Keeping *Sonic Hedgehog* under the thumb, genetic regulation of limb development

Sonic Hedgehog onder de duim houden,

genetische regulatie van

ledemaatontwikkeling

Dit promotieonderzoek kwam tot stand binnen de afdeling Klinische Genetica van het ErasmusMC te Rotterdam.

Dit proefschrift kwam mede tot stand met financiële steun van:
Erasmus Universiteit Rotterdam
Afdeling Klinische genetica
J.E. Jurriaanse Stichting

ISBN/EAN: 978-90-9024241-5

Cover: Ochtendtoilet, Jan Steen, 1663
The Royal Collection © 2008,
Her Majesty Queen Elizabeth II
Layout: Frank Wijgerse
Printed by: CPI-Wohrmamm Print Service, Zutphen

Keeping *Sonic Hedgehog* Under the Thumb, Genetic Regulation of Limb Development

Sonic Hedgehog Onder de Duim Houden,
Genetische Regulatie van Ledemaatontwikkeling

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. S.W.J. Lamberts

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

woensdag 17 juni 2009 om 13:45 uur

door

Elisabeth Mathilde Lodder

geboren te Utrecht

Promotiecommissie:

Promotor:

Prof.dr. B.A.Oostra

Overige leden:

Prof.dr. E.A. Dzierzak

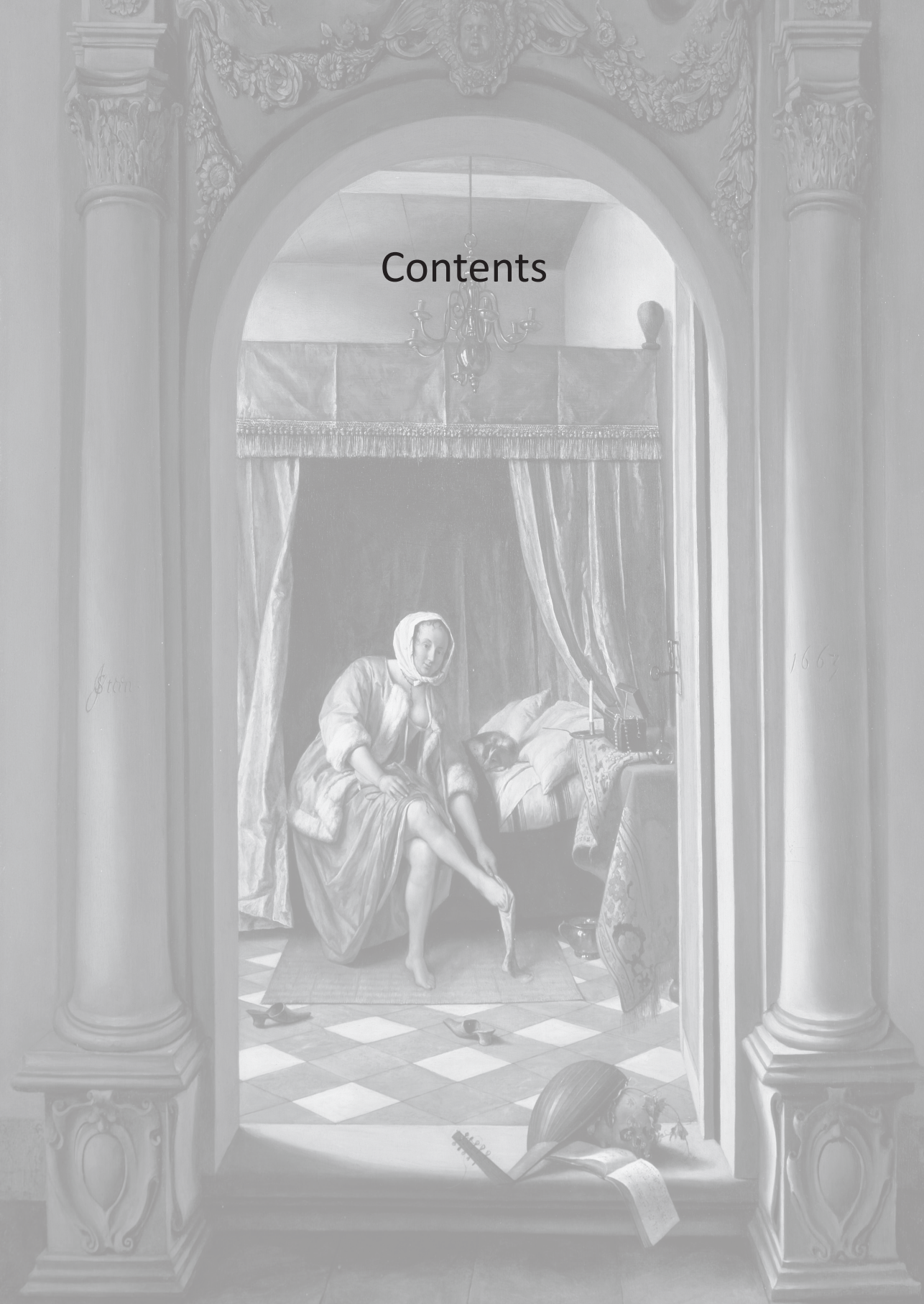
Prof.dr. S.E.R. Hovius

Prof.dr. J.N.J. Philipsen

Copromotor:

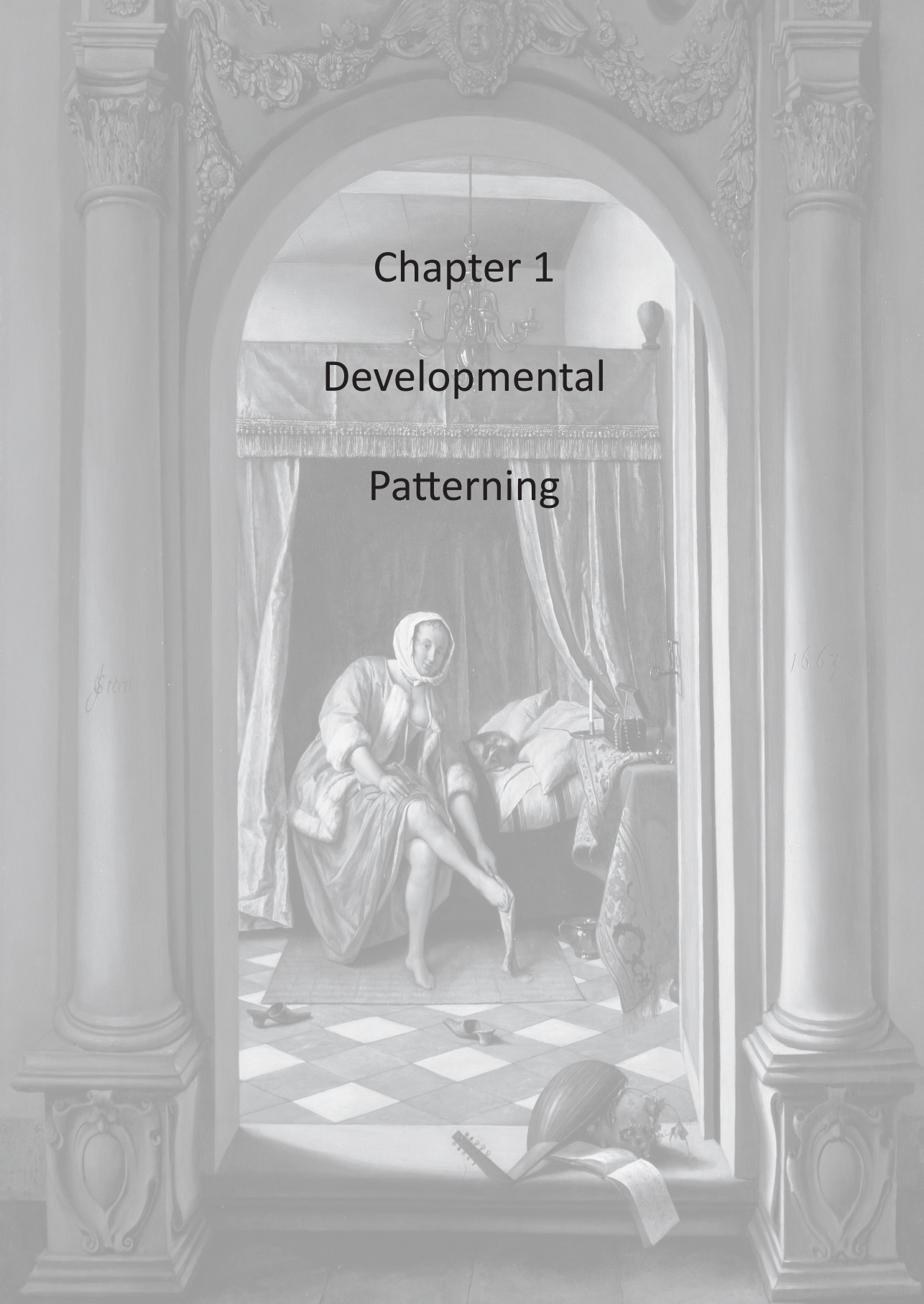
Dr. E. de Graaff

Contents



Chapter 1

Developmental Patterning



The limb as model system

The development of a single fertilised egg into a complete organism with all its different cell-types is a complicated process. Each cell needs to know its relative position to the rest of the body, in order to know whether it should become bone, muscle, blood vessel, skin or any of the other cell types present. Traditionally the limb has been used as a model to study the mechanisms involved in patterning. For one, it is accessible for manipulations in the chicken egg, a feature that has been extensively used in classical embryology. Moreover, the limb is not essential for embryonic survival, allowing experiments that destroy the limb organisation to be followed over time. As a result, the development of the limb has been well characterised. Genetic analyses in the mouse have also added to our insights in limb development. The basic mechanisms during the development of the upper limb in chick and mouse (and human) are similar. The following chapter will describe the results from experiments performed either in mouse or chick and can be extrapolated to the human situation. Where differences are known to exist, they are indicated specifically.

Before we start

In this thesis, I will discuss the limb bud as if it were a pre-existing entity without connection to the rest of the body. This of course is a simplification of reality. Many of the signalling molecules and mechanisms discussed below are essential for patterning other parts of the body besides the limb buds. For example, *Sonic Hedgehog* is expressed in the developing notochord where it triggers a signalling cascade involving Wnt signalling, BMPs and FGFs, patterning the neural tube above.

In mice, the limb buds start to protrude from the body wall at embryonic day 9-10, with the hind limbs lagging behind the forelimbs by approximately one day. How the initiation of the limb buds takes place is a highly disputed topic. It is clear that a feedback loop between Wnt signalling and FGF signalling is essential for the correct positioning of the four limbs[1,2]. How these pathways are regulated

individually and in concert remains largely unknown. The fore and hind limb specific T-Box transcription factors *Tbx5* and *Tbx4* are necessary for induction, as limb bud initiation fails in mice carrying targeted deletions of either *Tbx* gene[3,4]. Furthermore, a role for the *Hox* genes has been suggested as these genes are essential for the anterior-posterior patterning of the trunk. However, targeted deletions of almost all paralogues and entire clusters have now been generated and only one of these shows a minor repositioning of the limb bud initiation site[5,6]. How the positioning of the limb buds at the correct height along the body axis is regulated remains therefore unclear.

Signalling Centres

The patterning of the limb can be described following the three axes of the future limb (figure 1.1): proximal to distal (close to the body to the tips of the fingers/ toes), anterior to posterior (thumb to little finger) and dorsal to ventral (back of the hand to the palm). Each cell needs to "know" its position relative to these three axes. Positional information is conveyed by signalling centres on the borders of the axes. The proximal to distal (PD) axis is patterned by signals from the Apical Ectodermal Ridge (AER), an epithelial thickening on the dorsal-ventral border of the limb bud. The anterior to posterior (AP) patterning is regulated by a group of mesodermal cells on the posterior side of the limb bud named the Zone of Polarizing Activity (ZPA). Finally, the dorsal to ventral (DV) axis is patterned by signals from the ectoderm. All signalling centres will be discussed in detail in the following sections.

Proximal to distal

The Apical Ectodermal Ridge

Initially, the limb bud is a homogenous mass of mesodermal cells covered with a thin ectodermal layer. The first sign of patterning is the appearance of a thin epithelial thickening at the proximal tip of the limb bud: the Apical Ectodermal

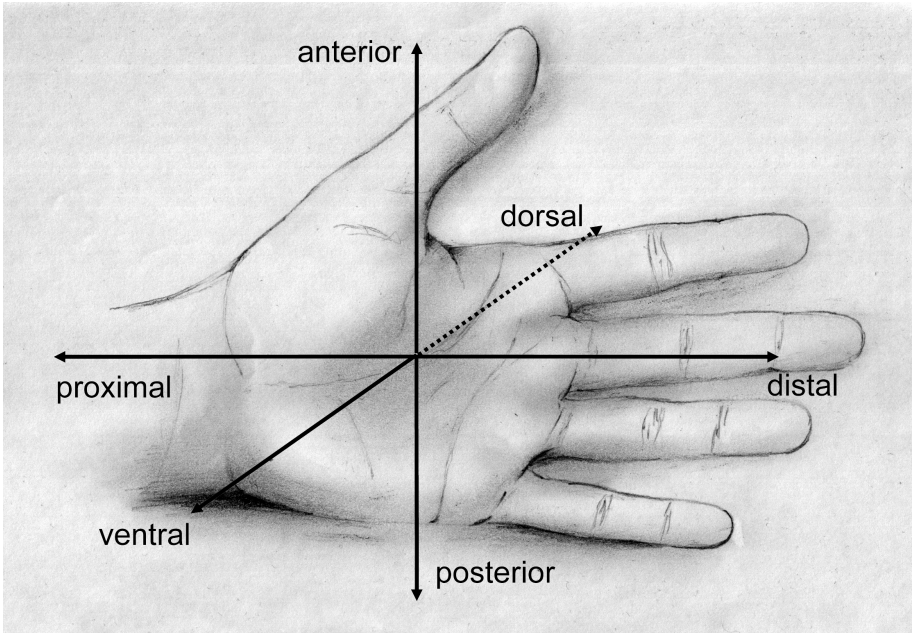


Figure 1.1 Orientation of the axes relative to the adult hand*: proximal to distal (PD) close to the body wall to the tip of the fingers; anterior to posterior (AP) thumb to the little finger; dorsal to ventral (DV) back of the hand to the palm (drawing courtesy of Harry Wijgerse).*

Ridge (AER). The AER is essential for proper patterning along the PD-axis. Early embryological studies in chicken showed that removal of the AER at different time points during development leads to truncation of the limb at sequentially more distal levels[7-9]. However, the AER itself does not pattern the proximal to distal axis, it is only essential for outgrowth as swapping of AERs between early and late stage limb buds has no effect on skeletal patterning[10]. This finding led to the Progress Zone model: this model proposes that there is a region just below the AER, called the progress zone, in which signals from the AER keep the cells in a highly proliferative, undifferentiated state. During development, due to growth, cells are pushed out of the progress zone and start to differentiate. The amount of time in the progress zone determines the identity: the longer a cell has spent in the progress zone the more distal its cell fate. This model can explain the progressive truncations after AER removal[11].

Much later, it was discovered that a group of signalling molecules called the fibroblast growth factors (FGFs) can compensate for AER removal. When a bead soaked in FGF protein is placed at the site of AER removal, the proximo-distal patterning is rescued[12,13]. Furthermore, four FGF family members are specifically expressed in the AER: *Fgf4, Fgf8, Fgf9 and Fgf17*[14]. Conditional genetic targeting strategies in mice have allowed the analysis of the role of FGF signalling on limb development. Individual loss of *Fgf4*[15], *Fgf9*[16] and *Fgf17*[17] has no effect on limb development. However, genetic targeting of *Fgf8* alone or in combination with one of the other *Fgf's* leads to increasingly severe truncations of the limb[14,18,19].

In the absence of FGF signalling from the AER, due to genetic targeting or the surgical removal of the AER, a zone of cell death is observed in the distal mesenchyme[14,20,21]. This zone of cell death is relatively constant in size (approx. 200 μM) between HH stage 18 and 22 in chicken (equivalent to mice embryonic day 10.5-12.5). After stage 24, AER removal does not lead to cell death but cell proliferation is reduced significantly.

A combination of AER removal experiments with cell labelling led to the proposal of the early specification model. Cell membrane labelling with lipophilic dyes resulted in a fate map that indicated that cells contribute to particular segments of the limb based on their position in the early limb bud. Cells that will form the humerus/femur are far from the AER while cells that will form more distal structures are closer to the AER[21]. The cells that are present in the zone of cell loss (after AER removal) change over time due to expansion of the limb bud, leading to progressively more distal truncations upon progressively later AER removal. However, these results can also fit in the Progress Zone model. If the cells that die are the cells in the progress zone, the remaining cells will need more time before they are forced out of the progress zone by proliferation[20,22]. Moreover, more detailed fate maps by Arques *et al.*[23] show limited restriction of cell movement along the A/P axis in contrast to the earlier results.

Importantly, the phenotype of combined targeted deletions of different FGFs described recently are at first sight difficult to reconcile with either a progressive specification of more distal cell fates or a pre-existing specification in the early limb bud. In the *Fgf8*$^{-/-}$, *Ffg4*$^{-/-}$, *Fgf9*$^{+/-}$ mice limbs form which contain proximal (humerus) and distal (phalanges) but no intermediate (radius and ulna) elements[19]. This contrasts with the progressively more distal truncations predicted by both the early specification and the Progress Zone model. Mariani *et al.* therefore proposed an intercalation model to explain their results[19]. This model presumes the presence of two signals: an early signal from the body wall (retinoic acid, RA) and a later signal from the AER. With RA specifying the most proximal parts of the limb and the FGFs from the AER the most distal cell fates. The intermediate parts are then formed by an interaction between both signals. The AER signalling should therefore be considered as instructive rather than permissive based on this model. The major problem with this model is that in chicken it does not seem to apply as transplantation of an early distal limb tip to an older proximal limb stub does not produce the intermediate limb structures[8].

The possible differences between species and overlap between seemingly opposing models ensures that thus far the debate is still ongoing. The one aspect all models agree upon is the presence of a group of undifferentiated cells below the AER. How and when these cells acquire their positional information is still uncertain. All three models can be adjusted to incorporate the available data (reviewed in Towers and Tickle, 2009)[24]. Therefore, as specific markers for the different fates are still missing, no definite conclusions can be drawn about which model best describes the proximal to distal patterning.

Anterior to Posterior

Zone of Polarizing Activity (ZPA)

Forty years ago a group of cells was discovered that functions as a signalling centre patterning the anterior to posterior axis in the limb. When performing transplantation experiments to study the role of cell death during limb development a striking phenotype was observed. When cells from the posterior side of the limb bud were transplanted to the anterior side of another limb bud a mirror image duplication occurred[25] (figure 1.2)[26]. Subsequent experiments showed that the effect of this zone of polarizing activity (the ZPA) was both dependent on time of signalling

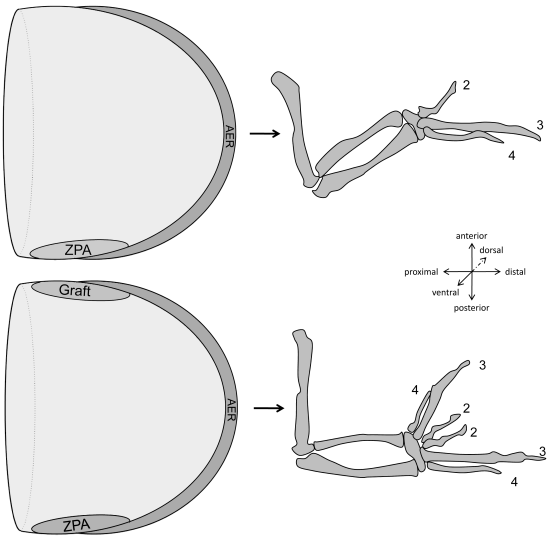


***Figure 1.2 The Zone of Polarising Activity**: a) in the normal limb bud a group of cells on the posterior side of the limb bud confers positional information along the AP axis resulting in a normal digit pattern in the chick wing bud (digits 2, 3 and 4); b) a graft of ZPA tissue on the anterior side of the limb bud results in a mirror image duplication of the digits (digits 4, 3, 2, 2, 3, 4) based on Saunders and Wolpert, 1968 and Honig and Summerbell, 1985[25,136].*

and on the dose (number of cells)[27]. This strongly suggested that the posteriorizing effect was based on a signalling morphogen that was secreted from the ZPA.

The first candidate for this morphogen was Retinoic Acid (RA). Beads with RA can mimic the phenotype of ZPA grafts on the anterior limb bud[28,29]. However, endogenous levels of RA in the ZPA are too low to induce these effects. The effect of RA turned out to be indirect: RA induces the activation of 5' members of the *HoxA* and *HoxD* cluster[6]. These in turn activate the real ZPA morphogen *Sonic Hedgehog*

(*Shh*). *Shh* was discovered as the true signal from the ZPA in the early nineties by Riddle *et al.*[30]. It was found because of its homology with the *Drosophila* patterning gene *hedgehog*.

Shh encodes a secreted signalling protein. After binding to its receptor Patched, Shh triggers a signalling cascade which blocks the proteolytic cleavage of a transcription activating form of Gli3 (Gli3A) into a transcription repressing form (*Gli3*R)[31]. The Shh gradient leads therefore to two opposing gradients of Gli3: GliR from anterior to posterior and *Gli3*A from posterior to anterior.

Shh is essential for limb development: in $Shh^{-/-}$ mice, truncated limbs develop that have only one digit, digit 1[32]. Interestingly, a deletion of *Gli3*, which leads to loss of both the activating and repressing forms, leads to the formation of many unpatterned digits[33]. This indicates that *Gli3* is necessary both to repress growth of the autopod and for patterning of the digits. Limbs of mice carrying both a *Shh* and a *Gli3* deletion also have many un-patterned digits, placing Gli3 downstream of Shh[34].

Sonic Hedgehog qualifies as a true morphogen: it is a secreted signalling protein, its effects are dose and time dependent and it can act over distances of several cell diameters. However, its mechanism of transport over these distances is unknown. Recently, Cre recombinase-mediated cell tracing experiments in mice shed a new light on *Shh* signalling[35]. In these experiments, it was shown that at later stages, half of the limb bud consists of cells that have expressed *Shh* at one point during development. Hence, (ex-) ZPA cells proliferate to form half of the autopod. This indicates that the digit identity is not (just) based on the level of SHH signalling but also on the amount of time a cell has expressed *Shh* itself. These results are further corroborated by studies in which the amount of SHH signalling is reduced while the duration of *Shh* expression is kept constant. Interestingly, these mice develop normal limbs[36,37].

The model of how the anterior to posterior axis is patterned has therefore changed from a solely morphogen based model to an integrated model in which

growth and displacement of cells from the ZPA is equally important (reviewed in Towers and Tickle 2009)[24]. Cells in the ZPA produce *Shh* which is then secreted. This signals to cells within its reach (the future digits 4 and part of digit 3 of the chick wing bud) to adopt a more posterior identity. *Shh* also stimulates proliferation forcing cells out of the region where signals from the AER maintain the expression of *Shh*[38,39]. The amount of time cells express *Shh* is therefore dependent on their distance from the ZPA: those that leave the ZPA early cease to express *Shh* earlier than those that leave the ZPA later. This difference in time of expression of *Shh* is the basis of the identity of the most posterior digits (part of digit 3 and digits 4, 5 in mice). The signals from the ZPA are therefore biphasic: an early dose dependent stage and a later growth and time dependent stage[38,39].

Dorsal to Ventral

Ectodermal-mesenchymal signalling

The signalling processes that govern the dorsal to ventral axis are relatively well characterised. Before the budding of the limb from the body wall, the lateral mesoderm imposes a dorsal or ventral identity upon the overlying ectoderm[40]. After this initial signal to the ectoderm, the signals from the ectoderm control the dorsal-ventral (DV) identity. Rotation of the ectodermal jacket results in a switch in DV identity of the mesoderm[41].

The key signalling molecules involved are Wnt7a in the dorsal ectoderm[42] and Bone Morphogenetic Proteins (BMPs) which activate Engrailed-1 (EN-1)[43] in the ventral ectoderm. Loss of *Wnt7a* expression leads to ventralisation of the limb bud[42], while loss of *En-1* leads to expansion of the dorsal domain due to the expansion of the *Wnt7a* expression pattern[44,45]. The signalling factors involved in the early signalling from the body wall are not yet known (for a review see Robert 2007)[46]. Wnt7a induces the expression of *Lmx1b*, which is essential for the dorsal identity in the dorsal mesoderm. This explains that the effects of *Wnt7a* and *En1* mutations are

not limited to the ectoderm but effect the mesoderm as well[47,48]. Targeted deletion of either *Wnt7a* or *En1* in a *Lmx1b* mutant background showed that Lmx1b functions as the sole functional readout of both ectodermal signals[49].

Clonal analysis of the mesenchyme of the developing limb bud showed that two distinct pools of mesenchyme exist along the DV axis. These pools of cells are separated by a clear plane which correlates with the expression domain of *Lmx1b*[23]. In the more proximal part of the limb chondrogenic condensation starts to develop at the border of the DV domains. Cells from both compartments contribute to these condensations and subsequent ossifications[23]. This indicates that the DV border does not represent a functional differentiation of cells with different fates, nor does the border represent a known signalling centre as none of the known signalling molecules are expressed specifically at the DV Plane[50].

Integration of the axes

Although the development of the limb can be described along the three axes of the limb bud, it is clear that integration of the positional information of all three axes is essential. Several feedback loops between the different axes have been described (see figure 1.3) and initiation of the different signalling centers is closely linked (for an extensive review see Niswander, 2002)[51].

AER and Dorsal Ventral coordination

A major role in the integration of the axes is played by BMP signalling (reviewed by Robert, 2007)[46]. BMP signalling in the early limb bud ectoderm results in activation of *En1* in the ventral ectoderm. BMP signalling also activates *Fgf8* in the AER through the activation of members of the *MSX* class of transcription factors[43,52]. Early positioning of both the AER and the dorsal and ventral ectodermal domains is therefore initiated through one set of signals, thus coordinating their relative positions.

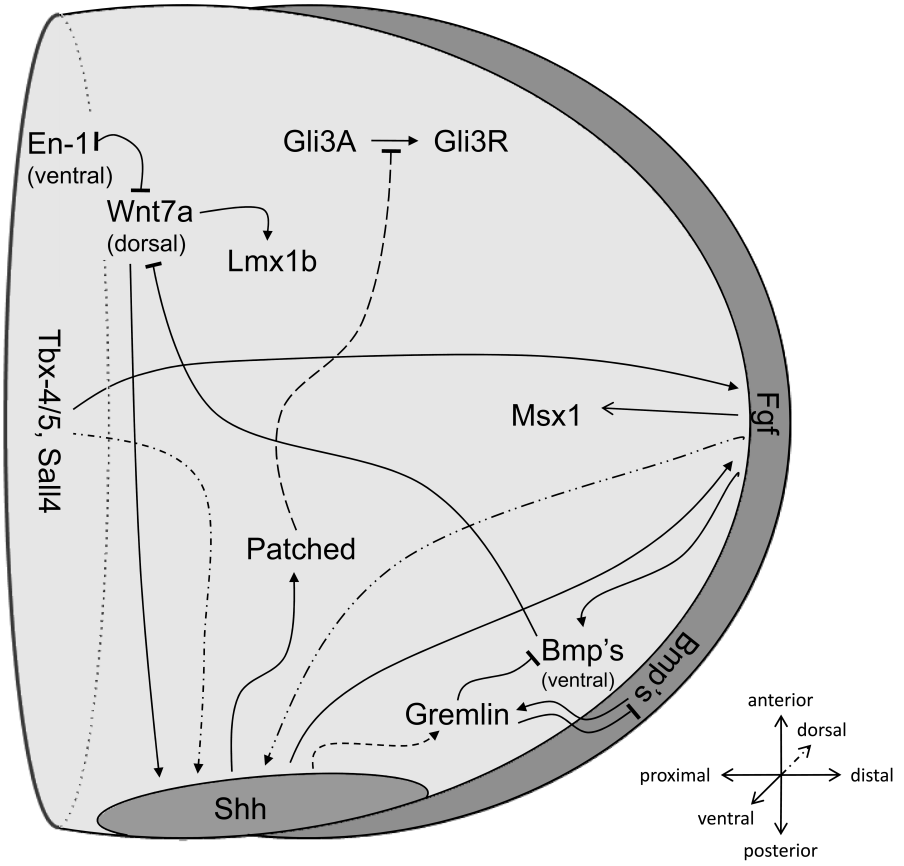


Figure 1.3 Interaction of signalling between the different axes*: the PD axis is patterned by signals from the AER (BMP signalling and FGF signalling activating Msx1 in the distal mesoderm keeping cells in a proliferative and undifferentiated state), Tbx-4/5 and Sall4 expression in the proximal mesoderm is essential for limb bud intitiation. The AP axis is patterned by Shh signalling from the ZPA, which through its receptor Patched inhibits the cleavage of the transcriptionally active form of Gli3 into the transcriptionally repressing form Gli3R. The DV axis is formed by the expression of En-1 in the ventral and Wnt7a in the dorsal ectoderm; Wnt7a activates Lmx1b in the dorsal mesoderm. The AP and PD axes are linked by a feedback loop between Shh and Fgf signalling. The AP axis feeds back on the DV and PD axes by inhibiting BMP signalling through the activation of the BMP inhibitor Gremlin by Shh. The DV axis is further linked to the AP axis by the activation of Shh expression by Wnt7a. Please note that for simplicity the Hox genes have been left out of this figure.*

ZPA and AER

Induction of both the AER and the ZPA is dependent on early expression of the *Hox* genes in the limb bud. An early phase of *Hox* gene expression in the limb bud follows a collinear activation that results in a "Russian doll" like expression pattern with more 3' genes being expressed sequentially more posterior. The *Hox* genes of paralogy groups 10-13 is essential for induction of the ZPA on the posterior rim of the limb bud[6]. This activation is probably direct, as biochemical evidence shows binding of Hoxd13 to the limb enhancer of *Shh*[53]. The induction of the AER is dependent on *Hox* paralogy groups 8-11. Loss of function of different *Hox* genes leads to loss of bones along the PD axis depending on the paralogy group that is affected[6]. The *Hox* genes therefore provide a coordinated mechanism for initiation of the two major signalling centres of the limb.

The ZPA needs the AER for its maintenance and *vice versa*. An indirect feedback loop between *Shh* and *Fgf-4* via BMP signalling and gremlin is essential for both AER and ZPA maintenance[54,55]. Disruption of this loop through the loss of *Shh* or *Fgf* expression leads not only to a reduction in the expected axis but also in the other axis. The limbs of *Shh*$^{-/-}$ mice do not only have a reduced number of digits, but the radius and ulna are also reduced in size. *In situ hybridisation* of early *Shh*$^{-/-}$ limb buds shows a regression of the AER and reduced expression of *Fgf4*[32,51,55,56]. Conversely, in mice carrying targeted deletions of Fgfs in the AER, not only the proximal to distal patterning is affected, but the number of digits, traditionally considered an AP patterning defect, is reduced as well [19,54].

Dorsal Ventral and Anterior Posterior interactions

The PD axis is not alone in its influence on the ZPA. In 2007 Nissim *et al.* described the role of the DV boundary in positioning of the ZPA. The expression of *Shh*, which defines the ZPA, is limited to a region close to the ectoderm in the middle of the DV axis[57]. Moreover, the expression of the transcription factors *Tbx2*

and *Tbx3* is limited to both the ZPA and a mirrored region on the anterior side of the limb bud[58,59]. This anterior expression domain is identical to the domain of ectopic *Shh* expression in polydactylous mouse mutants[60-62]. The DV ectodermal border is essential for the restriction of the expression of *Shh* and *Tbx2* to the posterior (and anterior in the case of *Tbx2*) margin. Grafts of this ectodermal region are sufficient to induce ectopic expression of *Tbx2.* Moreover, grafts could also induce ectopic *Shh* expression in the posterior half of the limb bud. These experiments show that the DV boundary is essential in positioning the ZPA to the most posterior margin of the limb bud[57].

Human mutations and mouse models

Studying the genetic background of congenital malformations of the limbs in humans and comparing them to mouse models is a good way to learn more about the developmental processes governing limb development. Over the years many human limb deformities have been characterised. In the London Dysmorphology Database around 2000 dysmorphological entries that include a limb phenotype have been described[63]. Not all of these have genetic causes, environmental influences have been found as well such as: maternal diabetes and drug use such as thalidomide.

Several different classifications of limb malformations exist, being either more clinically or more genetically oriented, or a mixture of both. The phenotypes observed in the distal part of the limb can be roughly subdivided in polydactyly (additional digits), syndactyly (lack of separation between digits), brachydactyly (shortened digits) and absence or deficiency of the larger bones (tibial dysplasia for example) or digits (oligodactyly). The malformation can be further subclassified based on location. Pre-axial polydactyly consists of extra fingers on the thumb side of the hand, while individuals with post-axial polydactyly have additional digits on the other side of the limb.

The last important classification is whether the limb malformations occur independently (isolated) or as part of syndrome involving more congenital malformations. As most genes that are involved in limb patterning are also involved in patterning other parts of the body, these syndromic malformations can teach us not only about the development of the limbs, but about the development of the rest of the body as well. The next section will highlight some of the phenotypes most relevant for the work presented in the remainder of this thesis.

Isolated limb malformations

Preaxial Polydactyly, Sonic Hedgehog

Shh is essential for proper limb development and is necessary for patterning many other organs including the CNS. The tight regulation of *Shh* expression is of utmost importance for proper pattern formation. Deregulation of *Shh* in the limb has been linked to the occurrence of isolated pre-axial polydactyly. Linkage and subsequent sequencing analysis of several families with preaxial polydactyly (PPD) led to the identification of a long range, *cis*-acting element (designated the ZRS for ZPA Regulatory Sequence). This element is responsible for the regulation of *Shh* expression in a limb specific manner[61,64]. Mice lacking the ZRS are viable and phenotypically normal except for a limb reduction defect identical to the limb phenotype seen in mice lacking *Shh* expression[56,65].

Point mutations in the ZRS lead to ectopic expression of *Shh* in the anterior margin of the limb bud and cause triphalangeal thumb and polysyndactyly (TPTPS, MIM #174500) in humans. Thus far 14 point mutations have been described in the ZRS in human, mice and cats with PPD[66]. Recently, Klopocki *et al.*[67] and Sun *et al.*[68] demonstrated that duplication of the ZRS can cause a phenotype very similar to the TPTPS caused by single nucleotide alterations in the ZRS.

Post-Axial Polydactyly, GLI3

Gli3 acts as both a transcriptional activator and a transcriptional repressor, depending on the presence or absence of *Shh* signalling[69]. This explains the diverse phenotypes associated with *Gli3* mutations: Greigs cephalopolysyndactyly syndrome (GCPS, MIM #175700) and Pallister Hall Syndrome (PHS, MIM # 146510) and possibly isolated pre- and postaxial polydactyly (reviewed by Biesecker in 2006)[70]. The role of *Gli3* in postaxial polydactyly (PPD) first became clear in the mouse mutant *Extra toes^{J} (Xt-j)*, in which a spontaneous intragenic deletion of *Gli3* was found to lead to the GCPS[33].

The phenotype of *Gli3* mutations is dependent on the position of the mutations within the *Gli3* gene. In general, truncating mutations of almost the entire gene and haplo-insufficiency due to large deletions and translocations involving the entire *Gli3* gene, lead to GCPS. On the other hand truncating mutations in the middle third of the gene lead to PHS. Whether the isolated pre- and post-axial phenotypes as reported by Radhakrishna *et al.*[71] are indeed isolated or whether they represent mild forms of GCPS is impossible to determine based on the clinical data available[70]. Mice, lacking Gli3 form unpatterned polydactylous limbs[34].

Syndactyly

Syndactyly consists of the lack of separation (webbing) of the digits. During development, controlled cell death separates the digits from one another. If this process does not take place properly, the digits will remain attached to each other. Syndactyly can occur on its own or in combination with other limb deformities such as polydactyly (both pre- and post-axial) or brachydactyly. Syndactyly has been classified in five different categories based on the involvement of the different digits[72,73]. For some forms the genetic defect has been found. In syndactyly type III, which is characterised by complete and bilateral syndactyly between the fourth and fifth fingers (only of the hands) mutations in the Gap junction protein alpha 1

(*GJA1)* gene have been described[74]. Syndactyly type III possibly represents a mild form of oculodentodigital dysplasia (ODDD), as *GJA1* mutations have been identified in ODDD. The mechanism by which GJA1, a member of the connexin protein family, can lead to syndactyly, remains to be determined.

Syndactyly type IV is a polysyndactyly with a complete cutaneous syndactyly of all fingers. A recent paper by Sun *et al.* shows that this phenotype can be caused by duplications of the ZRS (the limb specific *Shh* enhancer)[68].

Both type II and type V syndactyly can be caused by mutations in *HoxD13*[75-78], the most 5' member of the *HoxD* cluster. Synpolydactyly (Syndactyly type II) is an autosomal dominant inherited limb malformation with incomplete penetrance and variable expressivity. It is characterised by soft-tissue syndactyly between fingers 3 and 4 and between toes 4 and 5, with partial or complete digit duplication within the syndactylous web. Syndactyly type V is characterised by syndactyly with metacarpal and metatarsal synostosis (fusion of the bones)[72].

Brachydactyly

Brachydactylies, or shortening of the fingers/toes, can be subdivided in different classes[79,80], based on which phalanges are affected. All cases are inherited in an autosomal dominant manner. Type A consists of shortening of the middle phalanges and is subdivided in four types: A1-A4.

Brachydactyly type A1 (BDA1) is characterised by a uniform shortening of the middle phalanges in all digits with or without ankylosis (joint stiffness). In 2001, Gao *et al.* showed that BDA1 is caused by heterozygous mutations in the Indian hedgehog (*IHH*) gene[81]. Thus far, seven mutations have been identified, interestingly involving only 4 amino acids[82-86]. See chapter 6 of this thesis for more details. The IHH protein is involved in chondrocyte formation during limb development[87]. A short stature is often reported as a clinical feature in families with BDA1. It was therefore not surprising that homozygous mutations in *IHH* were identified in Acrocapitofemoral

dysplasia, an autosomal recessive skeletal dysplasia [88].

Type A2 (BDA2) consists of shortening of the middle phalanges, which is confined to the index finger and the second toe, all other digits being more or less normal. Because of a rhomboid or triangular shape of the affected middle phalanx, the end of the second finger usually deviates radially. It is caused by a mutation in the *BMPR1B* gene[89,90] or its ligand Growth Differentiation Factor 5 (GDF5)[91].

In type A3 (BDA3) brachydactyly, shortening is limited to the middle phalanx of the fifth finger. The rhomboid or triangular shape of the rudimentary middle phalanx results in a radial curvature (clinodactyly) of the fifth finger. Type A4 (BDA4) is not well described but is mainly characterised by brachymesophalangy of the 2nd and 5th digits. The genetic cause for BDA3 and BDA4 still remains to be found.

Type B brachydactyly is characterised, as the 4 A types, by short middle phalanges, but in addition the terminal phalanges are rudimentary or absent. Both fingers and toes are affected. Symphalangism is observed as well as mild syndactyly between the digits. Syndactyly in the feet usually involves the second and third toes. Type B brachydactyly is caused by mutations in the *ROR2* gene[92-94].

Brachydactyly type C is distinguished from the other brachydactyly types by the presence of short middle phalanges with relative sparing of the fourth phalanx; the usual presence of a shortened first metacarpal; and hypersegmentation of a digit, mostly involving the proximal phalanges of the second and third digits. Brachydactyly type C can be caused by mutations in either the *GDF5* gene[95,96], or its receptor *BMPR1B*[89] (also involved in BDA2[90]). The large overlap in the phenotype of BDA2, BDB and BDC is not surprising as all three genes are involved in GDF5 induced regulation of chondrogenesis[97].

Brachydactyly type D is characterised by short and broad terminal phalanges of the thumbs and big toes. In type E brachydactyly, shortening of the fingers is mainly in the metacarpals and metatarsals. Wide variability in the number of digits affected occurs from person to person, even in the same family. Skeletal malformations with

features overlapping those of brachydactyly types E and D can be caused by mutation in the *HOXD13* gene[98].

Human Syndromes with Preaxial Malformations

Holt-Oram Syndrome, Tbx5

The Holt-Oram Syndrome (HOS), also known as the Heart-Hand Syndrome I, is an autosomal dominant disorder and the most frequently occurring hand-heart syndrome with a prevalence estimated to be 0.95 per 100,000 total births. Mary Holt and Samuel Oram first described the Holt-Oram Syndrome in 1960[99] in four family members who were affected with heart disease in combination with skeletal malformations. The bilateral limb phenotype mainly involves the thumb, which may be triphalangeal, hypoplastic or absent. Radius deficiency and occasionally ulnar deficiency may also be seen in HOS[100].

The clinical manifestations vary, from subclinical radiographic findings to life-threatening disease. Other related types of heart-hand syndromes are known, such as the rare Heart-Hand syndrome type II, also known as Tabatznik's syndrome[101], and the Heart-Hand syndrome type III[102]. However, the limb deformity in Heart-Hand syndrome II and III is not restricted to pre-axial malformations of the hand.

The Holt-Oram syndrome is often caused by mutations in the T-box transcription factor *TBX5* located on chromosome 12q24.1[103] In some cases a distinction between a mild and severe phenotypes can be made based on the location of the mutation in the *TBX5* gene[104-106]. However, the presence of a *TBX5* mutation is not 100% predictive for the expression of the HOS phenotype[107], as within one family not all carriers of a mutation display the HOS phenotype. Moreover, *TBX5* mutations have also been found in patients with a non-HOS heart malformation[108]. Eighty-five percent of all Holt Oram cases are attributed to *de novo* mutations[109]. So far, in total over 70 missense and nonsense mutations have been reported in *TBX5*[103,110]. Most mutations are thought to cause haploinsufficiency. In the normal heart development pathway,

TBX5 heterodimerizes with the transcription factor NKX2.5 to activate downstream targets [111]. The co-activator required for functioning in the limb is yet to be identified [112].

The Holt-Oram syndrome is sometimes confused with Okihiro syndrome (Duane- Radial Ray syndrome). One of the common features of the Okihiro syndrome is an eye movement disorder (Duane anomaly), a specific form of strabismus (the inability to align both eyes) namely the absence of abduction, restricted adduction and retraction of the globe on attempted adduction. This syndrome can be coinherited with radial ray anomalies. A small number of Okihiro patients also manifest heart defects, particularly atrial septal defect, in combination with similar radial ray deficiencies as in HOS[113]. The list of associated features of the Okihiro syndrome is extensive: Duane anomaly, but also hearing loss, kidney defects, anal stenosis, pigmentary disturbances, external ear malformations and facial asymmetry in combination with radial ray anomalies are described[114].

Okihiro syndrome is caused by mutations in the *Sall4* gene[115]. In some cases a *Sall4* mutation is found in patients misdiagnosed with the Holt-Oram syndrome, who should instead be diagnosed with Okihiro Syndrome[116]. Further genetic analyses on *Sall4* can support the Okihiro syndrome if the phenotype is hardly indistinguishable from Holt-Oram Syndrome. *Sall4* and *Tbx5* act in the same genetic pathway, for both heart and limb development explaining the overlapping phenotypes[116]. Another participant in this pathway, *Sall1*, is mutated in Townes Brocks syndrome (TBS, MIM#107480) a similar syndrome, which encompasses radial-sided limb anomalies including thumb polydactyly and triphalangeal thumb[117].

