Screening for Novel Genes Contributing to the Transformed Status of Alveolar Rhabdomyosarcoma (ARMS)

by

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Onderzoek naar nieuwe genen die bijdragen aan de getransformeerde staat van Alveolaire Rhabdomyosarcoma (ARMS)

Thesis

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Ai miei nonni…

Contents:

Chapter 1

Scope of the thesis.

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Alveolar rhabdomyosarcoma (ARMS) is the second most common subtype of rhabdomyosarcoma, accounting for approximately 20% of cases [1]. ARMS is associated with the chromosomal translocations t(2;13)(q35;q14) and t(1;13)(p36;q14) resulting in the fusion proteins PAX3-FOXO1 and PAX7-FOXO1, respectively. The molecular mechanisms by which PAX3-FOXO1 contribute to ARMS formation remains to be fully determined [2]. However the data reported in this thesis demonstrate the functional interaction of novel ARMS associated genes with PAX3-FOXO1 and their contribution to the development of ARMS. The research performed in our laboratory is focused on defining the molecular biology behind PAX3-FOXO1-induced tumorigenesis. Published and unpublished data from this laboratory shows that expression of PAX3-FOXO1 alone is not sufficient for the transformation of primary mouse myoblasts (Lagutina, Pritchard and Grosveld, unpublished data) [3]. Abrogation of both the p53 and Rb pathways is required in order to allow PAX3-FOXO1 driven transformation (Lagutina, Pritchard and Grosveld, unpublished data,) [4,5]. My research is focused on the identification of these PAX3-FOXO1-complementing secondary events using a cDNA library screen. To this end, chapter 3 describes the identification of a novel gene named IRIZIO and its cooperation with PAX3-FOXO1 in transforming Arf-/- myoblasts *in vivo*. Similarly, chapter 4 illustrates the effect of lysil hydroxylase 1 (PLOD1) and its tumorigenic capacity when over expressed together with PAX3-FOXO1 in myogenic cells. Together, these results contribute to a better understanding of the mechanisms by which PAX3-FOXO1 and its cooperating proteins mediate myogenic cell transformation. Hopefully our findings will eventually contribute to improve the treatment of ARMS.

Chapter 2

Introduction.

2.1. Initial Characterization of RMS.

Rhabdomyosarcoma (RMS) consists of a group of pediatric soft tissue tumors related to the striated muscle lineage [6]. The name rhabdomyosarcoma is derived from the Greek word “rhabdo”, which means rod- or twig-shape and “myo” meaning muscle, which describes the histological appearance of this tumor. The first description of RMS was made by Weber in 1854, who described a localized enlargement of the tongue of a 21-year-old man, made up of striated muscle cells in all stages of differentiation [7]. Almost 100 years later Stout et al [8] recognized and described the unique morphology of rhabdomyosarcoma presenting a series of tumor samples

> *“made up of cells which have some of the characteristics of rhabdomyoblasts... The rhabdomyoblastic tumor cell is very variable in size and it assumes one of three different shapes. It is either rounded, strap-shaped or racquet-shaped… The tumor cells are sometimes vacuolated and are called spider or spider-web cells”*.

RMS is the most common soft tissue sarcoma in children with an annual incidence of seven per million children aged 15 years or younger [9,10].

2.2. Pathology of RMS.

RMS belongs to the group of small blue round cell tumors (abbreviated SBRCT). These tumors typically consist of undifferentiated cells and they are more typically seen in children than adults. Histopathological analysis of rhabdomyosarcoma is aimed at identifying the features of the skeletal muscle lineage using various techniques. Light microscopy and electron microscopy are employed for the identification of typical skeletal muscle features such as cross striations or macromere-related structures, including actins-myosin filaments and Z-bands. Immunohistochemistry is often used to confirm RMS diagnosis through the identification of skeletal muscle specific markers such as mood, myogenin and desman [6,11,12,13].

According to the International Classification of Rhabdomyosarcoma (ICR), RMS can be subdivided as follows: (i) alveolar rhabdomyosarcoma (ARMS)

and (ii) undifferentiated sarcoma, both characterized by a poorer prognosis, (iii) embryonal rhabdomyosarcoma (ERMS), typically having an intermediate prognosis and (iv) botrytis and spindle cell rhabdomyosarcoma (often considered a subset of ERMS) having a superior prognosis [6,14]. Alveolar rhabdomyosarcoma is generally characterized by dense aggregates of small, undifferentiated round cells that stain intensely with eosin. These cell aggregates are typically separated by fibrous septa, forming a structure resembling pulmonary alveoli (Fig 1). ARMS can also present without alveolar septation, representing the alveolar solid variant [15]. Undifferentiated sarcoma does not show any evidence of myogenesis or other differentiation. It typically lacks any defining cytologic or architectural features; cells are large, round with moderate cytoplasm [16,17]. Histologically ERMS is characterized by a heterogeneous cell population consisting of small round or spindle cells with hyperchromatic nuclei and/or of large tumor cells with abundant eosinophilic cytoplasm [18] (Fig. 2). Tumor cells can show all stages of myogenic differentiation from undifferentiated mesenchymal cells to elongated myoblasts, multinucleated my tubes, and fully differentiated myofibers [19]. The embryonal spindle cell variant (found in the paratesticular region and the head and neck), shows a stoma-rich appearance with elongated spindle-shaped cells. The botrytis variant appears as a polyploidy mass of dense tumor cells arising underneath the epithelial surface exclusively from the vagina and/or bladder [6,20]. Overall, ERMS and ARMS are considered the two major histological subtypes of RMS. ERMS accounts for over half of new RMS cases and affects patients of younger age, while ARMS is more commonly found in adolescents and accounts for 20-30% of cases [6,10].

RMS is believed to arise from primitive mesenchymal cells committed to the myogenic lineage [19], and can arise in any part of the body [6]. The first three intergroup rhabdomyosarcoma studies (IRS) report that 35% of all RMS tumors arise in the head (mainly orbit and parameningeal) or neck region, about 24% in the genitourinary system (bladder and prostate, paratesticular, vagina and uterus), 19% in the extremities and 22% in other sites

[6,21,22,23,24]. Generally RMS presents with a high rate of metastasis, with the alveolar variant presenting the most unfavorable outcome. The two most common sites of metastasis of RMS are the lungs (40% to 50% of the cases) and bone marrow (20% to 30%); less common sites are the bone (10%) and lymph node (up to 20 % depending on primary tumor location) [25,26,27]. Rarely, metastasis can be found in visceral organs (e. g. brain or liver) and generally in terminal-stage patients [6,28].

