituitary adenylate cyclase-activating polypeptide, also known as PACAP. It is expressed in the sub-ventricular zone and the dentate gyrus of the hippocampus of the adult mouse brain, which are sites where neurogenesis remains after birth. In addition the receptor was also found to be expressed in NSCs that were isolated from the adult mouse brain and maintained in

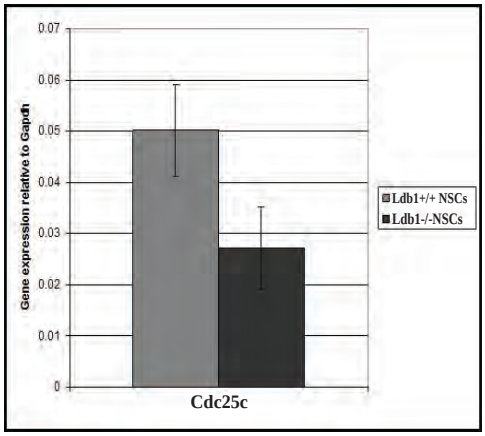
culture. PAC1 was shown to exert a proliferative effect on adult NSCs through the binding of its ligand PACAP. In addition the treatment of adult mice with PACAP demonstrated that the PAC1 ligand can induce proliferation in the sub-ventricular zone and the dentate gyrus of the hippocampus [66].

The expression of *Cxcr4* is lost in NSCs after the deletion of *Ldb1*. The chemokine receptor Cxcr4 and its ligand Sdf1 were shown to be involved in the development of the cerebellum [67, 68]. Closer inspection has provided additional evidence on the involvement of Cxcr4 in neural development. It is expressed in the CNS of the developing mouse and together with its ligand it is involved in cell migration and cell proliferation [69]. When the receptor and ligand are absent premature migration of the cerebellum granule cells has been described. In addition Sdf1 can influence the proliferative effect exerted by sonic hedgehog on these cells *in vivo* [70]. In Cxcr4 deficient mice the development of the dentate gyrus in the hippocampus shows some defects, notably a reduction in the number of cells that go through cell division and a premature differentiation of neurons [71]. Finally Sdf1 controls axonal outgrowth and branching in the granule cells of the cerebellum and in neurons of the hippocampus, thus Cxcr4 is, via the effect of its ligand, involved in the morphogenesis of neural cells [72, 73].

Cdc25c is a phosphatase involved in cell cycle regulation. When it is phosphorylated by Chk1 a site for the binding of the protein 14-3-3 is created and the phosphatase is inactivated [74-76]. A novel effect of the inactivation of Cdc25c, which is not expressed in the *Ldb1* null NSCs, on the regulation of cell cycle progression and the proliferation of adult rat neural progenitor cells has been described [77]. The exposure of neural progenitor cells to the HIV envelope glycoprotein gp120 has a profound effect on their proliferation. The mechanism behind the effect lies on a prolonged G1 phase that leads to cell cycle arrest and decreased proliferation. In more detail gp120 was found to activate a p38a MAPK-MAPKAPK2-Cdc25c cascade in adult neural progenitor cells, which leads to the phosphorylation and inactivation of Cdc25c. This cascade participates in the regulation of the DNA damage induced checkpoints G1/S and G2/M of the cell cycle [78]. Moreover another regulator of the G2/M checkpoint transition, *cyclin B1*, is down-regulated in the *Ldb1* NSCs [79].

The down-regulation of the genes involved in the G1/S and G2/M checkpoints prompted us to examine the progress of the *Ldb1* null NSCs through the cell cycle.





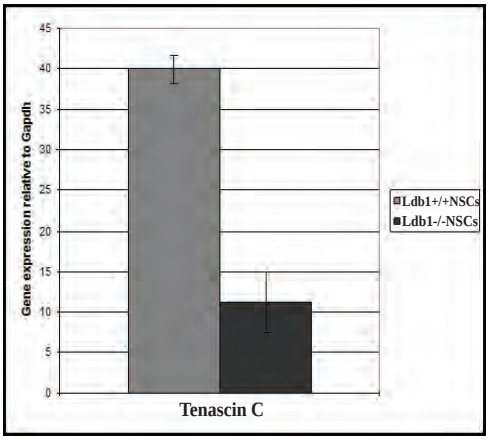


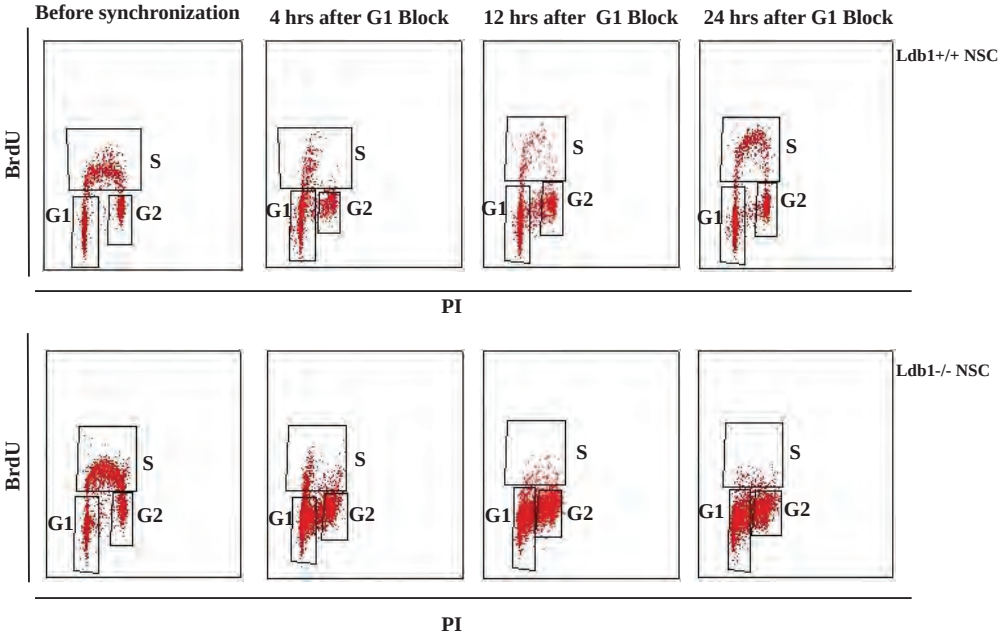
Figure 8. Confirmation of changes in gene expression detected with the microarray analysis of the wild type and *Ldb1* NSCs by quantitative real time PCR. The expression of each of the examined genes was normalized to the expression of *Gapdh*, which remains unchanged in the wild type and double knockout NSCs. RNA from three independently differentiated cell populations of wild type and *Ldb1* null NSCs was used.

Progress through the cell cycle is inhibited in *Ldb1* null NSCs

In order to investigate further the ability of NSCs to progress efficiently through the cell cycle in the absence of *Ldb1*, the cell cycle profile of wild type and *Ldb1* null NSCs was analyzed by FACScan. The cells were initially synchronized with the use of the cell cycle blocker mimosine, which can exert a reversible arrest at the late G1 phase of the cell cycle [80, 81]. The cells were incubated for 24 hours with 0.5mM of mimosine [82], after which they were released form the block and allowed to progress through the S phase and the rest of the cell cycle. At the following time points: 4, 12 and 24 hours after the release the wild type and *Ldb1* null NSCs were incubated with 1μM of BrdU for 10 minutes. The cells were harvested and labelled with the anti-BrdU FITC conjugated antibody and propidium iodine. BrdU is an analogue of thymidine that gets incorporated into newly synthesized DNA during the S phase and was used together with the anti-BrdU antibody to visualize the replication status of the cells, while the propidium iodine stain was used to determine

their DNA content with FACS analysis. Fig. 9 shows the cell cycle progression of the wild type and *Ldb1* null NSCs before synchronization and after the cells were released from the mimosine induced late G1 block. Before the synchronization there are no obvious differences between the wild type and knockout cells and 4 hours after the release from the block, the cells are slowly entering S phase. After 12 hours from the release, the wild type NSCs are able to progress through the cell cycle again efficiently, while 24 hours after the release the wild type NSCs have completely recovered from the block. That is not the case for the *Ldb1* null NSCs; they are unable to progress through the cell cycle even 24 hours after being released from the block. The cells are locked in G1 or G2 and no cells are going through the S phase of DNA replication.

It is still not completely understood how mimosine functions in order to exert a reversible block on mammalian cells at late G1. The expression and activity of a number of cell cycle regulators were shown to be affected after mimosine treatment, such as histone H1 kinase [89], p27 (Kip1) [90], p21 (CIP1) [91], cyclin D1[92]. In addition mimosine was shown to induce the expression of *Ndrg1* (*N-myc downstream regulated gene 1*) through a c-Jun/AP1 dependent process [93]. *Ndrg-1* is believed to be an inducer of G1 arrest and an inhibitor of cell proliferation. It is interesting to note here that the expression of *Ndrg1* is elevated in the *Ldb1* null NSCs (Table 1)



Ldb1+/+ NSCs	Before synchronization	4 hrs after G1 Block	12 hrs after G1 Block	24 hrs after G1 Block
G1	58.68%	80.10%	76.11%	68.83%
S	21.83%	5.73%	4.87%	13.80%
G2	17.6%	12.38%	14.38%	15.17%

Ldb1-/- NSCs	Before synchronization	4 hrs after G1 Block	12 hrs after G1 Block	24 hrs after G1 Block
G1	37.02%	57.41%	56.75%	56.26%
S	41.33%	9.85%	3.19%	2.99%
G2	18.41%	22.26%	35.42%	35.25%

Figure 9. The cell cycle profile of wild type and *Ldb1* null NSCs determined by FACScan. The cells were synchronized at the late G1 phase with the reversible cell cycle blocker mimosine for 24 hours and subsequently released. At the indicated time points the cells were labelled with BrdU and fixed. Their DNA content was determined with the propidium iodine staining (x-axis) and their replication status was determined via anti-BrdU labelling (y-axis), the gates indicate the G1, S and G2 populations. 4 hours after being released from the block the cells are slowly exiting G1 and entering S. 12 hours after the release the wild type NSCs begin to progress through the cell cycle again and 24 hours after the release they have completely recovered from the block. However the *Ldb1* null NSCs do not recover from the block even 24 hours after the release and they are locked in G1 and G2 without any cells going through the S phase of DNA replication.