VACTERL association

The VACTERL (Vertebral anomalies, Anal atresia, Cardiac abnormalities, Tracheo- Esophageal fistula (TEF), Renal anomalies, Limb anomalies) association is a combination of congenital anomalies of at least three of the mentioned organs

with limb malformations and is known to be a very heterogeneous disorder. This association has been expanded from the VATER association, to include cardiac anomalies and more specific limb anomalies[118]. The VACTERL association is often misdiagnosed as other syndromes share parts of its phenotypical spectrum. It occurs in nearly 1.6 per 10,000 births[118]. In a study by de Jong *et al.*[119], 70% of patients display non-VACTERL-type congenital anomalies in addition to the VACTERL spectrum. Limb defects include absent or displaced thumbs, polydactyly, syndactyly and forearm defects. A suggested risk factor for VACTERL is pre-existing maternal insulin-dependent diabetes, but too few cases have been studied to confirm this[120].

Little is known about the genetic cause of VACTERL, possible due to the high heterogeneity in phenotype. Kim *et al.* concluded from their study in mice on mutant *Gli* genes, that defective *Shh* signalling leads to the spectrum of VACTERL associated anomalies[121]. However, in humans, mutations involving *Shh* have been associated with holoprosencephaly instead of VACTERL[122] and as discussed above, mutations in *GLI3* lead to Greigs cephalopolysyndactyly syndrome and Pallister Hall syndrome.

Two families with VACTERL were found to have mutations in the proprotein convertase *PCSK5*[123]. Moreover, mice lacking *Pcsk5* display a VACTERL phenotype. The lack of functional PCSK5 protein leads to a lack of cleavage and thereby activation of Gdf11. During development, *Gdf11* is expressed in the anterior subectodermal mesoderm of the limb buds. Gdf11 is involved in chondrogenesis in chick limb buds[124], partially explaining the skeletal defects seen in VACTERL. However, mice deficient of Gdf11 do not have a limb phenotype[125].

Recently, a mutation in *HOXD13* was described to cause VACTERL in single individual, suggesting the involvement of signalling pathways downstream of Shh[126]. However, the limb phenotype bears more resemblance to the brachydactyly phenotype (described in a family carrying the same 'in frame' shortening of the polyalanine tract) than to the general limb phenotype in VACTERL[76].

Fanconi Anemia

Fanconi Anemia (FA) is a heterogenic autosomal or X-linked recessive disorder characterised by chromosomal instability and cancer susceptibility. FA can be divided into thirteen complementation groups (FA-A,-B,-C,-D1,D2,-E,-F,-G,-I,-J,-L,-M and –N) of which only Fanconi-B is not inherited autosomal. Apart from the risk of acute myeloid leukaemia, patients with FA have a high risk of developing solid tumours of head and neck, esophagus, liver, vulva and cervix[127]. Other associated features are pigmentary changes in the skin and malformations of the heart, kidney and aplasia of the radius or thumb deformity[128]. Growth retardation, microcephaly, or microphthalmia are also reported in patients with FA. In a study of Giampietro *et al.*[129] clinical data from 370 patients were analysed. Of these, approximately 50% had radial-ray abnormalities, ranging from bilateral absent thumbs and radii to a unilateral hypoplastic thumb or bifid thumb.

Amongst the thirteen complementation groups, only the gene responsible for FA-I has not been identified. Cells from FA patients experimentally demonstrate chromosomal instability and elevated sensitivity to DNA cross-linking agents, such as mitomycin C, diepoxybutane and cisplatin[130]. The proteins encoded by the FA genes form the FA complex, consisting of complex 1 and 2. Together with other proteins, this FA complex detects DNA damage or errors in replication. During the S phase of the normal cell cycle when a replication fork encounters a DNA cross-link, complex 1 is activated and responsible for the monoubiquination of FANCD2. FANCD2-Ub then interacts with BRCA2 in complex 2, which leads to repair of the cross-link[131]. The studies of Liu *et al.* implicated an important role for the tumor suppressor gene p53. When *FANCD2* is knocked out in zebrafish embryos, a proportion of cells undergo apoptosis mediated by *p53* resulting in developmental abnormalities[132].

However, the aetiology of the pre-axial malformations associated with FA remains unclear. Mouse mutants of three of the FA genes (A, C and D2) have been generated. Although many of the phenotypical aspects of FA, such as genomic

instability and reduced fertility are present, none of the mutants display a limb phenotype[133,134,135].

Conclusions

Although the patterning of the limb has been studied for over seven decades and the role of the signalling centres and their interactions has been extensively charted, much remains to be unravelled. Not only are the downstream effectors of the signalling centres still largely unknown, the molecular basis of the upstream transcriptional regulation of the signalling molecules involved in patterning the limb also remains elusive. In the next chapter a short introduction to the process of transcriptional regulation in general and more specifically of the regulation of *Shh* in the limb, will be given. Thereafter I will describe the results of studies to gain more insight in the patterning of the limb. These include characterisation of the genetic causes of human malformations and the study of the molecular basis of *Shh* transcriptional regulation in the limb.

References

1. Crossley, P.H., G. Minowada, C.A. MacArthur, and G.R. Martin, *Roles for FGF8 in the induction, initiation, and maintenance of chick limb development.* Cell, 1996. **84**(1): p. 127-136.
2. Cohn, M.J., J.C. Izpisua-Belmonte, H. Abud, J.K. Heath, and C. Tickle, *Fibroblast growth factors induce additional limb development from the flank of chick embryos.* Cell, 1995. **80**(5): p. 739-746.
3. Hasson, P., J. Del Buono, and M.P. Logan, *Tbx5 is dispensable for forelimb outgrowth.* Development, 2006.
4. Naiche, L.A. and V.E. Papaioannou, *Tbx4 is not required for hindlimb identity or post-bud hindlimb outgrowth.* Development, 2007. **134**(1): p. 93-103.
5. Kmita, M., B. Tarchini, J. Zakany, M. Logan, C.J. Tabin, and D. Duboule, *Early developmental arrest of mammalian limbs lacking HoxA/HoxD gene function.* Nature, 2005. **435**(7045): p. 1113-1116.
6. Zakany, J. and D. Duboule, *The role of Hox genes during vertebrate limb development.* Curr Opin Genet Dev, 2007. **17**(4): p. 359-366.
7. Saunders, J.W.J., *The proximo-distal sequence of origin of the parts of the chick wing and the role of the ectoderm.* J Exp Zool, 1948. **108**: p. 363-403.
8. Summerbell, D., J.H. Lewis, and L. Wolpert, *Positional information in chick limb morphogenesis.* Nature, 1973. **244**(5417): p. 492-496.
9. Summerbell, D., *A quantitative analysis of the effect of excision of the AER from the chick limb-bud.* J Embryol Exp Morphol, 1974. **32**: p. 651-660.
10. Rubin, L. and J.W. Saunders, Jr., *Ectodermal-mesodermal interactions in the growth of limb buds in the chick embryo: constancy and temporal limits of the ectodermal induction.* Dev Biol, 1972. **28**(1): p. 94-112.
11. Wolpert, L., C. Tickle, and M. Sampford, *The effect of cell killing by x-irradiation on pattern formation in the chick limb.* J Embryol Exp Morphol, 1979. **50**: p. 175-193.
12. Niswander, L., C. Tickle, A. Vogel, I. Booth, and G.R. Martin, *FGF-4 replaces the apical ectodermal ridge and directs outgrowth and patterning of the limb.* Cell, 1993. **75**(3): p. 579-587.

13. Fallon, J.F., A. Lopez, M.A. Ros, M.P. Savage, B.B. Olwin, and B.K. Simandl, *FGF-2: apical ectodermal ridge growth signal for chick limb development.* Science, 1994. **264**(5155): p. 104-107.

14. Sun, X., F.V. Mariani, and G.R. Martin, *Functions of FGF signalling from the apical ectodermal ridge in limb development.* Nature, 2002. **418**(6897): p. 501-508.

15. Sun, X., M. Lewandoski, E.N. Meyers, Y.H. Liu, R.E. Maxson, Jr., and G.R. Martin, *Conditional inactivation of Fgf4 reveals complexity of signalling during limb bud development.* Nat Genet, 2000. **25**(1): p. 83-86.

16. Colvin, J.S., R.P. Green, J. Schmahl, B. Capel, and D.M. Ornitz, *Male-to-female sex reversal in mice lacking fibroblast growth factor 9.* Cell, 2001. **104**(6): p. 875-889.

17. Xu, J., Z. Liu, and D.M. Ornitz, *Temporal and spatial gradients of Fgf8 and Fgf17 regulate proliferation and differentiation of midline cerebellar structures.* Development, 2000. **127**(9): p. 1833-1843.

18. Lewandoski, M., X. Sun, and G.R. Martin, *Fgf8 signalling from the AER is essential for normal limb development.* Nat Genet, 2000. **26**(4): p. 460-463.

19. Mariani, F.V., C.P. Ahn, and G.R. Martin, *Genetic evidence that FGFs have an instructive role in limb proximal-distal patterning.* Nature, 2008. **453**(7193): p. 401-405.

20. Rowe, D.A., J.M. Cairns, and J.F. Fallon, *Spatial and temporal patterns of cell death in limb bud mesoderm after apical ectodermal ridge removal.* Dev Biol, 1982. **93**(1): p. 83-91.

21. Dudley, A.T., M.A. Ros, and C.J. Tabin, *A re-examination of proximodistal patterning during vertebrate limb development.* Nature, 2002. **418**(6897): p. 539-544.

22. Wolpert, L., *Limb patterning: reports of model's death exaggerated.* Curr Biol, 2002. **12**(18): p. R628-630.

23. Arques, C.G., R. Doohan, J. Sharpe, and M. Torres, *Cell tracing reveals a dorsoventral lineage restriction plane in the mouse limb bud mesenchyme.* Development, 2007. **134**(20): p. 3713-3722.

24. Towers, M. and C. Tickle, *Growing models of vertebrate limb development.* Development, 2009. **136**(2): p. 179-190.

25. Saunders, J.W.J. and M.T. Gasseling, *Ectodermal-mesodermal interactions in the*

origin of limb symmetry, in *Epithlelial-Mesenchymal Interactions*, R.E. Fleischmajer and R. Billingham, Editors. 1968, Williams and Wilkins: Baltimore. p. 78-97.

26. Summerbell, D., *The zone of polarizing activity: evidence for a role in normal chick limb morphogenesis.* J Embryol Exp Morphol, 1979. **50**: p. 217-233.
27. Summerbell, D., *The control of growth and the development of pattern across the anteroposterior axis of the chick limb bud.* J Embryol Exp Morphol, 1981. **63**: p. 161-180.
28. Tickle, C., B. Alberts, L. Wolpert, and J. Lee, *Local application of retinoic acid to the limb bond mimics the action of the polarizing region.* Nature, 1982. **296**(5857): p. 564-566.
29. Summerbell, D., *The effect of local application of retinoic acid to the anterior margin of the developing chick limb.* J Embryol Exp Morphol, 1983. **78**: p. 269-289.
30. Riddle, R.D., R.L. Johnson, E. Laufer, and C. Tabin, *Sonic hedgehog mediates the polarizing activity of the ZPA.* Cell, 1993. **75**(7): p. 1401-1416.
31. te Welscher, P., M. Fernandez-Teran, M.A. Ros, and R. Zeller, *Mutual genetic antagonism involving GLI3 and dHAND prepatterns the vertebrate limb bud mesenchyme prior to SHH signaling.* Genes Dev, 2002. **16**(4): p. 421-426.
32. Chiang, C., Y. Litingtung, M.P. Harris, B.K. Simandl, Y. Li, P.A. Beachy, and J.F. Fallon, *Manifestation of the limb prepattern: limb development in the absence of sonic hedgehog function.* Dev Biol, 2001. **236**(2): p. 421-435.
33. Hui, C.C. and A.L. Joyner, *A mouse model of greig cephalopolysyndactyly syndrome: the extra-toesJ mutation contains an intragenic deletion of the Gli3 gene.* Nat Genet, 1993. **3**(3): p. 241-246.
34. Litingtung, Y., R.D. Dahn, Y. Li, J.F. Fallon, and C. Chiang, *Shh and Gli3 are dispensable for limb skeleton formation but regulate digit number and identity.* Nature, 2002. **418**(6901): p. 979-983.
35. Harfe, B.D., P.J. Scherz, S. Nissim, H. Tian, A.P. McMahon, and C.J. Tabin, *Evidence for an Expansion-Based Temporal Shh Gradient in Specifying Vertebrate Digit Identities.* Cell, 2004. **118**(4): p. 517-528.
36. Scherz, P.J., E. McGlinn, S. Nissim, and C.J. Tabin, *Extended exposure to Sonic*

hedgehog is required for patterning the posterior digits of the vertebrate limb. Dev Biol, 2007. **308**(2): p. 343-354.

37. Tabin, C.J. and A.P. McMahon, *Developmental biology. Grasping limb patterning.* Science, 2008. **321**(5887): p. 350-352.
38. Zhu, J., E. Nakamura, M.T. Nguyen, X. Bao, H. Akiyama, and S. Mackem, *Uncoupling Sonic hedgehog control of pattern and expansion of the developing limb bud.* Dev Cell, 2008. **14**(4): p. 624-632.
39. Towers, M., R. Mahood, Y. Yin, and C. Tickle, *Integration of growth and specification in chick wing digit-patterning.* Nature, 2008. **452**(7189): p. 882-886.
40. Geduspan, J.S. and J.A. MacCabe, *Evidence for the transmission of dorsoventral information to the ectoderm during the earliest stages of limb development.* Prog Clin Biol Res, 1986. **226**: p. 115-126.
41. MacCabe, J.A., J.W. Saunders, Jr., and M. Pickett, *The control of the anteroposterior and dorsoventral axes in embryonic chick limbs constructed of dissociated and reaggregated limb-bud mesoderm.* Dev Biol, 1973. **31**(2): p. 323-335.
42. Parr, B.A. and A.P. McMahon, *Dorsalizing signal Wnt-7a required for normal polarity of D-V and A-P axes of mouse limb.* Nature, 1995. **374**(6520): p. 350-353.
43. Ahn, K., Y. Mishina, M.C. Hanks, R.R. Behringer, and E.B. Crenshaw, 3rd, *BMPR-IA signaling is required for the formation of the apical ectodermal ridge and dorsal-ventral patterning of the limb.* Development, 2001. **128**(22): p. 4449-4461.
44. Cygan, J.A., R.L. Johnson, and A.P. McMahon, *Novel regulatory interactions revealed by studies of murine limb pattern in Wnt-7a and En-1 mutants.* Development, 1997. **124**(24): p. 5021-5032.
45. Loomis, C.A., E. Harris, J. Michaud, W. Wurst, M. Hanks, and A.L. Joyner, *The mouse Engrailed-1 gene and ventral limb patterning.* Nature, 1996. **382**(6589): p. 360-363.
46. Robert, B., *Bone morphogenetic protein signaling in limb outgrowth and patterning.* Dev Growth Differ, 2007. **49**(6): p. 455-468.
47. Chen, H., Y. Lun, D. Ovchinnikov, H. Kokubo, K.C. Oberg, C.V. Pepicelli, L. Gan, B. Lee, and R.L. Johnson, *Limb and kidney defects in Lmx1b mutant mice suggest an involvement of LMX1B in human nail patella syndrome.* Nat Genet, 1998. **19**(1): p.

51-55.

48. Riddle, R.D., M. Ensini, C. Nelson, T. Tsuchida, T.M. Jessell, and C. Tabin, *Induction of the LIM homeobox gene Lmx1 by WNT7a establishes dorsoventral pattern in the vertebrate limb.* Cell, 1995. **83**(4): p. 631-640.

49. Chen, H. and R.L. Johnson, *Interactions between dorsal-ventral patterning genes lmx1b, engrailed-1 and wnt-7a in the vertebrate limb.* Int J Dev Biol, 2002. **46**(7): p. 937-941.

50. Gamer, L.W., V. Ho, K. Cox, and V. Rosen, *Expression and function of BMP3 during chick limb development.* Dev Dyn, 2008. **237**(6): p. 1691-1698.

51. Niswander, L., *Interplay between the molecular signals that control vertebrate limb development.* Int J Dev Biol, 2002. **46**(7): p. 877-881.

52. Pizette, S., C. Abate-Shen, and L. Niswander, *BMP controls proximodistal outgrowth, via induction of the apical ectodermal ridge, and dorsoventral patterning in the vertebrate limb.* Development, 2001. **128**(22): p. 4463-4474.

53. Capellini, T.D., G. Di Giacomo, V. Salsi, A. Brendolan, E. Ferretti, D. Srivastava, V. Zappavigna, and L. Selleri, *Pbx1/Pbx2 requirement for distal limb patterning is mediated by the hierarchical control of Hox gene spatial distribution and Shh expression.* Development, 2006. **133**(11): p. 2263-2273.

54. Niswander, L., S. Jeffrey, G.R. Martin, and C. Tickle, *A positive feedback loop coordinates growth and patterning in the vertebrate limb.* Nature, 1994. **371**(6498): p. 609-612.

55. Panman, L., A. Galli, N. Lagarde, O. Michos, G. Soete, A. Zuniga, and R. Zeller, *Differential regulation of gene expression in the digit forming area of the mouse limb bud by SHH and gremlin 1/FGF-mediated epithelial-mesenchymal signalling.* Development, 2006. **133**(17): p. 3419-3428.

56. Chiang, C., Y. Litingtung, E. Lee, K.E. Young, J.L. Corden, H. Westphal, and P.A. Beachy, *Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function.* Nature, 1996. **383**(6599): p. 407-413.

57. Nissim, S., P. Allard, A. Bandyopadhyay, B.D. Harfe, and C.J. Tabin, *Characterization of a novel ectodermal signaling center regulating Tbx2 and Shh in the vertebrate limb.*

Dev Biol, 2007. **304**(1): p. 9-21.

58. Gibson-Brown, J.J., S.I. Agulnik, L.M. Silver, L. Niswander, and V.E. Papaioannou, *Involvement of T-box genes Tbx2-Tbx5 in vertebrate limb specification and development.* Development, 1998. **125**(13): p. 2499-2509.

59. Isaac, A., C. Rodriguez-Esteban, A. Ryan, M. Altabef, T. Tsukui, K. Patel, C. Tickle, and J.C. Izpisua-Belmonte, *Tbx genes and limb identity in chick embryo development.* Development, 1998. **125**(10): p. 1867-1875.

60. Blanc, I., A. Bach, and B. Robert, *Unusual pattern of Sonic hedgehog expression in the polydactylous mouse mutant Hemimelic extra-toes.* Int J Dev Biol, 2002. **46**(7): p. 969-974.

61. Lettice, L.A., S.J. Heaney, L.A. Purdie, L. Li, P. de Beer, B.A. Oostra, D. Goode, G. Elgar, R.E. Hill, and E. de Graaff, *A long-range Shh enhancer regulates expression in the developing limb and fin and is associated with preaxial polydactyly.* Hum Mol Genet, 2003. **12**(14): p. 1725-1735.

62. Masuya, H., T. Sagai, K. Moriwaki, and T. Shiroishi, *Multigenic control of the localization of the zone of polarizing activity in limb morphogenesis in the mouse.* Dev Biol, 1997. **182**(1): p. 42-51.

63. Winter, R.M. and M. Baraitser, *London Dysmorphology Database*. 2001, Oxford: Oxford University Press.

64. Maas, S.A. and J.F. Fallon, *Single base pair change in the long-range Sonic hedgehog limb-specific enhancer is a genetic basis for preaxial polydactyly.* Dev Dyn, 2005. **232**(2): p. 345-348.

65. Sagai, T., M. Hosoya, Y. Mizushina, M. Tamura, and T. Shiroishi, *Elimination of a long-range cis-regulatory module causes complete loss of limb-specific Shh expression and truncation of the mouse limb.* Development, 2005. **132**(4): p. 797-803.

66. Furniss, D., L.A. Lettice, I.B. Taylor, P.S. Critchley, H. Giele, R.E. Hill, and A.O. Wilkie, *A variant in the sonic hedgehog regulatory sequence (ZRS) is associated with triphalangeal thumb and deregulates expression in the developing limb.* Hum Mol Genet, 2008. **17**(16): p. 2417-2423.

67. Klopocki, E., C.E. Ott, N. Benatar, R. Ullmann, S. Mundlos, and K. Lehmann, *A*

microduplication of the long range SHH limb regulator (ZRS) is associated with triphalangeal thumb-polysyndactyly syndrome. J Med Genet, 2008. **45**(6): p. 370-375.

68. Sun, M.*, et al.*, *Triphalangeal thumb-polysyndactyly syndrome and syndactyly type IV are caused by genomic duplications involving the long-range, limb-specific SHH enhancer.* J Med Genet, 2008. **45**(9): p. 589-595.
69. Buscher, D., B. Bosse, J. Heymer, and U. Ruther, *Evidence for genetic control of Sonic hedgehog by Gli3 in mouse limb development.* Mech Dev, 1997. **62**(2): p. 175-182.
70. Biesecker, L.G., *What you can learn from one gene: GLI3.* J Med Genet, 2006. **43**(6): p. 465-469.
71. Radhakrishna, U.*, et al.*, *The phenotypic spectrum of GLI3 morphopathies includes autosomal dominant preaxial polydactyly type-IV and postaxial polydactyly type-A/B; No phenotype prediction from the position of GLI3 mutations.* Am J Hum Genet, 1999. **65**(3): p. 645-655.
72. Temtamy, S.A. and V.A. McKusick, *The genetics of hand malformations.* Birth Defects Orig Artic Ser, 1978. **14**(3): p. i-xviii, 1-619.
73. Winter, R.M. and C. Tickle, *Syndactylies and polydactylies: embryological overview and suggested classification.* Eur J Hum Genet, 1993. **1**(1): p. 96-104.
74. Paznekas, W.A., S.A. Boyadjiev, R.E. Shapiro, O. Daniels, B. Wollnik, C.E. Keegan, J.W. Innis, M.B. Dinulos, C. Christian, M.C. Hannibal, and E.W. Jabs, *Connexin 43 (GJA1) mutations cause the pleiotropic phenotype of oculodentodigital dysplasia.* Am J Hum Genet, 2003. **72**(2): p. 408-418.
75. Akarsu, A.N., I. Stoilov, E. Yilmaz, B.S. Sayli, and M. Sarfarazi, *Genomic structure of HOXD13 gene: a nine polyalanine duplication causes synpolydactyly in two unrelated families.* Hum Mol Genet, 1996. **5**(7): p. 945-952.
76. Zhao, X.*, et al.*, *Mutations in HOXD13 underlie syndactyly type V and a novel brachydactyly-syndactyly syndrome.* Am J Hum Genet, 2007. **80**(2): p. 361-371.
77. Kjaer, K.W., L. Hansen, H. Eiberg, A. Utkus, L.T. Skovgaard, P. Leicht, J.M. Opitz, and N. Tommerup, *A 72-year-old Danish puzzle resolved--comparative analysis of phenotypes in families with different-sized HOXD13 polyalanine expansions.* Am J

Med Genet A, 2005. **138**(4): p. 328-339.

78. Goodman, F.R., *Limb malformations and the human HOX genes.* Am J Med Genet, 2002. **112**(3): p. 256-265.

79. Fitch, N., *Classification and identification of inherited brachydactylies.* J Med Genet, 1979. **16**( ): p. 36-44.

80. Bell, J., *On brachydactyly and symphalangism.* Treasury of Human Inheritance, 1951. **Cambridge University Press**: p. 1-31.

81. Gao, B., J. Guo, C. She, A. Shu, M. Yang, Z. Tan, X. Yang, S. Guo, G. Feng, and L. He, *Mutations in IHH, encoding Indian hedgehog, cause brachydactyly type A-1.* Nat Genet, 2001. **28**(4): p. 386-388.

82. McCready, M.E., E. Sweeney, A.E. Fryer, D. Donnai, A. Baig, L. Racacho, M.L. Warman, A.G. Hunter, and D.E. Bulman, *A novel mutation in the IHH gene causes brachydactyly type A1: a 95-year-old mystery resolved.* Hum Genet, 2002. **111**(4-5): p. 368-375.

83. Kirkpatrick, T.J., K.S. Au, J.M. Mastrobattista, M.E. McCready, D.E. Bulman, and H. Northrup, *Identification of a mutation in the Indian Hedgehog (IHH) gene causing brachydactyly type A1 and evidence for a third locus.* J Med Genet, 2003. **40**(1): p. 42-44.

84. Liu, M., X. Wang, Z. Cai, Z. Tang, K. Cao, B. Liang, X. Ren, J.Y. Liu, and Q.K. Wang, *A novel heterozygous mutation in the Indian hedgehog gene (IHH) is associated with brachydactyly type A1 in a Chinese family.* J Hum Genet, 2006. **51**(8): p. 727-731.

85. Zhu, G., X. Ke, Q. Liu, J. Li, B. Chen, C. Shao, and Y. Gong, *Recurrence of the D100N mutation in a Chinese family with brachydactyly type A1: evidence for a mutational hot spot in the Indian hedgehog gene.* Am J Med Genet A, 2007. **143**(11): p. 1246-1248.

86. Lodder, E.M., A.J. Hoogeboom, J.H. Coert, and E. de Graaff, *Deletion of 1 amino acid in Indian hedgehog leads to brachydactylyA1.* Am J Med Genet A, 2008. **146A**(16): p. 2152-2154.

87. St-Jacques, B., M. Hammerschmidt, and A.P. McMahon, *Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation.* Genes Dev, 1999. **13**(16): p. 2072-2086.

88. Hellemans, J., P.J. Coucke, A. Giedion, A. De Paepe, P. Kramer, F. Beemer, and G.R. Mortier, *Homozygous mutations in IHH cause acrocapitofemoral dysplasia, an autosomal recessive disorder with cone-shaped epiphyses in hands and hips.* Am J Hum Genet, 2003. **72**(4): p. 1040-1046.

89. Lehmann, K., P. Seemann, J. Boergermann, G. Morin, S. Reif, P. Knaus, and S. Mundlos, *A novel R486Q mutation in BMPR1B resulting in either a brachydactyly type C/symphalangism-like phenotype or brachydactyly type A2.* Eur J Hum Genet, 2006. **14**(12): p. 1248-1254.

90. Lehmann, K.*, et al.*, *Mutations in bone morphogenetic protein receptor 1B cause brachydactyly type A2.* Proc Natl Acad Sci U S A, 2003. **100**(21): p. 12277-12282.

91. Kjaer, K.W., H. Eiberg, L. Hansen, C.B. van der Hagen, K. Rosendahl, N. Tommerup, and S. Mundlos, *A mutation in the receptor binding site of GDF5 causes Mohr-Wriedt brachydactyly type A2.* J Med Genet, 2006. **43**: p. 225-231.

92. Schwabe, G.C., S. Tinschert, C. Buschow, P. Meinecke, G. Wolff, G. Gillessen-Kaesbach, M. Oldridge, A.O. Wilkie, R. Komec, and S. Mundlos, *Distinct mutations in the receptor tyrosine kinase gene ROR2 cause brachydactyly type B.* Am J Hum Genet, 2000. **67**(4): p. 822-831.

93. Oldridge, M., A.M. Fortuna, M. Maringa, P. Propping, S. Mansour, C. Pollitt, T.M. DeChiara, R.B. Kimble, D.M. Valenzuela, G.D. Yancopoulos, and A.O. Wilkie, *Dominant mutations in ROR2, encoding an orphan receptor tyrosine kinase, cause brachydactyly type B.* Nat Genet, 2000. **24**(3): p. 275-278.

94. Bacchelli, C., L.C. Wilson, J.A. Cook, R.M. Winter, and F.R. Goodman, *ROR2 is mutated in hereditary brachydactyly with nail dysplasia, but not in Sorsby syndrome.* Clin Genet, 2003. **64**(3): p. 263-265.

95. Polinkovsky, A.*, et al.*, *Mutations in CDMP1 cause autosomal dominant brachydactyly type C.* Nat Genet, 1997. **17**(1): p. 18-19.

96. Galjaard, R.J., L.I. van der Ham, N.A. Posch, P.F. Dijkstra, B.A. Oostra, S.E. Hovius, E.J. Timmenga, G.J. Sonneveld, A.J. Hoogeboom, and P. Heutink, *Differences in complexity of isolated brachydactyly type C cannot be attributed to locus heterogeneity alone.* Am J Med Genet, 2001. **98**(3): p. 256-262.

97. Sammar, M.*, et al.*, *Modulation of GDF5/BRI-b signalling through interaction with the tyrosine kinase receptor Ror2.* Genes Cells, 2004. **9**(12): p. 1227-1238.

98. Johnson, D., S.H. Kan, M. Oldridge, R.C. Trembath, P. Roche, R.M. Esnouf, H. Giele, and A.O. Wilkie, *Missense mutations in the homeodomain of HOXD13 are associated with brachydactyly types D and E.* Am J Hum Genet, 2003. **72**(4): p. 984-997.

99. Holt, M. and S. Oram, *Familial heart disease with skeletal malformations.* Br Heart J, 1960. **22**: p. 236-242.

100. Newbury-Ecob, R.A., R. Leanage, J.A. Raeburn, and I.D. Young, *Holt-Oram syndrome: a clinical genetic study.* J Med Genet, 1996. **33**(4): p. 300-307.

101. Silengo, M.C., M. Biagioli, A. Guala, G. Lopez-Bell, and R. Lala, *Heart-hand syndrome II. A report of Tabatznik syndrome with new findings.* Clin Genet, 1990. **38**(2): p. 105-113.

102. Ruiz de la Fuente, S. and F. Prieto, *Heart-hand syndrome. III. A new syndrome in three generations.* Hum Genet, 1980. **55**(1): p. 43-47.

103. Mori, A.D. and B.G. Bruneau, *TBX5 mutations and congenital heart disease: Holt-Oram syndrome revealed.* Curr Opin Cardiol, 2004. **19**(3): p. 211-215.

104. Basson, C.T.*, et al.*, *Mutations in human TBX5 [corrected] cause limb and cardiac malformation in Holt-Oram syndrome.* Nat Genet, 1997. **15**(1): p. 30-35.

105. Basson, C.T., G.S. Cowley, S.D. Solomon, B. Weissman, A.K. Poznanski, T.A. Traill, J.G. Seidman, and C.E. Seidman, *The clinical and genetic spectrum of the Holt-Oram syndrome (heart-hand syndrome).* N Engl J Med, 1994. **330**(13): p. 885-891.

106. Basson, C.T.*, et al.*, *Different TBX5 interactions in heart and limb defined by Holt-Oram syndrome mutations.* Proc Natl Acad Sci U S A, 1999. **96**(6): p. 2919-2924.

107. Brassington, A.M., S.S. Sung, R.M. Toydemir, T. Le, A.D. Roeder, A.E. Rutherford, F.G. Whitby, L.B. Jorde, and M.J. Bamshad, *Expressivity of Holt-Oram syndrome is not predicted by TBX5 genotype.* Am J Hum Genet, 2003. **73**(1): p. 74-85.

108. Reamon-Buettner, S.M. and J. Borlak, *TBX5 mutations in non-Holt-Oram syndrome (HOS) malformed hearts.* Hum Mutat, 2004. **24**(1): p. 104.

109. Huang, T., *Current advances in Holt-Oram syndrome.* Curr Opin Pediatr, 2002. **14**(6): p. 691-695.

110. Borozdin, W*., et al.*, *Expanding the spectrum of TBX5 mutations in Holt-Oram syndrome: detection of two intragenic deletions by quantitative real time PCR, and report of eight novel point mutations.* Hum Mutat, 2006. **27**(9): p. 975-976.

111. Linhares, V.L., N.A. Almeida, D.C. Menezes, D.A. Elliott, D. Lai, E.C. Beyer, A.C. Campos de Carvalho, and M.W. Costa, *Transcriptional regulation of the murine Connexin40 promoter by cardiac factors Nkx2-5, GATA4 and Tbx5.* Cardiovasc Res, 2004. **64**(3): p. 402-411.

112. King, M., J.S. Arnold, A. Shanske, and B.E. Morrow, *T-genes and limb bud development.* Am J Med Genet A, 2006. **140**(13): p. 1407-1413.

113. Hayes, A., T. Costa, and R.C. Polomeno, *The Okihiro syndrome of Duane anomaly, radial ray abnormalities, and deafness.* Am J Med Genet, 1985. **22**(2): p. 273-280.

114. Kohlhase, J., L. Schubert, M. Liebers, A. Rauch, K. Becker, S.N. Mohammed, R. Newbury-Ecob, and W. Reardon, *Mutations at the SALL4 locus on chromosome 20 result in a range of clinically overlapping phenotypes, including Okihiro syndrome, Holt-Oram syndrome, acro-renal-ocular syndrome, and patients previously reported to represent thalidomide embryopathy.* J Med Genet, 2003. **40**(7): p. 473-478.

115. Kohlhase, J., M. Heinrich, L. Schubert, M. Liebers, A. Kispert, F. Laccone, P. Turnpenny, R.M. Winter, and W. Reardon, *Okihiro syndrome is caused by SALL4 mutations.* Hum Mol Genet, 2002. **11**(23): p. 2979-2987.

116. Koshiba-Takeuchi, K., J.K. Takeuchi, E.P. Arruda, I.S. Kathiriya, R. Mo, C.C. Hui, D. Srivastava, and B.G. Bruneau, *Cooperative and antagonistic interactions between Sall4 and Tbx5 pattern the mouse limb and heart.* Nat Genet, 2006. **38**(2): p. 175-183.

117. Kohlhase, J., A. Wischermann, H. Reichenbach, U. Froster, and W. Engel, *Mutations in the SALL1 putative transcription factor gene cause Townes-Brocks syndrome.* Nat Genet, 1998. **18**(1): p. 81-83.

118. Khoury, M.J., J.F. Cordero, F. Greenberg, L.M. James, and J.D. Erickson, *A population study of the VACTERL association: evidence for its etiologic heterogeneity.* Pediatrics, 1983. **71**(5): p. 815-820.

119. de Jong, E.M., J.F. Felix, J.A. Deurloo, M.F. van Dooren, D.C. Aronson, C.P. Torfs, H.A. Heij, and D. Tibboel, *Non-VACTERL-type anomalies are frequent in patients*

with esophageal atresia/tracheo-esophageal fistula and full or partial VACTERL association. Birth Defects Res A Clin Mol Teratol, 2008. **82**(2): p. 92-97.

120. Castori, M., R. Rinaldi, P. Capocaccia, M. Roggini, and P. Grammatico, *VACTERL association and maternal diabetes: a possible causal relationship?* Birth Defects Res A Clin Mol Teratol, 2008. **82**(3): p. 169-172.

121. Kim, J., P. Kim, and C.C. Hui, *The VACTERL association: lessons from the Sonic hedgehog pathway.* Clin Genet, 2001. **59**(5): p. 306-315.

122. Schinzel, A., *Catalogue of unbalanced chromosome aberrations in man*. 2nd ed. 2001, New York, USA: De Gruyter.

123. Szumska, D*., et al.*, *VACTERL/caudal regression/Currarino syndrome-like malformations in mice with mutation in the proprotein convertase Pcsk5.* Genes Dev, 2008. **22**(11): p. 1465-1477.

124. Gamer, L.W., K.A. Cox, C. Small, and V. Rosen, *Gdf11 is a negative regulator of chondrogenesis and myogenesis in the developing chick limb.* Dev Biol, 2001. **229**(2): p. 407-420.

125. McPherron, A.C., A.M. Lawler, and S.J. Lee, *Regulation of anterior/posterior patterning of the axial skeleton by growth/differentiation factor 11.* Nat Genet, 1999. **22**(3): p. 260-264.

126. Garcia-Barcelo, M.M., K.K. Wong, V.C. Lui, Z.W. Yuan, M.T. So, E.S. Ngan, X.P. Miao, P.H. Chung, P.L. Khong, and P.K. Tam, *Identification of a HOXD13 mutation in a VACTERL patient.* Am J Med Genet A, 2008. **146A**(24): p. 3181-3185.

127. Rosenberg, P.S., M.H. Greene, and B.P. Alter, *Cancer incidence in persons with Fanconi anemia.* Blood, 2003. **101**(3): p. 822-826.

128. Glanz, A. and F.C. Fraser, *Spectrum of anomalies in Fanconi anaemia.* J Med Genet, 1982. **19**(6): p. 412-416.

129. Giampietro, P.F., B. Adler-Brecher, P.C. Verlander, S.G. Pavlakis, J.G. Davis, and A.D. Auerbach, *The need for more accurate and timely diagnosis in Fanconi anemia: a report from the International Fanconi Anemia Registry.* Pediatrics, 1993. **91**(6): p. 1116-1120.

130. Kennedy, R.D. and A.D. D'Andrea, *The Fanconi Anemia/BRCA pathway: new faces in*

the crowd. Genes Dev, 2005. **19**(24): p. 2925-2940.

131. Rahman, N. and A. Ashworth, *A new gene on the X involved in Fanconi anemia.* Nat Genet, 2004. **36**(11): p. 1142-1143.

132. Liu, T.X., N.G. Howlett, M. Deng, D.M. Langenau, K. Hsu, J. Rhodes, J.P. Kanki, A.D. D'Andrea, and A.T. Look, *Knockdown of zebrafish Fancd2 causes developmental abnormalities via p53-dependent apoptosis.* Dev Cell, 2003. **5**(6): p. 903-914.

133. Wong, J.C., N. Alon, C. McKerlie, J.R. Huang, M.S. Meyn, and M. Buchwald, *Targeted disruption of exons 1 to 6 of the Fanconi Anemia group A gene leads to growth retardation, strain-specific microphthalmia, meiotic defects and primordial germ cell hypoplasia.* Hum Mol Genet, 2003. **12**(16): p. 2063-2076.

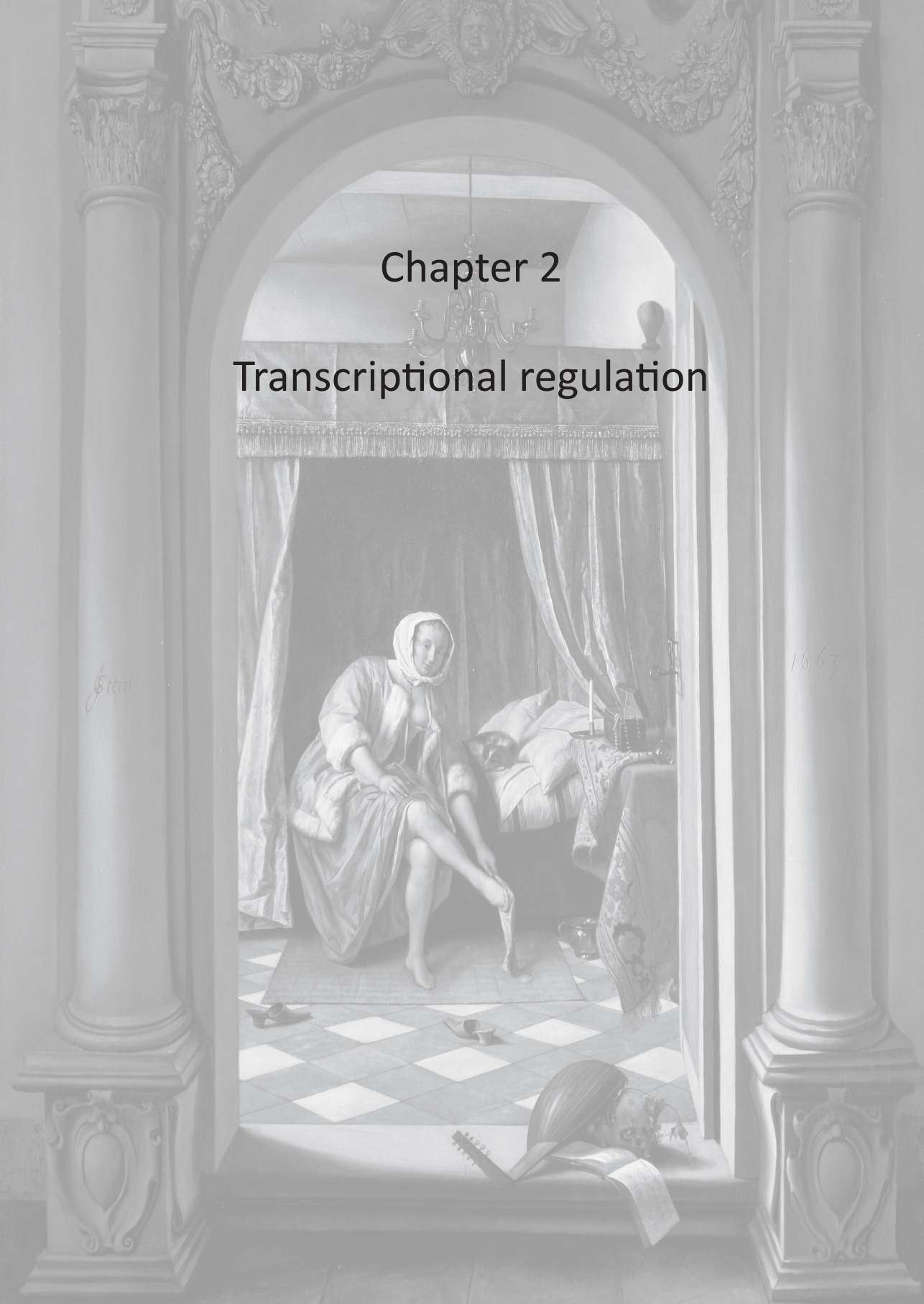
134. Chen, M*., et al.*, *Inactivation of Fac in mice produces inducible chromosomal instability and reduced fertility reminiscent of Fanconi anaemia.* Nat Genet, 1996. **12**(4): p. 448-451.

135. Houghtaling, S., C. Timmers, M. Noll, M.J. Finegold, S.N. Jones, M.S. Meyn, and M. Grompe, *Epithelial cancer in Fanconi anemia complementation group D2 (Fancd2) knockout mice.* Genes Dev, 2003. **17**(16): p. 2021-2035.

136. Honig, L.S. and D. Summerbell, *Maps of strength of positional signalling activity in the developing chick wing bud.* J Embryol Exp Morphol, 1985. **87**: p. 163-174.

Chapter 2

Transcriptional regulation



Introduction

As described in the previous chapter, misexpression of the signalling molecules that pattern the embryo often leads to developmental malformations. Therefore, transcription of these molecules needs to be tightly regulated to ensure the correct timing and positioning of expression. Besides the initiation of transcription, the effective level of mRNA in the cell is also regulated through mRNA degradation and differential splicing[1]. However, these post transcriptional processes fall outside the scope of this thesis and will not be discussed (for a recent review see Silva and Romao, 2009[2]). The molecular basis of transcriptional activation/repression is beginning to emerge. In the following chapter a short overview of the current knowledge about these processes will be given.

Transcription depends on chromatin modifications

Chromatin

The DNA in the nucleus is not just a randomly coiled string of nucleotides. To fit the 2 meters of DNA in a nucleus of 6 μm in diameter, the DNA is intricately folded and condensed (see figure 2.1). The level of folding differs between different regions of the DNA. Strongly compacted regions of heterochromatin are thought to prevent the transcription, while the more open structure of euchromatin facilitates transcriptional activation of genes[3].