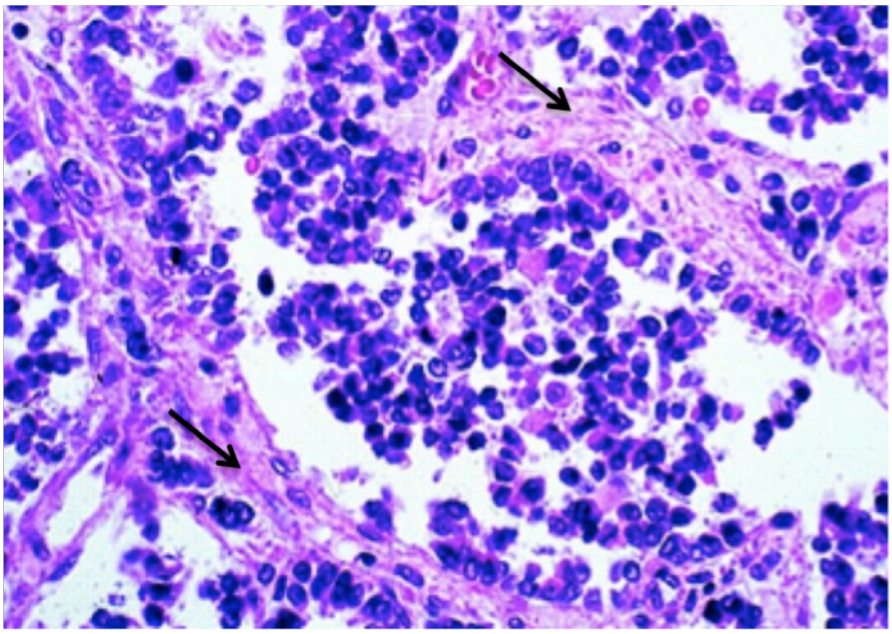

Figure 1: Hematoxylin and eosin staining of an ARMS section. The figure clearly shows small round cells with an intense blue-stained nucleus. Cell aggregates are generally separated by fibrous septae (black arrows). The figure was adapted from [29].

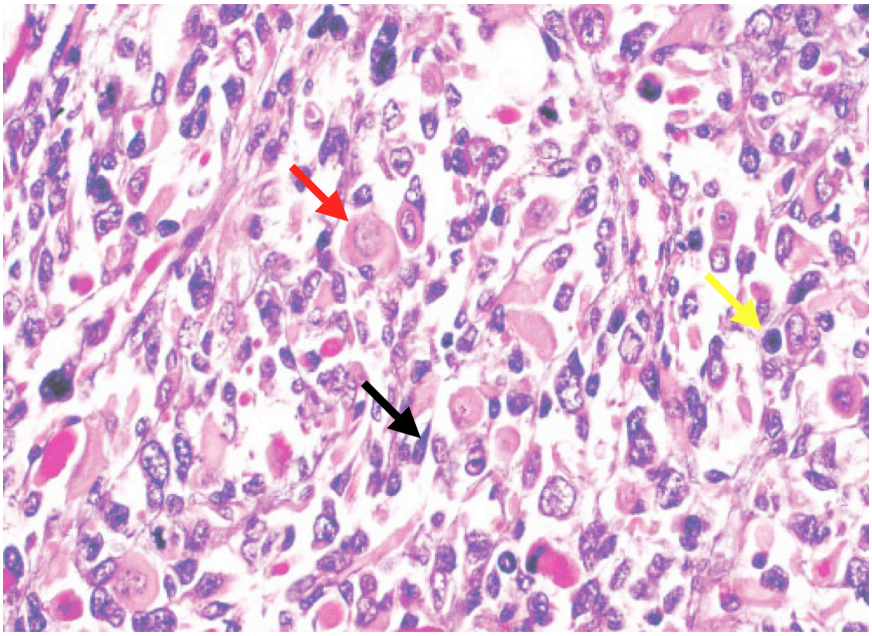

Figure 2: Hematoxylin and eosin staining of an ERMS section. ERMS cells represent different stages of skeletal muscle differentiation. Poorly differentiated cells are generally hyperchromatic round- (yellow arrow) or spindle-shaped (black arrows). More differentiated cells are larger with eosinophilic cytoplasm (red arrow). The image was adapted from [24].

2.3. Molecular biology of RMS.

Both ARMS and ERMS have typical, but distinct, genetic alterations. The ERMS subtype is characterized by loss of heterozygosity (LOH) at the short arm of chromosome 11 (11p15) [30]. Previous reports have shown that complete or partial LOH of this region can lead to the formation of various cancers such as breast, ovary, bladder, lung, testicles and Wilms' tumor [31]. The 11p15 locus harbors the insulin-like growth factor 2 (*IGF2*) gene that in mammals is maternally imprinted. IGF2 is a crucial fetal growth factor that binds the insulin-like growth factor 1 receptor (IGF-1R) [32]. Signaling through the IGF-1R has been associated with tumorigenesis and inhibition of apoptosis [33]. LOH with paternal dismay can lead to IGF2 over expression a phenomenon shown to be important for ERMS occurrence [34]. Interestingly, Anderson et al, [32] have shown that loss of imprinting (LOI) of the IGF2 gene has been detected in ARMS primary tumors, also resulting in higher IGF2 expression.

The molecular signature that most often characterizes the ARMS variant, involves the chromosomal translocations t(2;13)(q35;q14) or t(1;13)(p36;q14). Rarely, some ARMS cases presented the alternative chromosomal translocations t(X;2)(q13;q35), t(2;2)(p23;q35) and t(2;8)(q35;q13), which generate the fusion transcripts *PAX3-AFX, PAX3-NCOA1* and *PAX3-NCOA2,* respectively [35,36,37]. The majority of alveolar tumors express the *PAX3-FOXO1* fusion gene generated from the t(2;13) translocation. This aberrant event involves the fusion between the paired box family member *PAX3* located on chromosome 2 and the member of the fork-head family of transcription factors *FOXO1,* located on chromosome 13. A smaller portion of ARMS tumors present the variant translocation t(1;13), creating a fusion between *PAX7,* and *FOXO1,* encoding the chimeric protein PAX7-FOXO1 [38]. *PAX7* is the closest homolog of *PAX3* having a similar genetic locus organization and a similar, though distinct, skeletal muscle specific expression pattern [38,39,40]. In both cases, the break point occurs within the seventh intron of the PAX genes (17.5 kb in *PAX3* and 32 kb in *PAX7)* and the large first intron

of *FOXO1* (140kb), resulting in chimeric proteins possessing the N-terminal DNA binding domains (paired box and homeodomain) of PAX3 or PAX7 fused to the C-terminal potent transactivation domain of FOXO1 [38] (Fig.3). PAX3-FOXO1 can no longer bind to FOXO1 target genes as the forehead DNA binding domain is disrupted in the fusion protein [41,42,43,44].