Conclusion

The involvement of *Ldb1* in neural development was investigated, through the generation of knockout NSC lines. The severity of the phenotype seen in the *Ldb1*-/- mouse embryo, which is embryonic lethal after 9.5 dpc, is an indication of its importance in that developmental process.

Homogeneous populations of NSCs, maintained in adherent cultures, can be successfully obtained through the differentiation of ES cells. The obtained NSC populations have the ability to self-renew and they are pluripotent, being able to differentiate first towards both neuronal and astrocyte progenitors and consequently towards fully differentiated neurons and astrocytes under specific culture conditions. In order to reach a conclusion on the involvement of *Ldb1* in neural development, based on findings concerning its involvement in the generation of NSC lines *in vitro*, it is important to determine how similar these NSCs are to the ones that exist *in vivo*. As mentioned above NSCs, which are also called neuroepithelial cells, exist in the developing mouse embryo in the neural crest and the neural tube. In adult mice NSCs exist in niches, essential for regeneration of the CNS. They are found at the sub-ventricular zone of the forebrain and the hippocampus, which are the regions where neurogenesis still takes place [83, 84]. The NSCs that originate from differentiated ES cells mostly resemble endogenous radial glia, as they express the common markers RC2, Glast, BLBP, vimentin, Pax6, Emx2 [8, 36]. Radial glia cells develop from neuroepithelial cells that express Sox1 and provide guidance during the migration of neuroblasts, but are also the precursors of neurons, astrocytes and oligodendrocytes [85-88]. Taking into account the markers expressed, radial glia have a dorsal forebrain identity. On the other hand NSCs were also shown to express *Mash1* and *Olig2*, which are indicative of the ventral forebrain. However *Olig2* expression is thought to be an artefact, as it was shown to increase with FGF-2 treatment [8] . Interestingly both *Mash1* and *Olig2* are down-regulated in the *Ldb1* null NSCs. It should be taken into consideration that the NSCs which emerge from the differentiation of ES cell are to an extent an artificial cell population for any conclusions reached with the use of these cells as a model for neural development.

The deletion of *Ldb1* does not affect the self-renewal and proliferation of mouse ES cells or their morphology. Additionally the knockout ES cells retain their pluripotency as they are able to differentiate successfully towards NSCs. The emerging cells similarly do not suffer from a defect in their differentiation capacity, as they can efficiently generate astrocyte progenitors and fully differentiated astrocytes. However their proliferation capacity is severely affected as the cells have lost their ability to self-renew efficiently.

Furthermore although the *Ldb1* null NSCs express markers specific for the identification of these type of cells, while they do not express markers specific for more committed progenitors such as neuronal and astrocyte progenitors, nonetheless their morphology is different from the wild cells. We observed that the resulting phenotype of the knockout NSC lines cannot be fully replicated, which leads to the conclusion that the phenotype is semi-prevalent.

The proliferation defect seen in the *Ldb1* null NSCs can be attributed to the loss of *tenascin C*, *Mash1* and *PAC1* expression. In the case of *Mash1*, Ldb1 may be involved in the regulation of its expression in a manner similar to the control of the transcription of the *Drosophila* ortholog *ac*/*sc*.

However it is interesting to note the down-regulation of two important cell cycle regulators *cdc25c* and *cyclin B1*, which regulate the transition through the G1/S and G2/M cell cycle checkpoints. Based on this, the proliferation defect seen in the knockout NSCs can also be the result of cell cycle misregulation. Indeed the cell cycle profiling of the cells showed that both checkpoints are misregulated, since after synchronization with the use of mimosine at late G1, the knockout cells are unable to progress through the cell cycle and are instead stuck in G1 and G2. At this point it should be noted that although the knockout NSCs proliferate much slower than their wild type counterparts, they are able to increase their numbers to an extent and they can be maintained in culture. The proliferation phenotype is again semi-prevalent, which means that there are variations between the growth rates of the differentiated knockout NSC populations, although the growth rates are always slower in comparison to the wild type cells. The severity of the total cell cycle block seen after the synchronization of the cells, does not completely correlate with their proliferation phenotype and is probably the result of the mimosine treatment and the temporary arrest of the cells in late G1.

Taking the above observations into account we can conclude that, the slower proliferation described in the NSCs in the absence of *Ldb1* can be the result of misregulation of the G1/S and G2/M checkpoints, which leads to a longer progression through the cell cycle and much slower proliferation. The use of mimosine in order to synchronize the cells normally exerts a reversible block on late G1, from which the wild type NSCs can recover. However in the case of the *Ldb1* null NSCs; the use of mimosine has a more severe effect, probably in combination with the existing misregulation in the expression of other cell cycle regulators. Alternatively other pathways of cell cycle checkpoint control must be present to compensate for the absence of cdc25c and cyclin B1, however they fail to function after the cells have been temporarily arrested in late G1, leading to the cell cycle block seen after the release. It is possible that the *Ldb1* null NSCs can eventually recover from the mimosine block after a longer period of time and this should be investigated further.

In conclusion, *Ldb1* was successfully deleted in mouse ES cells, which were then differentiated towards NSCs. The differentiation model was used in order to investigate in more detail the role of *Ldb1* in neural development. The deletion did not have an effect on the ES cells or on their ability to generate a homogenous population of NSCs. Moreover the resulting NSCs were able to undergo further differentiation and generate astrocyte progenitors and mature astrocytes. However the lack of *Ldb1* expression did have a profound effect on the proliferation capacity of the cells, possibly due to the loss of genes that act downstream of *Ldb1* and have been previously shown to regulate self-renewal in neural stem and progenitor cells or due to a delay in cell cycle progression, supported by the total cell cycle arrest, which followed the temporary block of the *Ldb1* null NSCs in

late G1. The proliferation defect can explain the severe anterior truncation seen in the in the *Ldb1*-/- mouse embryos and the observed twisted shape of the neural tube, which is believed to be the result of a defective expansion of the neuroepithelium.

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References

1. Tam, P.P. and R.R. Behringer, *Mouse gastrulation: the formation of a mammalian body plan.* Mech Dev, 1997. **68**(1-2): p. 3-25.
2. Lee, K.J. and T.M. Jessell, *The specification of dorsal cell fates in the vertebrate central nervous system.* Annu Rev Neurosci, 1999. **22**: p. 261-94.
3. Trainor, P.A., *Specification of neural crest cell formation and migration in mouse embryos.* Semin Cell Dev Biol, 2005. **16**(6): p. 683-93.
4. Anderson, D.J., *Cellular and molecular biology of neural crest cell lineage determination.* Trends Genet, 1997. **13**(7): p. 276-80.
5. Evans, M.J. and M.H. Kaufman, *Establishment in culture of pluripotential cells from mouse embryos.* Nature, 1981. **292**(5819): p. 154-6.
6. Sato, M. and T. Nakano, *Embryonic stem cell.* Intern Med, 2001. **40**(3): p. 195-200.
7. Watt, F.M. and B.L. Hogan, *Out of Eden: stem cells and their niches.* Science, 2000. **287**(5457): p. 1427-30.
8. Pollard, S.M., et al., *Adherent neural stem (NS) cells from fetal and adult forebrain.* Cereb Cortex, 2006. **16 Suppl 1**: p. i112-20.
9. Luo, Y., et al., *Microarray analysis of selected genes in neural stem and progenitor cells.* J Neurochem, 2002. **83**(6): p. 1481-97.
10. Reynolds, B.A. and S. Weiss, *Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system.* Science, 1992. **255**(5052): p. 1707-10.
11. Garcion, E., et al., *Generation of an environmental niche for neural stem cell development by the extracellular matrix molecule tenascin C.* Development, 2004. **131**(14): p. 3423-32.
12. Gritti, A., et al., *Multipotential stem cells from the adult mouse brain proliferate and self-renew in response to basic fibroblast growth factor.* J Neurosci, 1996. **16**(3): p. 1091-100.
13. Bez, A., et al., *Neurosphere and neurosphere-forming cells: morphological and ultrastructural characterization.* Brain Res, 2003. **993**(1-2): p. 18-29.
14. Morcillo, P., et al., *Chip, a widely expressed chromosomal protein required for segmentation and activity of a remote wing margin enhancer in Drosophila.* Genes Dev, 1997. **11**(20): p. 2729-40.
15. Fernandez-Funez, P., et al., *The relative expression amounts of apterous and its co-factor dLdb/Chip are critical for dorso-ventral compartmentalization in the Drosophila wing.* Embo J, 1998. **17**(23): p. 6846-53.
16. Xu, Z., et al., *Identification of a TAL1 target gene reveals a positive role for the LIM domain-binding protein Ldb1 in erythroid gene expression and differentiation.* Mol Cell Biol, 2003. **23**(21): p. 7585-99.
17. Meier, N., et al., *Novel binding partners of Ldb1 are required for haematopoietic development.* Development, 2006. **133**(24): p. 4913-23.
18. Lahlil, R., et al., *SCL assembles a multifactorial complex that determines glycophorin A expression.* Mol Cell Biol, 2004. **24**(4): p. 1439-52.
19. Mukhopadhyay, M., et al., *Functional ablation of the mouse Ldb1 gene results in severe patterning defects during gastrulation.* Development, 2003. **130**(3): p. 495-505.
20. Finkelstein, R., et al., *The orthodenticle gene encodes a novel homeo domain protein involved in the development of the Drosophila nervous system and ocellar visual structures.* Genes Dev, 1990. **4**(9): p. 1516-27.
21. Simeone, A., et al., *A vertebrate gene related to orthodenticle contains a homeodomain of the bicoid class and demarcates anterior neuroectoderm in the gastrulating mouse embryo.* Embo J, 1993. **12**(7): p. 2735-47.
22. Ang, S.L., et al., *A targeted mouse Otx2 mutation leads to severe defects in gastrulation and formation of axial mesoderm and to deletion of rostral brain.* Development, 1996. **122**(1): p. 243-52.
23. van Meyel, D.J., et al., *Chip is an essential cofactor for apterous in the regulation of axon guidance in Drosophila.* Development, 2000. **127**(9): p. 1823-31.
24. Treisman, J.E., et al., *eyelid antagonizes wingless signaling during Drosophila development and has homology to the Bright family of DNA-binding proteins.* Genes Dev, 1997. **11**(15): p. 1949-62.
25. Heitzler, P., et al., *Enhancer-promoter communication mediated by Chip during Pannier-driven proneural patterning is regulated by Osa.* Genes Dev, 2003. **17**(5): p. 591-6.
26. Shawlot, W. and R.R. Behringer, *Requirement for Lim1 in head-organizer function.* Nature, 1995. **374**(6521): p. 425-30.