The most basic level of organisation is the wrapping of the DNA around nucleosomes (figure 2.1c). The nucleosomes are separated by small stretches of unwrapped linker DNA (figure 2.1e). The spacing of the nucleosomes on the DNA is dynamic and is important for the regulation of transcription. Each nucleosome consists of an octamer of the basic chromatin proteins: the histones. Generally, the octamer consists of two H2A-H2B dimers and two H3-H4 dimers (figure 2.1c), although different variants of these core histones exist. These latter are thought to

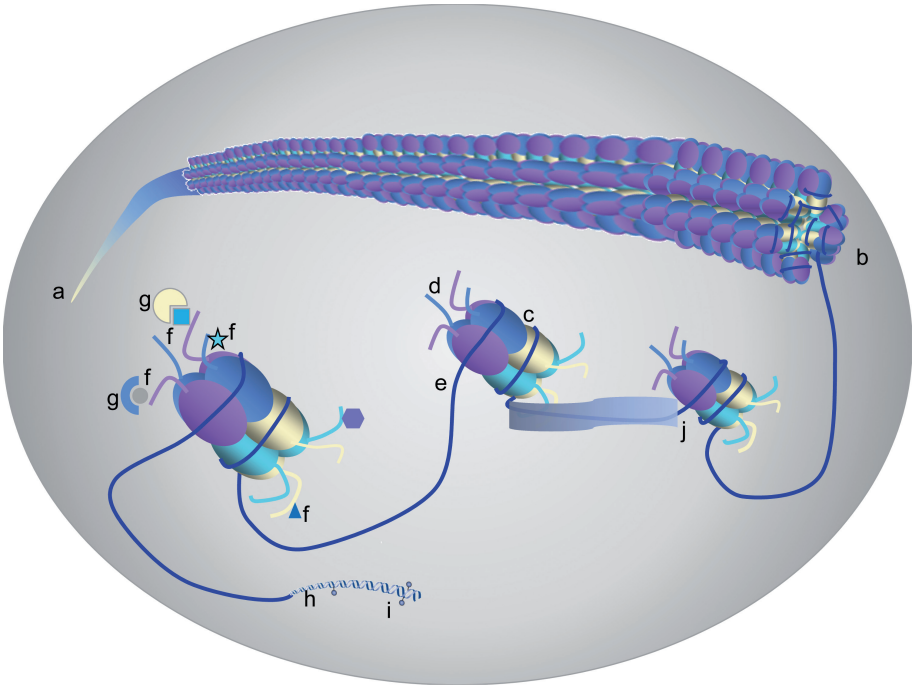


Figure 2.1 Organisation of the DNA into chromatin and its influence on transcription*: a) the localisation of a locus within the nucleus can influence its transcriptional potential; b) wrapping of the nucleosomes in higher order chromatin fiber; c) nucleosome: histone octamere, consisting of two two H2A-H2B dimers and two H3-H4 dimers whith 147 bp of DNA wrapped around it; d) histone tails protruding from the nucleosome; e) linker DNA in between two nucleosomes; f) diverse histone modifications (methylation, acetylation, phosphorylation, ubuiquitination and sumoylation); g) chromatin binding proteins interacting with specific histone modifications; h) DNA double helix; i) DNA methylation; j) linker histone H1.*

be important in the condensation of the chromatin of specific loci[4]. Around each histone octamer, 147 bp of DNA is wrapped 1.7 turns in a left handed coil[3].

Histones have a globular structure with a protruding N-terminal tail (figure 2.1d). This tail contains many sites that can be modified post-translationally (figure 2.1f,g). These modifications (such as methylation and acetylation) can influence the higher order folding of the DNA and therefore the accessibility of the DNA for transcription[5]. Histone modifications will be discussed in more detail in the next section.

The DNA was shown to be further compacted by the coiling up of the "beads on a string" structure into higher order condensations. This is partly achieved through linker histones of the H1 family (figure 2.1j), which bind to the nucleosomes and fold the DNA into a higher order structure called the "30 nm fibre" (figure 2.1b)(reviewed by Horn and Peterson, 2002 and Woodcock 2006)[6-8]. These linker-histones are absent on transcriptionally active chromatin and their presence inhibits transcription in *in vitro* studies[9]. Furthermore, decompaction of the DNA requires the eviction of H1 from the chromatin[10]. The stabilisation and compaction of the DNA in these higher order structures is essential for embryonic development, as reduction of the levels of linker-histones by 50% leads to embryonic lethality before day E11.5[11].

The regulation of the chromatin folding is an essential part of the transcriptional activation and repression of genes. Many proteins involved in the modification of the chromatin structure have been identified, including some of the general transcription factors that are essential for the assembly of the transcription machinery on the promoter of a gene[5].

Chromatin modifications regulation of transcription

At least eight different forms of modification of the histone tails exist (for a recent overview see Kouzarides, 2007[12]). Most of these involve the basic residues lysine and arginine in the histone tails. These amino acids are targets for methylation (both arginine and lysine can be mono- and bimethylated or trimethylated (only lysine)), deamination (of arginine residues) and acetylation (of lysines). These modifications can take place at over 60 different positions along the histone tail. Moreover, different modifications can be combined on one single tail. This allows for a wealth of combinatorial modifications, which has been named the histone code. Recently, it has become clear that the histone code is not a simple straightforward static combination of the different modifications. The different modifications influence each other and can be highly dynamic[13].

Acetylation

Acetylation is almost always associated with activation of transcription. The acetylation of the lysines in the N-terminal tails of the histones is performed by three classes of histone acetyl transferases (HATs): GNAT, MYST and CBP/p300[14]. Most of the enzymes acetylate diverse substrates but some show more specificity. By using mutated histone proteins in *in vitro* assembled chromatin[15], acetylation was shown to directly affect the compaction of the chromatin through neutralisation of the basic charge of the acetylated lysine.

The acetylation of the histones can be reversed by histone deactylation complexes (HDACs). There are three families of deacytelases that are involved in multiple signalling pathways. They are present in many repressive chromatin complexes. In general, the HDACs do not show much specificity for the different acetyl groups[12].

Methylation

Different lysines in the histone tails are methylated by different enzymes. The methyltransferases are, in contrast to the (de)acetylation enzymes, highly specific: the different residues are methylated by dedicated enzymes[16]. The mono-, di- and tri-methylation of the different lysines does not directly influence the compaction of the DNA. Instead it creates binding places that interact with different domains of chromatin binding proteins. These interactions are the basis of the functional output of these chromatin modifications.

Methylation at different residues has been associated with different functions of the DNA. For example, trimethylation of the 4th lysine of histone 3 (H3K4Me3) is associated with transcriptional start sites and like with trimethylation of H3K27, it is associated with active transcription[13]. In contrast trimethylation of H3K9 is associated with transcriptional repression and heterochromatin[12]. A recent report

by Kolasinska-Zwierz and colleagues shows that H3K36Me3 is not just associated with active transcription but is also related to splicing. This chromatin mark is overrepresented at exons and underrepresented at introns. Even more striking, its presence correlates with the frequency an exon is spliced into the final mRNA[17] indicating a very close interaction between transcription and the splicing machinery.

The effect of most methylation marks is not black and white but depends on the context. For example, both the H3K36Me3 mentioned above and methylation of H3K9 have a repressive effect when found in the promoter region of gene and a positive effect on elongation of transcription when found further downstream[18]. The more we learn about these modifications the more it becomes clear that context is very important for their functional relevance[12].

Besides histone methylation, the DNA itself can also be methylated (figure 2.1i). This DNA methylation is essential for proper regulation of transcription[5]. In the promoter region of many genes C/G rich regions can be found (CpG) islands. The cytosines of these CpG islands can be methylated, resulting in repression of transcription, that is most likely due to the recruitment of other chromatin modifications, such as histone deacetylation[13]. Mutations leading to hypermethylation of a promoter (such as the expansion of a CGG repeat in the 5' UTR of the *Fmr1* gene) can lead to disease (mental retardation due to the lack of expression of the *Fmr1* gene)[19].

Phosphorylation

Phosphorylation of histone residues has not yet been mapped as thoroughly as methylation. A described effect is the activation of c-jun and c-fos whose transcription is regulated through the NFKB pathway[20]. Chromatin immune precipitation (ChIP)-on-chip assays have shown the binding of many kinases to specific sites in the genome. This implies that the kinases that are known to be active in the cytoplasm

in many of the classical signal transduction pathways may have a direct effect on transcription[21].

Large modifications: ubiquitination and sumoylation

Like phosphorylation, little is known about the effect of these larger histone modifications on gene regulation. Ubiquitination and sumoylation are mutually exclusive, as they occupy the same histone tail residues. Ubiquitination of H2AK119 and H2BK120 appears to have opposite functions. While the first has been described to be involved in transcriptional repression, the latter appears to have a role in activation and elongation of transcription. The molecular basis of these processes is not clear[22-24].

General transcription factors are modifiers of the chromatin code

In contrast to prokaryotic gene transcription, eukaryotic RNA polymerase cannot function on its own. For proper transcription many co-factors are needed. These proteins are members of the general transcription complex and aid polymerase II in starting and continuing transcription. Many of these proteins have now been shown to be chromatin remodelling proteins, which remove repressive (such as H3K9Me) and place permissive (such as lysine acetylation) chromatin modifications. Furthermore, they interact with gene specific transcription factors to ensure proper timing and levels of expression [1].

Nuclear localisation

Chromosomes mostly do not intermingle in the nucleus but occupy their own restricted area, the chromosome territory[25]. These territories are not randomly organised with respect to one another. It is thought to be due to a stochastic process of chromosome decondensation within the limited space of the nucleus after

mitosis[26]. Fluorescent In Situ Hybridisation (FISH) is used to measure the localisation of a specific locus, visualising it with or without its chromosome territory. The position of the locus relative to the nuclear periphery and or its chromosome territory can then be determined. Although this technique can determine correlations between transcriptional activity and localisation, it is incapable of determining which is the cause and which the consequence[25].

Recently, much attention has been given to the observed differential localisation of actively transcribed regions in the centre of the nucleus and repressed/silent genomic regions at the nuclear periphery. The debate centres around the question whether the observed localisation of actively transcribed loci in "transcription factories" containing a high abundance of polymerase II is the result of an active process or a secondary result of chromatin decondensation and transcription of these loci[27-29].

The relation between active transcription and localisation at the nuclear centre is not one-on-one. Downregulation of expression of genes was observed around an integration site of repeats that relocate the locus to the nuclear periphery[30]. The observed effects are linked to the nuclear lamina as disruption of laminar stability leads to the upregulation of a large number of genes[31]. However, the nuclear periphery does not necessarily repress all transcription, as another locus did not show transcriptional repression[29]. Furthermore, transcriptionally inactive genes neighbouring active genes are found in the nuclear interior without being activated[32].

The localisation of loci relative to their chromosome territory has been widely studied. Although early results indicated a strong relationship between looping out of the chromosome territory and active transcription (reviewed by Cremer and Cremer in 2001[25]), more recent studies indicate that this is not a universal requirement for transcription and is most likely related to the genomic context of each locus[32,33].

Regulatory elements

Promoters, enhancers & insulators

Traditionally, DNA elements that control transcription levels are divided in promoters, enhancers/repressors and insulators. The promoter region of a gene is the DNA just upstream of the transcriptional start site where the polymerase complex assembles and gene specific transcription factors can bind. This region contains the TATA box, which is recognised by TFIID, one of the general transcription factors. Binding sites for gene specific transcription factors (facilitating/promoting transcription) can also be found in this area. These gene specific transcription factors are necessary to regulate the expression of a gene to ensure it is only transcribed when it is needed[5].

Enhancer/repressor is the general name for any DNA fragment that can influence the transcriptional activity of a gene, activating or repressing it respectively[5]. Enhancers can be located anywhere up to 1 megabase (Mb) upstream or downstream of the promoter of a gene. Some enhancer elements are highly specific for one target, while others can influence the expression of several or all genes in the vicinity[34,35]. In most cases of enhancer sharing, competition between the different promoters determines the relative influence of an enhancer on each specific promoter[36,37].

Insulators are a specific type of regulatory elements that exert their influence through the creation of a boundary between different parts of the DNA. They can prevent the spreading of heterochromatin into areas with a more open DNA structure. Moreover, they can restrict the effect of an enhancer to prevent it from inadvertently activating non-target genes[38,39]. One of the proteins known to be involved in insulator functioning is the CCCTC-binding factor CTCF. This protein mediates the formation of loops in the DNA, excluding interactions between DNA within the loop with DNA outside the loop[40,41]. Approximatly 15.000 CTCF insulator sites are found in the human genome, suggesting a rather widespread role of CTCF in the regulation of gene expression[42,43].

Conservation

Regulatory elements can be identified through multispecies comparisons of DNA sequences. Because these elements are essential for proper gene expression patterns, there is restriction against their mutation. This means that functional regulatory elements will be more conserved between species than non functional intervening DNA stretches[44]. Although many studies have been performed to systematically map such elements their characterisation has proven to be more difficult[45]. This is mainly due to the fact that even if the position of a conserved element is known, you do not know its function or even which gene it regulates. In addition, enhancer elements have been shown to locate up to 1 Mb away from their regulatory targets[46]. To complicate matters even further, their targets are not necessarily the genes they are flanked by[34]. Systematic approaches to address the function of these elements involve laborious construction of reporter plasmids that allow visualisation of the expression pattern generated by the enhancer. Unfortunately, this technique cannot be applied to identify repressive elements[47].

Quite a few regulatory elements of genes involved in limb development have thus far been characterised in some detail and their localisations differ per gene. For example, Beermann *et al.* have mapped new regulatory elements capable of reproducing most of the normal *Fgf-8* expression. Most of these elements were found in the 3' downstream region of the *Fgf-8* gene[48]. In contrast, many enhancer elements of the gene *Shh* characterised by Jeong *et al*. are located up to 500 kb upstream of the gene[49].

The presence of regulatory elements can sometimes be deduced from the phenotype of patients harbouring inversions or translocations. In some cases, no genes have been interrupted at the breakpoints indicating that the phenotype of these patients could be due to the disruption of proper expression patterns. Breakpoints upstream of *Shh* for example lead to holoprosencephaly (MIM #142945) due to separation of *Shh* and its central nervous system enhancers[49,50]. Separation

between a gene and its enhancers has been well documented for the *Sox9*, *HoxD* and *MAF* genes as well[46,51-57].

Small variations within regulatory elements could provide the basis for many of the phenotypic variations observed within and between species[5]. While pathogenic mutations within a developmental patterning gene generally have quite destructive results, mutations within a regulatory element, slightly affecting the affinity for a transcription factor for example, may produce small variations in phenotype[58].

Most enhancers consist of a group of smaller regulatory sequences of less than 100 bp (most likely transcription factor binding sites) within the larger regulatory region. Rastegar *et al.* recently showed that the order of and the distance between these smaller elements within enhancers, varies between different genes that are expressed in the zebrafish notochord[59]. Hare *et al.* showed that the same is true for the elements of the *eve* enhancers in different species of the fly. The produced expression patterns, however, appear to be the same[60]. These results indicate a functional flexibility within these enhancers. This will complicate the search for regulatory sequences, as less conserved regions may be functional if they contain several smaller conserved regions; these however are less likely to be found.

Distance

The distance between regulatory elements and their targets can be very large, quite often with other genes intervening between an enhancer and its target gene. This makes the task of assigning regulatory elements to specific target genes difficult. In an attempt to assess the average distance between enhancers and their targets in an unbiased manner Vavouri *et al.* worked with the assumption that paralogues genes have duplicated together with their enhancer sequences[61]. This allowed them to assign conserved non-coding elements to specific genes and therefore to calculate the distance between the enhancer and its target. Based on these calculations 30% of the enhancers lie within the first 100 kb of a gene but over 50% of all enhancers lie

more than 250 kb away from their targets.

Thus far, three gene specific enhancers have been mapped as far as 1 Mb from their transcriptional targets. Two of these the regulatory elements (of the *MAF* and *Sox9* genes) have been mapped based on the identical phenotype of patients bearing translocations (that separate the enhancer from its target gene) to patients that carry point mutations in either gene[56,57]. The third is the limb specific *Shh* enhancer: the ZRS[34,62]. This element will be discussed partly in the next section and in more detail in chapters 3 and 4. How these enhancers work over such long distances remains largely unknown.

Regulatory mutations in limb development

Regulatory elements have thus far been implicated in the limb defects observed in one inbred rat strain and five different mouse strains with limb defects. A very recent report by Liska *et al.* provides evidence that the preaxial polydactyly in the rat mutant *Lx* is caused by the deletion of a 155 kb region in an intron of the gene *Plzf* (promyelocytic leukemia zinc finger protein). This region contains a 3 kb conserved non-coding sequence. The deletion leads to a reduction in expression of *Plzf* and consequently to ectopic expression of its target genes *Hoxd10-13*, providing a clue to the aetiology of the polydactyly in these rats[63,64].

In mouse limb development five different mutations in enhancers have been described that cause a deregulation of expression patterns of patterning genes. The polydactylous mouse mutant *hemimelic extra toes (hx)* has a single nucleotide alteration within the limb *Shh* enhancer (the ZRS)[34]. This mutation causes ectopic expression of *Shh* on the anterior side of the limb bud and therefore polydactyly. The regulatory element involved was cloned partially due to the phenotype of the *Sasquatch* mouse. These polydactylous mice were accidentally created by the random insertion of a transgene close to the regulatory element, causing a duplication of the ZRS[34]. A third mouse strain in which *Shh* expression is disturbed is *dishevelled*. These

mice suffer from a more complicated change in *Shh* expression: besides ectopic expression at a later stage during limb development there is a loss of expression at the normal time and position. Due to a radiation induced inversion, *Shh* is separated from several of its long distance regulatory elements including the ZRS, causing a *Shh* null phenotype in homozygous state. On the other hand due to the same inversion *Shh* is ectopically induced in the phalangeal anlagen at E13.5-E14.5. This induces the expression of *Shh* targets such as *Gli3* and *patched*, leading to the brachydactyly phenotype observed in these mice[65].

The phenotype in *doublefoot* mice is caused by a 600 kb deletion *in cis* to Indian Hedgehog, starting 50 kb 5' of the gene and extending further away. This is thought to lead to the removal of a transcriptional repressor as *Ihh* is now ectopically expressed in the distal limb bud mesenchyme leading to the polydactylous phenotype[66,67].

Over the years, five different alleles causing the *limb deformity* (ld) phenotype have been identified. The limb defect of both hind and fore limbs in these animals is characterised by synostosis of the long limb bones (ulna and radius / tibia and fibula) and oligodactyly (fewer toes than normal) with syndactyly of the bones in the 4 paws[68]. Originally, the phenotype was attributed to the disruption of *Formin*[69]. However, more recently two new mutations were found that do not to disrupt the coding region of *Formin,* but instead affect the expression of the neighbouring gene *Gremlin. Gremlin* is a known signalling molecule, essential for the feedback loop between *Shh* and *Fgf4.* These results led to a re-evaluation of the *ld* mutations, uncovering a global control region (GCR) that is essential for the proper expression of *Gremlin*. This GCR is affected in all *ld* mutants[70].

All of the limb mutants described above disrupt the correct expression patterns of signalling molecules that are essential for proper limb development in some way. Although the genetic cause of these mutations has now been found, the molecular basis of how these mutations disrupt the normal initiation and repression of expression is far from clear.

Models of long distance regulation

As described in the preceding sections, regulatory elements are essential for proper regulation of gene transcription. Despite this essential role they can be located at considerable distances from their target genes. How these elements influence transcription over distances of several 100 kbs is not clear. Different models are starting to emerge. Although the basic ideas behind these models are completely different, they are not mutually exclusive and could well be operating in concert to generate the proper controls on transcriptional activation. The three models that have been characterised in most detail are chromatin remodelling, chromatin looping and regulation by non coding RNAs.

In the next sections, all three mechanisms will be discussed using the transcriptional regulation of the *Hox* clusters as example. Along the anterior-posterior axis of the embryo the homeotic transcription factors of the *Hox* clusters are expressed in a collinear fashion (the expression pattern of the genes along the AP axis reflects the position within the cluster with more 5' genes restricted to more posterior regions), which is essential for the anterior-posterior patterning[71]. In the limb bud only the *HoxA* and *HoxD* clusters play a major role, ectopic expression of 5' *HoxA/D* genes on the anterior side of the limb bud results in preaxial polydactyly (reviewed in Zakany and Duboule, 2007[72] and see chapter 1).

Chromatin remodelling and nuclear localisation

Ample evidence is available that restructuring of the chromatin is essential to establish the proper expression pattern of all the genes in the *Hox* clusters. Early evidence came from a study of the transcription factor *Plzf. Plzf* prevents ectopic expression of the *HoxD* cluster by recruiting the Polycomb Group (PcG) factors and HDACs. This leads to chromatin remodelling and silencing[73]. In 2004 Rastegar *et al.* observed sequential histone modifications at the *Hoxd4* regulatory sequences, starting at the neural enhancer followed by the *Hoxd4* promoter[74]. These sequential

modifications were compatible with a decondensation of the *Hoxd4* locus to allow active transcription. None of these modifications were observed in anterior tissue where *Hoxd4* remains silent, indicating an active role in establishing the *Hoxd4* expression.

The group of Bickmore studied the decondensation of the *HoxB* and *HoxD* loci upon transcription. Activation of the *HoxB* locus in ES cells and *in vivo* during development leads to the unwinding of a higher order chromatin organisation at this locus[75]. In these systems, the decondensation of the locus correlated with a relocation of the *HoxB* cluster outside the chromosome territory (CT). In contrast, induction of expression of the *HoxD* cluster leads to relocation outside the CT only along the main body axis; in the limb bud, however, the cluster is decondensed and partially transcribed but localises within the CT[33]. These results confirm the necessity of chromatin modifications at the *Hox* clusters for active transcription. However, they do not support the hypothesis that localisation outside the CT is essential for active transcription[25].

Contact/looping

Looping of the DNA (bringing regulatory sequences in close proximity to their transcriptional targets) has been described for several loci. The β-globin locus has been characterised in most detail. At this locus the Locus Control Region (LCR) interacts with the different promoters of the globin genes. This results in the switch of transcription of the embryonic globin genes to the transcription of the adult ones. This looping of the DNA forms an active chromatin hub, which is highly favourable of expression. Inside the formed loops RNA polymerase II can track along the DNA from the enhancers to the transcriptional start sites (reviewed in Noordermeer and de Laat, 2008[35]). The looping of this locus has been directly demonstrated by a technique called Chromatin Conformation Capture[76] (3C, for more details see chapter 7 General

Discussion). Recently, reports of interactions not only between enhancers and their targets but also between similarly expressed genes, even on different chromosomes have appeared[77-79]. However, the functional implications of these interactions *in trans* are far from clear[79].

Although no direct confirmation of looping has been published for any of the *Hox* clusters, the presence of DNA loops and their dynamics was demonstrated very elegantly by Montavon *et al.*. They use a combined approach measuring expression levels of the different *Hox* genes in the *HoxD* cluster. In the limb bud besides a spatial collinear expression pattern the level of expression of the different members of the *HoxD* cluster follows an anti-collinear pattern: *HoxD13* is expressed at the highest level, followed by *HoxD12*, *HoxD11* etc[72].

Montavon *et al.* applied mathematical modelling of these reverse collinear expression levels to predict the effect of specific targeted deletions and insertions in the cluster, measured the actual levels and used these to optimise the model, repeating the process in several rounds[36]. In the resulting model it is evident that a regulatory element situated 5' of the cluster, called the General Control Region (GCR) contacts a proximal enhancer (prox) which is situated in between the GCR and the Hox cluster (figure 2.2). This contact is followed by microscanning and activation of the different genes in the neighbourhood. The efficiency of this second step depends on gene specific promoter enhancer affinities and on the proximity of the promoter to the combined enhancers, producing the quantitative reverse collinear expression pattern in the developing limb[72,80].

Non coding RNAs

Recently, evidence was found for a new regulatory layer of expression control within the *Hox* clusters: non-coding RNAs. Rinn *et al.* provide evidence that a long non-coding RNA expressed from one *Hox* cluster can actively influence the expression

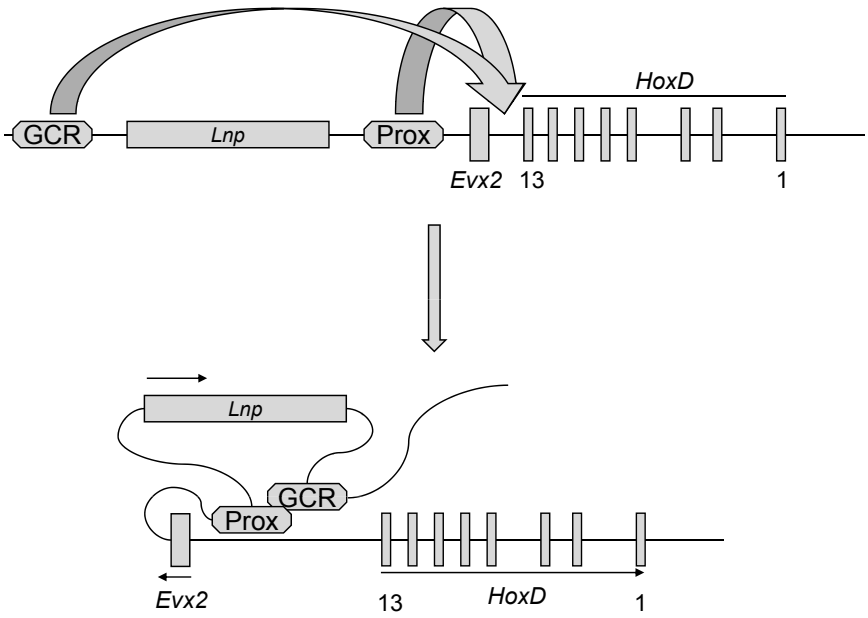


Figure 2.2 Looping and microscanning of the HoxD locus by the General Control Region (GCR) and the proximal enhancer (Prox)*: a) the GCR and Prox loop to the Evx-Hox intergenic region; b) from this position the enhancer complex scans the region and activates genes in the surrounding through a scanning process in which the vicinity of a gene and specific promoter enhancer affinity determine the efficiency of transcription. Based on Montavon et al. 2008*[36].

of another *Hox* cluster. The 2.2kb non-coding RNA HOTAIR is located within the *HoxC* locus. Strikingly, they find that HOTAIR represses transcription *in trans* along 40kb of the *HoxD* cluster. This repression is mediated through interactions with the Polycomb Repression Complex and H3K27 trimethylation of the locus[81].

More recently, Guttman *et al.* used a chromatin signature approach to identify long intergenic non-coding RNAs (lincRNAs) and identified approximately 1600 new lincRNAs. Most of these newly identified transcripts show a high degree of evolutionary conservation suggesting a selective constraint against mutation of these transcripts. HOTAIR was one of the identified lincRNAs validating the detection approach. Besides HOTAIR a new lincRNA in the *HoxC* cluster was identified further

downstream which was named Frigidair. Frigidair was identified based on its expression pattern as the expression of this lincRNA was inversely correlated the most with HOTAIR, suggesting a role in either *HoxD* activation or HOTAIR repression[82]. These results add an additional layer of complexity to the already complex regulation of transcriptional regulation of developmentally important genes.

Conclusions

Control of transcription of developmental patterning genes is extremely important as misexpression of these genes leads to congenital malformations. Transcriptional control is achieved at several levels ensuring subtle but stable control. Gene specific regulatory elements are bound by general and/or specific transcription factors which change the chromatin state of the locus, bind to other transcription factors causing DNA loops, interact with non-coding RNAs and probably influence transcription levels in ways still to be discovered. Knowledge of how these processes interact and work together to produce the dynamic expression patterns is essential for a full understanding developmental patterning of the limb.

References

1. Alberts, B., A. Johnson, J. Lewis, M. Raff, K. Roberts, and P. Walter, *How Cells Read the Genome: From DNA to Protein*, in *Molecular Biology of the Cell*, B. Alberts, Editor. 2008, Garland Science, Taylor & Francis Group: New York, USA. p. 329-410.
2. Silva, A.L. and L. Romao, *The mammalian nonsense-mediated mRNA decay pathway: to decay or not to decay! Which players make the decision?* FEBS Lett, 2009. **583**(3): p. 499-505.
3. Alberts, B., A. Johnson, J. Lewis, M. Raff, K. Roberts, and P. Walter, *DNA, Chromosomes and Genomes*, in *Molecular Biology of the Cell*, B. Alberts, Editor. 2008, Garland Science, Taylor & Francis Group: New York, USA. p. 195-262.
4. Loyola, A. and G. Almouzni, *Marking histone H3 variants: how, when and why?* Trends Biochem Sci, 2007. **32**(9): p. 425-433.
5. Alberts, B., A. Johnson, J. Lewis, M. Raff, K. Roberts, and P. Walter, *Control of Gene Expression*, in *Molecular Biology of the Cell*, B. Alberts, Editor. 2008, Garland Science, Taylor & Francis Group: New York, USA. p. 411-499.
6. Woodcock, C.L., *Chromatin architecture.* Curr Opin Struct Biol, 2006. **16**(2): p. 213-220.
7. Brown, D.T., T. Izard, and T. Misteli, *Mapping the interaction surface of linker histone H1(0) with the nucleosome of native chromatin in vivo.* Nat Struct Mol Biol, 2006. **13**(3): p. 250-255.
8. Horn, P.J. and C.L. Peterson, *Molecular biology. Chromatin higher order folding--wrapping up transcription.* Science, 2002. **297**(5588): p. 1824-1827.
9. Robinson, P.J. and D. Rhodes, *Structure of the '30 nm' chromatin fibre: a key role for the linker histone.* Curr Opin Struct Biol, 2006. **16**(3): p. 336-343.
10. Robinson, P.J., W. An, A. Routh, F. Martino, L. Chapman, R.G. Roeder, and D. Rhodes, *30 nm chromatin fibre decompaction requires both H4-K16 acetylation and linker histone eviction.* J Mol Biol, 2008. **381**(4): p. 816-825.
11. Fan, Y., T. Nikitina, E.M. Morin-Kensicki, J. Zhao, T.R. Magnuson, C.L. Woodcock, and A.I. Skoultchi, *H1 linker histones are essential for mouse development and affect nucleosome spacing in vivo.* Mol Cell Biol, 2003. **23**(13): p. 4559-4572.

12. Kouzarides, T., *Chromatin modifications and their function.* Cell, 2007. **128**(4): p. 693-705.

13. Berger, S.L., *The complex language of chromatin regulation during transcription.* Nature, 2007. **447**(7143): p. 407-412.

14. Sterner, D.E. and S.L. Berger, *Acetylation of histones and transcription-related factors.* Microbiol Mol Biol Rev, 2000. **64**(2): p. 435-459.

15. Shogren-Knaak, M., H. Ishii, J.M. Sun, M.J. Pazin, J.R. Davie, and C.L. Peterson, *Histone H4-K16 acetylation controls chromatin structure and protein interactions.* Science, 2006. **311**(5762): p. 844-847.

16. Bannister, A.J. and T. Kouzarides, *Reversing histone methylation.* Nature, 2005. **436**(7054): p. 1103-1106.

17. Kolasinska-Zwierz, P., T. Down, I. Latorre, T. Liu, X.S. Liu, and J. Ahringer, *Differential chromatin marking of introns and expressed exons by H3K36me3.* Nat Genet, 2009.

18. Vakoc, C.R., S.A. Mandat, B.A. Olenchock, and G.A. Blobel, *Histone H3 lysine 9 methylation and HP1gamma are associated with transcription elongation through mammalian chromatin.* Mol Cell, 2005. **19**(3): p. 381-391.

19. Verkerk, A.J., M. Pieretti, J.S. Sutcliffe, Y.H. Fu, D.P. Kuhl, A. Pizzuti, O. Reiner, S. Richards, M.F. Victoria, F.P. Zhang, and et al., *Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome.* Cell, 1991. **65**(5): p. 905-914.

20. Macdonald, N.*, et al.*, *Molecular basis for the recognition of phosphorylated and phosphoacetylated histone h3 by 14-3-3.* Mol Cell, 2005. **20**(2): p. 199-211.

21. Pokholok, D.K., J. Zeitlinger, N.M. Hannett, D.B. Reynolds, and R.A. Young, *Activated signal transduction kinases frequently occupy target genes.* Science, 2006. **313**(5786): p. 533-536.

22. Pavri, R., B. Zhu, G. Li, P. Trojer, S. Mandal, A. Shilatifard, and D. Reinberg, *Histone H2B monoubiquitination functions cooperatively with FACT to regulate elongation by RNA polymerase II.* Cell, 2006. **125**(4): p. 703-717.

23. Zhu, B., Y. Zheng, A.D. Pham, S.S. Mandal, H. Erdjument-Bromage, P. Tempst, and D. Reinberg, *Monoubiquitination of human histone H2B: the factors involved and their*

roles in HOX gene regulation. Mol Cell, 2005. **20**(4): p. 601-611.

24. Wang, M., D. Cheng, J. Peng, and C.M. Pickart, *Molecular determinants of polyubiquitin linkage selection by an HECT ubiquitin ligase.* Embo J, 2006. **25**(8): p. 1710-1719.
25. Cremer, T. and C. Cremer, *Chromosome territories, nuclear architecture and gene regulation in mammalian cells.* Nat Rev Genet, 2001. **2**(4): p. 292-301.
26. Parada, L.A., J.J. Roix, and T. Misteli, *An uncertainty principle in chromosome positioning.* Trends Cell Biol, 2003. **13**(8): p. 393-396.
27. Malyavantham, K.S., S. Bhattacharya, W.D. Alonso, R. Acharya, and R. Berezney, *Spatio-temporal dynamics of replication and transcription sites in the mammalian cell nucleus.* Chromosoma, 2008. **117**(6): p. 553-567.
28. Kininis, M. and W.L. Kraus, *A global view of transcriptional regulation by nuclear receptors: gene expression, factor localization, and DNA sequence analysis.* Nucl Recept Signal, 2008. **6**: p. e005.
29. Kumaran, R.I., R. Thakar, and D.L. Spector, *Chromatin dynamics and gene positioning.* Cell, 2008. **132**(6): p. 929-934.
30. Finlan, L.E., D. Sproul, I. Thomson, S. Boyle, E. Kerr, P. Perry, B. Ylstra, J.R. Chubb, and W.A. Bickmore, *Recruitment to the nuclear periphery can alter expression of genes in human cells.* PLoS Genet, 2008. **4**(3): p. e1000039.
31. Malhas, A., C.F. Lee, R. Sanders, N.J. Saunders, and D.J. Vaux, *Defects in lamin B1 expression or processing affect interphase chromosome position and gene expression.* J Cell Biol, 2007. **176**(5): p. 593-603.
32. Zink, D*., et al.*, *Transcription-dependent spatial arrangements of CFTR and adjacent genes in human cell nuclei.* J Cell Biol, 2004. **166**(6): p. 815-825.
33. Morey, C., N.R. Da Silva, P. Perry, and W.A. Bickmore, *Nuclear reorganisation and chromatin decondensation are conserved, but distinct, mechanisms linked to Hox gene activation.* Development, 2007. **134**(5): p. 909-919.
34. Lettice, L.A., S.J. Heaney, L.A. Purdie, L. Li, P. de Beer, B.A. Oostra, D. Goode, G. Elgar, R.E. Hill, and E. de Graaff, *A long-range Shh enhancer regulates expression in the developing limb and fin and is associated with preaxial polydactyly.* Hum Mol Genet,

2003. **12**(14): p. 1725-1735.

35. Noordermeer, D. and W. de Laat, *Joining the loops: beta-globin gene regulation.* IUBMB Life, 2008. **60**(12): p. 824-833.

36. Montavon, T., J.F. Le Garrec, M. Kerszberg, and D. Duboule, *Modeling Hox gene regulation in digits: reverse collinearity and the molecular origin of thumbness.* Genes Dev, 2008. **22**(3): p. 346-359.

37. Carvajal, J.J., A. Keith, and P.W. Rigby, *Global transcriptional regulation of the locus encoding the skeletal muscle determination genes Mrf4 and Myf5.* Genes Dev, 2008. **22**(2): p. 265-276.

38. West, A.G., M. Gaszner, and G. Felsenfeld, *Insulators: many functions, many mechanisms.* Genes Dev, 2002. **16**(3): p. 271-288.

39. Lunyak, V.V., *Boundaries. Boundaries...Boundaries???* Curr Opin Cell Biol, 2008. **20**(3): p. 281-286.

40. Bell, A.C., A.G. West, and G. Felsenfeld, *The protein CTCF is required for the enhancer blocking activity of vertebrate insulators.* Cell, 1999. **98**(3): p. 387-396.

41. West, A.G. and P. Fraser, *Remote control of gene transcription.* Hum Mol Genet, 2005. **14 Spec No 1**: p. R101-111.

42. Bao, L., M. Zhou, and Y. Cui, *CTCFBSDB: a CTCF-binding site database for characterization of vertebrate genomic insulators.* Nucleic Acids Res, 2007.

43. Kim, T.H., Z.K. Abdullaev, A.D. Smith, K.A. Ching, D.I. Loukinov, R.D. Green, M.Q. Zhang, V.V. Lobanenkov, and B. Ren, *Analysis of the vertebrate insulator protein CTCF-binding sites in the human genome.* Cell, 2007. **128**(6): p. 1231-1245.

44. Ovcharenko, I. and M.A. Nobrega, *Identifying synonymous regulatory elements in vertebrate genomes.* Nucleic Acids Res, 2005. **33**(Web Server issue): p. W403-407.

45. Visel, A., S. Prabhakar, J.A. Akiyama, M. Shoukry, K.D. Lewis, A. Holt, I. Plajzer-Frick, V. Afzal, E.M. Rubin, and L.A. Pennacchio, *Ultraconservation identifies a small subset of extremely constrained developmental enhancers.* Nat Genet, 2008. **40**(2): p. 158-160.

46. Pfeifer, D., R. Kist, K. Dewar, K. Devon, E.S. Lander, B. Birren, L. Korniszewski, E. Back, and G. Scherer, *Campomelic dysplasia translocation breakpoints are scattered over*

1 Mb proximal to SOX9: evidence for an extended control region. Am J Hum Genet, 1999. **65**(1): p. 111-124.

47. Pennacchio, L.A.*, et al.*, *In vivo enhancer analysis of human conserved non-coding sequences.* Nature, 2006. **444**(7118): p. 499-502.

48. Beermann, F., K. Kaloulis, D. Hofmann, F. Murisier, P. Bucher, and A. Trumpp, *Identification of evolutionarily conserved regulatory elements in the mouse Fgf8 locus.* Genesis, 2006. **44**(1): p. 1-6.

49. Jeong, Y., K. El-Jaick, E. Roessler, M. Muenke, and D.J. Epstein, *A functional screen for sonic hedgehog regulatory elements across a 1 Mb interval identifies long-range ventral forebrain enhancers.* Development, 2007. **133**(4): p. 761-772.

50. Belloni, E.*, et al.*, *Identification of Sonic hedgehog as a candidate gene responsible for holoprosencephaly.* Nat Genet, 1996. **14**(3): p. 353-356.

51. Dlugaszewska, B.*, et al.*, *Breakpoints around the HOXD cluster result in various limb malformations.* J Med Genet, 2005.

52. Wunderle, V.M., R. Critcher, N. Hastie, P.N. Goodfellow, and A. Schedl, *Deletion of long-range regulatory elements upstream of SOX9 causes campomelic dysplasia.* Proc Natl Acad Sci U S A, 1998. **95**(18): p. 10649-10654.

53. Hill-Harfe, K.L., L. Kaplan, H.J. Stalker, R.T. Zori, R. Pop, G. Scherer, and M.R. Wallace, *Fine Mapping of Chromosome 17 Translocation Breakpoints >/=900 Kb Upstream of SOX9 in Acampomelic Campomelic Dysplasia and a Mild, Familial Skeletal Dysplasia.* Am J Hum Genet, 2005. **76**(4).

54. Velagaleti, G.V., G.A. Bien-Willner, J.K. Northup, L.H. Lockhart, J.C. Hawkins, S.M. Jalal, M. Withers, J.R. Lupski, and P. Stankiewicz, *Position effects due to chromosome breakpoints that map approximately 900 Kb upstream and approximately 1.3 Mb downstream of SOX9 in two patients with campomelic dysplasia.* Am J Hum Genet, 2005. **76**(4): p. 652-662.

55. Bien-Willner, G.A., P. Stankiewicz, and J.R. Lupski, *SOX9cre1, a cis-acting regulatory element located 1.1 Mb upstream of SOX9, mediates its enhancement through the SHH pathway.* Hum Mol Genet, 2007. **16**(10): p. 1143-1156.

56. Leipoldt, M., M. Erdel, G.A. Bien-Willner, M. Smyk, M. Theurl, S.A. Yatsenko, J.R.

Lupski, A.H. Lane, A.L. Shanske, P. Stankiewicz, and G. Scherer, *Two novel translocation breakpoints upstream of SOX9 define borders of the proximal and distal breakpoint cluster region in campomelic dysplasia.* Clin Genet, 2007. **71**(1): p. 67-75.

57. Jamieson, R.V., R. Perveen, B. Kerr, M. Carette, J. Yardley, E. Heon, M.G. Wirth, V. van Heyningen, D. Donnai, F. Munier, and G.C.M. Black, *Domain disruption and mutation of the bZIP transcription factor, MAF,associated with cataract, ocular anterior segment dysgenesis and coloboma.* Hum. Mol. Genet., 2002. **11**(1): p. 33-42.

58. Woolfe, A*., et al.*, *Highly conserved non-coding sequences are associated with vertebrate development.* PLoS Biol, 2005. **3**(1): p. e7.

59. Rastegar, S., I. Hess, T. Dickmeis, J.C. Nicod, R. Ertzer, Y. Hadzhiev, W.G. Thies, G. Scherer, and U. Strahle, *The words of the regulatory code are arranged in a variable manner in highly conserved enhancers.* Dev Biol, 2008.

60. Hare, E.E., B.K. Peterson, V.N. Iyer, R. Meier, and M.B. Eisen, *Sepsid even-skipped enhancers are functionally conserved in Drosophila despite lack of sequence conservation.* PLoS Genet, 2008. **4**(6): p. e1000106.

61. Vavouri, T., G.K. McEwen, A. Woolfe, W.R. Gilks, and G. Elgar, *Defining a genomic radius for long-range enhancer action: duplicated conserved non-coding elements hold the key.* Trends Genet, 2006. **22**(1): p. 5-10.

62. Lettice, L.A*., et al.*, *Disruption of a long-range cis-acting regulator for Shh causes preaxial polydactyly.* Proc Natl Acad Sci U S A, 2002. **99**(11): p. 7548-7553.

63. Liska, F., P. Snajdr, L. Sedova, O. Seda, B. Chylikova, P. Slamova, E. Krejci, D. Sedmera, M. Grim, D. Krenova, and V. Kren, *Deletion of a conserved noncoding sequence in Plzf intron leads to Plzf down-regulation in limb bud and polydactyly in the rat.* Dev Dyn, 2009.