Expression of these chimeric proteins during early stages of myogenesis is thought to induce an abnormal myogenic growth/differentiation pattern, resulting in ARMS formation [10,43,45,46]. Identification of these specific translocations in ARMS allowed diagnostic techniques such as RT-PCR and fluorescence in situ hybridization (FISH) to be employed in the distinction between ERMS and ARMS, which can otherwise be difficult based on morphology alone [47]. ARMS clinical studies showed that PAX3-FOXO1 and PAX7-FOXO1-expressing tumors are associated with distinct clinical outcomes, with PAX7-FOXO1 positive tumors showing a better prognosis [48]. Understanding the functions of these chimeric proteins will clarify the molecular mechanism mediating ARMS formation and may provide therapeutic targets.

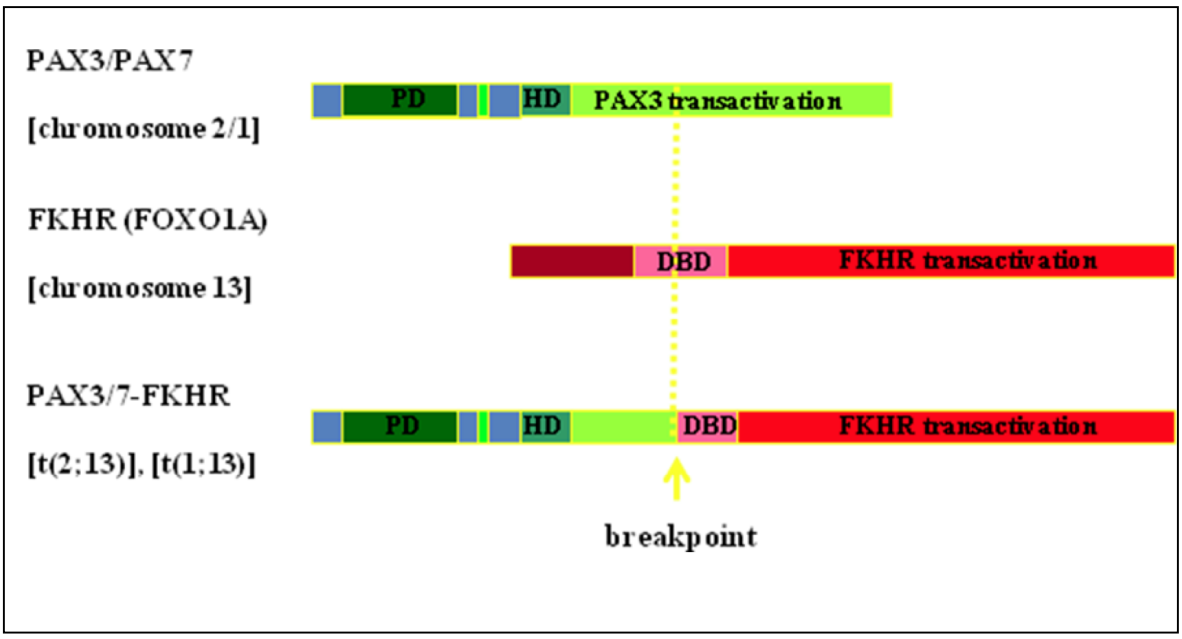

Figure 3: Schematic representation of the t(2;13) and t(1;13) translocation in ARMS. The resultant chimeric proteins contain the DNA binding domains of the PAX genes fused in frame with the transactivation domain of FOXO1, generating aberrant transcription factors having the DNA binding capacity of the PAX genes and the potent transcriptional transactivation activity of FOXO1 (Adapted from [38]).

2.4. Role of PAX3-FOXO1 in Alveolar Rhabdomyosarcoma.

The PAX3-FOXO1 fusion was first identified in 1993 [45] and cloned in the same year by two independent groups [43,46]. Since its cloning, many studies have been carried out to understand the role of PAX3-FOXO1 in rhabdomyosarcomagenesis. Early works performed in chicken fibroblasts or myogenic cell lines identified the monogenic potential of PAX3-FOXO1 by inducing anchorage independent growth and by inhibiting myogenic differentiation [49,50]. Further studies have examined many other aspects of PAX3-FOXO1-mediated cell transformation, showing that the monogenic effect of its expression aberrantly influenced cell proliferation, anchorage independent growth, cell survival and cell differentiation [51,52,53,54,55,56,57].

2.4.1 Effects of PAX3-FOXO1 on cellular proliferation.

Promotion of cell proliferation is a hallmark of many oncogenes including PAX3-FOXO1. The effects of PAX3-FOXO1 on cell proliferation and eventually on cell transformation were examined through experiments performed by Fredericks and colleagues. They engineered a specific repressor construct by fusing the PAX3 binding domain to the kruppel-associated box (KRAB) domain, a transcriptional repressor module present in the KOX1 gene, which is highly conserved in eukaryotic cells. Expression of this repressive construct in an RH30 ARMS cell line resulted in reduced cell proliferation, inhibition of anchorage-independent growth and inhibition of tumor formation in SCID mice [44]. More recently, repression of PAX3-FOXO1 expression has also been achieved by small interfering RNA (siRNA) technology. PAX3-FOXO1 was specifically knocked down in ARMS cell lines without affecting PAX3 or FOXO1 expression. In this study, depletion of the chimeric protein reduced cell motility, proliferation and induced differentiation in several ARMS cell lines (RH30, RH4, RH41), as indicated by increased expression of differentiation markers such as myogenin and myosin heavy chain (MyoHC) [58].

In addition, gene expression studies in NIH3T3 cells have shown that over expression of PAX3-FOXO1, but not PAX3, could up regulate *Igf2* and *Igfbp5* genes [59]. IGF2 and IGF1 have been shown to be necessary for myoblast proliferation and differentiation *in vitro* [60,61]. Increased expression of IGF2 has been detected in ERMS and ARMS primary tumor specimens compared to adult skeletal muscle, suggesting that increased IGF2 expression might play a key role in rhabdomyosarcoma formation [62]. As mentioned in paragraph 2.3, LOH at the short arm of chromosome 11 (11p15), which contains the *IGF2* gene, and LOI in the *IGF2* gene have also been associated with increased expression of IGF2 in ERMS and ARMS tumors, respectively. Blocking IGF-1R using specific monoclonal antibodies has been shown to reduce growth of RMS cell lines in vitro and in vivo [62,63].

The decreased level of the cell cycle kinas inhibitor p27Kip1 could also explain stimulation of cell proliferation in PAX3-FOXO1-expressing cells. Expression of the fusion protein, but not wild-type PAX3 in NIH3T3 fibroblast cells promotes proteasome-dependent degradation of p27Kip1 via increased expression the E3 ubiquitin lipase Skp2, which regulates p27Kip protein turnover. Skp2 was shown to be overexpressed at the protein and mRNA level either in PAX3-FOXO1 transducer NIH3T3 cells and in ARMS cell lines, suggesting that this E3 lipase might be a transcriptional target of PAX3-FOXO1 [64,65]. Chromatin immunoprecipitation experiments have shown that PAX3-FOXO1 can bind a 220 bp genomic sequence located 49kb downstream to the Skp2 gene and increased luciferase activity was measured in NIH3T3 cells co-transected with PAX3-FOXO1 and a reporter construct harboring this 220 bp sequence, suggesting that this cist-element is a PAX3-FOXO1 binding site and might be, at least in part, responsible for Skp2 up-regulation by PAX3-FOXO1 [66].