27. Nishioka, N., et al., *Ssdp1 regulates head morphogenesis of mouse embryos by activating the Lim1-Ldb1 complex.* Development, 2005. **132**(11): p. 2535-46.
28. Matthews, J.M. and J.E. Visvader, *LIM-domain-binding protein 1: a multifunctional cofactor that interacts with diverse proteins.* EMBO Rep, 2003. **4**(12): p. 1132-7.
29. Xu, Z., et al., *Single-stranded DNA-binding proteins regulate the abundance of LIM domain and LIM domain-binding proteins.* Genes Dev, 2007. **21**(8): p. 942-55.
30. Ostendorff, H.P., et al., *Dynamic expression of LIM cofactors in the developing mouse neural tube.* Dev Dyn, 2006. **235**(3): p. 786-91.
31. Hobert, O. and H. Westphal, *Functions of LIM-homeobox genes.* Trends Genet, 2000. **16**(2): p. 75-83.
32. Shirasaki, R. and S.L. Pfaff, *Transcriptional codes and the control of neuronal identity.* Annu Rev Neurosci, 2002. **25**: p. 251-81.
33. Thaler, J.P., et al., *LIM factor Lhx3 contributes to the specification of motor neuron and interneuron identity through cell-type-specific protein-protein interactions.* Cell, 2002. **110**(2): p. 237-49.
34. Zhao, Y., et al., *LIM-homeodomain proteins Lhx1 and Lhx5, and their cofactor Ldb1, control Purkinje cell differentiation in the developing cerebellum.* Proc Natl Acad Sci U S A, 2007. **104**(32): p. 13182-6.
35. Lee, S.K. and S.L. Pfaff, *Synchronization of neurogenesis and motor neuron specification by direct coupling of bHLH and homeodomain transcription factors.* Neuron, 2003. **38**(5): p. 731-45.
36. Conti, L., et al., *Niche-independent symmetrical self-renewal of a mammalian tissue stem cell.* PLoS Biol, 2005. **3**(9): p. e283.
37. Yamashita, T., et al., *Genomic structure and chromosomal localization of the mouse LIM domain-binding protein 1 gene, Ldb1.* Genomics, 1998. **48**(1): p. 87-92.
38. Ying, Q.L., et al., *Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture.* Nat Biotechnol, 2003. **21**(2): p. 183-6.
39. Scholer, H.R., et al., *Octamer binding proteins confer transcriptional activity in early mouse embryogenesis.* Embo J, 1989. **8**(9): p. 2551-7.
40. Lendahl, U., L.B. Zimmerman, and R.D. McKay, *CNS stem cells express a new class of intermediate filament protein.* Cell, 1990. **60**(4): p. 585-95.
41. Zimmerman, L., et al., *Independent regulatory elements in the nestin gene direct transgene expression to neural stem cells or muscle precursors.* Neuron, 1994. **12**(1): p. 11-24.
42. Goolsby, J., et al., *Hematopoietic progenitors express neural genes.* Proc Natl Acad Sci U S A, 2003. **100**(25): p. 14926-31.
43. Terskikh, A.V., et al., *From hematopoiesis to neuropoiesis: evidence of overlapping genetic programs.* Proc Natl Acad Sci U S A, 2001. **98**(14): p. 7934-9.
44. Bjornson, C.R., et al., *Turning brain into blood: a hematopoietic fate adopted by adult neural stem cells in vivo.* Science, 1999. **283**(5401): p. 534-7.
45. Harder, F., et al., *Erythroid-like cells from neural stem cells injected into blastocysts.* Exp Hematol, 2004. **32**(7): p. 673-82.
46. Malaterre, J., R.G. Ramsay, and T. Mantamadiotis, *Wnt-Frizzled signalling and the many paths to neural development and adult brain homeostasis.* Front Biosci, 2007. **12**: p. 492-506.
47. Montcouquiol, M., E.B. Crenshaw, 3rd, and M.W. Kelley, *Noncanonical Wnt signaling and neural polarity.* Annu Rev Neurosci, 2006. **29**: p. 363-86.
48. Sommer, L., *Multiple roles of canonical Wnt signaling in cell cycle progression and cell lineage specification in neural development.* Cell Cycle, 2004. **3**(6): p. 701-3.
49. Chesnutt, C., et al., *Coordinate regulation of neural tube patterning and proliferation by TGFbeta and WNT activity.* Dev Biol, 2004. **274**(2): p. 334-47.
50. Kitisin, K., et al., *Tgf-Beta signaling in development.* Sci STKE, 2007. **2007**(399): p. cm1.
51. Roussa, E., et al., *Transforming growth factor beta is required for differentiation of mouse mesencephalic progenitors into dopaminergic neurons in vitro and in vivo: ectopic induction in dorsal mesencephalon.* Stem Cells, 2006. **24**(9): p. 2120-9.
52. Louvi, A. and S. Artavanis-Tsakonas, *Notch signalling in vertebrate neural development.* Nat Rev Neurosci, 2006. **7**(2): p. 93-102.
53. Yoon, K. and N. Gaiano, *Notch signaling in the mammalian central nervous system: insights from mouse mutants.* Nat Neurosci, 2005. **8**(6): p. 709-15.
54. Tucker, R.P., et al., *Phylogenetic analysis of the tenascin gene family: evidence of origin early in the chordate lineage.* BMC Evol Biol, 2006. **6**: p. 60.

55. von Holst, A., et al., *Neural stem/progenitor cells express 20 tenascin C isoforms that are differentially regulated by Pax6.* J Biol Chem, 2007. **282**(12): p. 9172-81.
56. Joester, A. and A. Faissner, *Evidence for combinatorial variability of tenascin-C isoforms and developmental regulation in the mouse central nervous system.* J Biol Chem, 1999. **274**(24): p. 17144-51.
57. Gates, M.A., et al., *Cell and molecular analysis of the developing and adult mouse subventricular zone of the cerebral hemispheres.* J Comp Neurol, 1995. **361**(2): p. 249-66.
58. Bartsch, S., et al., *Expression of tenascin in the developing and adult cerebellar cortex.* J Neurosci, 1992. **12**(3): p. 736-49.
59. von Holst, A., *Tenascin C in stem cell niches: redundant, permissive or instructive?* Cells Tissues Organs, 2008. **188**(1-2): p. 170-7.
60. Garcion, E., A. Faissner, and C. ffrench-Constant, *Knockout mice reveal a contribution of the extracellular matrix molecule tenascin-C to neural precursor proliferation and migration.* Development, 2001. **128**(13): p. 2485-96.
61. McKay, R., *Stem cells in the central nervous system.* Science, 1997. **276**(5309): p. 66-71.
62. Cabrera, C.V., A. Martinez-Arias, and M. Bate, *The expression of three members of the achaete-scute gene complex correlates with neuroblast segregation in Drosophila.* Cell, 1987. **50**(3): p. 425-33.
63. Skeath, J.B. and S.B. Carroll, *Regulation of proneural gene expression and cell fate during neuroblast segregation in the Drosophila embryo.* Development, 1992. **114**(4): p. 939-46.
64. Jimenez, F. and J.A. Campos-Ortega, *Defective neuroblast commitment in mutants of the achaete-scute complex and adjacent genes of D. melanogaster.* Neuron, 1990. **5**(1): p. 81-9.
65. Guillemot, F., et al., *Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons.* Cell, 1993. **75**(3): p. 463-76.
66. Mercer, A., et al., *PACAP promotes neural stem cell proliferation in adult mouse brain.* J Neurosci Res, 2004. **76**(2): p. 205-15.
67. Ma, Q., et al., *Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice.* Proc Natl Acad Sci U S A, 1998. **95**(16): p. 9448-53.
68. Zou, Y.R., et al., *Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development.* Nature, 1998. **393**(6685): p. 595-9.
69. Lazarini, F., et al., *Role of the alpha-chemokine stromal cell-derived factor (SDF-1) in the developing and mature central nervous system.* Glia, 2003. **42**(2): p. 139-48.
70. Klein, R.S., et al., *SDF-1 alpha induces chemotaxis and enhances Sonic hedgehog-induced proliferation of cerebellar granule cells.* Development, 2001. **128**(11): p. 1971-81.
71. Lu, M., E.A. Grove, and R.J. Miller, *Abnormal development of the hippocampal dentate gyrus in mice lacking the CXCR4 chemokine receptor.* Proc Natl Acad Sci U S A, 2002. **99**(10): p. 7090-5.
72. Arakawa, Y., et al., *Control of axon elongation via an SDF-1alpha/Rho/mDia pathway in cultured cerebellar granule neurons.* J Cell Biol, 2003. **161**(2): p. 381-91.
73. Pujol, F., P. Kitabgi, and H. Boudin, *The chemokine SDF-1 differentially regulates axonal elongation and branching in hippocampal neurons.* J Cell Sci, 2005. **118**(Pt 5): p. 1071-80.
74. Furnari, B., et al., *Cdc25 inhibited in vivo and in vitro by checkpoint kinases Cds1 and Chk1.* Mol Biol Cell, 1999. **10**(4): p. 833-45.
75. Peng, C.Y., et al., *Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216.* Science, 1997. **277**(5331): p. 1501-5.
76. Sanchez, Y., et al., *Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25.* Science, 1997. **277**(5331): p. 1497-501.
77. Okamoto, S., et al., *HIV/gp120 decreases adult neural progenitor cell proliferation via checkpoint kinase-mediated cell-cycle withdrawal and G1 arrest.* Cell Stem Cell, 2007. **1**(2): p. 230-6.
78. Manke, I.A., et al., *MAPKAP kinase-2 is a cell cycle checkpoint kinase that regulates the G2/M transition and S phase progression in response to UV irradiation.* Mol Cell, 2005. **17**(1): p. 37-48.
79. Wang, X.W., et al., *GADD45 induction of a G2/M cell cycle checkpoint.* Proc Natl Acad Sci U S A, 1999. **96**(7): p. 3706-11.
80. Lalande, M., *A reversible arrest point in the late G1 phase of the mammalian cell cycle.* Exp Cell Res, 1990. **186**(2): p. 332-9.
81. Watson, P.A., et al., *Mimosine reversibly arrests cell cycle progression at the G1-S phase border.* Cytometry, 1991. **12**(3): p. 242-6.