64. Barna, M., P.P. Pandolfi, and L. Niswander, *Gli3 and Plzf cooperate in proximal limb patterning at early stages of limb development.* Nature, 2005. **436**(7048): p. 277-281.

65. Niedermaier, M*., et al.*, *An inversion involving the mouse Shh locus results in brachydactyly through dysregulation of Shh expression.* J Clin Invest, 2005. **115**(4): p. 900-909.

66. Babbs, C., D. Furniss, G.M. Morriss-Kay, and A.O. Wilkie, *Polydactyly in the mouse*

mutant Doublefoot involves altered Gli3 processing and is caused by a large deletion in cis to Indian hedgehog. Mech Dev, 2008. **125**(5-6): p. 517-526.

67. Crick, A.P., C. Babbs, J.M. Brown, and G.M. Morriss-Kay, *Developmental mechanisms underlying polydactyly in the mouse mutant Doublefoot.* J Anat, 2003. **202**(1): p. 21-26.

68. Zeller, R., A.G. Haramis, A. Zuniga, C. McGuigan, R. Dono, G. Davidson, S. Chabanis, and T. Gibson, *Formin defines a large family of morphoregulatory genes and functions in establishment of the polarising region.* Cell Tissue Res, 1999. **296**(1): p. 85-93.

69. Mass, R.L., R. Zeller, R.P. Woychik, T.F. Vogt, and P. Leder, *Disruption of formin-encoding transcripts in two mutant limb deformity alleles.* Nature, 1990. **346**(6287): p. 853-855.

70. Zuniga, A.*, et al.*, *Mouse limb deformity mutations disrupt a global control region within the large regulatory landscape required for Gremlin expression.* Genes Dev, 2004. **18**(13): p. 1553-1564.

71. Wolpert, L., J. Smith, T. Jessel, P. Lawrence, E. Robertson, and E. Meyerowitz, *Principles of Development*. 3rd ed. 2006, Oxford: Oxford University Press.

72. Zakany, J. and D. Duboule, *The role of Hox genes during vertebrate limb development.* Curr Opin Genet Dev, 2007. **17**(4): p. 359-366.

73. Barna, M., T. Merghoub, J.A. Costoya, D. Ruggero, M. Branford, A. Bergia, B. Samori, and P.P. Pandolfi, *Plzf mediates transcriptional repression of HoxD gene expression through chromatin remodeling.* Dev Cell, 2002. **3**(4): p. 499-510.

74. Rastegar, M., L. Kobrossy, E.N. Kovacs, I. Rambaldi, and M. Featherstone, *Sequential histone modifications at Hoxd4 regulatory regions distinguish anterior from posterior embryonic compartments.* Mol Cell Biol, 2004. **24**(18): p. 8090-8103.

75. Chambeyron, S. and W.A. Bickmore, *Chromatin decondensation and nuclear reorganization of the HoxB locus upon induction of transcription.* Genes Dev., 2004. **18**(10): p. 1119-1130.

76. Tolhuis, B., R.J. Palstra, E. Splinter, F. Grosveld, and W. de Laat, *Looping and interaction between hypersensitive sites in the active beta-globin locus.* Mol Cell, 2002. **10**(6): p.

1453-1465.

77. Spilianakis, C.G., M.D. Lalioti, T. Town, G.R. Lee, and R.A. Flavell, *Interchromosomal associations between alternatively expressed loci.* Nature, 2005. **435**(7042): p. 637-645.

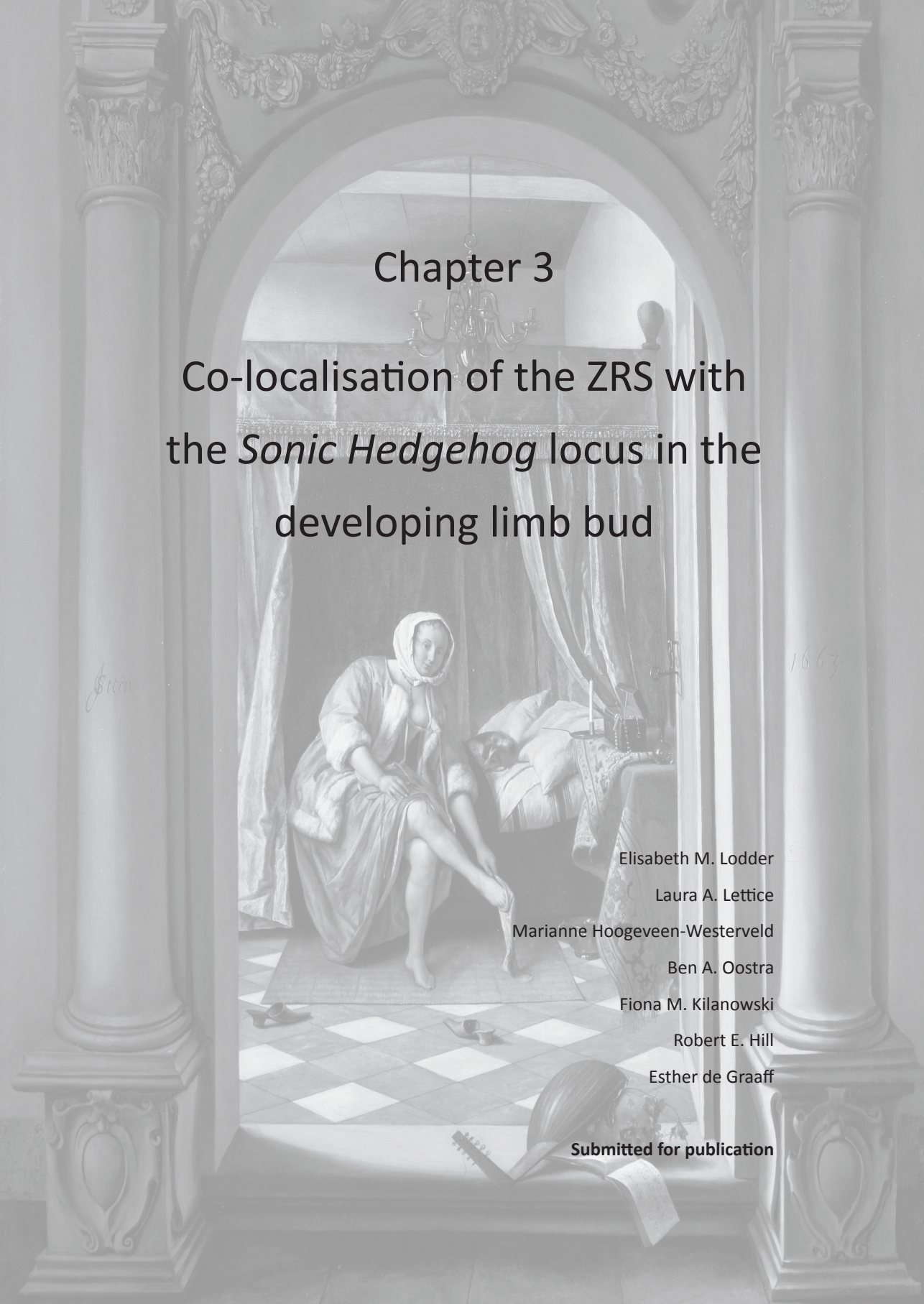
78. Simonis, M., P. Klous, E. Splinter, Y. Moshkin, R. Willemsen, E. de Wit, B. van Steensel, and W. de Laat, *Nuclear organization of active and inactive chromatin domains uncovered by chromosome conformation capture-on-chip (4C).* Nat Genet, 2006. **38**(11): p. 1348-1354.

79. Noordermeer, D., P. Klous, and W. de Laat, *Trans-activation of an endogenous mouse beta-globin gene by the human beta-globin LCR and a beta globin gene inserted in another chromosome*, in *Characterization of an ectopic beta-globin LCR, good neighbours and a distant friend*, D. Noordermeer, Editor. 2009: Rotterdam. p. 177-199.

80. Kmita, M., N. Fraudeau, Y. Herault, and D. Duboule, *Serial deletions and duplications suggest a mechanism for the collinearity of Hoxd genes in limbs.* Nature, 2002. **420**(6912): p. 145-150.

81. Rinn, J.L., M. Kertesz, J.K. Wang, S.L. Squazzo, X. Xu, S.A. Brugmann, L.H. Goodnough, J.A. Helms, P.J. Farnham, E. Segal, and H.Y. Chang, *Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs.* Cell, 2007. **129**(7): p. 1311-1323.

82. Guttman, M.*, et al.*, *Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals.* Nature, 2009.



Chapter 3

Co-localisation of the ZRS with the *Sonic Hedgehog* locus in the developing limb bud

Elisabeth M. Lodder
Laura A. Lettice
Marianne Hoogeveen-Westerveld
Ben A. Oostra
Fiona M. Kilanowski
Robert E. Hill
Esther de Graaff

Submitted for publication

Abstract

Sonic Hedgehog (Shh) is a major morphogen, expressed in the zone of polarizing activity (ZPA) of the developing limb bud, determining the anterior-posterior polarity. Recently, we identified a long distance regulatory element of *Shh* named the ZPA Regulatory Sequence (ZRS). The mechanism through which the ZRS, located in intron5 of another gene (*Lmbr1)* regulates *Shh* expression over a 1 Mb distance remains thus far unclear. Mice carrying partial deletions of the ZRS were generated to determine its function in the *in vivo* genomic location. Unexpectedly, the minimal region, capable of driving reporter expression, was not capable of driving *Shh* expression from its normal genomic position. The possibility of a direct interaction between the ZRS and the *Shh* gene was tested with cryoFISH. Limb specific co-localisation of the ZRS with the *Shh* gene was detected. This co-localisation occurs throughout the developing limb bud and not just in the ZPA, indicating its independence of active transcription of *Shh*. Interestingly, the co-localisation of the ZRS with the *Shh* gene is also present in the $ZRS^{-/-}$ knock-out pointing to the presence of a 2^{nd} long distance regulatory sequence in the vicinity of the ZRS which is responsible for the folding of the DNA in the limb bud. We generated knockouts of two candidate regions for the interaction between the ZRS and *Shh*; neither showed a limb phenotype. The location of the 2^{nd} long distance regulatory element of *Shh* remains therefore elusive.

Key words

Limb development

Sonic Hedgehog

Transcriptional regulation

Zone of polarizing activity

ZPA Regulatory Sequence

co-localisation

Introduction

The role of Sonic Hedgehog (Shh) in conferring positional information during development has been extensively described (reviewed in[1]). A major model system in which Shh plays an important role is the specification of the anterior-posterior axis in the limb. Normally, *Shh* is expressed in a group of cells called the Zone of Polarising Activity (ZPA) on the posterior side of the limb bud. Time and concentration of exposure to Shh determine the positional cell fate, the more Shh the more posterior the final structures will be[2,3]. A tight regulation of the expression is therefore crucial to the formation of a correctly patterned limb. We have identified a limb specific, *cis*-acting regulatory element of *Shh*[4].

The ZRS was identified through linkage analysis in families with pre-axial polydactyly (PPD, MIM#174500) in combination with the localisation of a transgene insertion in the mouse mutant Sasquatch. Point mutations in the ZRS were shown to be responsible for ectopic activation of Shh on the anterior side of the developing limb bud, giving rise to the PPD phenotype4-7.

The ZRS was also shown to be an enhancer of *Shh* on the posterior side of the limb bud, capable of driving the expression of a LacZ reporter construct in the ZPA[4]. The limb specific behavior of the ZRS is shown by the abolishment of *Shh* expression only in the ZPA in mice homozygous for a targeted deletion of the ZRS[8]. In addition, duplications of the ZRS result in pre-axial polydactyly, indicating a possible gain of function mechanism for the ectopic expression of *Shh* on the anterior side of the developing limb bud[9,10].

The main difficulty in elucidating the molecular mechanisms behind the role of the ZRS is its genomic location. The ZRS is located in intron 5 of another gene, *Lmbr1*, approximately 1 Mb upstream of *Shh*[4]. *Lmbr1* encodes an ubiquitously expressed transmembrane protein which does not appear to be involved in limb development[11]; although a targeted deletion of exon 1 of *Lmbr1*, including promoter and intronic sequence, showed a mild limb reduction phenotype[12]. The expression of

Lmbr1 is not influenced by either point mutations in the ZRS or by the deletion of the ZRS (data not shown). The testis specific expression of the only other described gene in between the ZRS and *Shh*, *RNF32*, is also unaffected by the ZRS (data not shown). The ZRS is therefore a highly specific long-range *cis* acting regulatory element of *Shh*.

The location of the ZRS contrasts with the other known regulatory elements of *Shh.* The enhancers for expression in other tissues such as the CNS and the floorplate mesenchyme lie within introns of S*hh* and up to 460kb upstream of the promoter region of *Shh*[13-15]. Although not many elements have been found to act over such long genomic distances the ZRS is not unique in this aspect: both *Maf* and *Sox9* have a regulatory element that lies far away, 1 Mb downstream and 900 kb upstream, respectively[16,17]. Unlike the ZRS these elements have not been mapped exactly but their presence is inferred from the phenotype of patients carrying genomic translocations. The regulation of *Shh* by the ZRS is therefore a very suitable system to study the mechanisms behind the extreme long distance regulation of transcription.

Several mechanisms have been proposed through which regulatory elements can facilitate transcription. They can mainly be divided in contact and non-contact models. In the models that assume no contact the enhancer is thought to work through the creation of a favourable environment for transcription, for example, by changes at the chromatin level such as methylation and acetylation[18]. In the last couple of years several papers have reported evidence for direct physical interactions between enhancers and the promoters of their target genes[18,19]. Because of the presence of unregulated genes in between the ZRS and the *Shh* gene such a direct interaction model is very attractive to explain the specificity of the long range regulation of *Shh.*

In this paper we describe experiments we performed to elucidate the mechanisms used by the ZRS to regulate *Shh* expression. We have tested whether the ZRS is directly interacting with the promoter of *Shh* using cryoFISH. Our results show a clear limb specific co-localisation of the ZRS with *Shh*, indicating that the ZRS is indeed in close proximity of the *Shh* promoter during limb development.

Furthermore, to gain more insight into which part of the ZRS is necessary for which function (repression and/or activation) of the ZRS, we created (partial) deletions of the ZRS *in vivo*. Surprisingly the minimal region sufficient for driving LacZ-reporter expression is not sufficient for *Shh* expression in the *in vivo* genomic context. The results of co-localisation studies in these (partial) deletions indicate the presence of a second long distance regulatory element mediating the proximity of the ZRS with the *Shh* locus. Targeted deletions of two likely candidate regions were generated, neither deletion caused abnormal limb development. It is therefore unlikely that these conserved elements are involved in the regulation of the chromatin folding of the ZRS towards *Shh.* The element responsible for the co-localisation of the ZRS with *Shh* remains therefore to be found.

Results

Co-localisation of the ZRS-region with the Shh-region

The ZRS co-localises with Shh in a limb specific manner

To test the possibility of interactions between the ZRS and the *Shh* promoter, we performed cryoFISH analysis which allows for relatively high resolution in the z-axis because the nuclei are sectioned in thin slices of approximately 200 nm (see figure 3.1a&b for a summary of the technique). As FISH probes we used fluorescently labeled BAC's overlapping the ZRS and the *Shh* gene (figure 3.1c). As negative control we measured co-localisation of the ZRS with a control probe 900 kb upstream of the ZRS and a probeset covering the gene *Maf* on mouse chromosome 8 and its downstream regulatory element (~1Mb from the transcriptional start site) because *Maf* is not expressed in either of our test tissues[16].

We first performed cryoFISH on sections of E11 hind limb buds and on sections of E14.5 fetal liver as a negative control. In E11 limb buds a significantly higher percentage of overlap is found between the ZRS-probe and the *Shh*-probe than

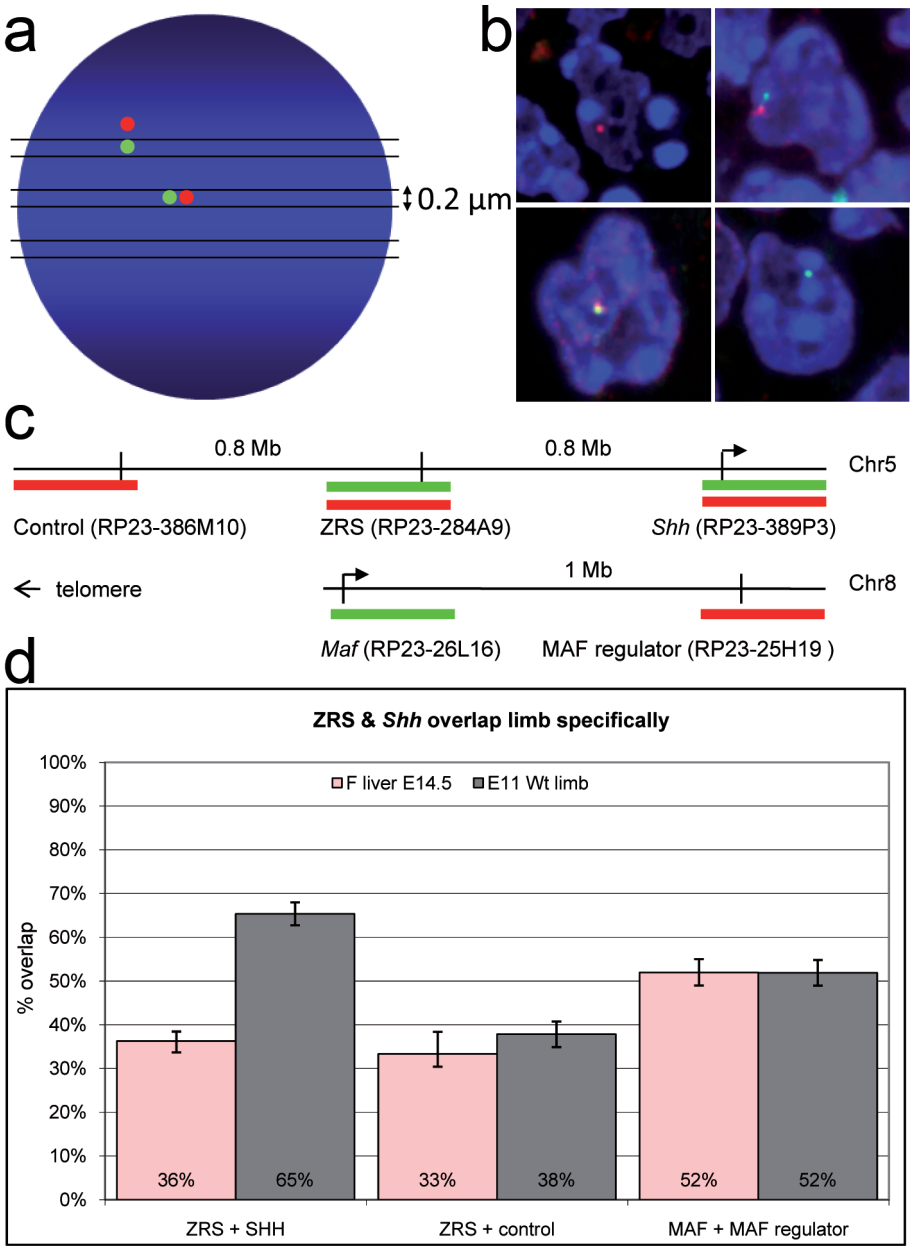


***Figure 3.1**: a) schematic view of a nucleus before dissection for cryoFISH, red & green dots indicate possible localisations of the two alleles. b) 4 examples of the possible visible signals clockwise starting top left: single red, separated red and green, single green, overlapping red and green. c) schematic overview of the localisation of the FISH probes. d) bar graph showing specific overlap of the ZRS with Shh in the developing limb bud.*

between the ZRS-probe and the control probe (65% vs 38%; p<0.0001, figure 3.1d). In contrast, in fetal liver both probe combinations show only background levels of co-localisation (36% and 33% resp. figure 3.1d). The ZRS is therefore co-localising specifically with *Shh* only in the developing limb bud. These results were confirmed in a dye swap control in which we labeled the probes with the opposite colours (data not shown). To further corroborate these results the percentage of overlap between the regulatory element of *Maf* and *Maf* itself was identical in E14.5 fetal liver and E11 limb bud (52%, figure 3.1d).

Co-localisation is not ZPA specific

The degree of co-localisation that was detected in the limb bud may be due to either a high level of co-localisation in the ZPA and background levels in the rest of the limb bud or reflected a high level of co-localisation throughout the limb bud. To determine this, we measured the level of co-localisation in different parts of the limb bud which were either expressing or not expressing *Shh*. The pre-axial polydactylous mouse strain *hemimelic extra toes (Hx)* has a point mutation in the ZRS which leads to ectopic expression of *Shh* on the anterior side of the developing limb bud (figure 3.2a)[20]. Sections of E11.5 *Hx/+* hind limbs therefore have two domains of *Shh* expression (figure 3.2b) instead of the single posterior domain found in wt limbs, reducing the number of sections that need to be made and circumventing the need to determine which side is posterior in a symmetrical section. We compared the co-localisation of the ZRS with the *Shh* gene between the regions expressing *Shh* on both sides of the limb and the middle part of the limb bud that does not express *Shh*. No significant difference was found (p=0.27; figure 3.2c), indicating that the observed high levels of co-localisation in the developing limb buds are most likely due to an even distribution of co-localisation throughout the limb bud.

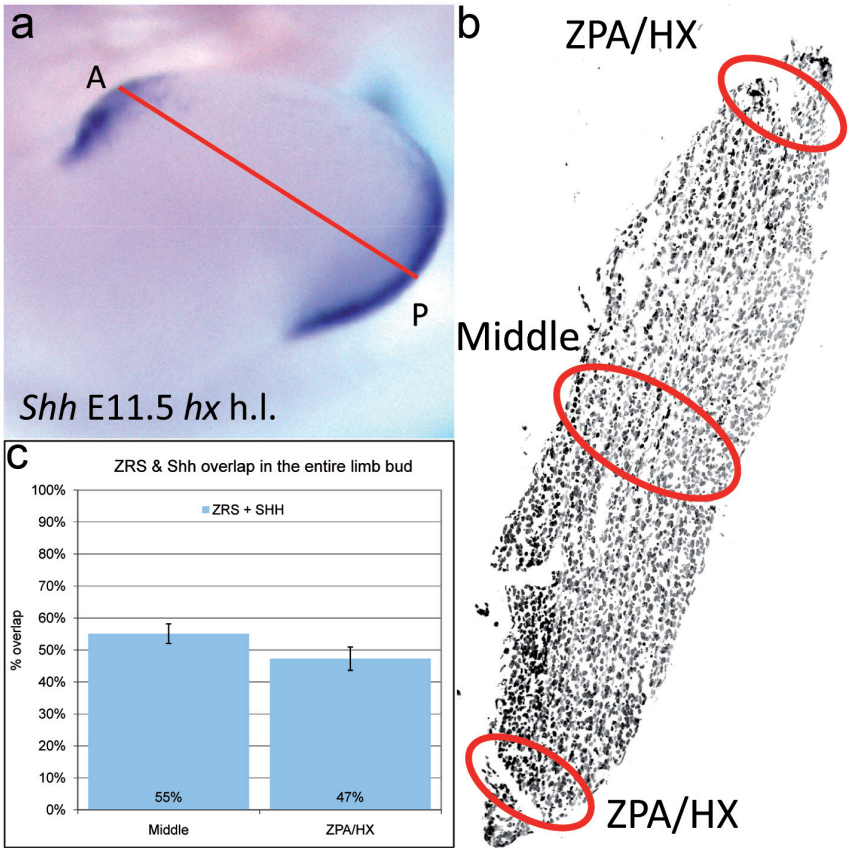


Figure 3.2 Co-localisation of Shh and the ZRS in the entire limb bud*: a) whole mount in situ hybridization showing Shh expression in an E11.5 hx/wt hind limb. b) section through an E11.5 hx/wt hind limb. c) bar graph showing the equal distribution of overlap between the ZRS and Shh in the limb.*

Co-localisation is independent of active Shh transcription

To determine a time frame for the co-localisation of the ZRS with *Shh* cryoFISH was performed on E9.5 fore limbs (in which *Shh* expression has just begun) and E13.5 hind limbs (in which *Shh* expression is undetectable). At both these stages there is a significantly higher overlap between the ZRS-probe and the *Shh*-probe than between the ZRS and the control ($p<0.0001$, figure 3.3a). The co-localisation at these stages does not differ from the co-localisation at E11, suggesting that this co-localisation is not dependent on active transcription of *Shh*. This is in agreement with the even distribution of co-localisation in the entire limb bud described above.

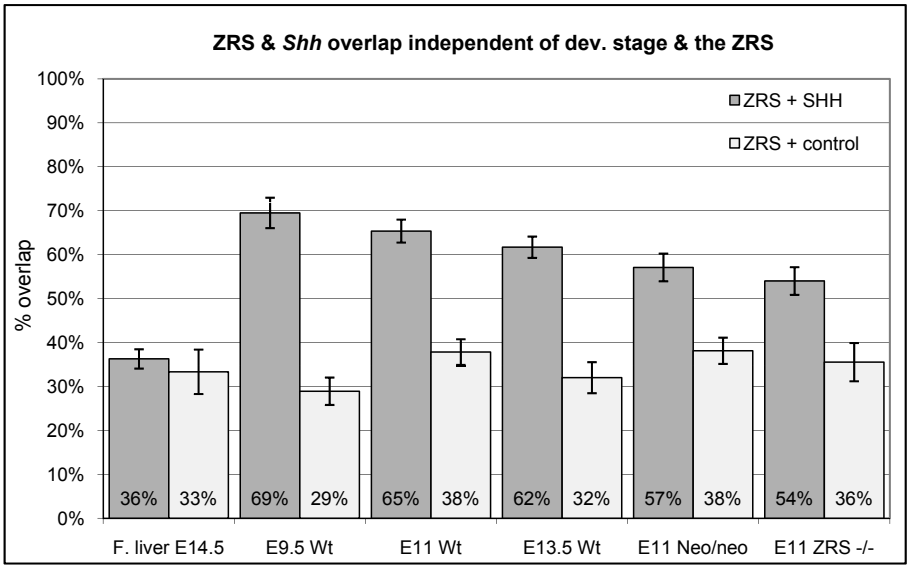


Figure 3.3 Bar chart showing limb specific overlap between the ZRS and Shh: *a) at different developmental ages and b) in different genetic backgrounds.*

(Partial) deletions

Deletion of the 3' ZRS results in loss of Shh expression

To determine the role of the ZRS in the co-localisation and to assess the roles of the different parts of the ZRS *in vivo* we created partial knock-outs. To this purpose a targeting construct was generated in which a neomycin cassette was inserted within the ZRS directly after the minimal region (MR) capable of driving LacZ expression in the ZPA. The 3' region of the ZRS was flanked with LoxP sites and the MR (5' region of ZRS) with *frt* sites (figure 3.4a bottom). This construct (ZRS^{neo}) allows for targeting two separate regions of the ZRS with only one homologous recombination event in ES-cells by crossing the mice with transgenic mice expressing either Flp-recombinase or Cre-recombinase.

Several chimeras, obtained from two independent targeted ES clones were bred to produce a F1 offspring. These F1 mice, heterozygous for the targeting construct ($ZRS^{neo/+}$), were indistinguishable from their wt littermates (data not shown). To

introduce the partial deletion of the 3' ZRS (leaving the MR intact) these mice were crossed with a mouse strain constitutively expressing Cre-recombinase under the control of the CAG promoter (a kind gift from A.P.M. de Wit). Unexpectedly, the heterozygous offspring ($ZRS^{del3'/+}$) was indistinguishable from their wt littermates (data not shown) and showed no signs of polydactyly. No skeletal aberrations were present; the mice were healthy and fertile.

To determine the effects of a homozygous deletion the heterozygous ($ZRS^{del3'/+}$) mice were interbred. Homozygous and heterozygous mice were born in normal Mendelian ratios. Surprisingly, the $ZRS^{del3'/del3'}$ mice show a phenotype that is comparable to that of the ZRS knockout mice described by Sagai *et al.*[8]: complete lack of all digits except digit 1, lack of longitudinal growth of the limbs and no other skeletal abnormalities (figure 3.4b, d). This indicates that the MR alone is not capable of driving *Shh* expression in the *in vivo* genomic context. This contrasts with the results of the LacZ reporter assay described previously[4], showing that the MR alone is capable of driving LacZ expression in the ZPA of transgenic mice. This contradicts the hypothesis that the MR contains all that is necessary for activation of the *Shh* gene and suggests that additional information and perhaps the whole of the highly conserved domain is essential for activity at the endogenous locus.

Deletion of the minimal region results in loss of Shh expression

In order to remove the MR we crossed $ZRS^{neo/wt}$ with mice expressing Flp recombinase from the Rosa locus (a kind gift of J. Charite). F1 mice heterozygous for the loss of the MR ($ZRS^{delMR/+}$) showed no skeletal aberrations or other phenotypic differences. Mice lacking the MR ($ZRS^{delMR/delMR}$) showed a ZRS knockout phenotype identical to the $ZRS^{del3'/del3'}$ phenotype (figure 3.4d) as was expected based on the activity of the MR in the LacZ reporter assays and the lack of activity of the 3' half of the ZRS in this same assay[4].

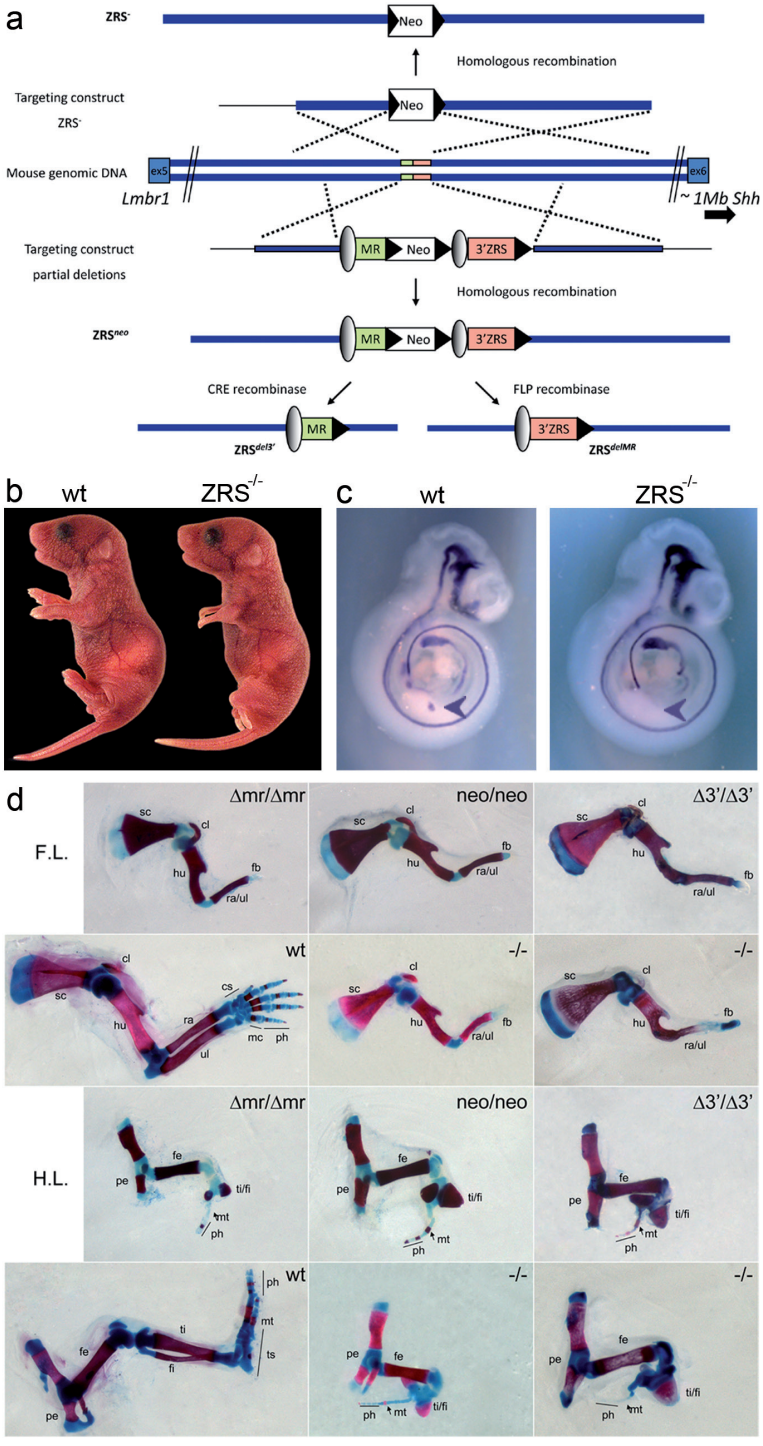
Insertion of Neomycin disturbs ZRS activation of Shh

To determine whether the loss of function phenotype in the ZRS$^{del3'/del3'}$ mice is due to the loss of elements in the 3' site of the ZRS or to the insertion of the neomycin cassette in an essential part of the ZRS we crossed the ZRS$^{neo/+}$ mice to homozygosity. The ZRS$^{neo/neo}$ mice showed the same phenotype as either of the partial deletions (figure 3.4d) suggesting that the phenotype is due to the insertion of the neomycin cassette in a part of the ZRS essential for its function in its normal genomic context.

To exclude the possibility of promoter competition of the ZRS, we tested the expression levels of neomycin in E10.5 ZRS$^{neo/+}$ embryos by RT PCR of anterior and posterior halves of E10.5 limb buds. Equal expression of neomycin in both halves of the limb bud was detected (figure 3.4e). We also tested the expression levels of *Shh*, *Lmbr1* and *Rnf32* to see whether their expression might be disrupted by the presence of the promoter of the neomycin cassette. Again, no differences were observed, besides the expected reduction in *Shh* level (figure 4e).

Deletion of the ZRS

Sagai *et al.* (2005) described the effects of a deletion of an 1167 bp region including the ZRS. The described phenotype is due to the complete loss of *Shh* expression in the developing limb buds. In order to see the effect of deletion of the entire ZRS on the co-localisation of the ZRS to *Shh*, we created a targeted deletion of the ZRS comprising only a smaller more conserved region originally described as the ZRS (942bp) by replacing it with a neomycin cassette (figure 3.4a top). Mice heterozygous for the loss of the ZRS showed no phenotypic differences from their wt littermates. Again, the homozygous ZRS$^{-/-}$ mice show a phenotype comparable to that described by Sagai and his colleagues: complete loss of expression of *Shh* in the developing limbs leading to an absence of digits other then digit 1 and truncation of the distal elongation of the limbs (figure 3.4b, d).


a
ZRS-
Neo
Homologous recombination
Targeting construct
ZRS-
Neo
Mouse genomic DNA
ex5
ex6
Lmbr1
~ 1Mb Shh
Targeting construct
partial deletions
MR
Neo
3'ZRS
Homologous recombination
ZRSneo
MR
Neo
3'ZRS
CRE recombinase
FLP recombinase
MR
3'ZRS
ZRSdel3'
ZRSdelMR
b
wt
ZRS-/-
c
wt
ZRS-/-
d
F.L.
Δmr/Δmr
neo/neo
Δ3'/Δ3'
wt
-/-
-/-
H.L.
Δmr/Δmr
neo/neo
Δ3'/Δ3'
wt
-/-
-/-

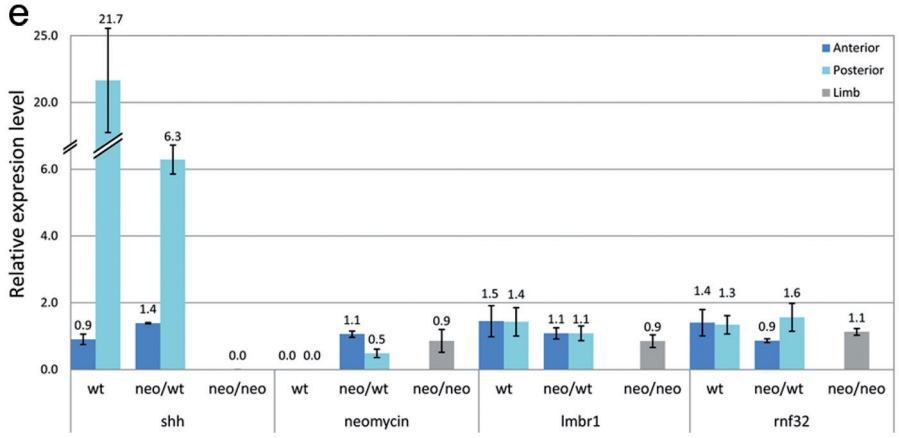


Figure 3.4 phenotype of different ZRS mutants: *a) schematic representation of targeting constructs used to generate a complete (top) or partial (bottom) deletion of the ZRS. Black triangles indicate LoxP sites, whereas the frt sites are represented by the graded ellipses. b) Limb reduction phenotype in ZRS-/- P0 mice. The phenotype is identical in all ZRS mutants. c) Whole mount in situ hybridization showing lack of Shh expression in the limbs only in all ZRS mutants. The arrowhead indicates the ZPA with and without Shh expression d) Skeletal staining of forelimb (F.L.) and hindlimb (H.L.) of newborn mice demonstrate the same limb reduction phenotype in the four mouse mutants. The different elements are labeled in the wildtype (wt): sc, scapula; pe, pelvis; cl, clavicle; hu, humerus; fe, femur; ra, radius; ul, ulna; ti, tibia; fi, fibula; ts, tarsals; mt, metatarsal; cs, carpals; mc, metacarpals; ph, phalanges, fb, floating skeletal element. Note that subtle differences in phenotype are not dependent on the genotype. e) Histograms showing relative expression levels of shh, neomycin, lmbr1 and rnf32 in anterior or posterior halves of E10.5 limbbuds (wt and ZRSneo/wt) and whole limbs (ZRSneo/neo), compared to expression in remaining tissue of the embryo's. Shh is not expressed in the limb buds of ZRSneo/neo mice. The difference in expression of Shh in anterior and posterior limb bud tissue reflects the high levels of expression of Shh in the ZPA detected by in situ hybridization. Note that the expression level of neomycin, is similar for the anterior and posterior halves.*

Loss of Shh expression in the ZRS deletions

To determine whether the $ZRS^{-/-}$ phenotype in the partial deletions and the $ZRS^{neo/neo}$ mice was due to loss of expression of *Shh* in the limb we performed whole mount *in situ* hybridizations of E10.5 embryos of all genotypes. The expression of *Shh* was indeed absent in the ZPA in all the homozygous (partial and complete) knock-outs and unaltered in the heterozygous embryos (figure 3.4c).

Co-localisation of the ZRS and Shh in the partial deletion mutants

Insertion of Neomycin does not interfere with co-localisation

As the insertion of the neomycin cassette in the ZRS disturbs the expression of *Shh* in the limb we hypothesized that this effect might be due to loss of direct interaction between the ZRS and the *Shh* gene. We therefore tested the co-localisation of the ZRS with *Shh* in the ZRS$^{neo/neo}$ mice with cryoFISH. Although the amount of overlap appears to be slightly lower in ZRS$^{neo/neo}$ hind limbs compared to E11 wt this difference is not statistically significant (p=0.2) (figure 3.3b). The co-localisation of the ZRS with *Shh* is apparently undisturbed by the insertion of the Neomycin cassette within the ZRS suggesting that the effect of the neomycin insertion must have a different molecular basis. This co-localisation of the ZRS and *Shh* in the ZRS$^{neo/neo}$ mice confirms the independence of the co-localisation upon active transcription of *Shh*, which is absent in ZRS$^{neo/neo}$ mice (figure 3.4e).

Co-localisation is ZRS independent

To determine the function of the ZRS in the co-localisation of the ZRS region with the *Shh* region in inter-phase limb bud nuclei we performed cryoFISH on hind limb buds from E11 ZRS$^{-/-}$ embryos. Again, a significant difference between the co-localisation of the ZRS-probe with the *Shh*-probe and the co-localisation of the ZRS-probe with the control probe was found ($p<0.0001$, figure 3.3b). Surprisingly, no significant differences between the ZRS$^{-/-}$ and the wild type limb buds (p=0.1) could be detected. The co-localisation of the ZRS with *Shh* in the ZRS$^{-/-}$ mice indicates that it is independent of the ZRS.

Other elements, therefore, are required for regulating the interaction of the ZRS and the Shh promoter. We investigated two candidate regions within the Lmbr1 gene, one region implicated in human acheiropodia and the second a multispecies conserved domain in intron 9. Acheiropodia (MIM#200500) is a rare recessive

condition in which the patients have truncations of all four limbs21. A 4-6kb deletion in the LMBR1 gene which removes exon 4 and surrounding DNA is associated with acheiropodia. Initially, these genetic data were interpreted as showing that the LMBR1 gene product had a role in limb development; however, subsequent identification of the ZRS and its role in limb specific Shh expression raised doubts about this initial interpretation. In addition, the limb defects seen in the acheiropodia patients are reminiscent of the limb phenotype of the ZRS knockout mouse8. Hence, a second compelling interpretation is that the acheiropodia deletion removes an important Shh regulator that may act in concert with the ZRS. In an attempt to make a mouse model for this condition, we deleted the corresponding region and additional DNA in mouse (38kb: Δexon4+ mutation) (figure 3.5). However, in contrast to the phenotype seen in human, the mouse deletion showed no limb defects or any other overt phenotype. Since there is no discernible limb abnormality, this deleted region in Lmbr1 does not regulate limbbud expression of the Shh gene and thus there appears to be no co-regulatory sequence in the acheiropodia corresponding region in mouse. This suggests that, in human, either the deletion reported for acheiropodia is secondary to the causative lesion or that mouse and human differ in this region.

Second, a region in intron 9 of the *Lmbr1* gene was deleted in the mouse genome. This region of 700bp contains a highly conserved element and in addition to the ZRS, is the only other conserved element within the *Lmbr1* gene found in vertebrates including fish (figure 3.5). Once again, this knockout had no affect on limb development in the mouse and no other overt phenotypes were seen. Analysis of both the Δexon4+ and the Δintron9 homozygous embryos detected normal levels of *Shh* RNA and in newborns skeletal analysis showed normal limb bone development (data not shown). Thus two candidate co-regulatory regions that reside near the ZRS do not seem to assist in regulating the expression of *Shh*.