2.4.2 Effects of PAX3-FOXO1 on cellular survival.

Deregulation of apoptotic signaling can play a primary role in cancer formation. It has been shown that PAX3-FOXO1 can influence cell survival by directly activating the expression of the anti-apoptotic BCL2 family member, BCL-XL

[44,57]. Conversely, it has also been shown that expression of PAX3-FOXO1 can induce apoptosis in cultured cells, causing a selection against cells expressing high levels of the fusion protein [67].

The tyrosine kinas MET (or cMet) plays a crucial role in several tumors by inducing proliferative and anti-apoptotic signals, angiogenesis and invasion [70,71,72], and is a known transcriptional target of PAX3 and PAX3-FOXO1 [73]. The cMet protooncogene mediates the pleiotropic action of the hepatocyte growth factor/scatter factor (HGF/SF) inducing a multiplicity of biological signals (for review see Goldberg and Rosen, 1993) [74] including migration of myogenic precursors into the limb buds [73,75]. cMet is aberrantly expressed in RMS tumors [73,76,77] and knock down of cMet in RMS cells affected cells proliferation, survival, invasiveness and tumor formation in a engraft model of RMS, suggesting that cMet may represent a therapeutic target for ARMS [78].

2.4.3 PAX3-FOXO1-depentent inhibition of myogenic differentiation.

One of the first indications that PAX3-FOXO1 can negatively affect the myogenic differentiation program derives from the work of Epstein and colleagues, performed in 1995 [50]. They found that over expression of PAX3 or PAX3-FOXO1 can inhibit the differentiation of C2C12 myoblasts, leading to the hypothesis that the tumorigenic effect of PAX3-FOXO1 could be caused, in part, by altered transcription of PAX3 target genes [2,59], given that both transcription factors possess the same DNA binding domains [43]. In addition, PAX3-FOXO1 possesses a more potent transcriptional activity than wild type PAX3 [79] and can activate genes not normally activated by Pax3 [59,80].

PAX family members play an important role during embryogenesis and can, if deregulated, also induce tumorigenesis [81]. PAX3 is considered one of the main regulators of the initial phases of embryonal myogenesis by activating the basic helix-loop-helix (bHLH) domain-containing myogenic regulatory factors (MRFs) Myf5 and MyoD [82]. This implies that deregulation of the PAX3 genetic program due to PAX3-FOXO1 expression might aberrantly

affect the myogenic process, leading to rhabdomyosarcoma formation. A recent study conducted in primary mouse myoblasts has shown that PAX3-FOXO1 has enhanced protein stability compared to wild type PAX3, normally silenced 24 hours after differentiation, leading to a failure in terminal differentiation of primary myoblasts [51]. It has also been reported that PAX3 transcriptional activity is normally reduced by direct protein-protein interaction with several co-repressors including human Daxx (hDaxx). hDaxx also binds PAX3-FOXO1 in various rhabdomyosarcoma cells but less efficiently and with almost no effect on the transcriptional activity of the chimeric protein, suggesting that this unresponsiveness to co-repressors also contributes to the enhanced transcriptional effect of the oncogene [52,83].

A mechanism that can partially explain the PAX3-FOXO1-mediated blockage of myoblast differentiation was suggested by the work of Roeb and colleagues [53]. They showed that the over expression of the fusion protein under the PAX3 promoter inhibits primary myoblast differentiation due to the down regulation of the cyclin-dependent kinase inhibitor p57, which normally stabilizes MYOD1 to promote myogenic differentiation. p57, like p21, binds and inactivates several G1 cyclin/Cdk complexes and is a tumor suppressor gene [84]. Reduction of p57kip2 was caused by the decreased level of the transcription factor EGR1, which is destabilized by physical interaction with PAX3-FOXO1 [53]. Interestingly, p57kip2 is located at 11p15, the chromosomal region paternally imprinted and affected by LOH in ERMS. Reduced levels of p57kip2 in both RMS subtypes have been proposed as a common mechanism that could affect rhabdomyosarcoma occurrence [53,54].

The evidence presented in this section sheds some light on how expression of PAX3-FOXO1 in early myoblasts might induce a deregulated myogenic program, leading to cellular transformation. Despite these oncogenic aspects, a conclusive mechanism defining the function of PAX3-FOXO1 as a transforming factor has not been elucidated yet. A summary of the different genetic mechanisms by which PAX3-FOXO1 can initiate cellular

transformation is illustrated in Figure 4. Understanding the role of PAX3-FOXO1 in ARMS tumorigenesis will help identify new potential molecular targets for the cure of this aggressive childhood disease.