82. Krude, T., *Mimosine arrests proliferating human cells before onset of DNA replication in a dose-dependent manner.* Exp Cell Res, 1999. **247**(1): p. 148-59.
83. Gage, F.H., et al., *Multipotent progenitor cells in the adult dentate gyrus.* J Neurobiol, 1998. **36**(2): p. 249-66.
84. Doetsch, F., et al., *Subventricular zone astrocytes are neural stem cells in the adult mammalian brain.* Cell, 1999. **97**(6): p. 703-16.
85. Alvarez-Buylla, A., J.M. Garcia-Verdugo, and A.D. Tramontin, *A unified hypothesis on the lineage of neural stem cells.* Nat Rev Neurosci, 2001. **2**(4): p. 287-93.
86. Gotz, M., *Glial cells generate neurons--master control within CNS regions: developmental perspectives on neural stem cells.* Neuroscientist, 2003. **9**(5): p. 379-97.
87. Merkle, F.T., et al., *Radial glia give rise to adult neural stem cells in the subventricular zone.* Proc Natl Acad Sci U S A, 2004. **101**(50): p. 17528-32.
88. Rakic, P., *Neuron-glia relationship during granule cell migration in developing cerebellar cortex. A Golgi and electronmicroscopic study in Macacus Rhesus.* J Comp Neurol, 1971. **141**(3): p. 283-312.
89. Feldman, S.T. and A. Schonthal, *Negative regulation of histone H1 kinase expression by mimosine, a plant amino acid.* Cancer Res, 1994. **54**(2): p. 494-8.
90. Wang, G., R. Miskimins, and W.K. Miskimins, *Mimosine arrests cells in G1 by enhancing the levels of p27(Kip1).* Exp Cell Res, 2000. **254**(1): p. 64-71.
91. Alpan, R.S. and A.B. Pardee, *p21WAF1/CIP1/SDI1 is elevated through a p53-independent pathway by mimosine.* Cell Growth Differ, 1996. **7**(7): p. 893-901.
92. Chang, H.C., et al., *Inhibitory effect of mimosine on proliferation of human lung cancer cells is mediated by multiple mechanisms.* Cancer Lett, 1999. **145**(1-2): p. 1-8.
93. Dong, Z., et al., *Modulation of differentiation-related gene 1 expression by cell cycle blocker mimosine, revealed by proteomic analysis.* Mol Cell Proteomics, 2005. **4**(7): p. 993-1001.

Chapter 5

Discussion

Discussion

The development of the mammalian embryo is a complex process characterized by a line of events that begin after fertilization and will eventually lead to the generation of all the cell types, tissues and organs of the adult animal. It is tightly controlled and involves the coordinated action of many signalling pathways and transcription regulators, which will direct the expression of genes that determine cell fate, differentiation and maturation. Cell cycle regulators and factors that control cell proliferation are also important in embryonic development and cell differentiation. From the generation of the zygote and the cleavage steps that lead from the two-cell stage to the blastocyst at 3.5 dpc, the number of regulatory factors that control embryonic development gradually increases. An important part of the correct programming in the initial stages of embryonic development is the contribution of maternal RNA, which starts to be degraded after the two-cell stage as genes that control embryonic development are switched on [1-4]. Regulatory factors, which are deposited as maternal RNA in the developing zygote prior to the onset of their expression, can be considered as master regulators of embryonic development. One such factor is the non-DNA binding Ldb1 as it was shown from studies done in *Drosophila* and mouse embryos [5, 6]. Its main function is to participate in the control of gene expression either by assisting the assemble of multi-protein complexes that act as transcription regulators or by facilitating long range interactions between gene promoters and regulatory elements often separated by large distances.

The mouse embryo has been extensively used as a model for the study of mammalian embryonic development. An important group of cells in the developing blastocyst are the ES cells, which are located in the ICM. These cells will give rise to the three germ layers of the developing embryo and subsequently to all the cell types, tissues and organs of the adult animal. ES cells can be derived from the blastocyst and maintained in adherent cultures *in vitro* for an unlimited period of time. They are characterized by their ability to remain in an undifferentiated state indefinitely through self-renewal and to differentiate towards any cell type under specific culture conditions. When grown in suspension ES cells form the spherical embryoid bodies that consist of cells of the three germ layers mimicking early embryonic development. However the comparison is somewhat restricted since embryoid bodies do not have a defined polarity or body plan. Despite this limitation ES cells and embryoid bodies are an important tool for the research into embryonic development and cell differentiation.

Ldb1 is involved at the earliest step of cell differentiation during hematopoiesis

The earliest step in mouse hematopoiesis is the emergence of the hemangioblast from *brachyury* expressing mesodermal cells that originate in the posterior of the primitive streak. It has been proposed that the specification of these mesodermal cells towards the hemangioblast lineage takes place prior to the cells leaving the primitive streak environment and migrating towards the extra-embryonic visceral yolk sac, where they will form the blood

islands by rapidly differentiating towards either the endothelial or the primitive erythroblast lineage via an intermediate step of hemogenic endothelium formation [7, 8].
The differentiation of ES cells into embryoid bodies has been proven as a useful tool for the study of the emergence and differentiation of the hemangioblast and Flk1 has been used as a marker to identify this cell. The BL-CFC from day 4 embryoid bodies has been identified as the *in vitro* equivalent of the hemangioblast as it can form blast colonies, which have the potential to generate both endothelial and hematopoietic progenitors *in vitro*.
The *Ldb1*-/- mouse is embryonic lethal after 9.5 dpc. Among the severe developmental defects, that we and others have observed, was the lack of hematopoiesis and the complete absence of blood islands in the extra-embryonic yolk sac. In addition no red blood cells were seen in circulation either in the yolk sac or the embryo proper at 8.5 and 9.5 dpc. Both primitive and definitive hematopoiesis are initiated in the yolk sac at 7.5 dpc and 8.25 dpc respectively, while circulation between the yolk sac and the embryo proper is established between 8.25 and 8.5 dpc as the newly formed heart begins to beat. However the main site for definitive hematopoiesis is considered the embryonic PSp/AGM region, where HSCs emerge at approximately 10.5 dpc just prior to the colonization of the fetal liver that takes place between 10.5 and 11.5 dpc. The severity of the effect that the deletion of *Ldb1* has on mouse hematopoietic development led to the conclusion that the observed phenotype is the result of a misregulation during the development of the hemangioblast.
In order to gain more insight into the role of *Ldb1* in hemangioblast regulation an *Ldb1* knockout ES cell line was differentiated towards embryoid bodies and the emergence and differentiation potential of the BL-CFCs was investigated in detail. We observed that *Ldb1* has a dual role in the development of the hemangioblast in that it is involved in the regulation of proliferation as well as differentiation. *Ldb1*-/- embryoid bodies grown in suspension for 4 days contain BL-CFCs , but their total number is approximately half compared to that seen in the *Ldb1*+/+ embryoid bodies. Furthermore these BL-CFCs do not form blast colonies in culture and eventually die.
Ldb1 can form homodimers and participate in multi-protein complexes through the binding of the LID domain on the two tandem LIM domain motif of LIM-HDs and LMOs. In hematopoietic cell lines Ldb1 forms a core complex with Gata-1or Gata-2, Lmo2 and the Scl/E2A heterodimer. Scl is a bHLH transcription factor that binds on the E-box DNA motif. It has been identified as an essential regulator of hematopoiesis as *Scl*-/- mouse embryos die by 10.5 dpc and show a similar phenotype with the *Ldb1*-/- embryos with respect to hematopoietic development. Scl is not involved in the initial emergence of the BL-CFCs. Instead it has been proposed that it functions in the subsequent differentiation step towards the formation of the blast colonies and the establishment of their hematopoietic potential. Recently in was proposed that Scl is involved in the differentiation of the hemangioblast towards the hemogenic endothelium, prior to the emergence of the primitive erythroblasts [8, 9].
The expression of *Scl* was down-regulated in BL-CFCs isolated from *Ldb1*-/- day 4 embryoid