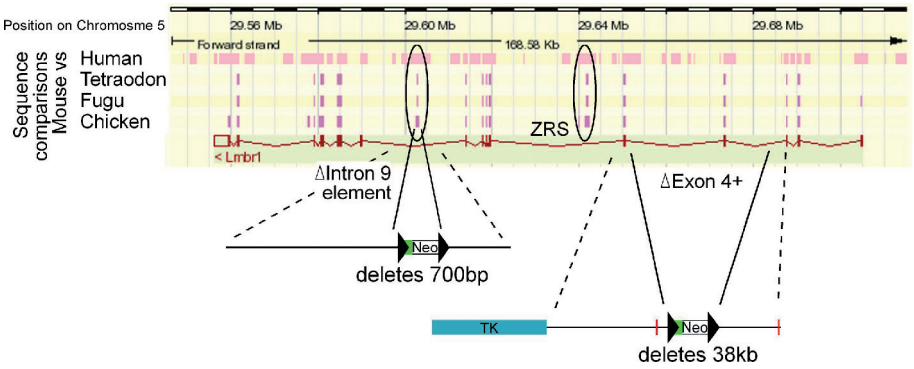


Figure 3.5 Deletions of the candidate ZRS co-regulators: *The horizontal red line is a depiction of the structure of the mouse Lmbr1 gene showing the position of the exons (red boxes). The positions of all multispecies conserved elements are shown above the gene diagram (taken from Ensembl mouse release 50). The only two conserved elements that are not exons are encircled and represent the ZRS and the intron 9 element that was deleted. The targeting constructs used are depicted below the gene and the approximate positions of the regions deleted are marked with solid lines and the ends of the arms of homology are marked with dotted ones. The position of exons 3 and 5 within the arms of the ΔExon4+ construct are marked by red bars. In both constructs, a neomycin gene driven by a PGK promoter (green box) and flanked by loxP sites (black triangles) was used to replace the deleted regions. In the case of ΔExon4+, the construct also carried a TK gene (blue rectangle) to allow for the negative selection of clones with non-homologous integration.*

Discussion

The ZRS co-localises with the Shh *locus*

Co-localisation

In the cryoFISH assay we observed that the BACs overlapping the ZRS and the *Shh* gene co-localise in a limb bud specific manner independent of developmental stage and genotype. The co-localisation of the ZRS with *Shh* appears to be independent of active transcription of *Shh* as it is observed throughout the limb bud and in ZRS$^{neo/neo}$ mice. It is specific for the ZRS and the *Shh* probes since the ZRS-probe and the control probe, which lies at the same distance from the ZRS only in the opposite direction, show much lower levels of co-localisation (figure 3.1a), excluding a general

compaction of mouse chromosome 5q in the developing limb bud. On the basis of these results we conclude that in the developing limb bud between developmental day 9.5 and 13.5 a chromatin organization is present in which the ZRS and the promoter region of *Shh* are spatially close to one another, thereby (presumably) facilitating *Shh* expression

How this chromatin state is reached or regulated remains unclear as it is independent of the presence of the ZRS itself, suggesting the presence of a second long range regulatory element in the vicinity responsible for this folding of the DNA. This unknown element needs to be located within or close to the region covered by the BAC used as a probe for the ZRS (*Lmbr1* is covered from ~100 kb upstream of exon 1 until intron 10, figure 3.1b), in order for its effect to have been picked up by our cryoFISH experiment. Although the cryoFISH assay is capable of detecting long range interactions very effectively, more subtle conformational changes within the areas covered by the BAC's will remain undetected. The presence of an unidentified, limb specific, long distance regulatory element in the vicinity of the ZRS would therefore explain the unexpected co-localisation of the ZRS and *Shh* in $ZRS^{-/-}$ limb buds.

Several likely candidate regions for this unknown regulatory element are present in the form of stretches of homology within introns 5 and 4 of LMBR1 which thus far have unknown functions. Of particular interest in this context is the deletion in a Brazilian family with recessive Acheiropody which covers exon 4 of *LMBR1* including flanking intronic sequence (1.2–2.5 kb 5' of exon 4 and 2.7–3.5 kb 3' of exon 4)[21]. This deletion does not include intron 5 in which the ZRS is located. Acheiropody is characterized by absent hand and feet, a phenotype very similar to that seen in the mice (partially) lacking the ZRS. To test whether this region indeed contains a *Shh* regulatory element we generated mice carrying a deletion of the region missing in these patients. No abnormal phenotype was found indicating that either the regulation of Shh is different between human and mice or the causative mutation in these patients is not the deletion of exon 4 of *LMBR1*. Lastly there is one

highly conserved element (conserved from fish to humans) present in intron 9 of *Lmbr1* which could be a likely candidate for the missing 2nd long distance element. However deletion of this region also gives no limb phenotype. It is therefore unlikely that this conserved element is involved in the regulation of the chromatin folding of the ZRS towards *Shh.* The location of the element responsible for the looping of the ZRS to *Shh* remains therefore still unknown.

Genomic distance important for ZRS function

The construct for the partial deletions of the ZRS was designed based on the results of the LacZ reporter assays. The minimal region sufficient for driving reporter expression in the correct region of the developing limb bud (as determined in[4]) was chosen as the minimal region in the partial deletions. The assumption was that this part of the ZRS should at least be capable of driving *Shh* expression on the posterior side of the limb bud[4]. Besides that, it could possibly also be active on the anterior side of the limb bud in the absence of putative repressing elements in the 3' half of the ZRS. If this was the case the deletion of the 3' half of the ZRS should give a dominant PPD phenotype. Surprisingly, the heterozygous mice were completely identical to their wild type littermates, showing no signs of pre-axial polydactyly. Only when bred to homozygosity it became clear that the introduced partial knock out interferes with normal *Shh* expression, in a way very similar to the complete ZRS knockout. Therefore the region of the ZRS sufficient of driving expression when close to its target gene is clearly not sufficient to drive expression in the *in vivo* genomic context, where there is a distance of almost 1 Mb between the ZRS and *Shh*. This difference could also be due to the difference in the promoter. In the LacZ reporter assays the MR was placed in front of the beta globin minimal promoter instead of the Shh promoter. Recent reports have shown that the specificity of enhancer elements to their own promoter may result in both false positive and false negative results in reporter assays using "alien" promoter sequences[22].

The phenotype of the ZRS$^{neo/neo}$ mice gives us a hint to the part of the ZRS that could be essential for the function of the ZRS over the almost 1 Mb distance in the normal genomic context. The nucleotides in between which the Neomycin cassette is inserted are not present in the minimal region effective in the LacZ reporter assay, but their disruption does lead to loss of *Shh* activation in the *in vivo* genomic context. The lack of expression in the presence of the complete, although interrupted, ZRS is unexpected. The disturbance of normal *Shh* expression could be due to competition with the *Shh* promoter of the HSV tK promoter of the neomycin cassette, as has been described for the pgk promoter (discussed in[22,23]). However, the HSV tK promoter is relatively weak compared to the pgk promoter which should prevent this problem[22,24]. If promoter competition did occur specific expression of *neomycin* in the ZPA would be expected instead of the ubiquitous low expression levels that were detected. The most likely explanation therefore, is that the insertion of the *neomycin* cassette disturbs a function of the ZRS itself, possibly a protein binding site. The function of this protein binding site can be one of many options: stabilization of the binding of the ZRS to the *Shh* promoter, activation of the transcription, recruiting transcription machinery proteins etc, etc. Sagai *et al.* (2005) describe a 'core' region: a minimal region of homology between human and Medaka. We inserted our *neomycin* cassette in this region, which could confirm the importance of this region in activating *Shh* expression.

A model for the regulation of Shh expression by the ZRS

Combining the data presented in this paper with the recent results published about the ZRS a model of *Shh* regulation is emerging in which the ZRS acts as a limb specific activator of *Shh* through direct interaction between the ZRS, *Shh* and an as yet elusive 2nd long distance element (figure 3.6). In this model throughout the developing limb bud a chromatin state is present in which the ZRS and the *Shh* promoter are in close proximity through folding of the DNA. This chromatin folding

is brought about by the binding of the unknown element X (indicated by bright blue in figure 3.6) to the *Shh* region, mediated by limb bud specific transcription factors. In the ZPA additional transcription factors are present that bind to the ZRS bringing it even closer to the *Shh* promoter and recruiting the transcriptional machinery, activating transcription of *Shh*. These differences in proximity between the ZPA and the rest of the limb bud may be too small to be picked up by the relatively low resolution of cryoFISH. Point mutations in the ZRS lead to ectopic expression of *Shh* through a gain of function of the ZRS, either through the loss of binding sites for

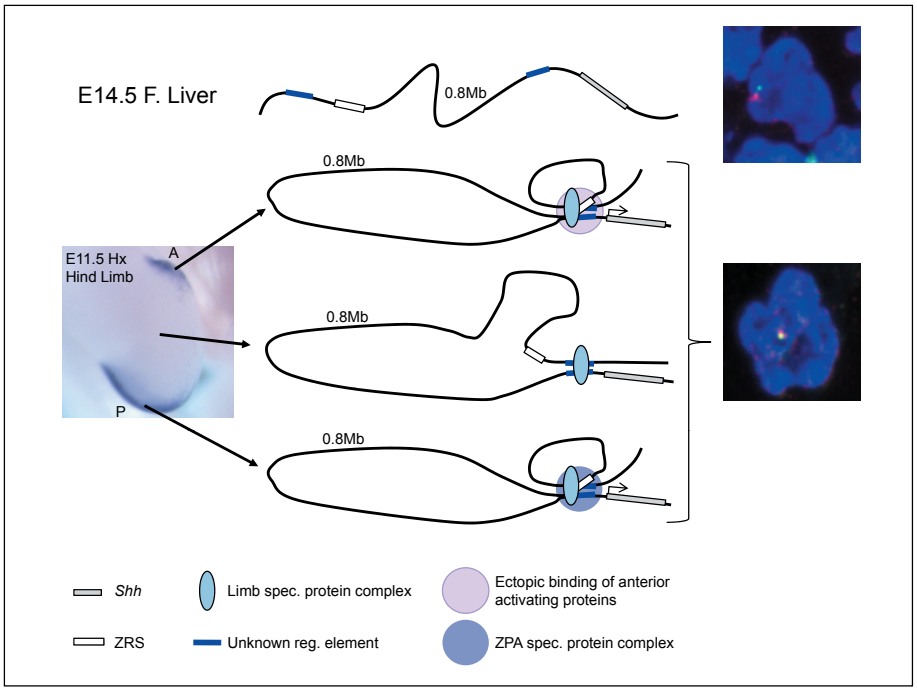


Figure 3.6 model for the regulation of Shh by the ZRS*: In E14.5 fetal liver the DNA is in a semi relaxed conformation in which the ZRS and Shh do not co-localise; in the developing limb the Shh promoter region is bound to an unknown long distance regulatory sequence in the vicinity of the ZRS mediated by limb specific transcription factors; on the posterior side ZPA specific proteins are recruited to the limb specific complex allowing transcriptional activation of Shh by the ZRS; on the anterior side point mutations in the ZRS allow ectopic activation of Shh through the aspecific binding of activating proteins.*

transcriptional repressors and/or through the loss of specificity for known posterior transcription factors, such as Hoxd13 and Hoxa12[25], allowing anterior transcription factors to bind and activate *Shh*. This ectopic expression is limited to the mirrored site of the ZPA through dependence upon signaling factors from the non-AER (apical ectodermal ridge) border of dorsal and ventral ectoderm[26]. The recent publications showing duplications of the ZRS to be the pathogenic mutation in families with pre-axial polydactyly fit the gain of function model for the ZRS[9,10]. In the model proposed above duplication of the ZRS would lead to a higher chance of binding of the ZRS to the *Shh* promoter and therefore to a higher chance of inducing *Shh* expression ectopically on the anterior side of the limb bud.

Conclusion

The ZRS is a transcriptional activator of *Shh* that appears to be co-localising with *Shh* in the limb bud. The minimal region of the ZRS as defined by a LacZ reporter assay is not sufficient for *Shh* transcription in the *in vivo* genomic context, in which the ZRS resides 1 Mb upstream of *Shh*. The co-localisation of the ZRS with the *Shh* gene is apparently independent of *Shh* expression or the ZRS itself. The co-localisation in the $ZRS^{-/-}$ limb bud is a strong indication of the presence of a second as yet elusive long distance genomic element that is responsible for the folding of the DNA in the limb bud. These results add an additional layer to the complexity of the long-range transcriptional regulation of *Shh* by the ZRS to specify a proper A/P axis in the developing limb bud.

Materials & Methods

CryoFISH

Dissection and fixation

CryoFISH was performed as described before[19] briefly, E14.5 liver, E9.5, 11 and E12.5 forelimbs and hind limbs were dissected in PBS on ice and carefully transferred to 8% paraformaldehyde for a fixation step of 2 hrs at 4°C (noon at the day the plug was found was set at E0.5). An equal volume of 2.3 M sucrose was added; the samples were left at 4°C overnight, after this the fixed tissue was immersed in 2.3 M Sucrose and left for minimally 4 hrs at 4°C. Fixed limbs were mounted on a specimen holder in an upright position on the proximal cutting edge (therefore with the AER on top) and snap-frozen in liquid nitrogen. Frozen limbs were stored in liquid nitrogen until sectioning.

Sectioning and hybridization

Ultrathin cryo-sections of approximately 200 nm were cut using a Reichert Ultramicrotome E equipped with a cryo-attachment (Leica). Sections were made parallel to the proximal cutting edge at the height of the ZPA. Using a loop filled with sucrose, sections were transferred to coverslips and stored at -20°C. For hybridization, sections were washed with PBS to remove sucrose, treated with 250 ng/ml RNase in 2X SSC for 1 h at 37°C, incubated for 10 min in 0.1 M HCl at room temperature, dehydrated in a series of ethanol washes and denatured for 8 min at 80°C in hybridization mix (50% formamide, 10% dextran sulfate, 2 X SSC, 50 mM phosphate buffer, pH 7.5) containing the labeled probes; the probes were allowed to hybridize at 37°C in a humid incubation chamber for 16 hrs (o/n). After post hybridization washes with 2X SSC, nuclei were counterstained with 20 ng/ml DAPI (Sigma) and mounted in VectaShield anti fading agent.

Probes and labeling

The following BAC's were used as probes (BACPAC Resources Centre, Oakland, California, USA): (RP23-284A9 (ZRS), RP23-389P3 (*Shh*), RP23-386M10 (1.6 Mb upstream of *Shh*), RP34-26L16 (*MAF*) and RP23-25H19 (*MAF* regulatory element) (figure 3.1c). The BAC's were directly labeled with either Chromatide Alexa594 dUTP (Invitrogen) or SpectrumGreen dUTP (Abbott Molecular) using nick translation using standard techniques.

Pictures, counting and statistics

Images were collected with a Zeiss Axio Imager Z1 epifluorescence microscope (100 plan apochromat, 1.4 oil objective), equipped with a charge-coupled device (CCD) camera and Isis FISH Imaging System software (Metasystems). A minimum of 250 ZRS-alleles (or the MAF regulator) were analyzed and scored (by a person not knowing the probe combination and the tissue in the sections) as overlapping, separate or single red or green spots. Percentages of overlap were calculated based on the number of times the signal from the ZRS-probe overlapped the signal from the other probe relative to the total number of signals from the ZRS probe. The t-test was used to determine significance; Bonferroni correction was used to compensate for the multiple testing problem.

Generation and analysis of recombinant mice

Targeting constructs and generation of the transgenic mice

To delete the entire ZRS, a construct was generated for homologous recombination in which a neomycin positive selection marker replaced the ZRS. For this a 2.9 kb fragment was amplified from wild type E14 ES cell DNA using primers 5'-catgcttgtctctcgaaaccc-3' and 5'-attaagagaggaagtgacctcag-3'. The loxP-flanked neomycin gene driven by the *HSV-TK* promoter was inserted in the *Vsp*I site. The 3'arm was obtained by amplification of a 5.8 kb fragment using primers

5'-aaatagtaaggacaggatcggc-3' and 5'-catcgatgtaccagaggcctgtaatcc-3'. This fragment was cloned behind the floxed HSV-TK neomycin cassette using *Eag*I (figure 3.4a top).

For the partial deletions, a construct for homologous recombination was created in such a way that with one construct two different parts of the ZRS could be deleted using either Flp- or Cre-recombinase. Homologous arms were obtained by PCR amplification as described above, with some modifications. For the 5' arm the 2.9 kb fragment, was joined using a *Hind*III site to an overlapping 1.6 kb amplification product of primers 5'-cacagtgtttacaaagatggc-3' and 5'-catattaaaacgatcttagttc-3', in which an frt site was inserted in the *Nde*I site. Next, a loxP-flanked neomycin resistance gene driven by the *HSV-TK* promoter, including an additional frt site 3' of neomycin, was inserted. The 3'arm of homology was extended by combining a 613 bp fragment obtained with primers 5'-tttctatcctgtgtcacagtt-3' and 5'-cacacccacccgtcacagaag-3', in which a loxP site was inserted in the *Bfu*AI site, with the above mentioned 5.8 kb fragment using EagI (figure 3.4a bottom). The targeting constructs were linearized with *Cla*I to facilitate homologous recombination of the plasmid with the ES cell genome.

As the deletion of exon 4 of *LMBR1* in the acheiropodia patients has not been mapped completely 38kb was removed which included exon 4, and most of introns 3 and 4, to ensure that sufficient sequence information was deleted from around exon 4. The construct that was generated (called pΔExon4+) contains a 5' homology arm that includes exon 3 and is comprised of a 3kb fragment generated by PCR using primers 5'-atgatcgcggccgctgaatatggcgatgagg-3' (containing a NotI site) and 5'-cagattccgcggaagatgaggatgctgtagtc-3' (containing a SacII site) which was subcloned into the corresponding sites of PGKneopA-loxP vector (the kind gift of Bill Skarnes). The 3' homology arm was generated from a 5.5 kb EcoRI fragment containing exon 5. A thymidine kinase gene was then added to allow for negative selection of incorrect targeting events. The targeting insert was released from the vector backbone using a KpnI digest.

The pΔIntron9 construct was built by recombineering using the protocols and reagents described by P Lui (the kind gift of Neal Copeland)[27]. Briefly, a 9.5kb genomic fragment from within intron 9 and containing ~700bp conserved element was retrieved by bacterial recombination from a *Lmbr1* containing BAC clone (RPCI-21 542N10) using a mini-targeting vector made with arms generated by PCR (using primers 5'-gcatatgcggccgcgccctgctgtgcaaggtac-3' and 5'-gcatatgaattcgaacttctaaagttatccagtac-3', and 5'-gcatatgaattcagtagacatgggcatgtggatc-3' and 5'-gcatatatcgataagtctggagcaattctcagtc-3'). A neomycin resistance gene cassette driven by the PGK promoter and flanked by loxP sites (from vector p452) was used to replace the conserved element by recombination. The homology arms from either side of the intron 9 conserved element were generated by PCR using the primers 5'-gatcatgcggccgctacaggcaacctcagtgttag-3' and 5'-gatcatggatccgcaataaagttatttccacaac-3', and 5'-gatcatgaattccttaaatttatataatggttgagtg-3' and 5'-gatcatgtcgactcaagtcgcgttctggatg-3'. The insert was isolated from the vector backbone using the unique NotI and ClaI sites flanking the retrieval arms.

Homologous recombination into the E14Tg2A line of ES cells, screening and generation of chimeras were performed using standard techniques[28,29].

Whole mount In situ hybridization and skeletal stainings

Embryos were stained for *Shh* expression as described before[30]. Skeletal stainings were performed as described earlier[28]

Real Time PCR analysis

RNA was isolated from anterior or posterior halves of E10.5 limb buds (wt and $ZRS^{neo/wt}$), whole limbs ($ZRS^{neo/neo}$), and remaining tissue of the embryo's using RNAbee (BioConnect, Huissen, the Netherlands) according to the manufacturers' protocol. Single strand cDNA synthesis was performed with SuperScript III (Invitrogen, Breda, the Netherlands). Relative expression levels were measured using iQ SYBR Green

Supermix (BioRad) on a ABI7300 Real time Cycler (Applied Biosystems). Primers were designed with the aid of PrimerExpress 1.0 (Applied Biosystems) software in such a way that forward and reverse primers were located in different exons, to exclude influences of genomic DNA contamination on the real time results. Primer sequences are available on request.

Acknowledgements

We thank R. Koppenol for his photographs of the mice strains, P. Klous and R. Willemsen for the help in setting up the cryoFISH experiment and F.M. Wijgerse for his time and effort counting the cryoFISH results. This work was supported by a grant from the Nederlandse organisatie voor Wetenschappelijk Onderzoek (016.056.320).

References

1. Ingham, P.W. and A.P. McMahon, *Hedgehog signaling in animal development: paradigms and principles.* Genes Dev, 2001. **15**(23): p. 3059-3087.
2. Dessaud, E., L.L. Yang, K. Hill, B. Cox, F. Ulloa, A. Ribeiro, A. Mynett, B.G. Novitch, and J. Briscoe, *Interpretation of the sonic hedgehog morphogen gradient by a temporal adaptation mechanism.* Nature, 2007. **450**(7170): p. 717-720.
3. Zhu, J., E. Nakamura, M.T. Nguyen, X. Bao, H. Akiyama, and S. Mackem, *Uncoupling Sonic hedgehog control of pattern and expansion of the developing limb bud.* Dev Cell, 2008. **14**(4): p. 624-632.
4. Lettice, L.A., S.J. Heaney, L.A. Purdie, L. Li, P. de Beer, B.A. Oostra, D. Goode, G. Elgar, R.E. Hill, and E. de Graaff, *A long-range Shh enhancer regulates expression in the developing limb and fin and is associated with preaxial polydactyly.* Hum Mol Genet, 2003. **12**(14): p. 1725-1735.
5. Lettice, L.A., A.E. Hill, P.S. Devenney, and R.E. Hill, *Point Mutations in a Distant Sonic Hedgehog Cis-Regulator Generate a Variable Regulatory Output Responsible for Preaxial Polydactyly.* Hum Mol Genet, 2007. **17**(7): p. 978-985.
6. Maas, S.A. and J.F. Fallon, *Single base pair change in the long-range Sonic hedgehog limb-specific enhancer is a genetic basis for preaxial polydactyly.* Dev Dyn, 2005. **232**(2): p. 345-348.
7. Lettice, L.A*., et al.*, *Disruption of a long-range cis-acting regulator for Shh causes preaxial polydactyly.* Proc Natl Acad Sci U S A, 2002. **99**(11): p. 7548-7553.
8. Sagai, T., M. Hosoya, Y. Mizushina, M. Tamura, and T. Shiroishi, *Elimination of a long-range cis-regulatory module causes complete loss of limb-specific Shh expression and truncation of the mouse limb.* Development, 2005. **132**(4): p. 797-803.

9. Klopocki, E., C.E. Ott, N. Benatar, R. Ullmann, S. Mundlos, and K. Lehmann, *A microduplication of the long range SHH limb regulator (ZRS) is associated with triphalangeal thumb-polysyndactyly syndrome.* J Med Genet, 2008. **45**(6): p. 370-375.

10. Sun, M.*, et al.*, *Triphalangeal thumb-polysyndactyly syndrome and syndactyly type IV are caused by genomic duplications involving the long-range, limb-specific SHH enhancer.* J Med Genet, 2008. **45**(9): p. 589-595.

11. Maas, S.A. and J.F. Fallon, *Isolation of the chicken Lmbr1 coding sequence and characterization of its role during chick limb development.* Dev Dyn, 2004. **229**(3): p. 520-528.

12. Clark, R.M., P.C. Marker, E. Roessler, A. Dutra, J.C. Schimenti, M. Muenke, and D.M. Kingsley, *Reciprocal mouse and human limb phenotypes caused by gain- and loss-of- function mutations affecting Lmbr1.* Genetics, 2001. **159**(2): p. 715-726.

13. Ertzer, R., F. Muller, Y. Hadzhiev, S. Rathnam, N. Fischer, S. Rastegar, and U. Strahle, *Cooperation of sonic hedgehog enhancers in midline expression.* Dev Biol, 2007. **301**(2): p. 578-589.

14. Jeong, Y., K. El-Jaick, E. Roessler, M. Muenke, and D.J. Epstein, *A functional screen for sonic hedgehog regulatory elements across a 1 Mb interval identifies long-range ventral forebrain enhancers.* Development, 2007. **133**(4): p. 761-772.

15. Epstein, D., A. McMahon, and A. Joyner, *Regionalization of Sonic hedgehog transcription along the anteroposterior axis of the mouse central nervous system is regulated by Hnf3-dependent and -independent mechanisms.* Development, 1999. **126**(2): p. 281-292.

16. Jamieson, R.V., R. Perveen, B. Kerr, M. Carette, J. Yardley, E. Heon, M.G. Wirth, V. van Heyningen, D. Donnai, F. Munier, and G.C.M. Black, *Domain disruption and mutation of the bZIP transcription factor, MAF,associated*

with cataract, ocular anterior segment dysgenesis and coloboma. Hum. Mol. Genet., 2002. **11**(1): p. 33-42.

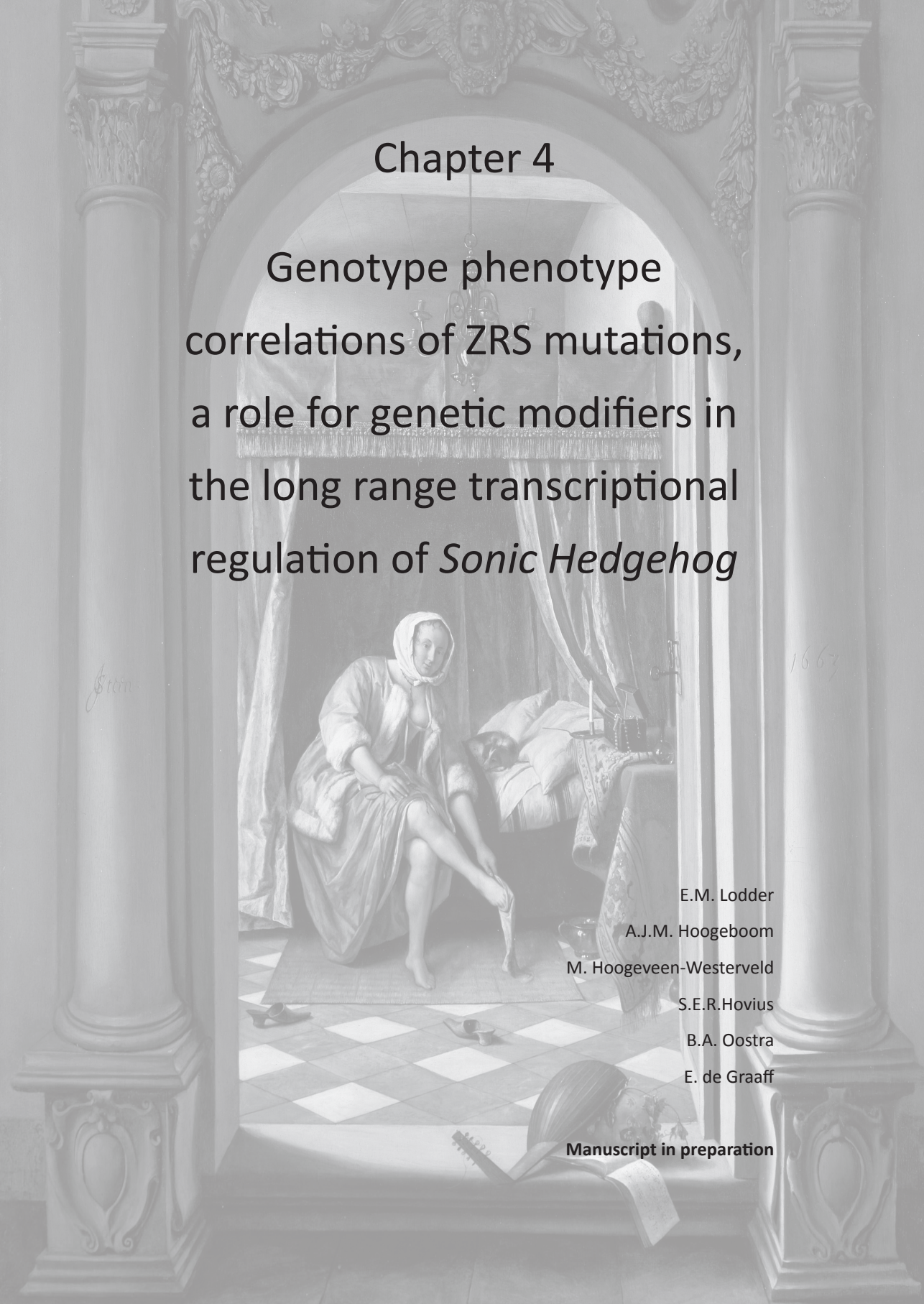
17. Bagheri-Fam, S., F. Barrionuevo, U. Dohrmann, T. Gunther, R. Schule, R. Kemler, M. Mallo, B. Kanzler, and G. Scherer, *Long-range upstream and downstream enhancers control distinct subsets of the complex spatiotemporal Sox9 expression pattern.* Dev Biol, 2006. **291**(2): p. 382-397.
18. Carter, D., L. Chakalova, C.S. Osborne, Y.F. Dai, and P. Fraser, *Long-range chromatin regulatory interactions in vivo.* Nat Genet, 2002. **32**(4): p. 623-626.
19. Simonis, M., P. Klous, E. Splinter, Y. Moshkin, R. Willemsen, E. de Wit, B. van Steensel, and W. de Laat, *Nuclear organization of active and inactive chromatin domains uncovered by chromosome conformation capture-on-chip (4C).* Nat Genet, 2006. **38**(11): p. 1348-1354.
20. Blanc, I., A. Bach, and B. Robert, *Unusual pattern of Sonic hedgehog expression in the polydactylous mouse mutant Hemimelic extra-toes.* Int J Dev Biol, 2002. **46**(7): p. 969-974.
21. Ianakiev, P., M.J. van Baren, M.J. Daly, S.P. Toledo, M.G. Cavalcanti, J.C. Neto, E.L. Silveira, A. Freire-Maia, P. Heutink, M.W. Kilpatrick, and P. Tsipouras, *Acheiropodia Is Caused by a Genomic Deletion in C7orf2, the Human Orthologue of the Lmbr1 Gene.* Am J Hum Genet, 2001. **68**(1): p. 38-45.
22. Carvajal, J.J., A. Keith, and P.W. Rigby, *Global transcriptional regulation of the locus encoding the skeletal muscle determination genes Mrf4 and Myf5.* Genes Dev, 2008. **22**(2): p. 265-276.
23. Olson, E.N., H.H. Arnold, P.W. Rigby, and B.J. Wold, *Know your neighbors: three phenotypes in null mutants of the myogenic bHLH gene MRF4.* Cell, 1996. **85**(1): p. 1-4.
24. Nakamura, S., S. Watanabe, M. Ohtsuka, T. Maehara, M. Ishihara, T. Yokomine, and M. Sato, *Cre-loxP system as a versatile tool for conferring*

increased levels of tissue-specific gene expression from a weak promoter. Mol Reprod Dev, 2008. **75**(6): p. 1085-1093.

25. Capellini, T.D., G. Di Giacomo, V. Salsi, A. Brendolan, E. Ferretti, D. Srivastava, V. Zappavigna, and L. Selleri, *Pbx1/Pbx2 requirement for distal limb patterning is mediated by the hierarchical control of Hox gene spatial distribution and Shh expression.* Development, 2006. **133**(11): p. 2263-2273.
26. Nissim, S., P. Allard, A. Bandyopadhyay, B.D. Harfe, and C.J. Tabin, *Characterization of a novel ectodermal signaling center regulating Tbx2 and Shh in the vertebrate limb.* Dev Biol, 2007. **304**(1): p. 9-21.
27. Liu, P., N.A. Jenkins, and N.G. Copeland, *A highly efficient recombineering-based method for generating conditional knockout mutations.* Genome Res, 2003. **13**(3): p. 476-484.
28. Lettice, L.A., L.A. Purdie, G.J. Carlson, F. Kilanowski, J. Dorin, and R.E. Hill, *The mouse bagpipe gene controls development of axial skeleton, skull, and spleen.* Proc Natl Acad Sci U S A, 1999. **96**(17): p. 9695-9700.
29. Mientjes, E.J., R. Willemsen, L.L. Kirkpatrick, I.M. Nieuwenhuizen, M. Hoogeveen-Westerveld, M. Verweij, S. Reis, B. Bardoni, A.T. Hoogeveen, B.A. Oostra, and D.L. Nelson, *Fxr1 knockout mice show a striated muscle phenotype: implications for Fxr1p function in vivo.* Hum Mol Genet, 2004. **13**(13): p. 1291-1302.
30. Hecksher-Sorensen, J., R.E. Hill, and L. Lettice, *Double labeling for whole-mount in situ hybridization in mouse.* Biotechniques, 1998. **24**(6): p. 914-916, 918.

Chapter 4

Genotype phenotype correlations of ZRS mutations, a role for genetic modifiers in the long range transcriptional regulation of *Sonic Hedgehog*



E.M. Lodder

A.J.M. Hoogeboom

M. Hoogeveen-Westerveld

S.E.R.Hovius

B.A. Oostra

E. de Graaff

Manuscript in preparation

Abstract

Mutations in a long range, limb specific, regulatory element (ZRS) of the embryonic patterning gene *Sonic Hedgehog* are involved in the development of preaxial polydactyly (PPD). Recently, duplications of the ZRS have been added to the spectrum of PPD causing mutations. We report here two novel duplications and three novel point mutations in the ZRS. Furthermore, we confirm the pathogenicity of a recently re-evaluated polymorphism in a large independent family, substantiating the role of this mutation in western European PPD. One novel point mutation (404G>C) involves a nucleotide that was previously described to be mutated (404G>A) emphasizing the importance of this nucleotide for correct functioning of the ZRS. The other three point mutations are only partially penetrant and are associated with a variable phenotype within the families. Finally, we present a review of all reported mutations involving the ZRS (17 point mutations and 9 duplications in humans, mice and cats including the ones reported here), highlighting the large phenotypic variability of the ZRS mutations. This variability indicates the presence of genetic modifiers that influence the phenotypic outcome of ZRS mutations.

Keywords

Preaxial polydactyly

Limb development

Congenital malformation

Sonic Hedgehog

Long distance transcriptional regulation

ZPA Regulatory Sequence (ZRS)

Introduction

Preaxial polydactyly type II or Triphalangeal-Thumb-Polysyndactyly (PPD II/ TPTPS) is a common congenital malformation of the limbs, which is the result of a disturbed patterning processes during limb development[1]. For a full set of five correctly patterned digits, positional information needs to be provided correctly along the three developmental axes: dorsal-ventral (DV), proximal-distal (PD) and anterior-posterior (AP). The patterning along the AP axis determines the number and identity of digits. Patterning defects along the AP axis generally lead to polydactyly (additional digits), preaxial in the case of additional digits on the thumb side of the hand/foot and postaxial when they are positioned at the opposite side[2].

During normal limb development a group of cells named the Zone of Polarizing Activity (ZPA) on the posterior (future little finger) side of the limb bud secretes SHH protein. This results in a gradient of this signalling molecule with high concentrations of SHH on the posterior side and almost no SHH on the anterior (future thumb) side. This gradient is essential for correct patterning of the AP axis. Ectopic expression of *SHH* on the anterior side leads to preaxial polydactyly[3,4].

Linkage analysis of several families with TPTPS led to the identification of a long range, *cis*-acting element (designated the ZRS for ZPA Regulatory Sequence) responsible for the regulation of *SHH* expression in a limb specific manner[5-7]. That the ZRS is essential for expression of *SHH* specifically in the developing limb was demonstrated in mice lacking the ZRS; they show a limb reduction defect, similar to the limb phenotype of *Shh* null mice, but are otherwise viable and phenotypically normal[8,9]. In contrast, point mutations in this ZRS lead to ectopic expression of *SHH* in the anterior margin of the limb bud and cause the human TPTPS phenotypes. So far, 14 different mutations have been described in the ZRS in human, mice and even cats with PPD[6,10-14]. Recently, duplications of the ZRS have been shown to cause TPTPS similar to the TPTPS caused by single nucleotide alterations in the ZRS[15,16].

In a recent report by Furniss *et al.* a single nucleotide substitution previously thought to represent a neutral polymorphism was shown to be the causative mutation in three linked families from southern England[13]. Here we report on a large Dutch family with a highly variable phenotype carrying the same nucleotide change, confirming the pathogenic nature of this mutation. Furthermore, we report 3 novel single nucleotide mutations and two novel duplications of the ZRS in small families and a sporadic patient. Together with the 14 point mutations and 7 duplications that are already published, these mutations reveal a complex genotype-phenotype correlation which suggests the presence of both genetic and environmental modifiers.

Clinical Report

Dutch2

The propositus (III-1 in figure 4.1b) was referred to us at the age of 4 months with TPT. Her mother had a small, wart-like thickening of her skin on both thumbs as a result of removal by ligature early in her life. No hand abnormalities were detected in the father, aunt and grandfather (I-1 and II-3, figure 4.1b). X-ray analysis of the latter confirmed the absence of ossal abnormalities of both thumbs (figure 4.2, D2 I-1). No other dysmorphologies were detected.

Dutch3

The propositus (VI-2 in figure 4.1a) was born as the second child of non-consanguineous parents after an uneventful pregnancy and delivery. He was referred to us at the age of 1.5 years with a left bifid thumb (figure 4.2 VI-2, taken at 1.5 years of age) and a widening of the thumb of the right hand (figure 4.2 VI-2). His height and weight were within the 95% confidence interval and no other dysmorphologies were visible. The family history was positive for thumb abnormalities. His father (V-1, figure 4.1a) was found to have a delta-phalanx bilaterally (figure 4.2 V-1), leading to

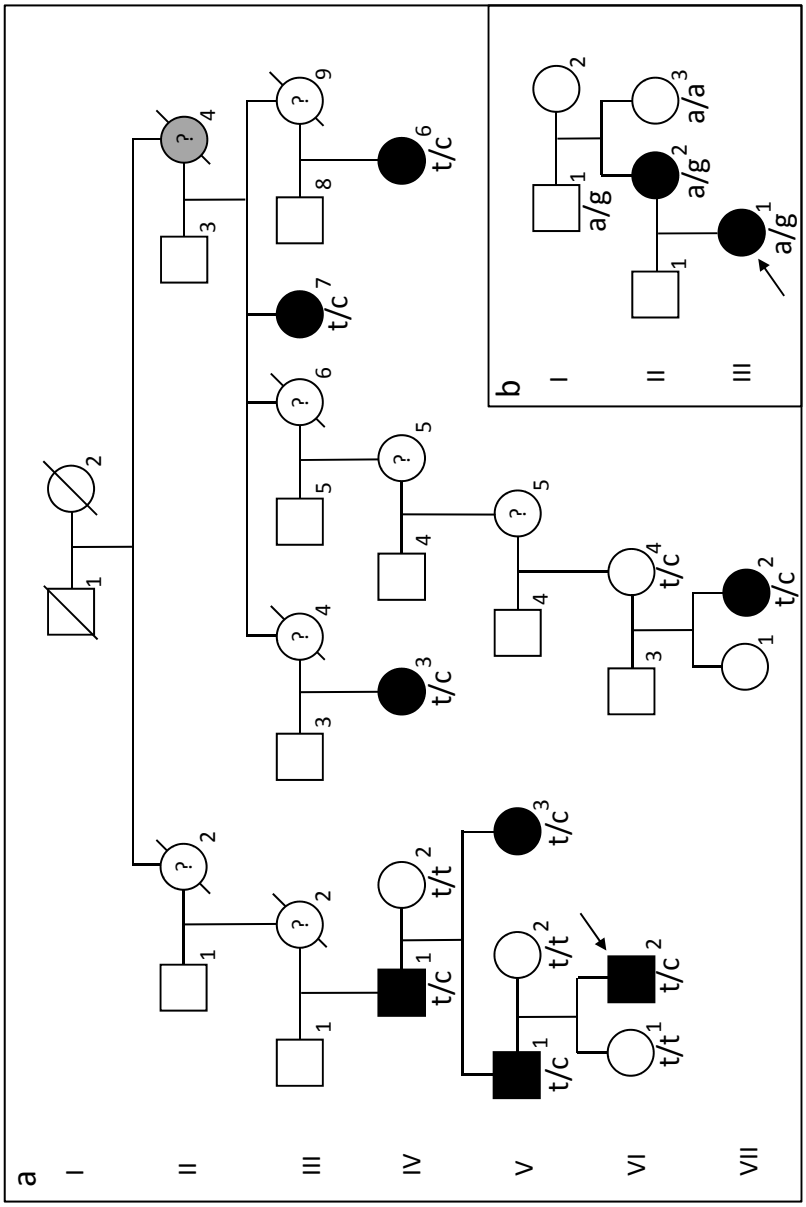
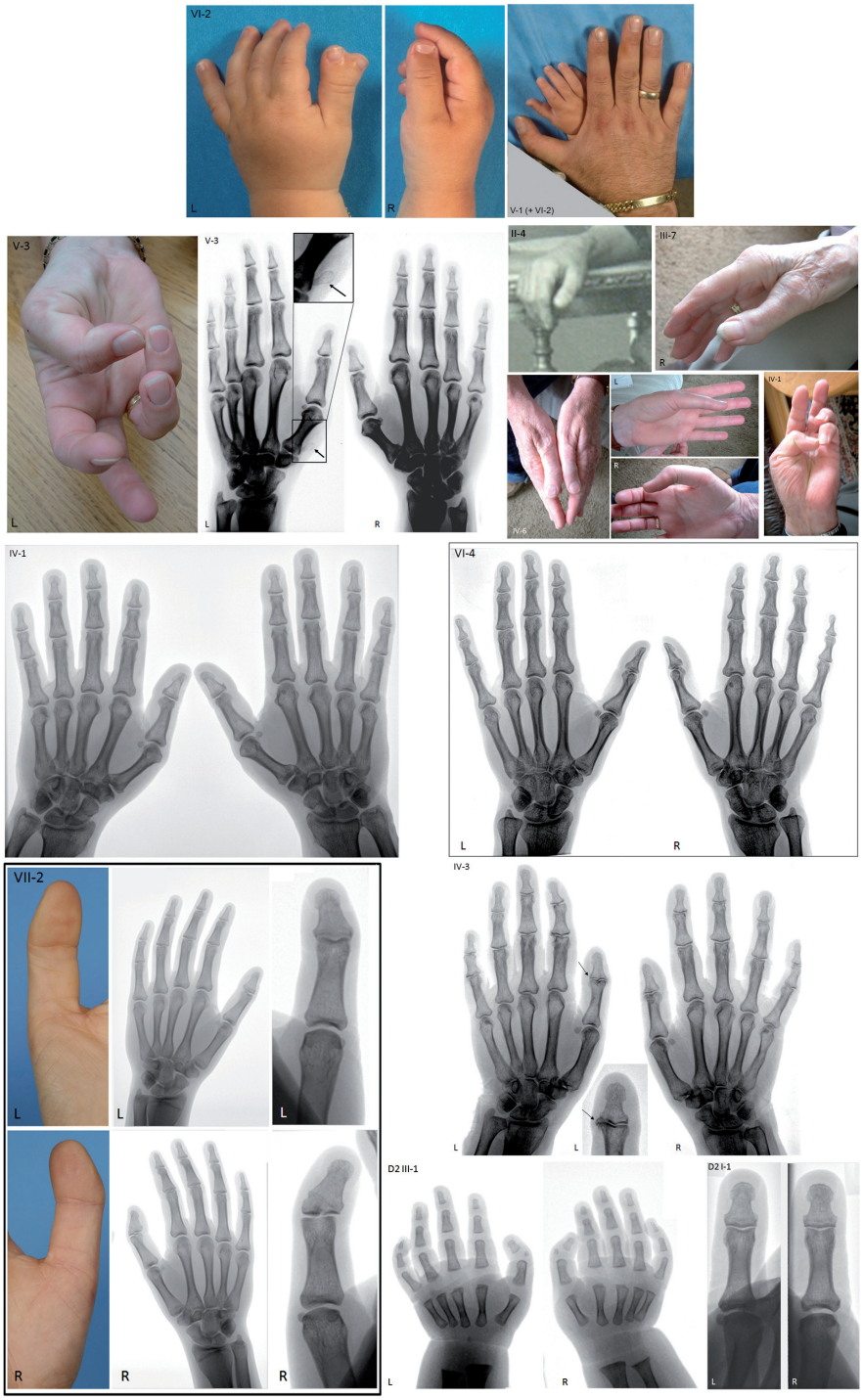


Figure 4.1 Pedigree structure and genotype *of a) the Dutch3 family showing 7 generations with 9 affected individuals(filled symbols). t/c indicates the mutation carriers; b) the Dutch2 family with 2 affected individuals in three generations, a/g indicates the mutation carriers; arrows indicate the probands.*

clinodactyly of the distal phalanx. His sister, individual V-3 (figure 4.1a), was found to have a non-opposable right thumb accompanied by thenar hypoplasia (figure 4.2 V-3). In contrast, her left thumb looked normal on the outside, but X-ray analysis displayed an additional bone-like structure next to the first metacarpal (figure 4.2 V-3). The grandfather of the propositus (IV-1, figure 4.1a) displayed a non-opposable thumb on the left (figure 4.2 IV-1), while no abnormality was found on his right hand. X-ray analysis of both hands demonstrated no obvious ossal malformations on either hand (figure 4.2 IV-1).