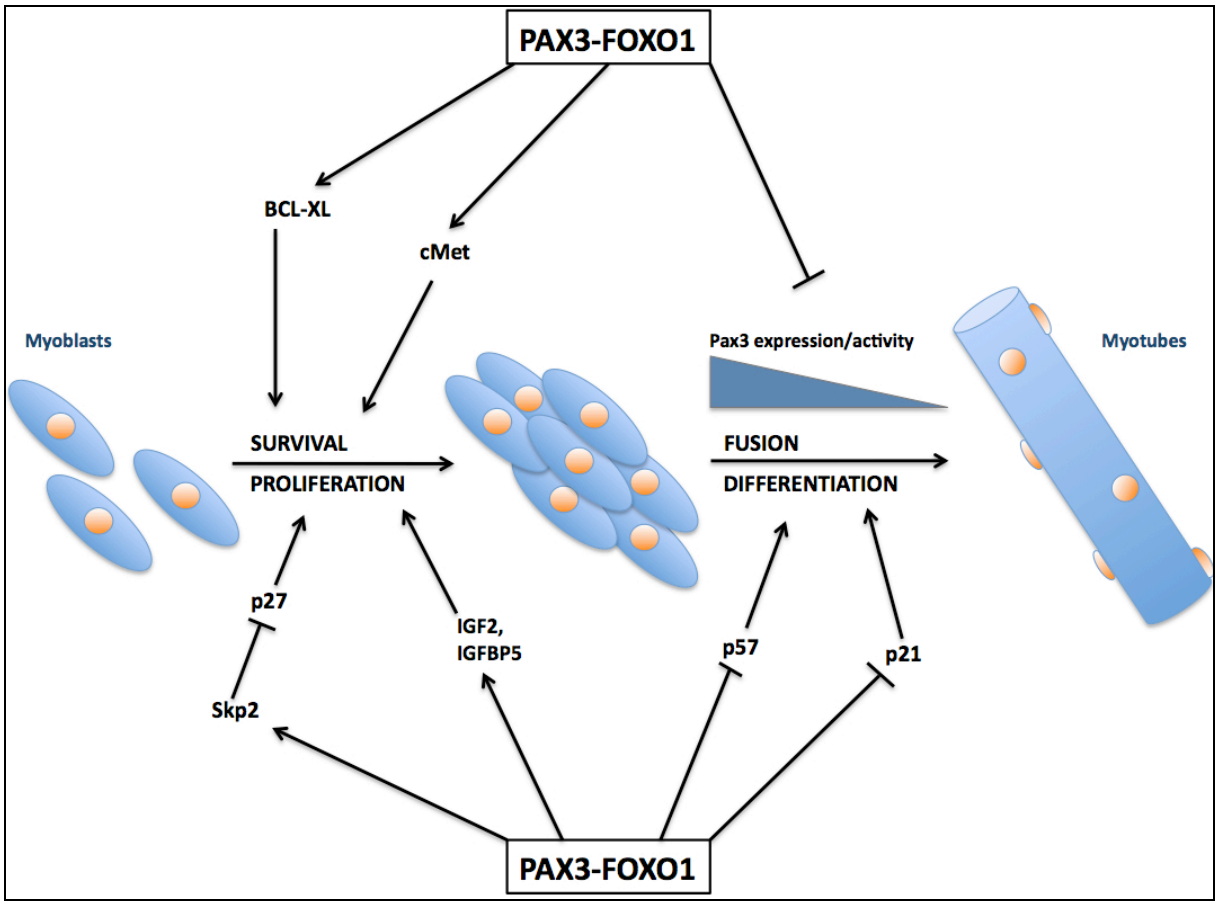

Figure 4: Schematic for the potential mechanism by which Pax3-FOXO1 expression can promote cell proliferation, cell survival and inhibit myogenic differentiation to promote the development of ARMS.

2.5. Genetic abnormalities in ARMS tumors.

ARMS tumor cells are thought to represent a primitive stage of myogenic differentiation, a consequence of the failure to convert undifferentiated myoblasts into fully mature muscle cells [85]. Previous studies have shown that expression of PAX3-FOXO1 alone is not sufficient to induce ARMS [3,4,86]. It is likely that a combination of altered molecular pathways affecting cell proliferation and cell survival together with PAX3-FOXO1 expression are required for ARMS development [87]. A summary of the additional genetic events that take part in ARMS tumorigenesis are illustrated in Fig. 5.

2.5.1. Secondary genetic events affecting cell survival.

One such factor contributing to ARMS development is p53. p53 is a transcription factor that, in response to cellular stress and/or oncogene activation, can induce many anti-proliferative and apoptotic signals [88]. MDM2 (HDM2) is a crucial negative regulator of p53 activity. It functions as an E3 ligase by directly binding the p53 transactivation domain and inducing p53 proteasomal degradation [89,90]. Inactivation of the p53 pathway occurs in many human malignancies [91,92], with a variable frequency among different tumor types [93]. Several studies have reported p53 mutations and MDM2 amplification in RMS primary tumors and cell lines [94,95,96,97,98]. Taylor et al. [98] have shown a p53 gene mutation in 1 out of 12 embryonal RMS biopsies (8.3%), while none of the four alveolar or the four undifferentiated and poorly differentiated subtypes showed p53 gene alterations. The study reported by Takahashi et al., [97] analyzed primary RMS specimens for p53-related abnormalities; 10 out of 45 samples (22.2%) had p53 gene abnormalities. p53 status studied in RMS cell lines showed a higher frequency of mutation: 66.7% of the embryonal and 50% of the alveolar cell lines had a p53 mutation (one alveolar line showed wild-type p53 but had MDM2 amplification). Predictably, mutations were more common in cell lines derived

from tumors previously exposed to chemotherapy compared to those derived from untreated tumors [97].

Further consistent with the role of p53 in ARMS development, genetic amplification of the 12q13-15 region, containing several oncogenes: *MDM2*, *GLI1*, *SAS* and *CDK4*, has been identified in 25-50% of ARMS tumors, and cell lines [98,99,100]. However, immunohistochemical analysis showed that MDM2 protein over expression was detected in a much smaller number of samples, 19.2% and 5.6% of ERMS and ARMS cases, respectively [97]. Although the report of Leushner et al. [101] shows that low levels of p53 and MDM2 were detected in 150 RMS tumors, there was no significant difference between the ARMS or ERMS subtypes, and no correlation was found with p53 or MDM2 expression and prognosis. Overall, these aberrations in the p53 pathway suggest a role for this pathway in RMS development.

The MDM2-p53 regulation is mediated by the action of the tumor suppressor $p19^{ARF}$ (ARF) encoded by the *INK4a/ARF* locus [102]. ARF stabilizes p53 by sequestering MDM2 in the nucleolus thereby preventing its ubiquitin ligase activity [103]. Although the *INK4a/ARF* locus is frequently mutated in human cancer including ERMS, a specific role for the tumor suppressor ARF in primary human ARMS formation still needs to be evaluated [104]

2.5.2. ARMS-associated mutations affecting the cellular proliferation.

The Rb pathway is another component believed to contribute to ARMS development. Disruption of the Rb (pRb) tumor suppressor pathway through the deregulation and inactivation of the cyclin dependent kinase (CDK) inhibitors p16 and p21 is often implicated in tumor formation [105]. p16 ($p16^{INK4}$) is the second tumor suppressor generated by an alternative open reading frame from the *INK4A/ARF* locus; it can inhibit the activity of cyclin dependent kinases 4 and 6 (CDK4/6), resulting in hypophosphorylated Rb, which in turn prevents the activation of the transcription factor E2F1, blocking cells in the G1 phase [106]. Loss of p16 has been detected in ERMS and ARMS cell lines and primary tumors, indicating that this CDK-inhibitor might be

involved in the development of childhood RMS [107,108]. The work of Iolascon et al, [107] looked for homozygous deletions of p16 in 12 primary RMS specimens (6 ARMS and 6 ERMS) and in 6 RMS cell lines, showing that the deletion is present in 25% of the primary tumor specimen (two ERMS and one ARMS) and in 100% of the cell lines. Moreover, the study of Obana et al. [108] showed reduced or absent p16 expression in 10 of 12 (83%) pediatric RMS tumors, suggesting that loss of p16 expression is associated with RMS development.