bodies as was the expression of a number of essential hematopoiesis regulatory factors. One such factor that we identified as acting downstream of *Ldb1* is *Fli1*. In the *Xenopus* embryo *Fli1* was shown to act upstream of *Scl* in the differentiation of the hemangioblast. The use of morpholinos did show that in the absence of *Fli1* hemangioblast cells are still able to emerge but their differentiation is blocked and they undergo apoptosis [10]. This phenotype is similar to the one observed in the *Ldb1*-/- embryoid bodies. Fli1 binds the *Scl* +19 enhancer element, which drives the expression of *Scl* in cells of mesodermal origin capable of differentiating towards blood, endothelium and smooth muscle cells as well as osteoprogenitors [11, 12]. The lack of expression of both *Fli1* and *Scl* can explain the inability of the *Ldb1*-/- BL-CFCs to undergo differentiation and form blast colonies, but it does not explain the reduced number of BL-CFCs in the *Ldb1*-/- embryoid bodies.

The hematopoietic regulator *Runx1* is expressed in the yolk sac between 7.5 dpc and 8.25 dpc, although its absence only affects the process of definitive hematopoiesis as it has been demonstrated in the knockout mouse. However the generation of a *Runx1* -/- ES cell line did show that contrary to previous conclusions *Runx1* may be involved in hemangioblast development since *Runx1*-/- embryoid bodies generated fewer blast colonies when compared to wild type ones. The differentiation potential of these blast colonies was impaired only towards definitive hematopoiesis, in accordance to previous observations of the role of *Runx1* in hematopoietic development [13]. Furthermore the core Ldb1/Scl/Lmo2/Gata-2 complex with the addition of Ets regulatory factors binds on the *Runx1* +23.5 enhancer, which was found to drive *Runx1* expression in hematopoietic tissues including the yolk sac [14]. We concluded that *Runx1* acts downstream of *Ldb1* in hemangioblast development, based on gene expression changes identified between *Ldb1*+/+ and *Ldb1*-/- BL-CFCs and that its down-regulation may result in the reduced number of Flk1+ BL-CFCs present in the *Ldb1*-/- embryoid bodies. Moreover from the gene expression arrays we identified *Ldb1* as the upstream regulator of a number of transcription factors, which are essential for the expression of many genes that will determine the hematopoietic cell fate.

It was interesting to observe that the Flk1+ BL-CFCs isolated from *Ldb1*-/- embryoid bodies show an increase in *brachyury* expression. Although *brachyury* is normally expressed in Flk1+ BL-CFCs that give rise to blast colonies [15], its elevated expression could be the cause of a misregulation in the specification of mesodermal cells towards the hemangioblast lineage in the posterior primitive streak. One explanation for the observed failure in hemangioblast development could be that the cells have maintained a mesodermal identity that hinders their differentiation.

Ldb is involved in neural development

The *Ldb1*-/- mouse embryo is characterized by truncation of the anterior, which leads to the absence of all head structures above the otic vesicle including the forebrain, midbrain and anterior hindbrain. A search for similar phenotypes in knockout mice did reveal that similar anterior truncation patterns have been observed due to the deletion of the genes

Otx2, *Lim1* and *Ssbp1* [16-19]. The LIM-HD factor Lim1 is an essential regulator of the head organizer [18] and can bind to Ldb1, while Ssbp proteins have been shown to protect Ldb1 from RLIM promoted ubiquitination and proteosome degradation. The homeobox factor Otx2 is expressed in the anterior visceral endoderm and it can bind to Lim1 [20]. Additionally Lim1, Ldb1 and Otx2 act synergistically on the promoter of *goosecoid* and regulate its expression in *Xenopus*. However we observed that the expression of *Otx2* is also dependent on the proper function of *Ldb1* and this is also the case in the *Xenopus* head organizer [21].

An additional defect in neural development identified in the *Ldb1*-/- mouse was the structure of the neural tube, which was twisted and this was attributed to a defective expansion of the neuroepithelium. In order to gain further insight in the role of *Ldb1* in neural development, *Ldb1*-/- ES cells were differentiated towards NSCs that resemble neuroepithelial radial glia cells. We observed that *Ldb1* is not necessary either for the generation of NSCs *in vitro* or for their differentiation potential at least towards the astrocyte lineage. Rather it is involved in maintaining their self-renewal capacity, an essential aspect of the stem cell identity. The *Ldb1*-/- NSCs exhibited a defect in proliferation from which they were not able to recover even after being kept in culture for prolonged periods of time.

Ldb1 is involved in the proliferation of progenitor cells

The data obtained from the differentiation of *Ldb1*-/- ES cells into embryoid bodies and NSCs have identified *Ldb1* as a regulator of progenitor cell proliferation. The self-renewal capacity of ES cells is not affected by the deletion; however as soon as the cells differentiate towards NSCs, a negative effect on their proliferation is immediately obvious. Similarly Flk1+ BL-CFCs isolated from *Ldb1*-/- embryoid bodies show a reduction of approximately 50% in number compared to wild type cells. The involvement of *Ldb1* in cell proliferation has been additionally reported in erythroid progenitor cell lines, the cerebellum and epithelial cells.

Scl/E2A and Scl/HEB heterodimers as part of an Ldb1 containing complex interact with Eto2, which functions as a negative regulator of Scl target genes and a positive regulator of erythroid progenitor cell proliferation. The stoichiometry of Eto2 to Scl within the complex is essential for its proper function. However it was determined that Eto2 is in fact dispensable for the terminal differentiation of erythroid cells. On the other had it is involved in the expansion of erythroid progenitors. An Scl/Eto2 complex was found to bind on the promoters of *Gfi1b* and *p21* and directly control their expression [22]. The expression of *Eto2* as well as *Gfi1b* was down-regulated in the *Ldb1*-/- BL-CFCs, which could explain their reduced number.

The Ldb1 core complex interacts with pRb (protein Retinoblastoma) and negatively regulates the expression of *c-kit* in human erythroid progenitor cell lines, through direct promoter binding [23]. pRb is a tumor suppressor that is involved in the regulation of cell proliferation and differentiation through its function in cell cycle progression [24, 25]. While it is not completely understood how pRb affects cell proliferation, it is known that during

the cell cycle it functions at the G1/S checkpoint [26]. It is also known that pRb interacts with members of the E2F family and specifically blocks their transactivation domain. Prior to the entry into S phase pRb releases the E2F factors, which regulate the expression of genes involved in the exit from G1 and progression into S [27].
Additionally the conditional deletion of *Ldb1* in the developing cerebellum of mouse embryos resulted in a reduction in the numbers of Purkinje cells at 18.5 dpc. The same phenotype was observed in the *Lhx1*/*Lhx5* double knockout mice and may be the result of a proliferation defect [28].
The conditional deletion of *Lmo4* in mice leads to a decrease in the proliferation of mammary epithelial cells by up to 50% [29]. Lmo4 forms a complex with Ldb1 that binds on the promoter of *Bmp7* and regulates its expression, with the additional involvement of HDAC2, in human breast cancer cells. The expression of *Bmp7* was reduced in the *Ldb1* -/- BL-CFCs.

Ldb1 in cancer

Ldb1 is involved in a type of human leukemia through a translocation that creates a fusion product with *Lmo1* [30]. While this is not surprising since most of the hematopoiesis regulators were initially identified as fusion products of chromosome rearrangements that cause leukemias, *Ldb1* is believed to be involved in the emergence of other types of cancer and this is to be expected if we consider its demonstrated involvement in the control of cell proliferation. *Ldb1* and *Lmo4* were found to be overexpressed at the invasive portion of the oral squamous carcinoma cells, particularly in less differentiated carcinoma cells, as well as in cervical lymph node metastasis. Oral squamous carcinomas are neoplasms of the head and neck that have a worldwide incidence of 300.000 cases annually and are characterized by a high mortality rate [31]. In addition *Lmo4* is overexpressed in 50% of sporadic breast cancer cases. The encoding protein was found to interact with the breast and ovarian cancer tumor suppressor BRCA1 and negatively regulate its function as transcription regulator [32, 33].

Common genes in hematopoietic and neural development

The expression analysis of *Ldb1*-/- BL-CFCs and *Ldb1*-/- NSCs revealed a number of genes that were differentially expressed in both cell types and can be seen in Table 1. Altogether 79 genes were identified as having an altered pattern of expression as the direct result of the *Ldb1* deletion in both types of progenitor cells. The majority of them, 54 or 68.4%, showed the same pattern of misregulation i.e. they were found to be either down-regulated or up-regulated in both BL-CFCs and NSCs. However 25 genes or 31.6% showed an opposite pattern of misregulation. From these ones, 20 genes or 80% were down-regulated in the BL-CFCs and up-regulated in the NSCs, while 5 genes or 20% were up-regulated in the BL-CFCs and down-regulated in the NSCs.
An overlap in gene expression patterns between mouse adult HSCs and neurospheres enriched in neural stem and progenitor cells has been described previously, as common genes expressed in both types of cells were identified with the use of expression arrays

[34].