A family portrait of a female ancestor (II-4, figure 4.1a) is suggestive for clinodactyly of the right thumb (figure 4.2, II-4), however no examination by a physician is available. Her daughter (III-7, figure 4.1a) had a broad distal phalanx of her right thumb, resulting in clinodactyly and immobility of this thumb. Please note the absence of skin folds at the junction of the distal and proximal phalanges (figure 4.2 III-7). Her left thumb was found to be normal. One generation lower, individual IV-6 (figure 4.1a) had complaints of stiff thumbs. In her right thumb active flexion was impaired, whereas in her left thumb flexion was not possible at all (figure 4.2 IV-6). Please note the scars on her wrist due to an unrelated operation. Both

Figure 4.2 Variable phenotype in families Dutch2 and 3 *in reading order: external phenotype of the propositus (VI-2) of family Dutch 3 (left L and right R); note the bifid left thumb and broadened right thumb. Right hands of V-1 and VI-2. Right hand of patient V-3; note the non opposable thumb. Autoradiographs of patient V-3; note additional bone-like structure next to the first metacarpal (enlarged in inset). The thumb of II-4 as seen on an old family photograph. Clinodactyly of the right thumb of III-7 and of both thumbs of IV-6; both thumbs of IV-6 showed impaired flexion. The non opposable left thumb of individual IV-1. Autoradiographs of IV-1 and VI-4 do not show any ossal abnormalities. The thumbs of patient VII-2 show clinodactyly; radiographs of the hands and thumbs of VII-2 note the abnormally shaped right distal phalanx whereas no ossal abnormality of the left thumb is apparent. Autoradiograph of patient IV-3 note the ossification of the terminal interphalangeal joint. Triphalangeal thumbs in the propositus of the Dutch 2 family (D2 III-1) and normal thumbs in the grandfather (D2 I-1).*


VI-2
L
R
V-1 (+ VI-2)
V-3
L
V-3
L
R
II-4
III-7
R
L
R
IV-6
IV-1
IV-1
VI-4
L
R
IV-3
L
L
R
VII-2
L
L
L
R
R
R
D2 III-1
L
R
D2 I-1
L
R

thumbs demonstrated clinodactyly of the distal phalanx (figure 4.2 IV-6, no X-rays available). Her cousin (IV-3, figure 4.1a) has a small ossification within the terminal interphalangeal cleft of the left thumb.

Individual VI-4 (figure 4.1a) had no complaints of her hands and X-ray analysis of both hands revealed no abnormalities (figure 4.2 VI-4). Her daughter (VII-2, figure 4.1a) occasionally suffered from 'painful thumbs' and was found to have clinodactyly of the distal phalanx of both thumbs (figure 4.2 VII-2). X-ray analysis demonstrated either a small delta phalanx attached to the distal phalanx or an enlarged, deltoid epiphysis in her right thumb, whereas no ossal abnormality of the left thumb was apparent. All affected individuals were phenotypically normal, besides for the abnormalities of the hands. The feet were unaffected in all.

Brazilian

The phenotype of the Brazilian family was previously described by Vargas *et al.*[17].

Australian

The propositus demonstrated preaxial polydactyly of the left hand with three phalanges in both radial digits. The triphalangeal thumb of the right hand was broad, with ulnar deviation of the distal phalanx. The phenotype of the mother was essentially the same, except for bilateral thenar hypoplasia, absent in her daughter. The grandfather of the propositus was unaffected (data not shown).

USA1

A woman came to the clinic during her second pregnancy. She displayed severe limb abnormalities affecting all four limbs. The upper extremities revealed bilateral triphalangeal thumbs, narrow palms and syndactyly of digits 2-5, for which she had multiple surgeries to maximize the function of the digits. Her left leg

had minor abnormalities of the tibia in addition to a smaller fibula. Her right leg was more severely affected, with an absent or severely hypoplastic tibia, and was amputated just below her knee early in her life. Both feet displayed polysyndactyly, with 6 toes on her left and 8 toes on her right foot (data not shown). According to the patient, there was no family history of limb anomalies. During her first pregnancy, an ultrasound around week 13 of gestation revealed severe fetal limb anomalies, which involved all four extremities. During her second pregnancy an ultrasound at 13 weeks of gestation suggested severe anomalies involving the lower limbs, with apparently normal upper extremities. The right femur appeared shortened and there was significant hypoplasia of the tibia and fibula of both legs, with probably clubbed feet bilaterally.

Dutch4

This affected child from Chinese descent has bilateral PPD with an additional thumb on both hands. The long bones of her arms were unaffected. In addition no abnormality was detected in her feet. No relatives were available for examination or testing.

Results

ZRS mutations

Reduced penetrance

The ZRS was screened for mutations by direct sequencing in all six patients/ families described in the previous section. In all three families with reduced penetrance (Dutch2, Dutch3 and Australian), mutations were found in the ZRS. The mother of the index patient of the Dutch2 family was heterozygous for a new mutation, in the first half of the ZRS(165A>G, figure 4.3). This variation was present not only in the affected mother and the index patient (see figure 4.1b) but also in her unaffected grandfather (see figure 4.2 D2 I-1), indicating a reduced penetrance of this mutation. This 165A>G mutation was absent in 150 unaffected Dutch controls, arguing that this novel variant is the pathogenic mutation in this small family.

In the Australian family a novel mutation 743T>G near the end of the ZRS was identified (figure 4.3). The affected index patient and her mother, as well as the unaffected grandfather were heterozygous. The mutation was absent in 120 Dutch and 90 Australian controls. This mutation appears to be the fourth mutation in the ZRS with reduced penetrance.

The 295T>C nucleotide change was recently re-evaluated by Furniss *et al.* to be the pathogenic mutation in three linked families from Southern England instead of being a neutral polymorphism[13]. This mutation is also present in all affected members and obligate, though unaffected, carriers of family Dutch3, confirming both the pathogenic nature, as well as the reduced penetrance, of this polymorphism (figure 4.3).

Hot spot

In the Brazilian family described by Vargas *et al.* we detected a mutation of the same nucleotide as the Cuban mutation reported in the original paper by Lettice *et*

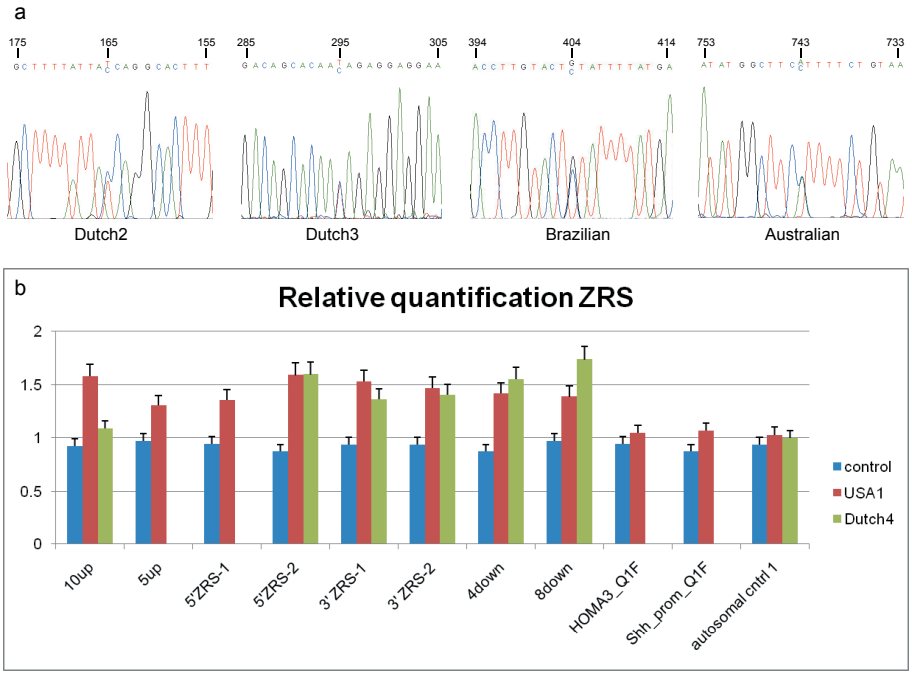


Figure 4.3 Novel ZRS mutations*: a) electropherograms of the Dutch2 165A>T mutation, the Dutch3/UK3 295T>C mutation, the Brazilian 404G>C and the Australian 743T>G mutation; b) qPCR results for the USA1 and Dutch4 patient showing the duplication of the ZRS.*

al. [6]. Instead of the 404G>A, as found in the Cuban family we detected a 404 G>C in all affected individuals (figure 4.3).

Duplications

In two of the patients no mutation in the ZRS was found (Dutch4 and USA1), except for the neutral polymorphism 3C>G (rs10254391) in Dutch 4 and the fetus of USA1. The two peaks showed an allele imbalance in the height of the peaks in the same manner as described by Sun *et al.*[15]. We therefore tested whether the presence of ZRS duplications could explain the observed phenotype: isolated TPTPS in the Dutch4 patient and the much more severe phenotype of PPD with tibial dysplasia in family USA1 (see clinical report). Subsequent experiments indeed revealed ZRS duplications in both samples. In family USA1, the duplication was determined by

quantitative (q)PCR with 8 independent primer combinations. The duplication spans at least 18 kb starting between 766-980 kb from *SHH* with an undetermined end point on the centromeric side (see figure 4.3&4.4).

The duplication in patient Dutch4 was confirmed by qPCR as well. Unfortunately, not enough material was available to determine the exact size of the duplication; however, it stretches at least 8.8 kb from the ZRS towards *SHH*. On the centromeric side the breakpoint is located within 10 kb from the ZRS (figure 4.3 & 4.4). As the patient is adopted and has a Chinese background with unknown family history we cannot exclude the possibility that the patient is related to one of the (Chinese) families reported by Sun *et al.* However, as all six families described by Sun *et al.* were found to carry independent duplications of the ZRS, it is likely that the duplication in the Dutch4 patient is independent as well.

Discussion

Above, we describe three novel ZRS point mutations and confirm one partially penetrant mutation reported by Furniss *et al.* in the UK3 families (see figure 4.4)[13]. The presence of the 295T>C mutation in a large Dutch family with variable phenotype confirms that this mutation is a frequent cause of TPTPS in Western Europe. Two of the newly described mutations are located outside the most conserved region of the ZRS (165A>G and 743T>G); these are both only partially penetrant.

The third mutation, 404G>C, involves the same nucleotide as the 404G>A mutation in a Cuban family originally described by Lettice *et al.*[6]. Interestingly, in both families affected individuals present with tibial dysplasia[17] (one individual has absent tibia but duplicated fibula) besides the TPT. These mutations lie only 2 bp from the ENU mutation M100081 406A>G in mouse[10], of which the limb malformation also involves the tibia. The involvement of the tibia in these mutations corresponds to the amount of ectopic *Shh/LacZ* expression as measured by a reporter assay[12]. In this assay the 404G>A, 406A>G mutations and the 558G>T (*Hx*) mutation which also

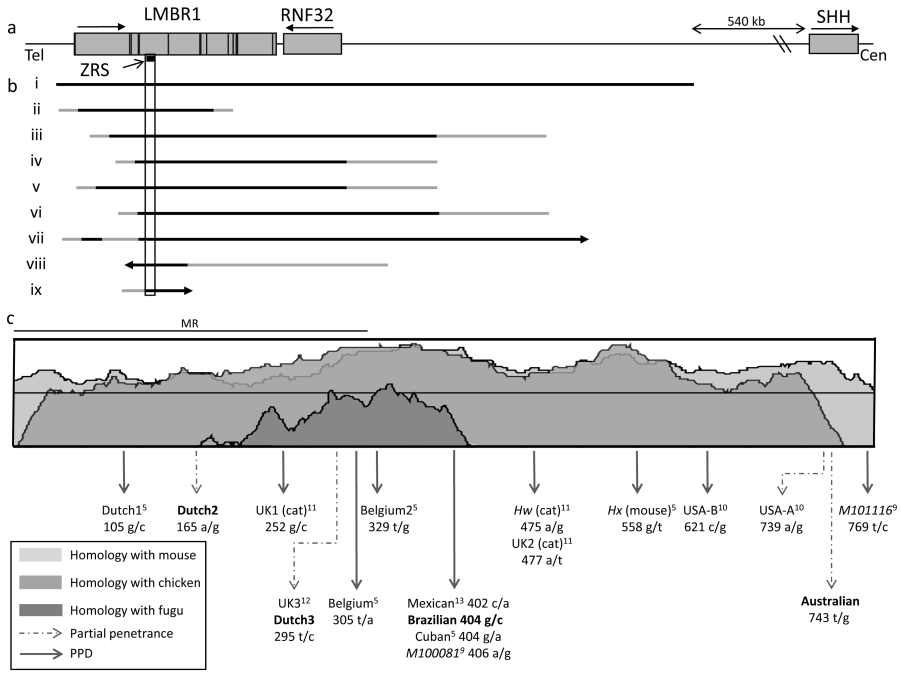


Figure 4.4 Mutational overview of the ZRS*: a) genomic context of the ZRS and SHH, arrows indicate direction of transcription cen: centromere, tel: telomere b) location of the duplications (black lines) of the ZRS, gray lines indicate the area that contains the border of the duplication, arrows indicate an unknown border; i duplication reported by Klopocki et al.[16] ii-vii duplications reported by Sun et al.[15] viii-ix duplications of patients USA1 and Dutch4 (this report), respectively; c) homology plot of the human ZRS compared to mouse (light gray), chicken (darker gray) and puffer fish (darkest gray) (http://genome.lbl.gov/vista/), MR indicates the minimal region capable of driving transcription of a LacZ reporter construct6 the locations of the known mutations (new mutations described in this report are in bold) are marked with arrows (dashed for partially penetrant mutations) nucleotide numbering indicates the (homologous) nucleotide in the human ZRS as originally adopted by Lettice et al.[6] superscript numbers refer to the original reports (see references). Note the distribution of the mutations throughout the entire ZRS.*

affects the tibia (depending on the mouse strain in homo- or heterozygous state) show an increased anterior LacZ expression as compared to the Belgian2 (329T>G) mutation. Furthermore, a mutation at position 402 (402C>T) in a small Mexican family with TPT with associated PPD was described by Sun *et al.* in an abstract for the 54th ASHG meeting in 2004[14]. Unfortunately, the description in the abstract does not

mention whether or not the phenotype involves the tibia. These mutations together form a mutational hotspot at the end of the ultra conserved region, with 4 reported mutations in a stretch of only 5 nucleotides. It therefore seems likely that these nucleotides represent a transcription factor binding site that is disrupted by all four mutations.

Duplications of the ZRS have been described in nine different families (including this report) and one mouse strain. These duplications are associated with a wide phenotypic spectrum of TPTPS/SD4 like phenotypes, ranging from mild TPTPS to severe syndactyly of all six digits resulting in a "cup-shaped" hand[15] to TPTPS with tibial dysplasia (family USA1 described above). Thus far, hardly any lower limb involvement was reported in combination with ZRS duplications: only in 4 out of the 19 affected individuals that were studied in detail in families 2 & 3, syndactyly of ray 4/5 was described by Sun *et al.*. Furthermore, the feet of the 50 affected individuals from families 1, 4 & 5 were normal[15]. Klopocki *et al.* only mention that the feet were less severely affected in the members of the family they describe[16]. These cases would therefore indicate a larger role for ZRS duplications in the arms compared to the limbs as is found with most point mutations described. In contrast, in family USA1 described here, tibial dysplasia was present in both the mother and foetus, while the large bones of the arm were unaffected. This is similar to the tibial dysplasia described for point mutations in the mutational hotspot within the ZRS as discussed above.

Two phenotypes described in the literature are hard to reconcile with the current model for ZRS regulation of SHH transcription. In both cases this is most likely due to incomplete genetic analysis of the patients. The first is a patient with acheiropody, a rare recessive condition in which the patients have truncations of all four limbs[18], reminiscent of the phenotype in mice lacking the ZRS[8]. The patients harbour a deletion of exon 4 of *LMBR1* which does not cover the ZRS. However, as the patients' DNA was analysed before the discovery of the ZRS, mutations involving

the ZRS may have been missed. The second unexplained phenotype was found in a patient with a translocation t(5;7) which separates the ZRS from *SHH*. Unexpectedly, this patient has preaxial polydactyly[7]. In contrast, mice heterozygous for a targeted deletion of the ZRS show no phenotype and homozygous mice have acheiropody as described above. Most likely, the phenotype in this patient is either caused by ectopic induction of *SHH* by enhancers near the translocation breakpoint on chromosome 5 or by a recessive point mutation in the ZRS on the normal chromosome 7 which was missed because no mutation analysis of the ZRS was performed.

A major problem with all described ZRS mutations is the wide range of clinical variability within families carrying the same mutation and to some extent, within individuals between left and right extremities. This clinical variability adds to the difficulty in assigning genotype phenotype correlations, especially as only for some of the mutations large well-documented families are available that help in assessing the variability of a certain mutation. The described variation points towards the presence of genetic and environmental modifiers influencing the phenotypic outcome of the same mutation. The differences between left and right within an individual may be due to either environmental influences within the womb, or to stochastic differences of transcriptional activation. The genetic variation may consist of slightly different levels between individuals of transcription factors that bind to the ZRS and regulate *SHH* transcription. Thus far, the only interaction partners of the ZRS that have been described are *HOXD10* and *HOXD13*[19]. The discovery of other ZRS interaction partners may prove helpful in the search for genetic modifiers of the ZRS phenotypes.

Material and methods

We studied 1 sporadic patient and several affected individuals from a large Dutch family and several smaller families all with congenital preaxial limb malformations. For a full description of the phenotypes see the clinical report. Informed consent was obtained from all individuals. Genomic DNA samples were isolated from peripheral blood and from a fibroblast cell line of the USA1 fetus following standard protocols. An 1123 bp fragment covering the ZRS was amplified by standard Polymerase Chain Reaction (PCR) (primer sequences and PCR conditions as described before[6]). Direct sequencing of both strands of the PCR products was performed using Big Dye Terminator 3.1 (Applied Biosystems). Fragments were loaded on an ABI3100

Supplemental table 1: qPCR primers and results

name	Sequence 5'-3'	location	USA1	Dutch4
10upF	TCCACAATGTTCACAGCATCTTC	10 kb 5' from the ZRS	Duplication	Normal
10upR	TTGAAATGAACGAGGAGCTGC			
5upF	GGCCAAATACCCTCCCAATG	5 kb 5' from the ZRS	Duplication	ND
5upR	CCTTTTCCTCCCTGGCACA			
5'ZRS-1F	TCTTAGGGCTCAGGGAACATCTT	5' ZRS	Duplication	ND
5'ZRS-1R	TCTGGAGTGGAGGAGGGAGA			
5'ZRS-2F	CAGCAACTTAATGAAAGTGGGAGAA	5' ZRS	Duplication	Duplication
5'ZRS-2R	ACTCCCAGAAAAATCTTGGGTTT			
3' ZRS-1F	TGGCACATGCGCATATTTGGC	3' ZRS	Duplication	Duplication
3' ZRS-1R	CACTAAGATCAAAACATGACC			
3' ZRS-2F	TTCCAAAAGGAAGCTGAAGTA	3' ZRS	Duplication	Duplication
3' ZRS-2R	CCCCAATTGAGAGCACTAGT			
4downF	GCATGCGCACACCTTGC	4 kb 3' of the ZRS	Duplication	Duplication
4downR	GTCAGCGCCTACAGATGAGCT			
8downF	CAGCTTCTCGGGAGGCAA	8 kb 3' of the ZRS	Duplication	Duplication
8downR	CACCAAAAGTGAAGTGCAGTGG			
HOMA3_Q1F	TTGCATTACCTCATCCTCCAGTT	220 kb 3' of the ZRS	Normal	ND
HOMA3_Q1R	GCCTTGTTCTGTGATATGTTGAGC			
Shh_prom_Q1F	AGCACCTACCATTCCAGGGA	Shh transcriptional start	Normal	ND
Shh_prom_Q1R	GAGGGAGGAGCGCATGTCT			
autosomal control 1F	TGAACGACTTCGGCCTGTC	autosomal control 1	Normal	Normal
autosomal control 1R	TCACGCCTCTCTCAAATCCTC			
autosomal control 2F	AGCTCAAGTCTACCAGCATCTGG	autosomal control 2	Normal	ND
autosomal control 2R	GGATGGTAGGCATTGTGCTCA			

sequence analyzer and analyzed with DNA Sequencing Analysis (ver.3.7) and SeqScape (ver.2.1) software (Applied Biosystems). Genomic duplication of the ZRS was tested with Real Time PCR using iQ SYBR Green Supermix (BioRad) on an ABI7300 Real time Cycler (Applied Biosystems). Primers were designed with the aid of PrimerExpress 1.0 (Applied Biosystems) software, at four independent positions within the ZRS and at regularly spaced intervals around the ZRS. Relative DNA concentrations were determined using the ddCT method with an unaffected individual and an autosomal genomic control region as reference. Primer sequences are listed in supplemental table 1.

Acknowledgements

We thank the patients and the family for their cooperation and R. Koppenol for his work on the illustrations. This work was supported by a grant from the Nederlandse organisatie voor Wetenschappelijk Onderzoek to EdG (016.056.320).

References

1. Balci, S., M. Demirtas, B. Civelek, M. Piskin, O. Sensoz, and A.N. Akarsu, *Phenotypic variability of triphalangeal thumb-polysyndactyly syndrome linked to chromosome 7q36.* Am J Med Genet, 1999. **87**(5): p. 399-406.
2. Towers, M. and C. Tickle, *Growing models of vertebrate limb development.* Development, 2009. **136**(2): p. 179-190.
3. Riddle, R.D., R.L. Johnson, E. Laufer, and C. Tabin, *Sonic hedgehog mediates the polarizing activity of the ZPA.* Cell, 1993. **75**(7): p. 1401-1416.
4. Blanc, I., A. Bach, and B. Robert, *Unusual pattern of Sonic hedgehog expression in the polydactylous mouse mutant Hemimelic extra-toes.* Int J Dev Biol, 2002. **46**(7): p. 969-974.
5. Heutink, P., J. Zguricas, L. van Oosterhout, G.J. Breedveld, L. Testers, L.A. Sandkuijl, P.J. Snijders, J. Weissenbach, D. Lindhout, S.E. Hovius, and et al., *The gene for triphalangeal thumb maps to the subtelomeric region of chromosome 7q.* Nat Genet, 1994. **6**(3): p. 287-292.
6. Lettice, L.A., S.J. Heaney, L.A. Purdie, L. Li, P. de Beer, B.A. Oostra, D. Goode, G. Elgar, R.E. Hill, and E. de Graaff, *A long-range Shh enhancer regulates expression in the developing limb and fin and is associated with preaxial polydactyly.* Hum Mol Genet, 2003. **12**(14): p. 1725-1735.
7. Lettice, L.A.*, et al.*, *Disruption of a long-range cis-acting regulator for Shh causes preaxial polydactyly.* Proc Natl Acad Sci U S A, 2002. **99**(11): p. 7548-7553.
8. Sagai, T., M. Hosoya, Y. Mizushina, M. Tamura, and T. Shiroishi, *Elimination of a long-range cis-regulatory module causes complete loss of limb-specific Shh expression and truncation of the mouse limb.* Development, 2005. **132**(4): p. 797-803.
9. Chiang, C., Y. Litingtung, E. Lee, K.E. Young, J.L. Corden, H. Westphal, and P.A. Beachy, *Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function.* Nature, 1996. **383**(6599): p. 407-413.
10. Masuya, H.*, et al.*, *A series of ENU-induced single-base substitutions in a long-range cis-element altering Sonic hedgehog expression in the developing mouse limb bud.* Genomics, 2006.

11. Gurnett, C.A., A.M. Bowcock, F.R. Dietz, J.A. Morcuende, J.C. Murray, and M.B. Dobbs, *Two novel point mutations in the long-range SHH enhancer in three families with triphalangeal thumb and preaxial polydactyly.* Am J Med Genet A, 2007. **143**(1): p. 27-32.

12. Lettice, L.A., A.E. Hill, P.S. Devenney, and R.E. Hill, *Point Mutations in a Distant Sonic Hedgehog Cis-Regulator Generate a Variable Regulatory Output Responsible for Preaxial Polydactyly.* Hum Mol Genet, 2007. **17**(7): p. 978-985.

13. Furniss, D., L.A. Lettice, I.B. Taylor, P.S. Critchley, H. Giele, R.E. Hill, and A.O. Wilkie, *A variant in the sonic hedgehog regulatory sequence (ZRS) is associated with triphalangeal thumb and deregulates expression in the developing limb.* Hum Mol Genet, 2008. **17**(16): p. 2417-2423.

14. Sun, M., Q. Liu, F.X. Wu, W.H.Y. Luo, E.W. Jabs, and X. Zhang. *Linkage Analysis and Mutation Screening in Families With Triphalangeal Thumb and Associated Hand Malformations*. in *ASHG 54th Annual Meeting*. 2004. Toronto Convention Center, Toronto, Canada.

15. Sun, M.*, et al.*, *Triphalangeal thumb-polysyndactyly syndrome and syndactyly type IV are caused by genomic duplications involving the long-range, limb-specific SHH enhancer.* J Med Genet, 2008. **45**(9): p. 589-595.

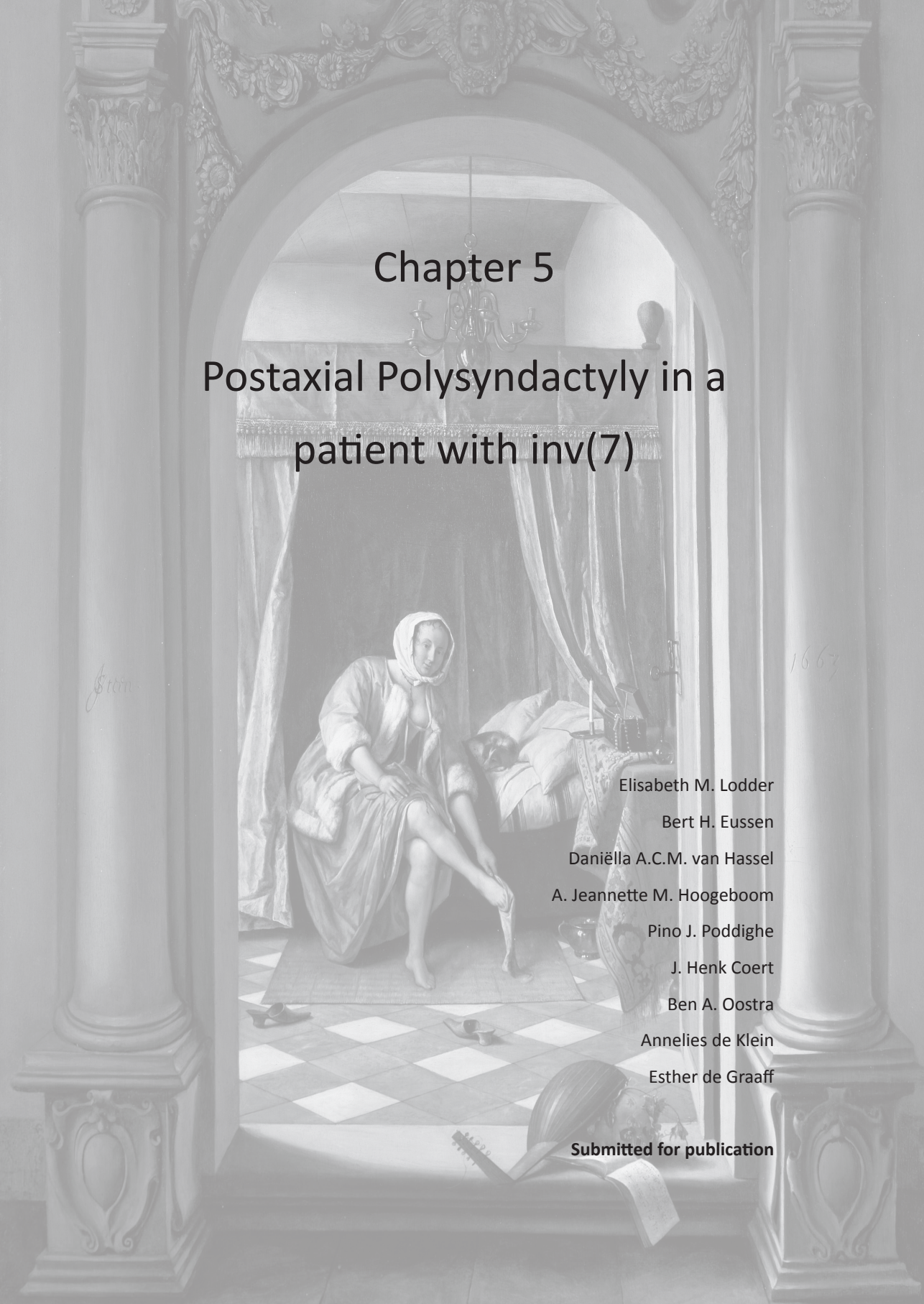
16. Klopocki, E., C.E. Ott, N. Benatar, R. Ullmann, S. Mundlos, and K. Lehmann, *A microduplication of the long range SHH limb regulator (ZRS) is associated with triphalangeal thumb-polysyndactyly syndrome.* J Med Genet, 2008. **45**(6): p. 370-375.

17. Vargas, F.R., R.L. Pontes, J.C. Llerena Junior, and J.C. de Almeida, Absent tibiae--polydactyly--triphalangeal thumbs with fibular dimelia: variable expression of the Werner (McKusick 188770) syndrome? Am J Med Genet, 1995. 55(3): p. 261-264.

18. Ianakiev, P., M.J. van Baren, M.J. Daly, S.P. Toledo, M.G. Cavalcanti, J.C. Neto, E.L. Silveira, A. Freire-Maia, P. Heutink, M.W. Kilpatrick, and P. Tsipouras, *Acheiropodia Is Caused by a Genomic Deletion in C7orf2, the Human Orthologue of the Lmbr1 Gene.* Am J Hum Genet, 2001. **68**(1): p. 38-45.

19. Capellini, T.D., G. Di Giacomo, V. Salsi, A. Brendolan, E. Ferretti, D. Srivastava, V.

Zappavigna, and L. Selleri, *Pbx1/Pbx2 requirement for distal limb patterning is mediated by the hierarchical control of Hox gene spatial distribution and Shh expression.* Development, 2006. **133**(11): p. 2263-2273.



Chapter 5

Postaxial Polysyndactyly in a patient with inv(7)

Elisabeth M. Lodder
Bert H. Eussen
Daniëlla A.C.M. van Hassel
A. Jeannette M. Hoogeboom
Pino J. Poddighe
J. Henk Coert
Ben A. Oostra
Annelies de Klein
Esther de Graaff

Submitted for publication

Abstract

Apparently balanced chromosomal inversions may lead to disruption of developmentally important genes at the breakpoints of the inversion, causing congenital malformations. Characterisation of such inversions may therefore lead to new insights in human development. Here, we report on a *de novo* inversion of chromosome 7 (p15.2q36.3) in a patient with postaxial polysyndactyly. The breakpoints do not disrupt likely candidate genes for the limb phenotype observed in the patient. However, on the p-arm the breakpoint separates the *HoxA* cluster from a gene desert containing several conserved non-coding elements, suggesting that a disruption of a *cis*-regulatory circuit of the *HoxA* cluster could be the underlying cause of the phenotype in this patient.

Key Words

Pericentric chromosomal inversion

postaxial polysyndactyly

congenital malformation

chromosome 7

HoxA cluster

Introduction

Pericentric inversions are relatively common chromosomal abnormalities with an incidence 0.012% in newborns[1]. These are generally without consequence for the carrier[1]. However, *de novo* chromosomal inversions in patients with congenital malformations can be the causative factor of the malformation through disruption of developmentally important genes at the breakpoints[2].

Postaxial polydactyly (MIM #174200) is a congenital malformation in which additional digits are present on the posterior (little finger) side of the hand and or feet. Syndactyly of the additional digits and the most posterior digits is often observed[3]. Here, we describe a patient with postaxial polysyndactyly carrying an inversion on chromosome 7. Chromosomal abnormalities of human chromosome 7 are mapped and annotated in order to get a better understanding of the functioning of entire chromosomes[4]. The chromosome 7 database (http://www.chr7.org/) lists 23 chromosomal aberrations that are associated with polydactyly. Seven of these aberrations are related to two genes that are important in limb development: *GLI-Kruppel family member 3 (GLI3)* on the p-arm and *SONIC HEDGEHOG (SHH)* on the q-arm. Both genes lie in the vicinity of the suspected breakpoints of the inversion in our patient*;* this prompted us to investigate the inversion in more detail.

We carefully mapped the breakpoints on both arms of chromosome 7, using Fluorescent In Situ Hybridisation (FISH) followed by Southern blot analysis. We cloned the chromosomal breakpoints by Polymerase Chain Reaction and direct sequencing. Neither breakpoint disrupts a gene that is likely to be involved in limb development. On the q-arm the inversion breakpoint lies in an intergenic non-conserved region. On the p-arm the inversion disrupts *NFE2L3* a member of the erythroid Cap'n collar transcription factors[5]. However, this breakpoint is located 1 Mb telomeric of the *HOXA* gene cluster resulting in a separation of the *HOXA* cluster and a gene desert harbouring several conserved non-coding elements. This suggests that a disruption

of a *cis*-regulatory circuit of the *HOXA* cluster could be the underlying cause of the phenotype in this patient.

Materials and methods

Case and family history

The patient was born at 37 weeks gestational age after an uneventful pregnancy as the first child of non-consanguineous parents, without a family history of congenital malformations. His birth weight was 2630 gram (normal for gestational age). The boy presented with postaxial polydactyly and syndactyly 3-6 of both hand and feet (figure 5.1). Autoradiography showed a shortened ulna and overgrown radial head on both sides. An extension limitation of the left knee was observed, due to a congenital flexion-contraction (98°) with webbing of the fossa poplitea. The left knee also presented with a lateral patella luxation. On examination at 19 months his length was 79 cm (-2 standard deviatons (sd) for the average population) and his weight was 8.4 kg (< -2 sd weight length ratio). At 4 years and 9 months his length was 96.4 cm (-3.5 sd for the population average/-2.5 sd for his target height), his weight was 13.8 kg (-1 sd weight length ratio), and his OFC was 50.3 cm (between 0 and -1 sd). No other major or minor congenital malformations were found. His psychomotoric development was normal. Both parents were unaffected. Informed consent was obtained for the patient and his parents.

Karyotyping

Karyotyping was performed on GTG-banded metaphases obtained from peripheral blood cultures, of the patient and his parents, using standard procedures[6]. Results were described in accordance with the ISCN 2005[7].

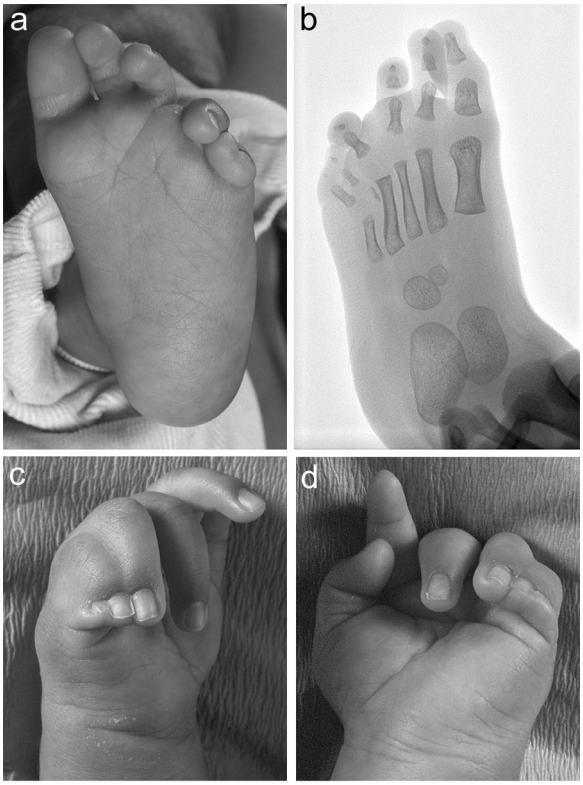


Figure 5.1 phenotype of the patient: *a) Postaxial polydactyly of the left foot with syndactyly of the 5th and 6th rays b) x-ray radiograph of the left foot c,d) photographs of the right (c) and left (d) hand showing the postaxial polydactyly with syndactyly of the 4th-6th rays.*

FISH

Fluorescent In Situ Hybridisation (FISH) analysis was performed as described before[8]. Bacterial Artificial Chromosomes (BAC's) and Fosmids were ordered from BACPAC Resource Center (BPRC) at the Children's Hospital Oakland Research Institute in Oakland (http://bacpac.chori.org/). The relevant BAC-clones and Fosmids are shown in figure 5.3. Hybridisation locations were validated on reference human metaphase spreads.

Affymetrix SNP array

DNA from the patient was isolated from peripheral blood according to standard procedures and hybridized on the 250K NspI Single Nucleotide Polymorphism array (Affymetrix, Santa Clara, CA) according to the manufacturer's protocols. Hybridisation

intensities were compared to a well characterized set of reference individuals.

Southern blotting

Southern blotting was performed according to standard protocols[9]. Probes were amplified using radioactive Polymerase Chain Reaction (PCR), using the following primer combinations. Q40: fw 5'-cttctaatgcctgacaatgagg-3', rev 5'-ggccagcgggtctaacctc-3'; P15: fw 5'-ggcttcctgtagtgtgaattttg3', rev 5'-gaactcttagacacaatatcc-3'; P20: fw 5'-ggtgtcagcccagaaggaga-3', rev 5'-ccagggctaccatgatgacagc-3'. PCR conditions are available on request.

PCR and Sequencing of the breakpoints

To sequence the breakpoints of the inversion we designed primers in the vicinity of the suspected breakpoints and deletions for PCR amplification. PCR conditions are available on request. The resulting PCR products were sequenced using Big Dye Terminator 3.1 (Applied Biosystems). Fragments were loaded on an ABI3100 sequence analyser and analyzed with DNA Sequencing Analysis (ver.3.7) and SeqScape (ver.2.1) software (Applied Biosystems). Primer sequences: 7p-breakpoint: fw 5'-gggctggtttcttcagggcacag-3', rev 5'-tgaaaatcccactgtgaacgc-3'; 7q-breakpoint: fw 5'-cgcacaaaccaggtggaagcc-3', rev 5'-ctcattgtcaggcattagaagc-3'; Δ200: fw 5'-gcacatcttgcaccgccc-3' rev 5'-caacctccacctcccagctg-3'; Δ773: fw 5'-ccataagacagagtactgaggg-3' rev 5'-taagtcttccagagagaaagaag-3'.

Real Time PCR cDNA analysis

RNA was isolated from Epstein–Barr virus (EBV)-transformed lymphoblastoid cell lines[10] of the patient and healthy controls using RNAbee (BioConnect, Huissen, the Netherlands) according to the manufacturers protocol. Single strand cDNA synthesis was performed with SuperScript III (Invitrogen, Breda, the Netherlands). Relative expression levels were measured using iQ SYBR Green

Supermix (BioRad) on a ABI7300 Real time Cycler (Applied Biosystems). Primers were designed with the aid of PrimerExpress 1.0 (Applied Biosystems) software in such a way that forward and reverse primer were located in different exons, to exclude influences of genomic DNA contamination on the real time results. Primer sequences: *NFE2L3*: fw1 5'-aaggcacccgcggaac-3', rev1 5'-atccacagcttcgtgcttttc-3'; fw2 5'-atgagagacatctgaatgggacag-3', rev2 5'-agatgccctccagtgaattttc-3'; *UBE3C*: fw 5'-gaagaaaggcgaaggttgaaaaa-3', rev 5'-tgcacttctttggatggaatattg-3'; *HNRNPA2B1*: fw 5'-gcttaagctttgaaaccacagaaga-3', rev 5'-tgcttgcaggatccctcatta-3'; *HOXA7*: fw 5'-cccgcttcctgtcaggtc-3', rev 5'-gctctgcagtgacctcgc-3'; *HOXA11*: fw 5'-cgtcttccggccacactga-3', rev 5'-ctagctcccggatctggtact-3'; *HOXA13*: fw 5'-caaatgtactgccccaaagagc-3', rev 5'-ataggagctggcatccgagg-3'; *SHH*: fw 5'-atgaagaaaacaccggagcg-3', rev 5'- tcatcaccgagatggccaaag-3'.

Results

The karyotype of the patient revealed two abnormalities: a duplication of the Y chromosome and an inversion on chromosome 7 (47,XYY,inv(7)(p15q36). Karyotyping of the parents revealed no abnormalities; the inversion of chromosome 7 is therefore *de novo.* The 47, XYY genotype is common (incidence 1:1000-1:500 in newborn males) and is not associated with any overt adverse phenotypes[11]. Therefore, the *de novo* inversion of chromosome 7 most likely causes the polysyndactyly seen in the patient. We aimed to determine the precise breakpoints of the inversion at DNA level. Different techniques were used, narrowing down the breakpoint areas with each step.

Based on the karyoptype we mapped the breakpoints using Fluorescent In Situ Hybridisation (FISH). Several BAC's were tested on 7p15 and 7q36 (data not shown). The breakpoints were first mapped to the regions covered by BAC's RP11-66O14 on the p-arm and RP11-51L24 on the q-arm (data not shown). The breakpoints were further fine-mapped with fosmids covering the BAC's. On the short arm of chromosome 7 the

breakpoint was mapped to the region between fosmid G248P81511A6 which stayed on the p-arm and fosmid G248P89547A12 which showed a signal on the q-arm of the inverted chromosome (figure 5.2c). The breakpoint on the q-arm was mapped to fosmid G248P89016G3, which gave a split signal on the inverted chromosome 7 (figure 5.2b). To exclude large genomic imbalances at the inversion breakpoints we performed a SNP array on DNA from the patient. The average probe spacing of the array is 10 kb at 7p15.2 and 15kb at 7q36.3. No deletions or duplications were found (figure 5.2a).