Involvement of p53 and pRb in PAX3-FOXO1-driven tumorigenesis has been confirmed by several *in vitro* and *in vivo* studies using human or mouse experimental models [4,5,97,109,110]. Experiments performed in our laboratory have shown that defective p53 and pRb pathways are required to induce transformation in PAX3-FOXO1 expressing mouse myoblasts. Although the expression of the fusion protein alone in $Arf^{-/-}$ mouse myoblasts failed to generate tumors in NOD/SCID mice, expression of the PAX3-FOXO1 *Ink4a/Arf*$^{-/-}$ myoblasts or co-expression of the chimeric gene with the novel gene IRIZIO [111], produced tumors after injection into mice.

Consistent with our data, Naini et al. [5] showed that the transformation of normal human myoblasts required expression of PAX3-FOXO1, over expression of hTERT and MycN, and down-regulation of p16INK4A/p14ARF. Moreover, Keller et al, [4] were able to induce ARMS in their mouse model only when the mice lacked *INK4a/ARF* and *p53* loci.

2.5.3. Other genetic events affecting ARMS tumorigenesis.

In addition to the loss of the p53 and/or pRb pathways, several additional genes have been implicated in ARMS development. Up-regulation of p21 by the muscle regulatory transcription factor (MRF) MYOD has been shown to have an important role in myoblast differentiation, promoting cell cycle withdrawal and terminal myogenic differentiation [112]. Although mutations in the p21 gene are rarely seen in RMS [10,113], decreased levels of p21 expression have been detected in RMS, with ARMS expressing the lowest

amount [114]. Recently it has been shown that PAX3-FOXO1 controls the expression of p21 through the transcription factor EGR1 [115]. PAX3-FOXO1 interacts with and destabilizes EGR1 by enhancing its proteasomal degradation [53] and EGR1 directly controls the expression of p21[116]. Reactivation of p21 in ARMS cell lines has been shown to induce apoptosis and reduce *in vivo* tumor growth [115], re-enforcing the concept that aberrant cell cycle regulation is an important component of PAX3-FOXO1 induced tumorigenicity.

The MYC oncogene family consists of three distinct nuclear proteins (C-, N- and L- MYC) that play an important role in cell proliferation, apoptosis, and differentiation [117,118,119,120,121]. MYC members are often deregulated in human cancers [122,123] and several studies have reported C-MYC amplification in RMS cell lines [124] and N-MYC amplification in RMS primary tumors and cell lines [12,125,126,127,128,129,130]. Dias et al. [12] and Driman et al. [126] have shown N-MYC gene amplification in 66.6% and 42.9% of ARMS tumors respectively, but no gene amplification was detected in ERMS tumors. Both studies also report that there was no difference in clinical behavior between N-MYC amplified and non-amplified tumors [12,126]. Similarly, the work of Pandita et al. [130] showed that N-Myc is amplified in 50% of the ARMS tumors analyzed, without any difference in survival rate between patients harboring amplified and unamplified tumors. Toffolatti et al. [125] analyzed N-Myc over expression at the RNA and protein level in RMS cell lines (5 ERMS and 5 ARMS), showing that the protein was present in all cell lines, with higher levels in the ARMS subtype. Amplification of N-MYC in neuroblastoma tumors strongly predicts poor clinical outcome [126,131]. Hachitanda et al [128] detected N-MYC amplification in 60% of the ARMS and 25.6% of the ERMS tumors; the survival rate of ARMS patients was 66% for the non-amplified and 11% for the N-MYC amplified cases. Together, these data suggest that N-MYC amplification plays a role in RMS formation, especially in the alveolar subtype, and in some cases N-MYC amplification is a useful prognostic factor of poor outcome [128].

Finally, mutations in the RAS gene family, which comprises three members H-RAS, K-RAS and N-RAS, is characteristic of many human cancers [132]. Although RAS pathway activation has been found predominantly in ERMS tumors [133,134], *in vitro* studies using mesenchymal stem cells (MSC) expressing PAX3-FOXO1 have shown that the presence of constitutively active H-RAS can lead to the formation of tumors highly resembling ARMS when both the p53 and RB pathways are also inactivated [110], indicating that activation of RAS downstream targets might have an important function in ARMS formation. Notably, increased Ras activity diminishes pRB control via up-regulation of D cyclins [135].

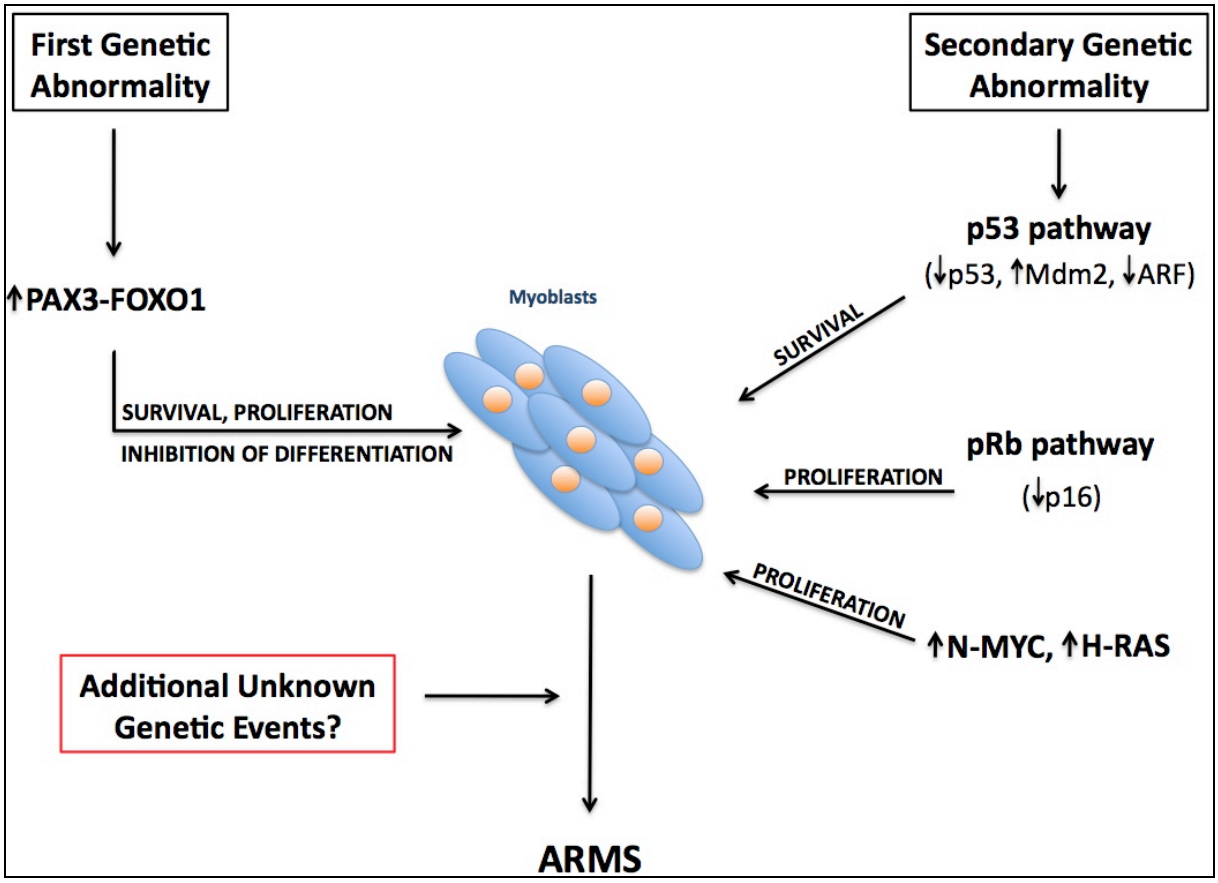

Figure 5: Summary of the genetic events that take place during myogenesis and that might lead to ARMS formation. The proliferative and differentiation-suppressive effects of PAX3-FOXO1 are complemented by secondary mutations affecting cell proliferation and cell survival and by additional unknown genetic events leading to transformation of primary myoblasts.