Proliferating hematopoietic progenitor cells from the bone marrow of adult mice positive for the HSCs markers CD34, Sca-1, c-kit and AA4.1, were shown to express genes that identify neural cells. When these cells were transplanted into the brain of adult mice they survived for 14 months and differentiated into distinct lineages of neuronal cells while beginning to express specific markers. The expression of CD34 was lost approximately 2 months after the transplantation took place [35].

Furthermore NSCs isolated from neurospheres and injected in pre-implantation blastocysts were found to contribute to hematopoietic tissues and express markers specific for erythroid cells as well as globins during the embryonic development of the hosts [36].

HSCs and NSCs share the ability of self-renewal as a common characteristic, which is also essential for their specification as stem cells. Their self-renewal capacity may be regulated by the same genes expressed in both cells types. One such gene could be *Ldb1*, which is involved in the control of the proliferation of both BL-CFCs and NSCs, although the hierarchy through which *Ldb1* functions in this process remains to be investigated.

The identification of factors that are involved in the regulation of stem and progenitor cell proliferation, maintenance and differentiation can be useful in the ongoing research effort towards the establishment of successful therapeutic methods for the cure of many disorders and pathological conditions.

Table 1. List of the genes that show differential expression in *Ldb1*-/- BL-CFCs and *Ldb1*-/- NSCs compared with wild type BL-CFCs and NSCs respectively. The fold change (up-regulation or down-regulation) in the expression of each gene in the knockout cells is given. Bold characters indicate the genes that have the lowest q-values. Grey squares distinguish the genes that show opposite patterns of differential expression in the *Ldb1*-/- BL-CFCs and the *Ldb1*-/- NSCs.

Gene Symbol	Gene Title	Fold Change BL-CFCs	Fold Change NSCs
Nnat	neuronatin	0.58	**0.0214**
Ednrb	endothelin receptor type B	0.612	**0.0361**
Aldoc	aldolase 3, C isoform	0.311	**0.0687**
Lrrn1	leucine rich repeat protein 1, neuronal	0.523	**0.0886**
Atp1a2	ATPase, Na+/K+ transporting, alpha 2 polypeptide	0.652	**0.0896**
Lfng	LFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase	0.6	**0.0925**
Mest	mesoderm specific transcript	**0.046**	**0.107**
Ddx3y	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	**0.033**	**0.109**
Eif2s3y	eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked	0.016	**0.117**
Zcchc12	zinc finger, CCHC domain containing 12	0.655	**0.12**
Lmo1	LIM domain only 1	1.899	**0.127**
Bcas1	breast carcinoma amplified sequence 1	0.661	**0.131**
Smpdl3a	sphingomyelin phosphodiesterase, acid-like 3A	0.538	**0.138**
Rbmx	RNA binding motif protein, X chromosome	0.338	**0.139**
Fzd9	frizzled homolog 9 (Drosophila)	0.643	**0.142**
Cxcr4	chemokine (C-X-C motif) receptor 4	0.662	**0.149**
Rnd2	Rho family GTPase 2	**0.214**	**0.153**
Slc1a3	solute carrier family 1 (glial high affinity glutamate transporter), member 3	0.522	**0.16**
Nrarp	Notch-regulated ankyrin repeat protein	1.661	**0.161**
Lgr5	leucine rich repeat containing G protein coupled receptor 5	0.509	0.161
Tnc	tenascin C	0.655	**0.176**
Tspan12	tetraspanin 12	0.431	**0.195**
Hoxd8	homeo box D8	0.42	0.228
Tagln3	transgelin 3	0.621	0.231
Hes6	hairy and enhancer of split 6 (Drosophila)	1.776	0.235
Sox21	SRY-box containing gene 21	1.679	0.236
Bcan	Brevican	0.538	**0.238**
Smpdl3b	sphingomyelin phosphodiesterase, acid-like 3B	0.56	0.238
Kcnc1	potassium voltage gated channel, Shaw-related subfamily, member 1	1.717	0.245

Gene Symbol	Gene Title	Fold Change BL-CFCs	Fold Change NSCs
Sema6a	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6A	0.648	0.249
Homer2	homer homolog 2 (Drosophila)	0.516	0.258
Mcart6	mitochondrial carrier triple repeat 6	0.666	0.286
B3gnt2	UDP-GlcNAc: betaGal beta-1,3-N-acetylglucosaminyltransferase 2	0.596	3.293
Smad3	MAD homolog 3 (Drosophila)	0.664	3.34
Cd151	CD151 antigen	0.637	3.516
Mmp14	matrix metallopeptidase 14 (membrane-inserted)	1.857	3.518
Esd	esterase D/formylglutathione hydrolase	1.526	3.608
Pcolce	procollagen C-endopeptidase enhancer protein	1.5	3.666
Npr2	natriuretic peptide receptor 2	0.658	3.717
Rora	RAR-related orphan receptor alpha	0.558	3.81
Nr1d1	nuclear receptor subfamily 1, group D, member 1	0.648	3.839
Myh9	myosin, heavy polypeptide 9, non-muscle	2	4
Bace2	beta-site APP-cleaving enzyme 2	2.242	4
Cd9	CD9 antigen	2.611	4.153
Ablim1	actin-binding LIM protein 1	0.569	4.212
Ndrg1	N-myc downstream regulated gene 1	0.621	4.722
Anxa5	annexin A5	1.663	4.755
Nfat5	nuclear factor of activated T-cells 5	2	4.839
Hebp1	heme binding protein 1	1.677	**4.878**
Lpp	LIM domain containing preferred translocation partner in lipoma	1.662	4.901
Ero1lb	ERO1-like beta (S. cerevisiae)	0.651	4.932
Lmna	lamin A	1.882	**5.209**
Bmp2	bone morphogenetic protein 2	0.523	5.239
Cited2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	0.653	5.244
Arhgap6	Rho GTPase activating protein 6	**0.5**	**5.29**
Epha2	Eph receptor A2	1.612	5.322
Zfhx3	zinc finger homeobox 3	2.1	**5.324**
Spnb2	spectrin beta 2	0.555	5.471
Crlf1	cytokine receptor-like factor 1	**5.757**	5.504
Tgm2	transglutaminase 2, C polypeptide	**0.5**	5.724
Dlc1	deleted in liver cancer 1	1.547	**5.947**
Trib3	tribbles homolog 3 (Drosophila)	1.549	6.377
Socs3	suppressor of cytokine signaling 3	0.59	6.764

Gene Symbol	Gene Title	Fold Change BL-CFCs	Fold Change NSCs
Cgnl1	cingulin-like 1	0.571	6.872
Sbno2	strawberry notch homolog 2 (Drosophila)	1.565	6.923
Rbpms	RNA binding protein gene with multiple splicing	1.51	7.052
Efnb2	ephrin B2	**0.522**	**7.586**
Gbp2	guanylate nucleotide binding protein 2	**2.515**	8.752
Tgfbi	transforming growth factor, beta induced	2.244	9.623
Serpinf1	serine (or cysteine) peptidase inhibitor, clade F, member 1	1.558	**10.368**
Anxa2	annexin A2	1.769	**10.523**
Srxn1	sulfiredoxin 1 homolog (S. cerevisiae)	0.619	**10.567**
Ifi35	interferon-induced protein 35	0.653	**11.143**
Ass1	argininosuccinate synthetase 1	1.5	**11.851**
Adm	adrenomedullin	0.645	**13.65**
Timp1	tissue inhibitor of metalloproteinase 1	1.531	**15.113**
S100a6	S100 calcium binding protein A6 (calcyclin)	2.536	19.75
Meg3	maternally expressed 3	1.54	40.338
Clu	clusterin/similar to clusterin	1.799	**72.57**

Future prospects

The expression profile of two distinct types of progenitor cells, BL-CFCs and NSCs in the absence of *Ldb1* has been determined. From our data we concluded that *Ldb1* is an essential factor of cell differentiation during hematopoiesis, as well as a regulator of cell proliferation during both hematopoiesis and neural development. Although a large number of transcription factor showed misregulation due to the deletion of *Ldb1*, its direct targets in these two specific cell populations remain to be identified, in order to shed more light into its role in cell fate specification.

Long range interactions, which describe the communication between promoters and distant regulatory elements, are achieved through the physical bending of the DNA molecule and the formation of a loop-like structure. Such interactions have been shown to be important in gene expression regulation during cell differentiation. For example the expression of genes of the α- and β-globin loci as well as the Th2 locus depends on such interactions during cell differentiation. Ldb1 is believed to function as a facilitator of long range interactions.

ES cells deficient for *Ldb1* show no phenotype, which means that their self-renewal and differentiation capacities are not affected. As soon as the cells differentiate towards lineage restricted progenitors a phenotype is obvious. It is possible that interactions between promoters and distant regulatory elements are not involved in the maintenance of ES cells and that the expression of ES cell specific genes is driven by promoters or regulatory elements located in close proximity to the transcription initiation site. During differentiation the necessity of more distantly located regulatory elements for transcription initiation or repression increases and factors such as *Ldb1* begin to actively participate in the regulation of gene expression.

It will be interesting to determine whether Ldb1, which is expressed in ES cells, participates in any protein complexes in these cells and if it regulates the expression of any genes. We also aim to compare the positioning of Ldb1 binding sites in the genome of ES cells, BL-CFCs and NSCs, in order to determine whether Ldb1 begins to influence long range interactions, at the onset of cell differentiation and not before. Finally we would like to investigate whether long range interactions are initiated as a mechanism of transcription regulation and gene expression during the differentiation of ES cells towards more restricted cell lineages.