Within the regions on both arms indicated by the fosmids we designed regularly spaced probes for Southern blot analysis. On the q-arm probe Q40 showed different sized bands for 7 different restriction enzymes. Based on these restriction fragments the breakpoint on q was mapped to a 200 bp region (figure 5.2d and data not shown). Furthermore, the size of the aberrant fragments from the inverted chromosome 7 was used to determine the position of the breakpoint on the p-arm. On the p-arm probes P15 and P20 showed double bands with multiple restriction enzymes (figure 5.2d). The difference between the abnormal and the normal band size for probe P15 and P20 was the same for all restriction enzymes (approx. 200 bp and approx. 800 bp respectively) indicating the presence of small heterozygous deletions in the patient.

Next, we designed PCR primers around the 2 deleted regions and both breakpoints to determine their exact locations. On the q-arm, the breakpoint lies 20kb before the transcriptional start site of Ubiquitin Protein Ligase E3C (*UBE3C*) in a non-conserved region (figure 5.3). *UBE3C* is a "Homologous to E6AP C-Terminus" family member involved in substrate protein selection for ubiquitin ligation[12].

The deletion detected by probe P15 is a complex rearrangement of a highly repetitive region in intron 1 of *NFE2L3*, partially covering Variation_28613[13] resulting in the deletion of approximately 200 bp. The deletion detected by P20 is a 773bp deletion in intron 2 of *NFE2L3*. See figure 5.3 for an overview of the positions of the deletions in *NFE2L3*. Both deletions are not *de novo* as the father carries the

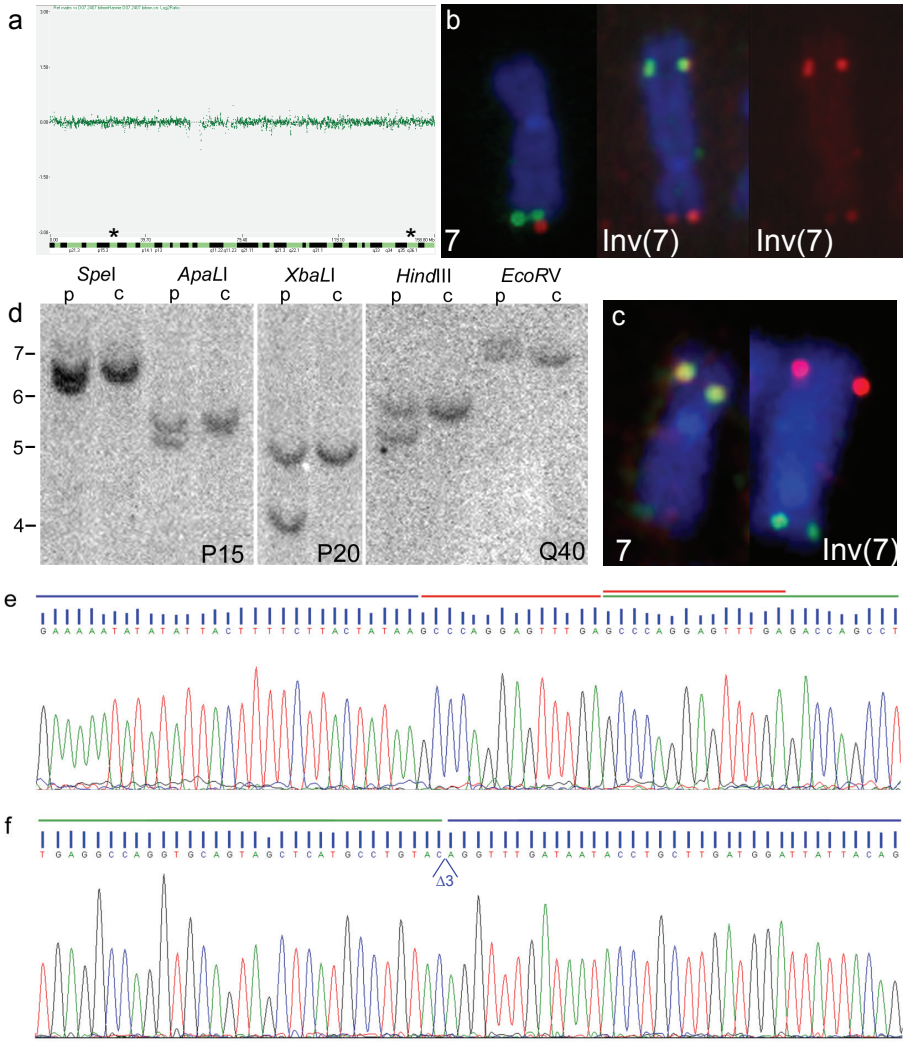


Figure 5.2 Detailed mapping of the inversion breakpoints: *a) Affymetrix SNP array results for chromosome 7 revealing no large deletions or duplications; breakpoints of the inversion are indicated by stars b) FISH analysis of the breakpoint on 7q showing G248P85855B2 (green signal swaps to 7p) and G248P89016G3 (red, split signal) c) FISH analysis of the breakpoint on 7p showing separation of fosmids G248P81811A6 (red stays on p) and G248P89547A12 (green, swaps to q) d) Southern blot analysis of probe P15, P20 and Q40 showing abnormal sized bands in the patient (p) and not in the control (c); e + f) sequence analysis of the breakpoints 7p (e, rev) and 7q (f, rev) green, blue and red line indicate sequence from 7p, 7q and duplicated sequence from 7p respectively.*

Δ773 deletion and the mother the Δ200 deletion (data not shown). Moreover both deletions involve highly repetitive intronic regions indicating it is highly unlikely that either deletion causes the polysyndactyly.

The breakpoint on the p-arm of Chromosome 7 lies within intron 1 of *NFE2L3*. The inversion of chromosome 7 in this patient leads therefore to the disruption of *NFE2L3* (figure 5.3). The sequences of the breakpoints revealed that the inversion is not completely balanced. On the p-arm a 14 nucleotide duplication of a repeat sequence was detected. On 7q the inversion has resulted in the deletion of 3 nucleotides (figure 5.3). It is unclear whether a relationship exists with either of the small deletions. The distance between the breakpoint and the deletions is too large to be able to determine whether the deletion is on the paternal or the maternal chromosome.

We tested the expression levels of the genes surrounding the breakpoints (*NFE2L3*, *HNRNPA2/B*1 and *UBE3C*) and of candidate genes in the vicinity: (Sonic Hedgehog (*SHH)* and genes in the *HOXA* cluster) in EBV transformed cell lines of the patient and controls. No changes in expression level were detected for *UBE3C*, *HNRNPA2B1* and *SHH* and the expression level of *NFE2L3* was reduced to 50% as expected (data not shown). The level of expression of *HOXA7, HOXA11* and *HOXA13* was too variable in all (control and patient) EBV cell lines to allow conclusions to be drawn (data not shown).

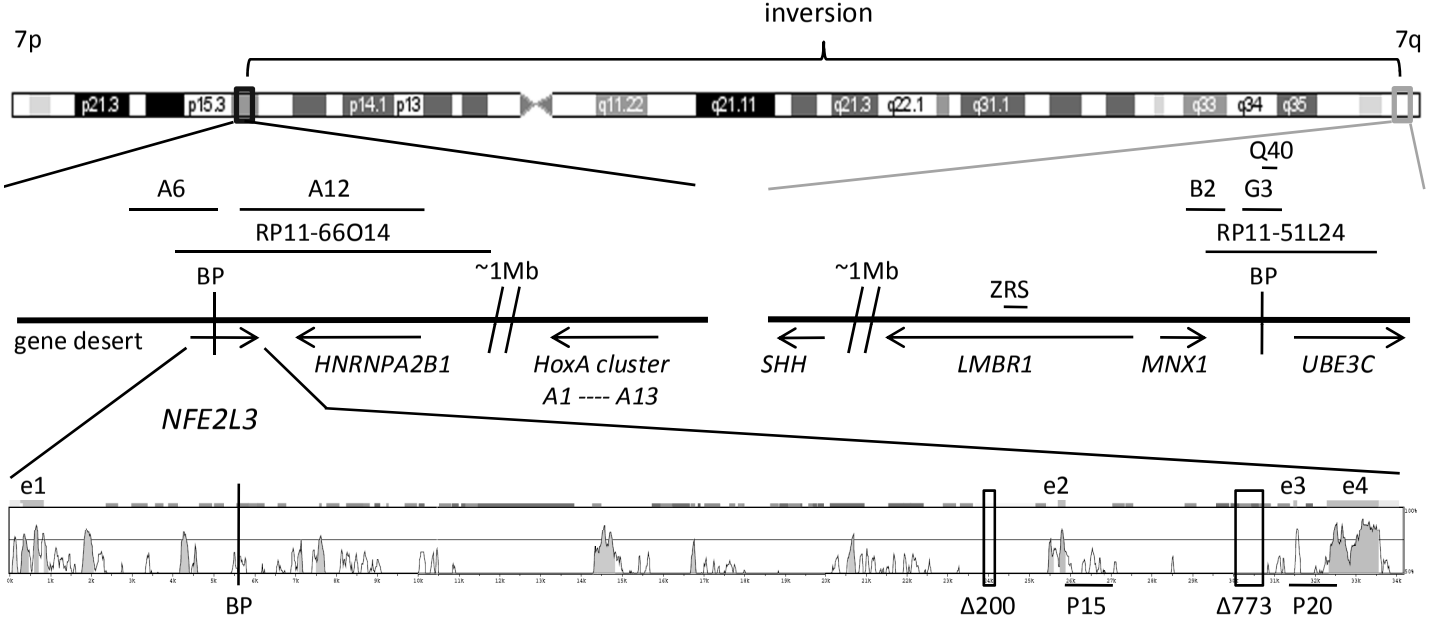


Figure 5.3 Overview of the inversion on chromosome 7*: From top to bottom: karyogram of chromosome 7 (based on the Ensembl genome browser http://www.ensembl.org/). Black rectangle: area of the breakpoint on 7p; grey rectangle: area of the breakpoint on 7q. Locations of the BAC's (RP11-66O14 and RP11-51L24) and fosmids A6 (G248P81811A6), A12 (G248P89547A12), B2(G248P85855B2) and G3 (G248P89016G3) and probe Q40 are indicated by lines. Genomic context (not to scale) of the breakpoints (BP), indicating the position of genes flanking the breakpoints and those of particular interest due to their role in limb development (ZRS: limb specific SHH cis-regulatory element). Homology plot of NFE2L3 (http://www.gsd.lbl.gov/vista/); stretches of homology >75% between human and mouse are indicated in grey. The line marks the position of the breakpoint, the rectangles indicate the deletions, e1-4 mark the positions of the exons and probes P15 and P20 are indicated by lines.*

Discussion

We determined the exact positions of a the breakpoints of a chromosome 7 inversion in a patient with polysyndactyly. These breakpoints disrupt only one gene: *NFE2L3*. *NFE2L3* is a member of the Cap'n' collar erythroid transcription factor family[5] and is suggested to have a role in the oxidative stress response[14,15]. During development, *NFE2L3* is expressed in mesodermal derivatives in avian embryo's; no expression of *NFE2L3* in the limb buds is detected[16]. Mice homozygous for a knockout allele of *NFE2L3* (deleting exon 3 & 4) lack a limb phenotype[17]. Therefore, the disruption of the *NFE2L3* gene by the inversion and the two inherited small intronic deletions are very unlikely to cause the limb phenotype observed in the patient.

As the phenotype could not be explained by the disruption of *NFE2L3* we hypothesized that disruption of a *cis*-regulatory circuit of a developmentally important gene could cause the phenotype. The p-breakpoint of the inversion lies between a gene desert containing several multispecies conserved non coding elements (figure 5.3) and the HoxA cluster. The homeobox transcription factors of the *HOXA* and *HOXD* cluster are known to play important roles during development. One of their functions is the patterning of the anterior to posterior axis of the limbs. Correct timing and position of expression is essential for the correct activation of downstream targets such as *SHH*, Fibroblast Growth Factors and Bone Morphogenetic Proteins[18]. Slight misregulation of the expression of the *HOX* genes can cause limb malformations, such as polydactyly [19,20].

The distance between the conserved non coding elements in the gene desert and the HoxA cluster ranges from 1-1.8 Mb. Although these distances are relatively large, regulatory elements that function over such distances have been described, especially in the context of developmentally specific enhancers[21-24]. Recent work by Duboule *et al.* has shown that the distance between the *HOXD* cluster and its regulatory elements is dependent upon the time in evolution when the expression pattern was acquired. Elements regulating the basic expression cascade along the

trunk are close to the cluster whereas elements regulating later expression such as in the limb are located further away[25]. How the expression of the HOXA cluster is regulated in the limb is thus far unknown. The inversion on chromosome 7 may suggest the presence of long distance regulatory elements of the HoxA cluster in the gene desert telomeric of *NFE2L3*.

In summary, we have mapped the breakpoints of a *de novo* inversion on chromosome 7 in a patient with postaxial polysyndacytyly. The breakpoints do not seem to disturb developmentally important genes. Therefore the most likely explanation of the polysyndactyly is the disruption of a *cis*-regulatory circuit of gene(s) that are involved in limb development. This may indicate the presence of a long distance regulatory element of the HoxA cluster within a gene desert telomeric of breakpoint on the short arm of chromosome 7. Future genetic analysis of polydactyly in both mice and human will be necessary to confirm the existence of such a regulatory element.

Acknowledgements

We thank the patient and his family for their cooperation, Wilfred van IJcken (Erasmus MC Department of Biomics) for microarray hybridisations. EdG is supported by a grant from the Dutch Scientific Organisation (NWO, 016.056.320)

References

1 Morel F, Laudier B, Guerif F *et al*: Meiotic segregation analysis in spermatozoa of pericentric inversion carriers using fluorescence in-situ hybridization. *Hum Reprod* 2007; **22**: 136-141.

2 Lybaek H, Oyen N, Fauske L, Houge G: A 2.1 Mb deletion adjacent but distal to a 14q21q23 paracentric inversion in a family with spherocytosis and severe learning difficulties. *Clin Genet* 2008; **74**: 553-559.

3 Castilla EE, Lugarinho R, da Graca Dutra M, Salgado LJ: Associated anomalies in individuals with polydactyly. *Am J Med Genet* 1998; **80**: 459-465.

4 Scherer SW, Cheung J, MacDonald JR *et al*: Human chromosome 7: DNA sequence and biology. *Science* 2003; **300**: 767-772.

5 Kobayashi A, Ito E, Toki T *et al*: Molecular cloning and functional characterization of a new Cap'n' collar family transcription factor Nrf3. *J Biol Chem* 1999; **274**: 6443-6452.

6 van den Berg C, Van Opstal D, Brandenburg H *et al*: Accuracy of abnormal karyotypes after the analysis of both short- and long-term culture of chorionic villi. *Prenat Diagn* 2000; **20**: 956-969.

7 Lisa G. Shaffer NT: ISCN 2005: An International System for Human Cytogenetic Nomenclature (2005). Basell, S. Karger, 2005.

8 Eussen BH, van de Laar I, Douben H *et al*: A familial inverted duplication 2q33-q34 identified and delineated by multiple cytogenetic techniques. *Eur J Med Genet* 2007; **50**: 112-119.

9 Sambrook J, Fritsch E, Maniatis T: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor NY, Cold Spring Harbor Laboratory Press, 1989.

10 Bliek JB, de Klein A, Luider TM *et al*: New approach for the identification of folate-related pathways in human embryogenesis. *Cell Mol Biol (Noisy-le-grand)* 2004; **50**: 939-944.

11 Rives N, Simeon N, Milazzo JP, Barthelemy C, Mace B: Meiotic segregation of sex chromosomes in mosaic and non-mosaic XYY males: case reports and review of the literature. *Int J Androl* 2003; **26**: 242-249.

12 Wang M, Cheng D, Peng J, Pickart CM: Molecular determinants of polyubiquitin

linkage selection by an HECT ubiquitin ligase. *Embo J* 2006; **25**: 1710-1719.

13 Levy S, Sutton G, Ng PC *et al*: The diploid genome sequence of an individual human. *PLoS Biol* 2007; **5**: e254.

14 Sankaranarayanan K, Jaiswal AK: Nrf3 negatively regulates antioxidant-response element-mediated expression and antioxidant induction of NAD(P)H:quinone oxidoreductase1 gene. *J Biol Chem* 2004; **279**: 50810-50817.

15 Chowdhury I, Mo Y, Gao L, Kazi A, Fisher AB, Feinstein SI: Oxidant stress stimulates expression of the human peroxiredoxin 6 gene by a transcriptional mechanism involving an antioxidant response element. *Free Radic Biol Med* 2008.

16 Etchevers HC: The cap 'n' collar family member NF-E2-related factor 3 (Nrf3) is expressed in mesodermal derivatives of the avian embryo. *Int J Dev Biol* 2005; **49**: 363-367.

17 Derjuga A, Gourley TS, Holm TM *et al*: Complexity of CNC transcription factors as revealed by gene targeting of the Nrf3 locus. *Mol Cell Biol* 2004; **24**: 3286-3294.

18 Zakany J, Duboule D: The role of Hox genes during vertebrate limb development. *Curr Opin Genet Dev* 2007; **17**: 359-366.

19 Montavon T, Le Garrec JF, Kerszberg M, Duboule D: Modeling Hox gene regulation in digits: reverse collinearity and the molecular origin of thumbness. *Genes Dev* 2008; **22**: 346-359.

20 Goodman FR: Limb malformations and the human HOX genes. *Am J Med Genet* 2002; **112**: 256-265.

21 Lettice LA, Heaney SJ, Purdie LA *et al*: A long-range Shh enhancer regulates expression in the developing limb and fin and is associated with preaxial polydactyly. *Hum Mol Genet* 2003; **12**: 1725-1735.

22 Bien-Willner GA, Stankiewicz P, Lupski JR: SOX9cre1, a cis-acting regulatory element located 1.1 Mb upstream of SOX9, mediates its enhancement through the SHH pathway. *Hum Mol Genet* 2007; **16**: 1143-1156.

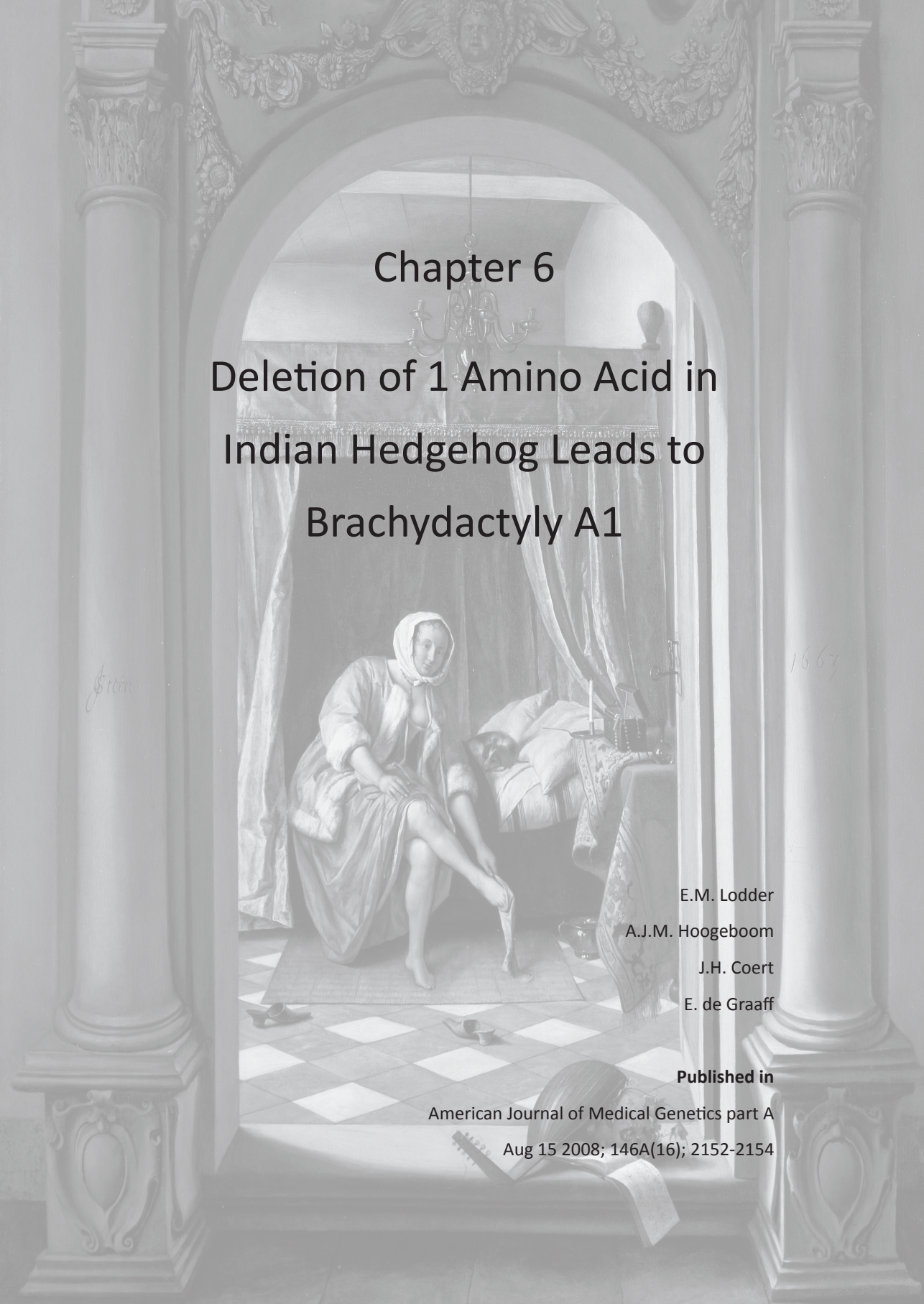
23 Jamieson RV, Perveen R, Kerr B *et al*: Domain disruption and mutation of the bZIP transcription factor, MAF,associated with cataract, ocular anterior segment dysgenesis and coloboma. *Hum. Mol. Genet.* 2002; **11**: 33-42.

24 Bagheri-Fam S, Barrionuevo F, Dohrmann U *et al*: Long-range upstream and downstream enhancers control distinct subsets of the complex spatiotemporal Sox9 expression pattern. *Dev Biol* 2006; **291**: 382-397.

25 Spitz F, Duboule D: Global control regions and regulatory landscapes in vertebrate development and evolution. *Adv Genet* 2008; **61**: 175-205.

Chapter 6

Deletion of 1 Amino Acid in Indian Hedgehog Leads to Brachydactyly A1



E.M. Lodder

A.J.M. Hoogeboom

J.H. Coert

E. de Graaff

Published in

American Journal of Medical Genetics part A

Aug 15 2008; 146A(16); 2152-2154

Abstract

Brachydactyly type A1 is a limb malformation characterized by a uniform shortening of the middle phalanges in all digits. Mutations in the Indian Hedgehog (*IHH*) gene were shown to be the cause of this autosomal dominant disorder. The IHH protein is known to be an important signaling molecule involved in chondrocyte formation. So far, only missense mutations in *IHH* have been reported to cause BrachydactylyA1. We report here on the first deletion in *IHH*, p.delE95*,* causing mild BrachydactylyA1 in a small Dutch family. This brings the total number of different mutations found to cause BDA1 to seven.

Keywords

Brachydactyly type A1

limb development

congenital malformation

Indian Hedgehog

Introduction

Brachydactyly type A1 (BDA1) is characterized by a uniform shortening of the middle phalanges in all digits with or without symphalangism. Additionally, short stature is often reported as a clinical feature in families with BDA1[1,2].

In 2001, Gao *et al*. showed that BDA1 is caused by mutations in the Indian hedgehog (*IHH*) gene[3]. The IHH protein is involved in chondrocyte formation during limb development. Thus far eight different missense mutations in *IHH* have been reported. Six of these mutations cause BDA1 and two cause Acrocapitofemoral Dysplasia[3-10].

All of the mutations in patients with BDA1 reported to date are missense mutations. Based on the previously described mutations we studied the genomic sequence of *IHH* in a Dutch family with mild brachydactyly.

Materials en methods

We studied three generations of a Dutch family with BDA1 (figure 6.1a). The project was approved by the local ethical authorities. DNA samples were isolated from peripheral blood (individuals I-3, II-1, II-2 and II-4), buccal swabs (I-2 and III-3), or saliva (III-1 and III-2), following standard protocols.

All three exons and intron-exon boundaries of *IHH* were amplified by PCR (primer sequences and PCR conditions are available on request). Direct sequencing of both strands of the PCR products was performed using Big Dye Terminator 3.1 (Applied Biosystems). Fragments were loaded on an ABI3100 sequence analyzer and analyzed with DNA Sequencing Analysis (ver.3.7) and SeqScape (ver.2.1) software (Applied Biosystems).

Clinical report

The proband (individual III-3, figure 6.1a) was the third child of non-consanguineous parents following a normal pregnancy and delivery. At the age of five months he was referred for congenital hand malformations with camptodactyly of the third fingers. On examination his weight was 6.2 kg (20th centile) and his length was 63 cm (25th centile), and his OFC was 42.2 cm (40th centile). There were no dysmorphic findings other than his hands. He had brachydactyly and ulnar deviation of all fingers with a rotational deformity of the second fingers. At re-examination at 2.5 years his middle finger lengths were left 3.3 cm and right 3.5 cm and his hand length was 9.5 cm in both hands (middle finger to hand length 34.7 % and 36.8 % respectively, well below the 3rd centile). No fusion of the phalanges was seen nor were the middle phalanges completely absent; therefore the BDA1 was judged to be mild. His feet were normal on external examination. Radiographs revealed shortened middle phalanges of the hands (figure 6.1b) and missing ossification nodes of the middle phalanges of both fourth toes.

The other affected members of the family also showed no dysmorphic findings other than their hands, the middle finger to hand ratios were all below the 3rd centile (I-3: 36.2%; II-4: 38.5%; III-1: 34.2%; III-2: 37.5%; III-3: 34.7%). Radiographs of the hands of patients II-4, III-1 and III-2 reveal shortened middle phalanges and shortened proximal phalanges in the thumb in III-2, a common feature in BDA1 (fig 1b). In contrast the middle finger to hand length ratios of the unaffected family members were within the 50th centile ((I-2: 42.4%; II-1: 42.9%; II-2: 41.7%). All family members including the non-affected were of short stature, but within the 95% confidence interval: their lengths fall within the lower 16th centile (I-1: 1.60m; I-2: 1.50m; II-1: 1.60m; II-2: 1.60m; II-3; 1.63m; II-4: 1.63m; III-1: 1.35m (age 11)), except patient III-2 whose length of 1.13m at the age of 8.5 years is below the 2nd centile.

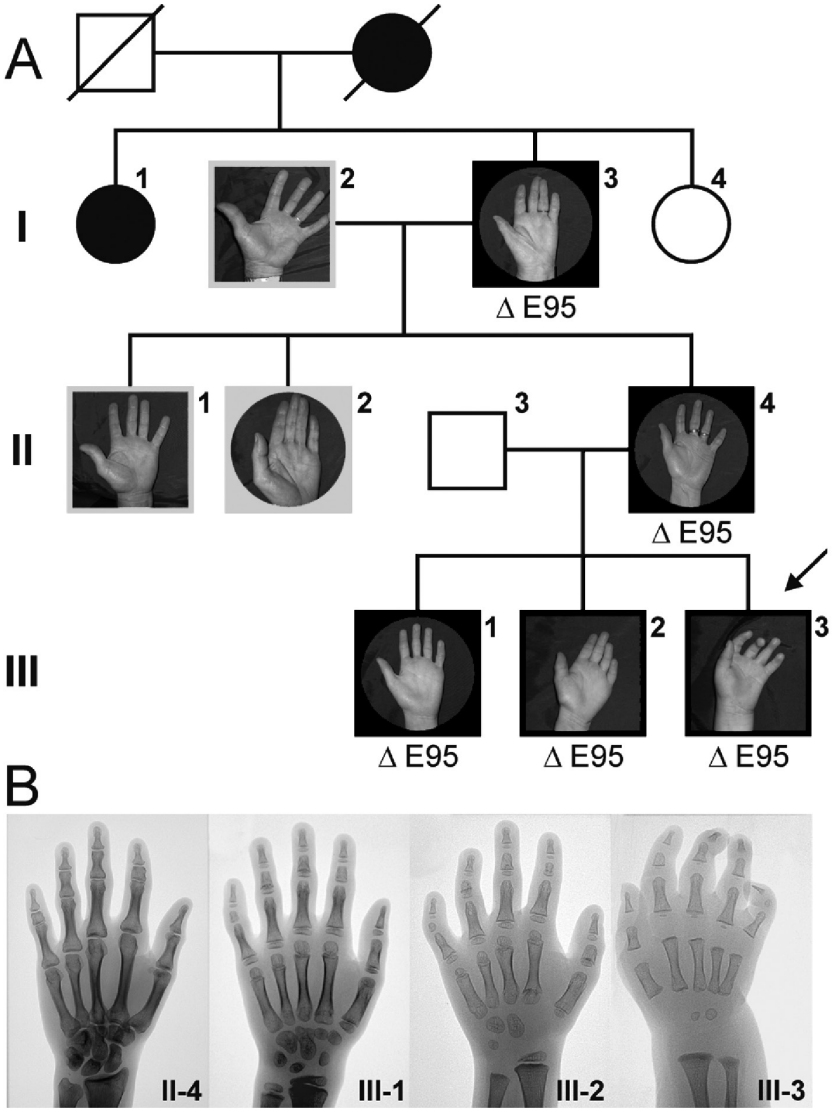


Figure 6.1 Pedigree structure and phenotype in a family with BDA1*: a) pedigree structure, the left hand of all investigated family members is shown, affected members indicated by dark grey lining; arrow indicating the proband. Note the shortness of the fingers relative to the length of the hand in the patients compared to the unaffected family members (I-2: 42.4%; I-3: 36.2%; II-1: 42.9%; II-2: 41.7%; II-4: 38.5%; III-1: 34.2%; III-2: 37.5%; III-3: 34.7%) b) x-ray radiographs of the hands of patient II-4 (left hand), patient III-1 (left hand), patient III-2 (left hand) and the proband (patient III-3 right hand) showing the shortened middle phalanges.*

Results and discussion

Sequencing of *IHH* in an affected family member showed a heterozygous change (c.283_285delGAG, which predicts p.del95E) (figure 6.2). Besides two known SNP's in exon 3 (rs3731881 & rs394452), no other coding variations were found. Sequence analysis in all family members revealed perfect segregation of c.283_285delGAG with BDA1. Again, no other coding variations were found.

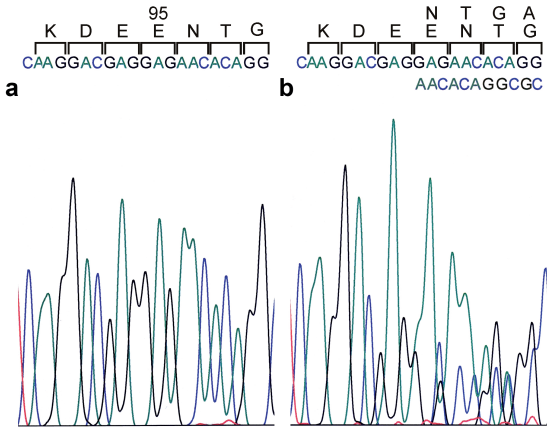


Figure 6.2

Sequence electropherograms *of normal (a) and mutated (b) IHH. Both DNA and amino acid sequences are shown. The heterozygous deletion of GAG at position 283-285 results in a one amino acid deletion (p.delE95).*

In order to predict a possible effect of this deletion on the structure of the IHH protein, we performed modeling of the IHH protein using Swissmodel (http://swissmodel.expasy.org/)[11]. The E95 residue was predicted to be located on the edge of a groove important for the interaction between IHH and its receptor Patched (PTC). Modeling of the normal IHH and two previously identified missense mutations (E95K and E95G) showed no difference between normal IHH and the known mutations. However, the E95 deletion predicted the loss of a loop on the edge of the groove (figure 6.3). This suggests that the deletion of this conserved amino acid is the cause of BDA1 in this family.

Thus far, seven mutations have been found to cause BDA1, involving only four amino acids, all of which are located at the predicted site of interaction between IHH and PTC. The two mutations causing Acrocapitofemoral Dysplasia are located far away from this groove on the opposite site of IHH[6]. The mildness of the phenotype in this

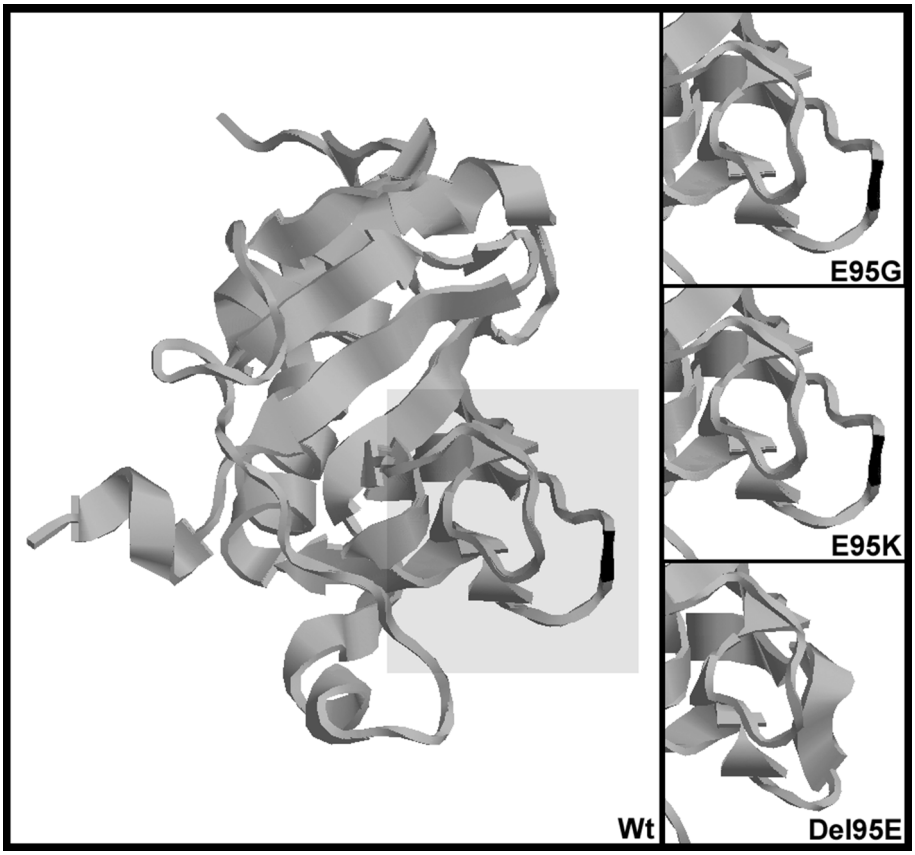


Figure 6.3 Protein models of IHH *made with Swissprot, wild type IHH (complete protein, gray square indicates the region of interest), detail of: the E95G mutation, the E95K mutation and del95E. Residue E95 is indicated in black. Note the loss of the fold in the model for delE95.*

family suggests that although the folding of the groove is altered, this does not lead to a complete loss of function of the IHH protein. In contrast, the E95K mutation causes a more severe phenotype with missing and fused phalanges[3], indicating that change of charge at this position influences the function of IHH more than the complete loss of the glutamic acid. However, since there is an incomplete understanding of the way IHH interacts with PTC it is difficult to judge why the two different mutations have a different effect[12] on the formation of the digits. Unfortunately no phenotypic details are available for the E95G mutation, since these could shed a broader light on the function of the IHH protein in digit formation[7,13].

Although clinodactyly and ulnar deviation have been associated with BDA1, to our knowledge, camptodactyly has not[1,14]. Present data do not allow us to determine whether this is an uncommon manifestation of BDA1 or coincidental. Due to the short stature of the unaffected family members it is impossible to draw conclusions about the coincidence of BDA1 with short stature in this family.

In conclusion, we report the first deletion in *IHH* leading to BDA1. This brings the total number of mutations found to cause BDA1 to seven. Three of these mutations involve the E95 residue, indicating the importance of this highly conserved amino acid for proper IHH signaling. We predict that more E95 mutations will be found in families with BDA1.

Acknowledgements

We thank the family for their cooperation and R. Koppenol for his work on the illustrations. This work was supported by a grant from the Nederlandse organisatie voor Wetenschappelijk Onderzoek (016.056.320).

References

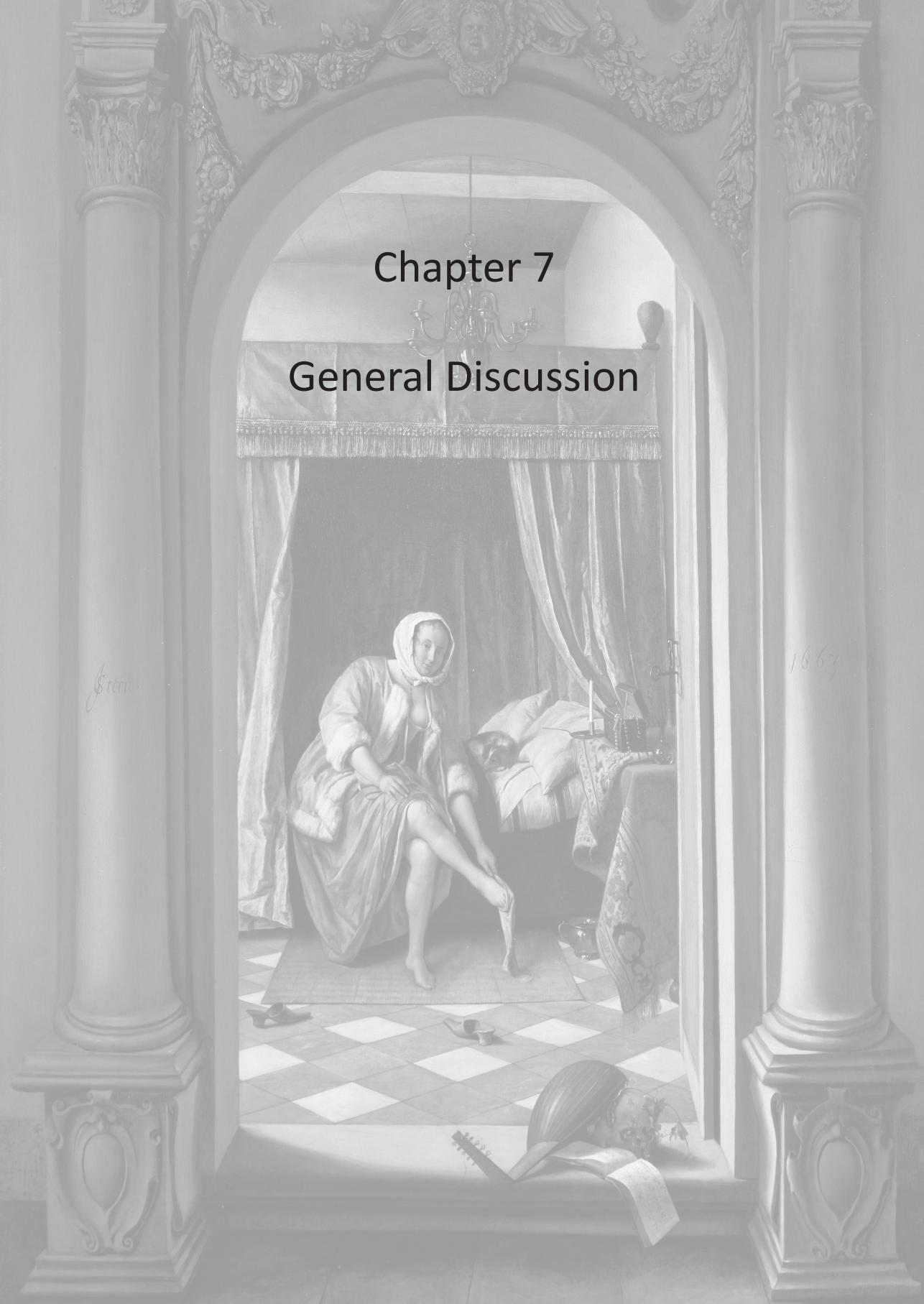
1. Fitch, N., *Classification and identification of inherited brachydactylies.* J Med Genet, 1979. **16**( ): p. 36-44.
2. Bell, J., *On brachydactyly and symphalangism.* Treasury of Human Inheritance, 1951. **Cambridge University Press**: p. 1-31.
3. Gao, B., J. Guo, C. She, A. Shu, M. Yang, Z. Tan, X. Yang, S. Guo, G. Feng, and L. He, *Mutations in IHH, encoding Indian hedgehog, cause brachydactyly type A-1.* Nat Genet, 2001. **28**(4): p. 386-8.
4. Gao, B. and L. He, *Answering a century old riddle: brachydactyly type A1.* Cell Res, 2004. **14**(3): p. 179-87.
5. Giordano, N.*, et al.*, *Mild brachydactyly type A1 maps to chromosome 2q35-q36 and is caused by a novel IHH mutation in a three generation family.* J Med Genet, 2003. **40**(2): p. 132-5.
6. Hellemans, J., P.J. Coucke, A. Giedion, A. De Paepe, P. Kramer, F. Beemer, and G.R. Mortier, *Homozygous mutations in IHH cause acrocapitofemoral dysplasia, an autosomal recessive disorder with cone-shaped epiphyses in hands and hips.* Am J Hum Genet, 2003. **72**(4): p. 1040-6.
7. Kirkpatrick, T.J., K.S. Au, J.M. Mastrobattista, M.E. McCready, D.E. Bulman, and H. Northrup, *Identification of a mutation in the Indian Hedgehog (IHH) gene causing brachydactyly type A1 and evidence for a third locus.* J Med Genet, 2003. **40**(1): p. 42-4.
8. Liu, M., X. Wang, Z. Cai, Z. Tang, K. Cao, B. Liang, X. Ren, J.Y. Liu, and Q.K. Wang, *A novel heterozygous mutation in the Indian hedgehog gene (IHH) is associated with brachydactyly type A1 in a Chinese family.* J Hum Genet, 2006. **51**(8): p. 727-31.
9. McCready, M.E., A. Grimsey, T. Styer, S.M. Nikkel, and D.E. Bulman, *A century later Farabee has his mutation.* Hum Genet, 2005. **117**(2-3): p. 285-7.
10. McCready, M.E., E. Sweeney, A.E. Fryer, D. Donnai, A. Baig, L. Racacho, M.L. Warman, A.G. Hunter, and D.E. Bulman, *A novel mutation in the IHH gene causes brachydactyly type A1: a 95-year-old mystery resolved.* Hum Genet, 2002. **111**(4-5): p. 368-75.
11. Arnold, K., L. Bordoli, J. Kopp, and T. Schwede, *The SWISS-MODEL workspace: a*

web-based environment for protein structure homology modelling. Bioinformatics, 2006. **22**(2): p. 195-201.

12. Visel, A., S. Minovitsky, I. Dubchak, and L.A. Pennacchio, *VISTA Enhancer Browser--a database of tissue-specific human enhancers.* Nucleic Acids Res, 2007. **35**(Database issue): p. D88-92.
13. Mastrobattista, J.M., P. Dolle, S.H. Blanton, and H. Northrup, *Evaluation of candidate genes for familial brachydactyly.* J Med Genet, 1995. **32**(11): p. 851-4.
14. Armour, C.M., M.E. McCready, A. Baig, A.G. Hunter, and D.E. Bulman, *A novel locus for brachydactyly type A1 on chromosome 5p13.3-p13.2.* J Med Genet, 2002. **39**(3): p. 186-8.