2.6. Genetic RMS mouse models.

Genetic alterations affecting the expression of oncogenes and/or tumor suppressor genes in normal tissues are thought to be the initial steps for cellular transformation. Additional molecular abnormalities in the pre-cancerous cell and interactions with the surrounding environment are also required for tumor progression [136]. As mentioned above, the genetic hallmarks of ARMS is the presence of the PAX3-FOXO1 or PAX7-FOXO1 fusion proteins [43]. Genetically engineered mouse models have been used to mimic human cancers [137]. During the past 10 years, several ARMS murine models have been generated in order to understand the pathogenesis of this disease.

In 2001 Anderson and colleagues [135] generated transgenic mice expressing PAX3-FOXO1 under the control of mouse *Pax3* promoter/enhancer sequences. Transgenic animals did not form tumors but showed hind-limb skeletal muscle defects together with abnormal migration of neural crest cells, due to the interference of ectopic PAX3-FOXO1 with endogenous Pax3 expression early during myogenesis [138]. This result also indicated that PAX3-FOXO1 expression alone is insufficient to elicit tumor formation in the mouse [135], which supports the results seen *in vitro* (section 2.5).

A year after the publication of Anderson's work, another mouse ARMS model was generated by Lagutina et al. [3]. To better mimic the human disease, a Pax3-FOXO1 construct was inserted into the *Pax3* locus, generating a knock-in mouse having the chimeric gene under the control of the endogenous *Pax3* regulatory elements. Similar to the Anderson model, Pax3-FOXO1 heterozygous mice did not develop malignancy, but showed multiple abnormalities involving lungs, heart, tongue and diaphragm and died around the time of birth. The phenotype observed in this mouse model showed similarities with the phenotype observed in the splotch mutant mice, in which *Pax3* is homozygously inactivated, indicating that Pax3-FOXO1 expression has a dominant negative effect on endogenous Pax3 activity during embryogenesis [3].

Conditional Pax3-FOXO1 knock-in mouse models using the bacteriophage P1 Cre/LoxP recombination system [139] has also been employed for the generation of ARMS mouse models [4,188,189]. In 2004 Keller et al. [4] generated a Cre-mediated conditional knock-in of Pax3-FOXO1 by inserting the transactivation domain of FOXO1 into the *Pax3* locus, allowing normal expression of Pax3 until activation of Cre recombinase generated the *Pax3-FOXO1* fusion gene. Conditional *Pax3-FOXO1* mice were bred with mouse strains specifically designed to express Cre in myogenic cells under the control of the middle/late myogenic differentiation factor Myf6 (MRF4) or the paired box transcription factor Pax7. In the latter case, PAX3-FOXO1 expression in $Pax7^{+}$ myogenic cells resulted in a dominant negative effect of the chimeric protein on the endogenous PAX7, generating mice having minor birth defects and a reduction in muscle mass due to a decreased number of Pax7-expressing satellite cells. However these animals did not develop ARMS tumors [189]. It is important to note that, even though it was known that PAX3-FOXO1 requires additional mutations to acquire a transformed phenotype, no additional genetic alterations were introduced into this mouse model.

In contrast, Keller et al [4] were able to recapitulate ARMS tumor formation in mice where Cre expression was driven by the promoter of the myogenic transcription factor Myf-6. Tumor formation, however, occurred at a low frequency: only 1/228 mice developed an ARMS-like tumor at one year of age [4]. Although, ARMS tumors present PAX3-FOXO1 gene amplification very rarely [129], an increased rate of tumor formation was observed when homozygous Pax3-FOXO1-expressing mice were crossed with animals carrying homozygous deletion of *Ink4a/Arf* or *p53*, reinforcing the concept that PAX3-FOXO1 expression alone is not sufficient to generate neoplasia [4].

Previous reports have led to the general concept that chromosomal translocations occur between transcriptionally active chromatin loci [190,191] indicating that the PAX3-FOXO1 translocation likely occurs in a cell that is actively transcribing both PAX3 and FOXO1 genes. Although *Pax3* is not expressed in adult muscle fibers [138], Keller et al. [4] suggest that the cell of

origin for ARMS may be terminally differentiated Myf6-expressing muscle fibers, rather than in myogenic progenitor cells. The authors propose a dedifferentiation mechanism whereby PAX3-FOXO1 expression in nascent skeletal muscle fibers would induce those cells to reverse terminal cell cycle withdrawal and return into a more undifferentiated state. This model would be consistent with the poorly differentiation status of human ARMS [192], However it seems more likely, given the somewhat poor characterization of Myf6 expression during myogenesis [193,194], that Myf6 expression may occur in a small number of actively proliferating $PAX3^{+}$ myogenic precursor cells. It may be these cells that give rise to ARMS in this model. This hypothesis would also account for the low frequency of ARMS development in this model.

The idea that mouse mesenchymal stem cells (MSC) might be the cell of origin of ARMS has been recently proposed by Anderson and colleagues [159,195]. They have shown that PAX3-FOXO1 expression in MSC enhanced cell growth and induced the expression of the myogenic markers MyoD and Myogenin but not Myf5, as seen in primary ARMS tumor cells. Inactivation of p53 and pRb or activation of the RAS pathway resulted in a tumorigenic phenotype when PAX3-FOXO1 expressing MSC cells were subcutaneously injected into mice, generating tumors showing a histological, immunohistochemical and gene expression profile similar to human ARMS [159].

Collectively these models show that the timing, localization, and level of PAX3-FOXO1 expression are critical to the development of ARMS, while avoiding developmental defects. Despite the development of these multiple ARMS mouse models, the identity of the cell of origin of alveolar rhabdomyosarcoma remains elusive. The model of the Capecchi group clearly shows that PAX3-FOXO1 can induce tumorigenesis *in vivo*, but additional mouse models are required to fully recapitulate ARMS formation. Generation of a temporally regulated PAX3-FOXO1 expression in myogenic cells using a tamoxifen-inducible Cre/ER system [196] could be a promising way to investigate the oncogenic potential of PAX3-FOXO1 during different stages of muscle

formation either before or after birth, and fully recapitulate ARMS formation in a mouse model.