References

1. Clegg, K.B. and L. Piko, *Poly(A) length, cytoplasmic adenylation and synthesis of poly(A)+ RNA in early mouse embryos.* Dev Biol, 1983. **95**(2): p. 331-41.
2. Giebelhaus, D.H., J.J. Heikkila, and G.A. Schultz, *Changes in the quantity of histone and actin messenger RNA during the development of preimplantation mouse embryos.* Dev Biol, 1983. **98**(1): p. 148-54.
3. Johnson, M.H., *The molecular and cellular basis of preimplantation mouse development.* Biol Rev Camb Philos Soc, 1981. **56**(3): p. 463-98.
4. Pratt, H.P., V.N. Bolton, and K.A. Gudgeon, *The legacy from the oocyte and its role in controlling early development of the mouse embryo.* Ciba Found Symp, 1983. **98**: p. 197-227.
5. Mukhopadhyay, M., et al., *Functional ablation of the mouse Ldb1 gene results in severe patterning defects during gastrulation.* Development, 2003. **130**(3): p. 495-505.
6. Morcillo, P., et al., *Chip, a widely expressed chromosomal protein required for segmentation and activity of a remote wing margin enhancer in Drosophila.* Genes Dev, 1997. **11**(20): p. 2729-40.
7. Huber, T.L., et al., *Haemangioblast commitment is initiated in the primitive streak of the mouse embryo.* Nature, 2004. **432**(7017): p. 625-30.
8. Lancrin, C., et al., *The haemangioblast generates haematopoietic cells through a haemogenic endothelium stage.* Nature, 2009. **457**(7231): p. 892-5.
9. D'Souza, S.L., A.G. Elefanty, and G. Keller, *SCL/Tal-1 is essential for hematopoietic commitment of the hemangioblast but not for its development.* Blood, 2005. **105**(10): p. 3862-70.
10. Liu, F., et al., *Fli1 acts at the top of the transcriptional network driving blood and endothelial development.* Curr Biol, 2008. **18**(16): p. 1234-40.
11. Pimanda, J.E., et al., *Transcriptional link between blood and bone: the stem cell leukemia gene and its +19 stem cell enhancer are active in bone cells.* Mol Cell Biol, 2006. **26**(7): p. 2615-25.
12. Pimanda, J.E., et al., *Gata2, Fli1, and Scl form a recursively wired gene-regulatory circuit during early hematopoietic development.* Proc Natl Acad Sci U S A, 2007. **104**(45): p. 17692-7.
13. Lacaud, G., et al., *Runx1 is essential for hematopoietic commitment at the hemangioblast stage of development in vitro.* Blood, 2002. **100**(2): p. 458-66.
14. Nottingham, W.T., et al., *Runx1-mediated hematopoietic stem-cell emergence is controlled by a Gata/Ets/SCL-regulated enhancer.* Blood, 2007. **110**(13): p. 4188-97.
15. Fehling, H.J., et al., *Tracking mesoderm induction and its specification to the hemangioblast during embryonic stem cell differentiation.* Development, 2003. **130**(17): p. 4217-27.
16. Acampora, D., et al., *Forebrain and midbrain regions are deleted in Otx2-/- mutants due to a defective anterior neuroectoderm specification during gastrulation.* Development, 1995. **121**(10): p. 3279-90.
17. Ang, S.L., et al., *A targeted mouse Otx2 mutation leads to severe defects in gastrulation and formation of axial mesoderm and to deletion of rostral brain.* Development, 1996. **122**(1): p. 243-52.
18. Shawlot, W. and R.R. Behringer, *Requirement for Lim1 in head-organizer function.* Nature, 1995. **374**(6521): p. 425-30.
19. Nishioka, N., et al., *Ssdp1 regulates head morphogenesis of mouse embryos by activating the Lim1-Ldb1 complex.* Development, 2005. **132**(11): p. 2535-46.
20. Nakano, T., et al., *OTX2 directly interacts with LIM1 and HNF-3beta.* Biochem Biophys Res Commun, 2000. **267**(1): p. 64-70.
21. Mochizuki, T., et al., *Xlim-1 and LIM domain binding protein 1 cooperate with various transcription factors in the regulation of the goosecoid promoter.* Dev Biol, 2000. **224**(2): p. 470-85.
22. Goardon, N., et al., *ETO2 coordinates cellular proliferation and differentiation during erythropoiesis.* Embo J, 2006. **25**(2): p. 357-66.
23. Vitelli, L., et al., *A pentamer transcriptional complex including tal-1 and retinoblastoma protein downmodulates c-kit expression in normal erythroblasts.* Mol Cell Biol, 2000. **20**(14): p. 5330-42.
24. Lipinski, M.M. and T. Jacks, *The retinoblastoma gene family in differentiation and development.* Oncogene, 1999. **18**(55): p. 7873-82.
25. Classon, M. and E. Harlow, *The retinoblastoma tumour suppressor in development and cancer.* Nat Rev Cancer, 2002. **2**(12): p. 910-7.
26. Weinberg, R.A., *The retinoblastoma protein and cell cycle control.* Cell, 1995. **81**(3): p. 323-30.
27. Giacinti, C. and A. Giordano, *RB and cell cycle progression.* Oncogene, 2006. **25**(38): p. 5220-7.
28. Zhao, Y., et al., *LIM-homeodomain proteins Lhx1 and Lhx5, and their cofactor Ldb1, control Purkinje cell differentiation in the developing cerebellum.* Proc Natl Acad Sci U S A, 2007. **104**(32): p. 13182-6.

29. Wang, N., et al., *The LIM-only factor LMO4 regulates expression of the BMP7 gene through an HDAC2-dependent mechanism, and controls cell proliferation and apoptosis of mammary epithelial cells.* Oncogene, 2007. **26**(44): p. 6431-41.
30. Valge-Archer, V., A. Forster, and T.H. Rabbitts, *The LMO1 and LDB1 proteins interact in human T cell acute leukaemia with the chromosomal translocation t(11;14)(p15;q11).* Oncogene, 1998. **17**(24): p. 3199-202.
31. Mizunuma, H., et al., *The LIM-only protein, LMO4, and the LIM domain-binding protein, LDB1, expression in squamous cell carcinomas of the oral cavity.* Br J Cancer, 2003. **88**(10): p. 1543-8.
32. Visvader, J.E., et al., *The LIM domain gene LMO4 inhibits differentiation of mammary epithelial cells in vitro and is overexpressed in breast cancer.* Proc Natl Acad Sci U S A, 2001. **98**(25): p. 14452-7.
33. Sum, E.Y., et al., *The LIM domain protein LMO4 interacts with the cofactor CtIP and the tumor suppressor BRCA1 and inhibits BRCA1 activity.* J Biol Chem, 2002. **277**(10): p. 7849-56.
34. Terskikh, A.V., et al., *From hematopoiesis to neuropoiesis: evidence of overlapping genetic programs.* Proc Natl Acad Sci U S A, 2001. **98**(14): p. 7934-9.
35. Goolsby, J., et al., *Hematopoietic progenitors express neural genes.* Proc Natl Acad Sci U S A, 2003. **100**(25): p. 14926-31.
36. Harder, F., et al., *Erythroid-like cells from neural stem cells injected into blastocysts.* Exp Hematol, 2004. **32**(7): p. 673-82.

Chapter 6

Summary

Samenvatting

Summary

Mammalian embryonic development, for which the mouse has been extensively used as a model, is a complex process that begins with the generation of the zygote and leads to the formation of the adult animal body tissues and organs. Signalling pathways and transcription factors that regulate cell differentiation are essential for embryonic development. The transcription regulator Ldb1 and its involvement in two distinct differentiation pathways, hematopoiesis and neural development, is the subject of study of this thesis.

Hematopoiesis takes place in two distinct waves in the extra-embryonic tissues and the embryo proper. Initially primitive hematopoiesis gives rise to large nucleated erythroblasts and is followed closely by definitive hematopoiesis that gives rise to hematopoietic stem cells. HSCs generated mainly in the PSp/AGM region seed the fetal liver between 10.5 dpc and 11.5 dpc, which from then on acts as the main hematopoietic site of the mouse embryo. Shortly before birth hematopoiesis shifts to the bone marrow, which acts as the main hematopoietic organ throughout adult life. HSCs are able to differentiate towards the erythroid, lymphoid and myeloid lineages. Hematopoietic development is closely associated with vascular development that gives rise to the vascular network of the mouse embryo.

Neural development begins as ectodermal cells under the influence of signals from the notochord begin to differentiate and become neuroectodermal cells, giving rise to the neural plate, which after folding will generate the neural tube that forms the brain and spinal cord. Similarly to the hematopoietic tissues and organs that contain stem cells on which they rely throughout adult life for regeneration, the central nervous system contains stem cells protected in specific niches that serve the same purpose.

The regulation of gene transcription is an essential process in cell differentiation and development. Multiprotein complexes that assemble on genomic DNA promoter and enhancer sites are involved in controlling transcription initiation. Such a complex that binds on E-box/GATA elements and consists of an Scl/E47 heterodimer, Gata-1, Lmo2 and Ldb1 was found to assemble in erythroid cells. The deletion of each of the above genes in mice leads to severe defects in hematopoiesis, which indicates that they are essential regulators of that process. *Ldb1* is an eleven exon highly conserved gene that is ubiquitously expressed in embryonic and adult tissues. It does not bind DNA, but is able to interact with LIM domains, present in a large number of regulatory proteins, essential for differentiation and development. The *Drosophila* ortholog of *Ldb1* functions by enabling long range interactions through a process of bringing an enhancer and a promoter in close proximity and thus indirectly facilitating the initiation of gene transcription.