Chapter 7

General Discussion



General Discussion

The aim of the work presented in this thesis was to elucidate the molecular background of limb developmental defects. Different approaches were used to unravel (parts of) the processes that are responsible for the patterning of the limbs during development. Mutational analyses of patients with congenital limb malformations (brachydactyly and polydactyly (both pre- and postaxial)) identified novel mutations in: a known signalling molecule (*IHH* described in *chapter 6*), a known regulatory element (the long distance regulatory element of *Shh,* the ZRS, see *chapter 4*), and pointed towards a possible novel regulatory mechanism of limb developmental signalling molecules (the *HoxA* cluster, discussed in *chapter 5*). Besides these patient-based approaches, functional studies of the ZRS revealed a co-localisation with the promoter region of the *Shh* locus (*chapter 3*), providing further insight into how this regulatory element may exert its influence over a 1 Mb distance.

Genotype Phenotype Correlations

The search for candidate genes causing human disease/congenital malformations can generally be separated in two approaches depending on the family history of the patients. In large families with several affected individuals, linkage analysis can be applied to determine the genomic region carrying the disease causing alteration. In sporadic cases where no family history is available or the patient is the first in the family with the disease, far less options are available. Without visible chromosomal aberrations, the only available option is screening of known loci. Although this approach will (of course) never reveal new genes involved in the phenotype, it may help to improve our knowledge of how the known genes are functioning in limb development.

In *chapter 6* a novel mutation in a family with Brachydactyly type A1 (BDA1), a single amino acid deletion in *Indian Hedgehog (IHH),* is described. This mutation is the first deletion to be found in *IHH* giving interesting new information about the interaction between the IHH protein and its receptor Patched. All mutations in *IHH* that lead to BDA1 are located within the interaction domain of IHH that allows it to bind to Patched. Interestingly, the phenotype caused by this novel deletion is milder than a missense mutation of the same (negatively charged) amino acid, suggesting that the change of charge at this position has a more deleterious effect on the interaction than the deletion of the amino acid[1,2].

The results of screening for mutations in the ZRS in patients with diverse forms of pre-axial polydactyly resulted in the discovery of several new point mutations and two novel duplications of the ZRS (described in *chapter 4*). These novel mutations in combination with the mutations described in literature emphasise the complexity of this regulatory element, as it is thus far impossible to see a clear genotype-phenotype correlation. For example, the ZRS 295T>C mutation gives a very mild phenotype with incomplete penetrance in two families (UK3 and Dutch3). In contrast, the 404G>A/C mutation results in PPD and tibial dysplasia. The high phenotypic heterogeneity within and between individuals carrying the same mutation indicates that genetic modifiers and environmental influences play an essential role in the manifestation of the final phenotype.

The variation in phenotype between the different point mutations within the ZRS was suggested to be caused by differences in the induced level of ectopic Shh expression, as was found by reporter assays studying the effect of the point mutations on LacZ expression in transgenic mice[3]. The reason why one mutation would result in a higher level of ectopic *Shh* expression than the other is not clear. Most likely this is based on the affinity of transcription factors for the introduced binding site and/or the amount of loss of affinity for anterior transcription repressing factors. Future research will hopefully shed more light on this question when the protein

interaction partners of the ZRS are found. Thus far the only described interactions are with the *Hoxd10* and *Hoxd13* transcription factors: both were shown to activate a ZRS-luciferase reporter construct and to interact with the ZRS in E10.5 limb buds[4]. How these interactions are involved in the regulation of *Shh* remains to be investigated.

The presence of large chromosomal aberrations in some patients is very useful in the search for genetic defects causing limb malformations. In *chapter 5* a *de novo* inversion of chromosome 7 in a patient with post-axial polydactyly is characterised. No likely candidate genes are interrupted at either breakpoint of the inversion, most likely indicating the disruption of a cis-regulatory transcriptional control circuit. On 7p the inversion disrupts *NFE2L3*, a gene that is not expressed in the developing limb bud in chicken and of which targeted deletions do not give a phenotype in mice[5,6]; on 7q no genes are interrupted. Both breakpoints are situated approximately 1-2 Mb away from genes known to be involved in limb development. On the short arm of chromosome 7 the breakpoint separates the *HoxA* cluster from a gene desert containing several conserved non-coding sequences. On the long arm the breakpoint lies 1.5 Mb upstream of *Shh*. The inversion therefore does not separate *Shh* from the ZRS but it could separate it from thus far unknown other regulatory elements. This option, however, is less likely as the synteny between the human and mouse chromosome is disrupted close to the inversion breakpoint. The region between the inversion breakpoint on 7q and the loss of synteny does not contain likely candidate conserved non-coding sequences (http://www.ensembl.org/)[7]. Hence, the most likely option is that the *HoxA* cluster is separated from a regulatory element within the gene desert upstream of *NFE2L3,* thereby disrupting transcriptional control during limb development. Furthermore, both the *HoxA* cluster and *Shh* may be influenced by regulatory sequences normally involved in regulating the other gene(s).

Transcriptional (long distance) regulation

The regulation of transcription over long distances can be mediated through the formation of DNA loops bringing regulatory sequences in close proximity to their target genes. As described in *chapter 3* this is the case for *Shh* and the ZRS. Surprisingly, the looping is not dependent on the presence of the ZRS, suggesting the presence of more long distance elements involved in the regulation of *Shh* expression. A recent paper by Amano and colleagues[8] confirms these results in most respects. However, at first glance some of their conclusions seem to be at odds with the model based on our results. In order to evaluate both studies, more information about the techniques used is essential.

DNA looping, measurements

Fluorescent In Situ Hybridisation

Different forms of Fluorescent In Situ Hybridization (FISH) are essential for visualization of the nuclear localisation of genomic loci. Normal FISH suffers from a lack of resolution due to the limitations of normal light fluorescent microscopy and fixation techniques that destroy the nuclear architecture. Different strategies to overcome these limitations have been described in literature. For all FISH techniques probe size is an important variable: the smaller the probe the higher the resolution but the lower the signal strength. Generally, Bacterial Artificial Chromomes (BACs) (on average 200 kb) or Fosmids (~50 kb) are used but smaller probes have been applied successfully.

Three-dimensional FISH (3D-FISH) aims to preserve nuclear structure as much as possible through fast fixation and mild permeabilisation, followed by confocal microscopy[9]. Unfortunately, the amount of time required for analysis of one nucleus limits the amount of loci that can be analysed and the resolution in the z-plane of the acquired 3D image is still low (~400 nm). Furthermore, although cells are fixed with paraformaldehyde, structural changes of the nucleus occur and local chromatin

organisation may be compromised[9,10].

Cryo-FISH[10] overcomes the low resolution in the z-plane of confocal microscopy with sectioning of the nuclei into thin (< 200 nm) slices separating any signals that are further apart in the z-plane into different sections (see fig 7-1). As the nuclei are sectioned, permeability of the nucleus to the FISH probes is no longer an issue and stronger fixation can be used. This results in a better preservation of the nuclear architecture. This can be observed clearly as in the sections the nuclei have preserved their irregular appearance (see figure 3-1) in contrast to the spherical nuclei observed in 3D-FISH. Furthermore, cryo-FISH allows the visualisation of nuclear architecture in cells in their normal context as the entire tissue is fixed instead of the single cell suspensions needed for 3D-FISH.

Chromosome Conformation Capture

Molecular evidence of direct interaction fo two genomic loci can be obtained through Chromosome Conformation Capture or 3C (for an overview of the technique see Simonis *et al.* 2007[11]). In short, this technique uses cross-linking agents such as paraformaldehyde to link any protein DNA complex to any other complex that happens to be in the vicinity at the time of cross-linking. Relative cross-linking frequency (as measured by qPCR with primers on both the target and the tested DNA) is used as a measure of the average distance between two loci at the time of crosslinking. Normal 3C will only pick up relatively frequent interactions as others will be lost against the background of random cross-linking of non-interacting DNA fragments. Control experiments are therefore essential for any 3C experiment[12].

Long distance interactions of the ZRS with Shh

In *chapter 3* we describe the measurement of co-localisation between *Shh* and its long distance regulatory element the ZRS with cryo-FISH. In the developing limb the ZRS and *Shh* co-localise in 65% percent of the loci. A control probe the same

distance from the ZRS as *Shh* but in the opposite direction co-localises in only 35% of the loci with the ZRS. This same amount of background co-localisation was found in E14.5 fetal liver, a tissue that does not express *Shh* and is therefore a good negative control. Our results show that this co-localisation is independent of the ZRS itself, as it is present in limbs of mice homozygous for a targeted deletion of the ZRS. These results are confirmed by the recently published study of Amano *et al.*[8] in which the co-localisation of the ZRS with *Shh* is measured by 3D-FISH.

Our cryo-FISH experiments do not show a difference between the different areas of the limb bud, but a homogenous distribution of co-localisation throughout the limb bud. In contrast, Amano *et al.* show a significant difference between the *Shh* expressing Zone of Polarising Activity (ZPA) and the middle of the limb bud (which remains significant after correcting for the subdivision in distance categories below the resolution of a confocal microscope). This difference most likely indicates the presence of a less constrained chromosomal loop in the central part of the limb bud that is lost during the preparation for 3D-FISH and preserved in the cryo-FISH. Amano *et al.* allow the limb bud cells to grow in a single cell suspension for half an hour at 37°C before fixation for 3D-FISH. As normal biological context and cell-cell interactions are lost under these conditions, it is highly likely that DNA-DNA interactions that are not extremely tight will be lost. This does fit the model we propose in *chapter 3* (figure 3-6) in which a general interaction between the ZRS and vicinity of the *Shh* gene is present in the entire limb bud. This poised state allows the formation of an even closer interaction in the ZPA and the anterior margin of the limb bud, which may be essential for *Shh* expression. It is this closer chromatin loop that is most likely the interaction measured by Amano *et al.* Our experiments reveal both the less tight interactions in the entire limb bud and the interaction in the ZPA and anterior margin. This could also explain why we do not find a difference between the interaction frequency at E10.5 and E13.5, while the interaction observed by Amano *et al.* is lost by E12.5 (at this stage the anterior margin of the limb bud is still capable

of expressing *Shh* as seen in the limb buds of *Hx* mice[13]).

The existence of interactions in the entire limb bud is further corroborated by the fivefold increase in cross-linking frequency between the ZRS and *Shh* in the entire limb bud found in a 3C experiment by Amano *et al.*. This increase in cross-linking frequency would not be possible if the interaction would only exist in cells from the ZPA and the anterior margin, as the signal would be lost against the background of the rest of the limb bud. This is especially so since only a small number of ZPA cells at any given time transcribe *Shh,* as shown by pre-mRNA FISH results in the same paper[8].

Interestingly, no interaction between *Shh* and a forebrain specific enhancer SBE4[14] was detected in E10.5 forebrain[8]. Either this regulatory region functions without need for interaction with the *Shh* promoter, or the signal is not picked up against the background of non-interacting cells in the forebrain, as SBE4 only directs expression in a small portion of forebrain cells.

A final interesting observation in this paper is the localisation of the *Shh* gene relative to its chromosome territory (CT). *Shh* is looped out of the chromosome 5 territory in only a portion of the ZPA cells, consistent with an intermittent expression from each *Shh* locus resulting in relocalistion relative to the CT. However, the absence of this relocalisation relative to the CT in $ZRS^{-/-}$ limb buds almost certainly does not reflect a necessity of the ZRS for this relocalisation as suggested by the authors. Based on results in other studies[15,16] it is far more likely that the relocalisation relative to the CT reflects chromatin decondensation upon active transcription rather than active relocalisation of the locus to a “transription factory” in which the ZRS would play a role.

In *chapter 3* the roles of different parts of the ZRS are examined using targeted deletions of different regions. Based on the results of reporter assays in which (parts of) the ZRS were placed before a β-globin minimal promoter and the LacZ reporter gene, a targeting construct was made that allowed the deletion of two halves of the

ZRS *in vivo* using either cre- or flp recombinase. Unexpectedly, both halves turned out to be essential for *in vivo Shh* activation. This contrasts to the situation in the reporter constructs in which the first half of the ZRS was capable of driving reporter expression in transgenic mice[17]. This result can be due to promoter efficiency as the LacZ reporter expression also continues expression for a short period after the *Shh* expression has ceased. The other more interesting option is that the 2nd half of the ZRS is dispensable only when directly in front of the target gene and is essential for expression initiation in the normal genomic context where 1 Mb separates the ZRS from *Shh.*

Concluding remarks

All studies described in this thesis not only add to our knowledge about normal limb development and how this delicate process may go awry, they all add additional layers of complexity to the current models of how cells adopt their different cell fates during the pattering off the limbs. How all these processes interact will be an interesting topic for future research.

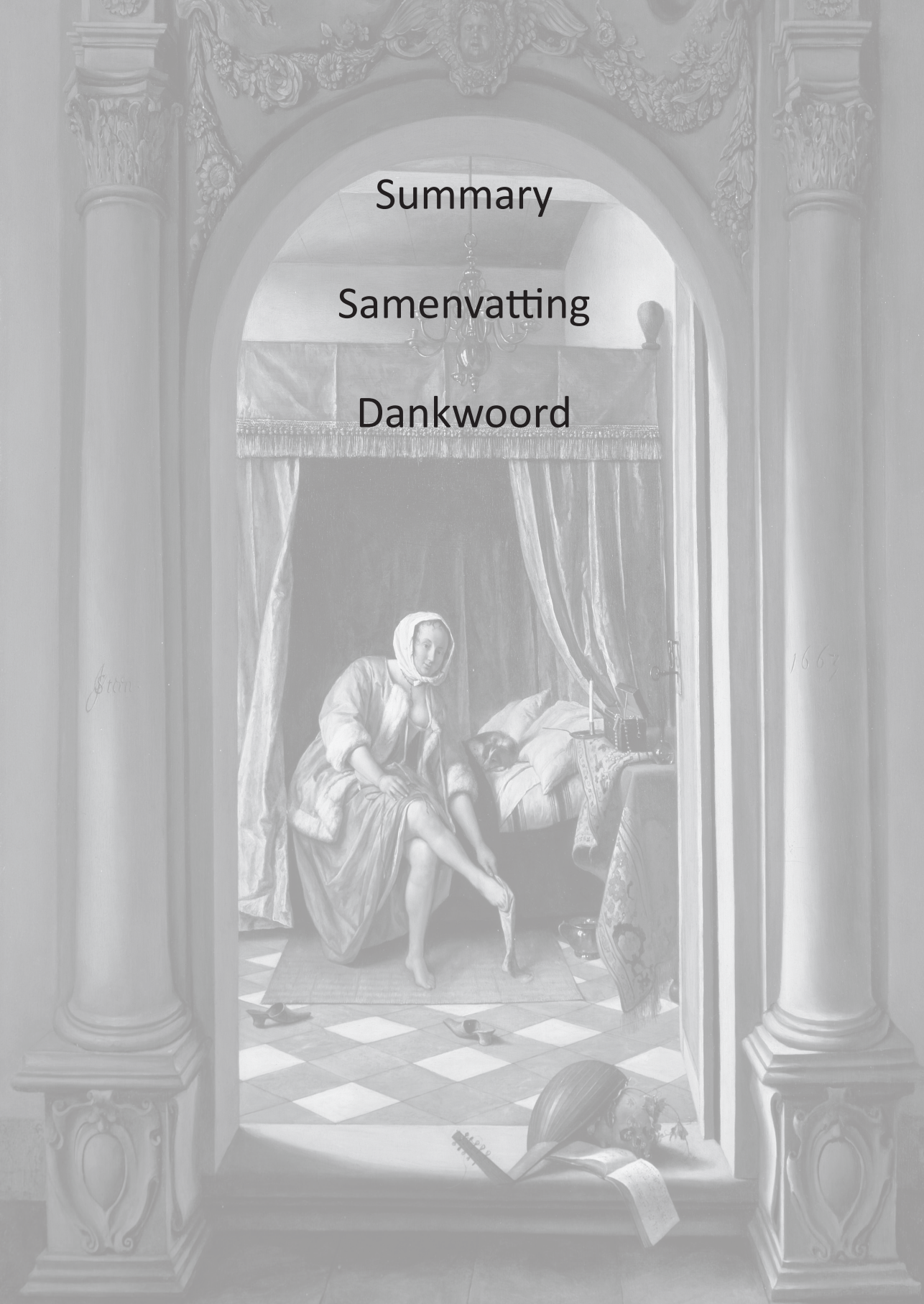
It would be especially interesting to find the transcription factors that bind to the ZRS, and to see if and how their interaction is changed when the ZRS is mutated. Studying the looping of the ZRS towards *Shh* in the different parts of the limb bud and mapping the different conformational states of this transcriptional complex, may become feasible in the near future with techniques like 3C requiring less material. Last but not least exploring the genetic defects of patients with unexplained phenotypes involving the ZRS in more detail (with techniques unavailable at the time of their original analysis) possible may turn out to be very revealing.

References

1. Kirkpatrick, T.J., K.S. Au, J.M. Mastrobattista, M.E. McCready, D.E. Bulman, and H. Northrup, *Identification of a mutation in the Indian Hedgehog (IHH) gene causing brachydactyly type A1 and evidence for a third locus.* J Med Genet, 2003. **40**(1): p. 42-44.
2. Gao, B., J. Guo, C. She, A. Shu, M. Yang, Z. Tan, X. Yang, S. Guo, G. Feng, and L. He, *Mutations in IHH, encoding Indian hedgehog, cause brachydactyly type A-1.* Nat Genet, 2001. **28**(4): p. 386-388.
3. Furniss, D., L.A. Lettice, I.B. Taylor, P.S. Critchley, H. Giele, R.E. Hill, and A.O. Wilkie, *A variant in the sonic hedgehog regulatory sequence (ZRS) is associated with triphalangeal thumb and deregulates expression in the developing limb.* Hum Mol Genet, 2008. **17**(16): p. 2417-2423.
4. Capellini, T.D., G. Di Giacomo, V. Salsi, A. Brendolan, E. Ferretti, D. Srivastava, V. Zappavigna, and L. Selleri, *Pbx1/Pbx2 requirement for distal limb patterning is mediated by the hierarchical control of Hox gene spatial distribution and Shh expression.* Development, 2006. **133**(11): p. 2263-2273.
5. Derjuga, A., T.S. Gourley, T.M. Holm, H.H. Heng, R.A. Shivdasani, R. Ahmed, N.C. Andrews, and V. Blank, *Complexity of CNC transcription factors as revealed by gene targeting of the Nrf3 locus.* Mol Cell Biol, 2004. **24**(8): p. 3286-3294.
6. Etchevers, H.C., *The cap 'n' collar family member NF-E2-related factor 3 (Nrf3) is expressed in mesodermal derivatives of the avian embryo.* Int J Dev Biol, 2005. **49**(2-3): p. 363-367.
7. Hubbard, T.J.*, et al.*, *Ensembl 2009.* Nucleic Acids Res, 2009. **37**(Database issue): p. D690-697.
8. Amano, T., T. Sagai, H. Tanabe, Y. Mizushina, H. Nakazawa, and T. Shiroishi, *Chromosomal Dynamics at the Shh Locus: Limb Bud-Specific Differential Regulation of Competence and Active Transcription.* Dev Cell, 2009.
9. Solovei, I., A. Cavallo, L. Schermelleh, F. Jaunin, C. Scasselati, D. Cmarko, C. Cremer, S. Fakan, and T. Cremer, *Spatial preservation of nuclear chromatin architecture during three-dimensional fluorescence in situ hybridization (3D-FISH).* Exp Cell Res, 2002.

276(1): p. 10-23.

10. Branco, M.R. and A. Pombo, *Intermingling of chromosome territories in interphase suggests role in translocations and transcription-dependent associations.* PLoS Biol, 2006. **4**(5): p. e138.
11. Simonis, M., J. Kooren, and W. de Laat, *An evaluation of 3C-based methods to capture DNA interactions.* Nat Methods, 2007. **4**(11): p. 895-901.
12. Dekker, J., *The three 'C' s of chromosome conformation capture: controls, controls, controls.* Nat Methods, 2006. **3**(1): p. 17-21.
13. Blanc, I., A. Bach, and B. Robert, *Unusual pattern of Sonic hedgehog expression in the polydactylous mouse mutant Hemimelic extra-toes.* Int J Dev Biol, 2002. **46**(7): p. 969-974.
14. Jeong, Y., K. El-Jaick, E. Roessler, M. Muenke, and D.J. Epstein, *A functional screen for sonic hedgehog regulatory elements across a 1 Mb interval identifies long-range ventral forebrain enhancers.* Development, 2007. **133**(4): p. 761-772.
15. Ragoczy, T., M.A. Bender, A. Telling, R. Byron, and M. Groudine, *The locus control region is required for association of the murine beta-globin locus with engaged transcription factories during erythroid maturation.* Genes Dev, 2006. **20**(11): p. 1447-1457.
16. Morey, C., N.R. Da Silva, P. Perry, and W.A. Bickmore, *Nuclear reorganisation and chromatin decondensation are conserved, but distinct, mechanisms linked to Hox gene activation.* Development, 2007. **134**(5): p. 909-919.
17. Lettice, L.A., S.J. Heaney, L.A. Purdie, L. Li, P. de Beer, B.A. Oostra, D. Goode, G. Elgar, R.E. Hill, and E. de Graaff, *A long-range Shh enhancer regulates expression in the developing limb and fin and is associated with preaxial polydactyly.* Hum Mol Genet, 2003. **12**(14): p. 1725-1735.



Summary

Samenvatting

Dankwoord

Summary

During the development of a single fertilised egg into a complete human body or animal, it is essential for cells to learn their position within the body. Some cells will become skin, others muscle and again others bone or one of the many other cell types. Furthermore, it is important that growth does not occur at the same rate in all directions so shapes can appear such as a head and legs, instead of a large sphere of cells. For this, a growing embryo needs information about the axes of the body: from head to tail, from front to back and from left to right. Each cell in the body needs to "know" its position relative to the rest of the cells.

During the development of the limbs, the same applies. An arm or leg has three axes as well: from close to the body wall to the tips of the fingers or toes, from the back of the hand to the hand palm, and from the thumb to the little finger. If you want to know how these axes develop, studying the development of limbs has many advantages. Arms and legs are not essential for initial survival. In contrast, when something is wrong with, for example, the development of the head an embryo will most likely die. For this reason, limb development has been used as a model system for pattern formation for a long time. A detailed description of limb development is given in chapter 1. For the correct patterning of the axes it is essential that the genes in the cell nucleus are turned on at the correct time and place. In chapter 2 an overview of the processes involved in the turning on and off of genes is given.

A large part of the work described in this theses concerns Sonic Hedgehog. During the development of the limbs, the protein Sonic Hedgehog is produced only on the future little finger side of the limb. Sonic Hedgehog is essential to make sure that fingers develop on this side of the hand, while on the thumb side where no Sonic Hedgehog is available a thumb will form. The restriction of Sonic Hedgehog to the little finger side of the limb is regulated by a piece of DNA named the ZRS. This ZRS controls the expression of the gene that is essential for the production of the Sonic Hedgehog protein. In chapter 4 a couple of families are described in which small

errors (mutations) in the ZRS have been found. The consequence of these mutations is that Sonic Hedgehog is now not only produced on the little finger side of the limb but on the future thumb side as well. As a result, the hands (and sometimes feet) of the affected individuals show abnormalities on the thumb side. Depending on the mutation these abnormalities range from an additional phalange in the thumb to complete extra digits.

In the DNA the ZRS is located very far away from the gene coding for Sonic Hedgehog, much further than most other known regulatory areas in the DNA. An important question is therefore: how does the ZRS control whether this gene is turned on or off over such a large distance? You can picture the DNA as a long thin thread containing the genes. Because this thread is so long, it needs to be folded to fit inside the nucleus of a cell. Due to this folding it is possible that the ZRS comes into close proximity of the Sonic Hedgehog gene. In this way, it could theoretically come close enough to control whether the gene is turned on or off. Our measurements described in chapter 3 confirm that this is indeed the case. The ZRS folds towards the Sonic Hedgehog gene specifically in the developing limbs.

Besides the work on the ZRS, this thesis describes the analysis of a patient with additional digits on both hands and feet (chapter 5) and of a family with short fingers (chapter 6). The patient described in chapter 5 has a chromosomal inversion. A chromosomal inversion occurs when the DNA breaks at two positions and is glued back together with the middle part put in backwards. In this patient, both breakpoints have been mapped exactly. Surprisingly, neither breakpoint disrupts a gene that could explain the additional fingers of the patient. It is therefore most likely that due to the inversion a gene has become separated from a regulatory region in the DNA. This would cause this gene to be turned on or off at the wrong time and or position, which could lead to the additional fingers of the patient.

In the family with short fingers described in chapter 6 we tested whether we could find a mutation in a known gene (Indian Hedgehog). In the literature this gene

has already been described to be involved in the development of short digits. In the family described in chapter 6 we found a new mutation in this gene that causes their hand malformation. Thus far, in all families described only small alterations of the gene were present. In this family however, a small part of the gene was missing. This missing piece of DNA overlaps the position of two other known mutations. This is an indication of the importance of this part of the protein.

All chapters of this thesis describe small steps to learn more about the development of the limbs, summarised and discussed in chapter 7. These steps involve research on the genetic background of limb malformations in patients (chapters 4, 5, and 6), besides the more fundamental research into how the found mutations cause the axes of the limb to be wrongly defined (chapter 3).

Samenvatting

Tijdens de ontwikkeling van een enkele bevruchte eicel tot een compleet mens of dier is het van belang dat cellen hun positie in het lichaam leren kennen. Sommige moeten huidcellen worden, andere spier, weer andere bot of een van de vele andere celtypen. Daarnaast is het belangrijk dat niet in iedere richting even veel groei plaats vindt zodat er vormen kunnen ontstaan zoals een hoofd en benen, in plaats van een grote bol van cellen. Een groeiend embryo heeft hiervoor informatie nodig over de assen van het lichaam: van kop naar staart, van voor naar achter en van links naar rechts. Iedere cel in het lichaam moet “weten” waar hij zich bevindt ten opzichte van alle andere cellen.

Ook tijdens de ontwikkeling van de ledematen is dit nodig. Een arm of been heeft ook drie assen: van dichtbij het lichaam tot de puntjes van de vingers, van de handpalm naar de rug van de hand en van de duim naar de pink. Als je wilt onderzoeken hoe deze assen ontstaan heeft het onderzoek naar ledematen voordelen ten opzichte van het onderzoek naar de grote lichaamsassen. Armen en benen zijn in eerste instantie niet essentieel om te blijven leven. Terwijl als er iets mis gaat met de ontwikkeling van bijvoorbeeld een hoofd, een embryo meestal niet levensvatbaar is. Om deze reden zijn armen en benen al heel lang in gebruik als modelsysteem voor het onderzoek naar het ontstaan van assen tijdens de ontwikkeling. Een gedetailleerde beschrijving van de ledemaatontwikkeling staat beschreven in hoofdstuk 1. Voor het vormen van de assen is het van groot belang dat de genen in de celkern op het juiste moment aan en uitgeschakeld worden. In hoofdstuk 2 is een overzicht gegeven van de bekende processen die een rol spelen bij dit aan- en uitschakelen.

Een groot deel van het werk beschreven in dit proefschrift gaat over het eiwit Sonic Hedgehog. Tijdens de ontwikkeling van de handen/voeten wordt Sonic Hedgehog alleen gemaakt aan de kant van de hand waar later de pink/kleine teen ontstaat. Sonic Hedgehog zorgt er voor dat de vingers aan de pinkkant van de hand vingers worden. Aan de duimkant is het niet aanwezig, hierdoor wordt de duim een

duim en geen vinger. Dat Sonic Hedgehog alleen aan de pinkkant wordt gemaakt wordt geregeld door een stukje DNA dat de ZRS is genoemd. Deze ZRS zorgt ervoor dat het gen dat nodig is om dit eiwit te maken alleen aan staat aan de toekomstige pinkkant. In hoofdstuk 4 worden een aantal families beschreven waarin kleine foutjes (mutaties) in de ZRS zijn gevonden. Deze mutaties leiden ertoe dat Sonic Hedgehog niet alleen aan de pinkkant wordt gemaakt maar ook aan de duimkant. Dit heeft als gevolg dat de handen van deze mensen afwijkingen hebben aan de duimkant van de hand. Afhankelijk van de mutatie verschilt dit van extra kootjes in de duim tot complete extra vingers.

De ZRS ligt in het DNA heel erg ver van het gen voor Sonic Hedgehog gen vandaan, veel verder dan de meeste andere bekende regelstukken in het DNA. Een belangrijke vraag voor mijn onderzoek was dan ook: hoe regelt de ZRS, over zo'n lange afstand, of het gen voor Sonic Hedgehog aan of uit staat? En hoe leiden de hierboven beschreven mutaties beschreven tot het extra aanzetten van Sonic Hedgehog? DNA kun je zien als een lange dunne sliert waarin de genen liggen opgeslagen. Omdat DNA zo lang is, ligt het opgevouwen in de kern van de cel. Door deze vouwing is het mogelijk dat de ZRS naar het gen voor Sonic Hedgehog vouwt. Op deze manier zou de ZRS dicht genoeg in de buurt van Sonic Hedgehog kunnen komen om het aan dan wel uit te zetten. Uit onze metingen beschreven in hoofdstuk 3 blijkt dat dit inderdaad het geval is. De ZRS vouwt specifiek in de ontwikkelende ledematen naar het gen voor Sonic Hedgehog en zet het op die manier op de juiste plaatsen aan en uit.

Naast het werk aan de ZRS beschrijf ik in dit proefschrift onderzoek naar een patiënt met extra vingers aan beide handen en voeten (hoofdstuk 5) en een familie met korte vingers (hoofdstuk 6). De patiënt beschreven in hoofdstuk 5 heeft een inversie van een chromosoom. Dat betekent dat zijn DNA op twee plaatsen gebroken is waarna het middelste stuk er achterstevoren weer tussen is geplakt. Na onderzoek hebben we de exacte locaties van de twee breukpunten kunnen bepalen. Onverwacht valt geen van de twee breukpunten in een gen waarvan het uitschakelen zou kunnen

verklaren waarom de patiënt extra vingers heeft. We denken daarom dat in deze patiënt door de inversie een gen gescheiden is van een stuk DNA dat regelt wanneer het aan dan wel uit moet staan. Hierdoor zal dit gen op het verkeerde moment aan of uit gaan wat kan leiden tot de handafwijking van de patiënt.

In de familie met de korte vingers die wordt beschreven in hoofdstuk 6 hebben we gekeken of we een mutatie konden vinden in een aan Sonic hedgehog verwant gen: Indian Hedgehog. In andere families waren al foutjes in dit gen gevonden die de verkorte vingers veroorzaakten. In de familie van hoofdstuk 6 hebben we een nieuwe mutatie gevonden die in deze familie de korte vingers veroorzaakt. In alle families die tot nu toe waren beschreven bestond de mutatie uit een kleine verandering in het DNA. In de familie in hoofdstuk 6 ontbreekt een klein stukje van het DNA op exact dezelfde plek waar in 2 andere families veranderingen waren gevonden. Dit wijst erop dat exact dit deel van het eiwit noodzakelijk is voor het goed functioneren van dit eiwit.

Alle hoofdstukken van dit proefschrift beschrijven kleine stapjes om meer te weten te komen over de ontwikkeling van ledematen, samengevat en bediscusieerd in de discussie (hoofdstuk 7). Aan de hand van patienten met aangeboren afwijkingen van de armen of benen (hoofdstukken 4, 5 en 6). Daarnaast, ook op basis van functionele studies, om te achterhalen hoe de mutaties gevonden in patiënten leiden tot het verkeerd definieren van een van de assen in de ledematen (hoofdstuk 3).

Curriculum Vitae

Personalia

Name:	Elisabeth Mathilde Lodder
Date/place of birth:	October 31, 1977, Utrecht, the Netherlands

Employment

2004 – 2009	PhD student, dep. Clinical Genetics, Erasmus MC Rotterdam, promotor: Prof. dr. B.A. Oostra; co-promotor: dr. E. de Graaff : Keeping *Sonic Hedgehog* under the thumb, genetic regulation of limb development
2001 – 2003	IT Professional, PinkRoccade IT Management

Education

1996 - 2000	University of Utrecht: Biology, doctorandus (MSc) **Six months research project (2):** University College London, MRC Laboratory for Molecular Cell Biology,: Mapping of the binding site of glyt1E/F on the intracellular loop of the GABA-C receptor subunit r1. (Prof. dr. S. Moss and Prof. dr. A. Verkleij) **Literature study:** University of Utrecht dep. of theoretical biology Homeotic changes and the evolution of novel bodyplans. (Prof. dr. P. Hogeweg) **Six months research project (1):** Hubrecht laboratory of the KNAW, Utrecht: Hox gene regulation in Xenopus laevis. (dr. R. Morgan and Prof. dr. A. J. Durston)
1996 - 1998	University of Utrecht: History, propedeuse (Bachelor)
1990 - 1996	Herman Jordan Lyceum Zeist, VWO

Awards

2009	NVHG Annual Award 2009

List of Publications

Felix, J.F., A.A. de Munck, E.M. de Jong, **E.M. Lodder**, S. Swagemakers, R.R. de Krijger, P.J. van der Spek, D. Tibboel, A. de Klein and R.J. Rottier (2007). *Gene-expression analysis of tracheo-oesophageal fistulas. Aetiological Studies* in Oesophageal Atresia/ Tracheo-Oesophageal Fistula Acombined genetic and environmental approach: PhD Thesis. J. F. Felix. Rotterdam: 87-113.

van Gils, W., **E. M. Lodder**, H. W. Mensink, E. Kilic, N. C. Naus, H. T. Bruggenwirth, W. van IJcken, D. Paridaens, G. P. Luyten and A. de Klein (2008). *Gene expression profiling in uveal melanoma: two regions on 3p related to prognosis.* Invest Ophthalmol Vis Sci 49(10): 4254-4262.

Kilic, E., W. van Gils, **E. Lodder**, H. B. Beverloo, M. E. van Til, C. M. Mooy, D. Paridaens, A. de Klein and G. P. Luyten (2006). *Clinical and cytogenetic analyses in uveal melanoma.* Invest Ophthalmol Vis Sci 47(9): 3703-3707.

Lodder, E. M., A. J. Hoogeboom, J. H. Coert and E. de Graaff (2008). *Deletion of 1 amino acid in Indian hedgehog leads to brachydactylyA1.* Am J Med Genet A 146A(16): 2152-2154.

Lodder, E.M., L.A. Lettice, M. Hoogeveen-Westerveld, B.A. Oostra, F.M. Kilanowski, R.E. Hill, and E. de Graaff (2009) *Co-localisation of the ZRS with the Sonic Hedgehog locus in the developing limb bud* Submitted

Lodder, E.M., B.H. Eussen, D.A.C.M. van Hassel, A.J.M. Hoogeboom, P.J. Poddighe, J.H. Coert, B.A. Oostra, A. de Klein, E. de Graaff (2009) *Postaxial Polysyndactyly in a patient with inv(7)* Submitted

PhD Portfolio

Name PhD student: Elisabeth Lodder Erasmus MC Department: Clinical Genetics Research School: MGC	PhD period: 2004-2009 Promotor: Prof.Dr. B.A. Oostra Supervisor: Dr. E. de Graaff

1. PhD training	**Year**	**Hours**
General academic skills		
- Biomedical English Writing and Communication	2007	80
- Laboratory animal science	2005	120
- Post-Doc retraite, Heeze, the Netherlands	2008	24
Research skills		
- Biomedical research techniques	2004	40
- Courses in Practical DNA Microarray Analysis, Berlin	2004	40
- Clinical methods for Data-analysis	2004	160
- Transgenesis and gene targeting	2005	40
- Experimental approach to molecular and cell biology	2005	40
- From development to disease	2005	24
International conferences		
- Third-sixth Winter Schools of the International Graduiertenkolleg, "Transcriptional control in developmental processes" Kleinwalsertal, Austria (oral presentations each year)	2005-2008	200
- Chromatin Mediated Biological Decisions, Marburg Germany (poster)	2006	24
- Designing the Bodyplan: Developmental Mechanisms, Leiden, the Netherlands (poster)	2007	40
- 10th international conference on limb development and regeneration, Madrid, Spain (oral presentation)	2008	40
Seminars and workshops		
- PhD workshops: Leuven, Luik, Oxford, Maastricht (oral presentation in 2007, other years poster)	2004-2007	120
2. Teaching activities		
Supervising practicals		
- Haplotype analysis (teaching + supervision of practical)	2005-2008	60 60
- Virtual genecloning (supervision of practical)		
Lectures		
- Molecular basis of limb development (for MSc students)	2006	4

Dankwoord

Vijf jaar lang heb ik hard gewerkt om tot dit moment te komen, mijn boekje is af! Dat mijn AIO tijd een hele leuke gezellige en zeer leerzame tijd was heb ik te danken aan de vele mensen met wie ik heb samengewerkt, of die op de achtergrond altijd in waren voor gezelligheid, spelletjes of gewoon een pot thee op de bank. Al deze mensen wil ik hier bedanken hoewel ik bang ben dat dat niet gaat lukken: de plek en mijn geheugen zijn nu eenmaal niet toereikend. Bij deze dus mijn excuses aan hen die ik per ongeluk onvermeld laat.

Esther, al vanaf ons eerste gesprek was het hartstikke gezellig. In de afgelopen vijf jaar hebben we samen met Marianne heel erg veel werk verzet, ook al was dat helaas niet altijd met het resultaat waar we op hoopten. Jouw oneindige doorzettingsvermogen en vertrouwen dat alles uiteindelijk op zijn pootjes terecht zou komen hielpen mij door de zwaardere momenten heen. Ik wil je bedanken voor al je kennis, gezelligheid en de vele gesprekken over van alles en nog wat.

Daarnaast natuurlijk mijn dank aan Ben voor mijn plekje op de afdeling klinische genetica. Maar ook voor het meedenken en de opbouwende hulp bij het uitwerken van mijn wilde plan voor een nieuwe techniek. Ik was ook erg blij met de mogelijkheid om, naast mijn eigen project, data-analyses voor anderen op de afdeling te kunnen doen. Hiervoor wil ik ook Peter van der Spek hartelijk bedanken. De eerste periode van mijn AIO tijd heb ik bij jou op de afdeling veel geleerd.

Alle leden van mijn (grote en kleine) commissie wil ik van harte bedanken voor de tijd die ze stoppen in het lezen en beoordelen van mijn boekje. Elaine, bedankt voor de discussies over: “staat hier eigenlijk wel wat ik wil zeggen?”; Sjaak, de vier jaar “Winterschule” in Kleinwalsertal waren erg leuk, erg fijn dat je het ieder jaar opnieuw wilt regelen! Professor Hovius fijn dat U de tijd heeft genomen om in de kleine commissie plaats te nemen en mijn boekje te beoordelen. Annelies, bedankt voor alle mensen die je naar me toestuurde met hun data. Wouter en Jacqueline wat leuk dat jullie naar Rotterdam komen voor mijn promotie.

Marianne en Paulien, voor mij stond al heel lang vast dat ik dat ik jullie als paranimfen wilde. Zoveel als jij in die drie dagen per week voor elkaar krijgt Marianne blijft me verbazen. Zonder jou was ons handengroepje wel heel klein. Paulien, al bijna 30 jaar kennen we elkaar nu, je hebt mijn complete opleiding meegemaakt, van peuterspeelzaal tot nu. Ik ben heel erg blij dat jullie me terzijde willen staan tijdens mijn verdediging!

Niets van wat in dit boekje staat was mogelijk geweest zonder de medewerking van de families en patienten die in mijn boekje en in die van mijn voorgangers beschreven worden, heel erg hartelijk dank daarvoor! Jeannette Hoogeboom, heel veel dank voor het vinden van de families, de huisbezoeken, het meedenken en de gezellige discussies.

Uit de uiteindelijk FISH resultaten die in dit boekje staan valt niet meer af te leiden hoeveel werk is gaan zitten in het uitzoeken en optimaliseren van de juiste techniek voor ons specifieke model. Heel veel mensen hebben hieraan een bijdrage geleverd: Berna Beverloo, An Langeveld, Ellen van Drunen, Mariette van de Corput, Petra Klous, Rob Willemsen, Gert van Capellen: heel erg hartelijk bedankt hiervoor! Daarnaast wil ik ook graag Bert Eussen en Hannie Douben bedanken, voor onder andere de SNP arrays.

Emine, Janine, Walter, Edwin en Femke bedankt voor jullie vertrouwen in mij, om jullie data uit handen te geven zodat ik ermee aan de slag kon. Jeannette, bedankt voor alle kleine en grote regeldingen en de gezellige praatjes bij de koffie. Pim, Leo, Mario, Sjozef bedankt voor alle eerste hulp bij computerongelukken en het vele malen herinstalleren van mijn (nog steeds instabiele) pc. Ook wil ik de mensen van het EDC bedanken voor hun goede zorgen, die we ons boven alleen realiseren als het een keertje wel misgaat.

De dagelijkse routine op het lab: het lunchen met de hele groep, het meezingen met de radio van de meer en minder getalenteerden onder ons, de rondjes koffie (ondanks de nieuwe automaat ken ik nog steeds de nummers van de meeste uit

mijn hoofd), ik zal het gaan missen. Guido bedankt voor alle primers, discussies over real-time PCR en je vrolijke gemopper op AIOs zoals ik. Ook de rest van de mensen aan "de andere kant van de muur" Bianca, Erik, Christan, Doro, Marina, Francesca, Tianna en Isa, bedankt voor de gezelligheid en de aandacht tijdens de werkbesprekingen. De FraX groep bij wie we de laatste tijd zaten Edwin, Ingeborg, Renate, Christina, Ronald, Josien en Femke ook jullie wil ik bedanken voor de gezelligheid, de nuttige en minder nuttige discussies (Herma en Judith!). Met z'n allen hebben jullie gezorgd dat ik altijd met plezier naar het lab kwam.

Ook al heb ik jullie de laatste jaren minder gezien dan me lief was, zonder familie en vrienden zou ik dit niet gekund hebben. JWG-ers, die ik intussen alleen maar zie zonder dat er een telescoop of deelnemer in de buurt is: Petra, Toineke, sterkte de komende maanden op naar de 31ste oktober... (Hugo ben je nu eigenlijk wel lid of niet?), Laurens, Cristel, Marcel H en Marcel V, Joyce. Eindelijk meer tijd voor spelletjes, 't Stichtse en andere gezelligheid. Simone, bedankt voor de vele potten thee met chocola dat blijven we erin houden!

Mijn "nieuwe" familie in Asten en Tilburg dankjewel voor de interesse en het iedere keer maar weer luisteren naar mijn verhalen over extra teentjes en vingers. Hopelijk kunnen jullie de Nederlandse samenvatting een beetje volgen, maar ik wil het gerust nog een keer uitleggen. Harry dankjewel voor je mooie hand!

Toke en Jan bedankt voor alle steun de afgelopen jaren, dat er nog maar vele mooie mogen volgen! Quirijn, heel veel plezier en geluk samen met Marcella. Hopelijk zullen er nog vele spelletjes avonden komen (citroentje!).

HAI Frank! Dankjewel, voor al je avonden die je actief in dit boekje hebt gestopt: tellen, opmaken, programmeren, maar nog heel veel meer voor alle avonden en dagen waarop je dat niet deed.

Liefs,

E.