The *Ldb1*-/- mouse suffers from a variety of defects, which include lack of yolk sac hematopoiesis and vascular development, absence of heart and foregut, anterior truncation, duplication of the posterior axis characterized by four rows of somites in approximately 1/3 of the knockout embryos. When the gene is deleted in embryonic stem cells, their

hematopoietic potential is lost. Embryoid bodies that arise from the differentiation of *Ldb1* -/- ES cells contain a decreased number of BL-CFCs that fail to develop into fully grown blast colonies. These cells that are identified through the expression of *Flk1* are considered as the *in vitro* equivalent of the hemangioblast, the earliest precursor of hematopoietic and endothelial progenitors. These observations indicate that hematopoiesis is impaired at an early developmental stage in the *Ldb1*-/- mouse embryos and embryoid bodies. Moreover in order to gain an insight into the involvement of *Ldb1* in neural development, *Ldb1*-/- ES cells were differentiated into neural stem cells that were found to suffer from a proliferation defect, while their differentiation potential was unaffected.

Changes in gene expression patterns were examined in *Ldb1*-/- mouse embryos, Flk1+ cells isolated from *Ldb1*-/- embryoid bodies and *Ldb1*-/- neural stem cells with the use of microarrays, in order to identify genes and regulatory pathways that act downstream of *Ldb1* and whose misregulation can explain the phenotypes observed due to its deletion. The gene expression analysis did reveal the misregulation of signalling pathways involved in development and cell differentiation as well as changes in the transcribed level of transcription factors involved either in hematopoiesis or neural development. The majority of genes that showed altered expression due to the deletion of *Ldb1* were downregulated. However a smaller number of genes showed elevated expression in the absence of *Ldb1* in accordance with previous observations that the gene can participate in transcription activation as well as repression complexes.

Samenvatting

De muis is een erg veel gebruikt model om de embryonale ontwikkeling van zoogdieren te bestuderen: een complex proces dat begint met de vorming van de zygote en resulteert in de formatie van de volgroeide dierlijke weefsels and organen. Signalering pathways en transcriptiefactoren betrokken bij de regulatie van celdifferentiatie zijn essentieel voor de embryonale ontwikkeling. Het transcriptie-regulerende Ldb1 en zijn betrokkenheid bij twee verschillende differentiatie pathways, namelijk hematopoiese en neurale ontwikkeling, is het onderwerp van deze dissertatie.

Hematopoiese vindt plaats in twee verschillende golven in het extra-embryonale weefsel en het eigenlijke embryo. Initieel resulteert de primitieve hematopoiese in het ontstaan van erytroblasten met grote nuclei, op de voet gevolgd door de definitieve hematopoiese die resulteert in het ontstaan van hematopoietische stamcellen. HSCs vanuit voornamelijk de PSp/AGM regio infiltreren de foetale lever op 10.5 dpc en 11.5 dpc, die vanaf dan dienst doet als de belangrijkste hematopoietische locatie van het muis embryo.

Kort voor de geboorte verplaatst de hematopoiese zich naar het beenmerg, dat verder dienst doet als het hematopoietische orgaan gedurende de volwassen levensperiode. HSCs zijn in staat zich te differentiëren in cellen van de erythroïde, lymfoïde en myeloïde typen. De hematopoietische ontwikkeling is nauw geassocieerd met de vasculaire ontwikkeling die leidt tot het ontstaan van het vasculaire netwerk in het embryo van de muis.

De neurale ontwikkeling begint als ectodermale cellen, onder invloed van signalen afkomstig van de notochord, beginnen te differentiëren en neuroectodermale cellen worden. Deze cellen vormen de neurale plaat, die na vouwing de neurale buis vormt. De neurale buis vormt op zijn beurt de hersenen en ruggenmerg. Vergelijkbaar met de hematopoietische weefsels en organen, waarin stamcellen verantwoordelijk zijn voor regeneratie tijdens de volwassen levensperiode, bevat het centrale zenuwstelsel in specifieke niches beschermde stamcellen die dezelfde rol vervullen.

De regulatie van gentranscriptie is een essentieel proces in celdifferentiatie en ontwikkeling. Multi-proteïne complexen die zich vormen op genomische DNA promotor en enhancer posities zijn betrokken bij het controleren van de initiatie van transcriptie. Een dergelijk complex dat E-box/GATA elementen bindt, bestaande uit een Scl/E47 heterodimeer, Gata-1, Lmo2 en Ldb1, is aangetroffen in erythroïde cellen. Verwijdering van elk van de bovengenoemde genen in muizen resulteert in ernstige defecten tijdens de hematopoiese, wat doet vermoeden dat ze essentiële regulatoren zijn van dat proces. *Ldb1* is een gen dat bestaat uit elf exonen, sterk geconserveerd is en overal in de embryonale en volwassen weefsels tot expressie komt. Het bindt niet direct aan DNA, maar is in staat om een interactie aan te gaan met de op regulatoire eiwitten veel voorkomende LIM domeinen. Deze regulatoire eiwitten zijn essentieel voor differentiatie en ontwikkeling. De *Drosophila* ortholoog van *Ldb1* speelt een belangrijke rol bij het mogelijk maken van lange-afstand interacties, waarbij een enhancer en een promoter dichtbij elkaar gebracht worden. Op

deze manier faciliteert *Ldb1* de initiatie van gentranscriptie.
De *Ldb1-/-* muis lijdt aan verscheidene defecten, waaronder afwezigheid van hematopoiese en vasculaire ontwikkeling in de dooierzak, afwezigheid van het hart en het eerste deel van de darmen, groeiafwijkingen (anterior) en duplicatie van de as (posterior), gekarakteriseerd door vier rijen somieten in ongeveer 1/3 van de knockout embryo's. Wanneer het gen verwijderd wordt in embryonale stamcellen verliezen deze hun hematopoietische potentie. Embryonale lichamen ontstaan uit gedifferentieerde *Ldb1-/-* ES cellen bevatten gereduceerde aantallen BL-CFCs, die zich tevens niet kunnen ontwikkelen tot volgroeide blastcel kolonies. Deze cellen, geïdentificeerd door de expressie van *Flk1*, worden beschouwd as het *in vitro* equivalent van de hemangioblast, de vroegste voorloper van hematopoietische en endotheliale precursors. Deze observaties laten zien dat hematopoiese verstoord is in een vroeg ontwikkelingstadium in *Ldb1-/-* muis embryo's en embryonale lichamen. Daarnaast, om inzicht te krijgen in de betrokkenheid van *Ldb1* bij neurale ontwikkeling, zijn *Ldb1-/-* ES cellen gedifferentieerd in neurale stamcellen. Deze cellen bleken een proliferatie defect te bezitten, terwijl hun potentie tot differentiatie ongewijzigd was.
Veranderingen in genexpressie patronen zijn onderzocht in *Ldb1-/-* muis embryo's, Flk1+ cellen geïsoleerd uit *Ldb1-/-* embryonale lichamen en *Ldb1-/-* neurale stamcellen met behulp van microarrays, om zo genen en regulatoire pathways downstream van *Ldb1* te identificeren, wiens misregulatie de geobserveerde fenotypen na verwijdering van *Ldb1* kan verklaren. De genexpressie analyse onthulde inderdaad misregulatie van signalering pathways betrokken bij ontwikkeling en celdifferentiatie, evenals veranderingen in expressieniveaus van transcriptiefactoren betrokken bij hematopoiese of neurale ontwikkeling. Het merendeel van de genen die een veranderde expressie lieten zien na verwijdering van *Ldb1* waren verminderd geëxpresseerd. Een klein aantal genen liet echter een verhoogde expressie zien in de afwezigheid van *Ldb1*, in overeenstemming met voorgaande observaties waarin het gen onderdeel kan zijn van zowel transcriptioneel activerende als onderdrukkende complexen.

Curriculum vitae

Name: Athina Mylona
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Department of Cell Biology, Erasmus Medical Centre, Rotterdam, The Netherlands.
Postdoctoral research

2003-2009
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PhD studies: The diverse role of *Ldb1* in cell differentiation and mouse embryonic development.

2002-2003
University of Oxford, United Kingdom, Wellcome Trust Centre for Human Genetics.
Research Assistant: “Investigation of the association between smaller than 1Mb rearrangements/deletions in the chromosomal telomeric regions and learning disabilities in children, with the use of Comparative Genome Hybridisation. Development of a novel diagnostic method, based on DNA arrays”

2001-2002
Imperial College London, United Kingdom, Department of Medical and Community Genetics, Kennedy Galton Centre.
MSc in Human Molecular Genetics

1998-2001
Queen Mary (University of London), United Kingdom, School of Biological Sciences.
BSc in Genetics

List of Publications

Genomic structure and cloning of two transcript isoforms of human Sp8.
Milona MA, Gough JE, Edgar AJ
BMC Genomics 2004, 5:86 (November 2004)

Expression of alternatively spliced isoforms of human Sp7 in osteoblast-like cells.
Milona MA, Gough JE, Edgar AJ
BMC Genomics 2003, 4:43 (November 2003)

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PhD Portfolio Summary
Summary of PhD training and teaching activities

Name PhD student: Maria-Athina Mylona Erasmus MC Department: Cell Biology Research School: Postgraduate school Molecular Medicine	PhD period: October 2003-December 2009 Promotor: Prof.dr. F.G. Grosveld

1. PhD training

	Year
General academic skills	
- Radiation Protection Course (level 5b), Rotterdam	2005
- Laboratory animal science (Artikel 9), Utrecht	2004
- Master's Molecular Medicine	2003-2004
In-depth courses	
- Analysis of microarray gene expression data, Rotterdam	2005
- In Vivo Imaging 'From Molecule to Organism', Rotterdam	2005
Presentations	
- EUrythron Meeting, Lisbon, Portugal	2009
International conferences	
- Seventh joint MGC-Cancer Research UK Graduate Student Conference, Oxford, UK (poster)	2006

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