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Chapter 3

Identification of Putative PAX3-FOXO1 Transcriptional Targets Through Microarray Analysis.

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Manuscript in preparation

Abstract and introduction.

Rhabdomyosarcoma (RMS) belongs to the group of round blue cell tumors and is considered the most common soft tissue sarcoma in children aged 15 years or younger [1,2]. RMS arises from immature mesenchymal cells committed to the myogenic cell lineage [3]. According to the histopathology classification, RMS can be subdivided in two main subtypes, alveolar (ARMS) and embryonal (ERMS), with ARMS having a more unfavorable prognosis [4,5]. The most common molecular abnormality characterizing ARMS is the presence of the t(2;13)(q35;q14) or t(1;13)(p36;q14) chromosomal translocations, which fuse the paired box family members *PAX3* or *PAX7*, to a member of the fork-head family of transcription factors *FOXO1* [6]. Expression of the chimeric proteins, PAX3- or PAX7-FOXO1, during the early phases of myogenesis is believed to initiate an aberrant genetic program leading to the failure of skeletal muscle differentiation and subsequent ARMS formation [2,7,8,9]. Various studies have shown that PAX3-FOXO1 expression is associated with increased cell proliferation, cell survival and inhibition of cell differentiation [10,11,12,13,14,15,16]. Due to the nature of the t(2;13)(q35;q14) , PAX3-FOXO1 maintains the PAX3 DNA binding domains fused to the transactivation domain of FOXO1 [7], which has been shown to have a stronger transcriptional activity than the PAX3 transactivation domain it replaces [17,18]. Given that both the normal and fusion transcription factor can activate similar target genes [19], it has been proposed that the aberrantly increased activation of PAX3 targets due to PAX3-FOXO1 expression may be the cause of ARMS [16,17,18,20]. There is also evidence in the literature that PAX3-FOXO1 is capable of activating genes not normally activated by PAX3 [19,21]. Despite these observations the mechanism underlying the oncogenic potential of PAX3-FOXO1 has not been discovered yet.

It is clear that identification of PAX3-FOXO1 target genes has a crucial role in understanding the mechanism by which it contributes to tumor pathogenesis. Using a microarray analysis performed on wild type mouse primary myoblasts stably expressing PAX3-FOXO1, PAX3 or empty vector, we show that PAX3-

FOXO1 specifically regulates genes involved in cell cycle progression, cellular invasiveness, and tumor cell metastasis. Further, we present a comparison between our results and results obtained by others in primary tumor samples, tumor cell lines or non-physiologically relevant cell types. We observed a mere 10.6% overlap in genes whose expression is altered in our primary myoblast system relative to other reports. This result suggests that the use of a mouse primary myoblast system, not burdened by additional genetic alterations other than PAX3-FOXO1, might represent a more appropriate experimental system to identify direct downstream targets of PAX3-FOXO1.

Materials and Methods

Isolation of mouse primary myoblasts.

Isolation of *Arf-/-* primary myoblasts has been described previously [22]. Myoblasts were cultured in Ham's F10 medium supplemented with 20% fetal calf serum, human basic fibroblast growth factor (bFGF) 1 ng/ml (Promega, Madison, WI), penicillin/streptomycin (100 µ/ml and 100 µg/ml, respectively) (Gibco-Invitrogen Corporation, Grand Island, NY) and plated on collagen-coated dishes (BD Biosciences, San Jose CA) at 37°C, 5% CO_2 and 6% O_2.

Retrovirus production, primary myoblast cell transduction and cell sorting.

The MSCV-IRES-GFP construct was generously provided by E. Vanin, of St. Jude Children's Research Hospital. Cloning of MSCV-PAX3-IRES-GFP and MSCV-PAX3-FOXO1-IRES-GFP has been described previously [10]. Retroviruses were generated by transiently transfecting the retroviral constructs into Phoenix-Eco cells and primary myoblasts were transduced as previously described [23]. Myoblasts were FACS sorted for GFP expression 48 hours post-transduction and expanded as described above.

RNA preparation for microarray.

RNA was extracted from low-passage mouse primary myoblasts stably expressing MSCV-IRES-GFP, MSCV-IRES-PAX3-GFP or MSCV-IRES-PAX3-FOXO1-GFP using Trizol reagent according to the manufacturer's instructions (Invitrogen Corporation, Grand Island, NY). RNA purity and integrity were determined by using the FlashGel system (Rockland ME, USA). We performed three independent experiments each time using freshly isolated myoblasts and de novo retrovirus transductions.

Microarray and data analysis.

Myoblast RNA were hybridized to MOE-430a Affymetrix arrays and the data were analyzed with the Local Pooled Error (LPE) t-test [24] and a False Discovery Adjustment (Benjamini-Hochberg) was applied to the p-values to account for multiple hypothesis testing. A threshold of >2-fold or >8-fold change plus a False Discovery Rate <5% was set to identify significantly changed probe sets.

Culture of cell lines and Western blot analysis.

ERMS (RD, JR1, RH6, RH2) and ARMS (RH30, RH4, RH41 RH3, RH28) cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum and penicillin/streptomycin (100U/ml and 100µg/ml, respectively, Gibco-Invitrogen Corporation, Grand island, NY). Cells were lysed in RIPA buffer supplemented with protease (Roche, Indianapolis, IN) and phosphatase (Sigma St. Louis, MO) inhibitor cocktail. Proteins were separated by 10% SDS-PAGE and transferred onto nitrocellulose. Membranes were then blocked in 3% non-fat milk in TBS-Tween (w/v) and probed with Skp2 (1:1000, Cell Signaling, Beverly, MA) and GAPDH (1.5000, Chemicon-Millipore Billerica, MA) antibodies. The optical density of Skp2 and GAPDH bands were measured using the ImageJ software (http://rsbweb.nih.gov/ij/index.html).

Mice.

C57/BL6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were maintained at St. Jude Children's Research Hospital following the Institutional Animal Care and Use Committee guidelines.

Results and discussion

Identification of differentially expressed genes in PAX3-FOXO1 expressing myoblasts.

To elucidate the gene regulatory program driven by PAX3-FOXO1, we performed a microarray analysis by comparing whole cell RNA isolated from primary mouse myoblasts stably transduced with retroviral vectors encoding PAX3, PAX3-FOXO1 or GFP. To identify significant changes in the gene expression profile in the PAX3 or PAX3-FOXO1 myoblasts arrays we applied a Local Pooled Error (LPE) test [24] on genes with a threshold of >2-fold change plus a False Discovery Rate (FDR) <5%. This produced 116 differentially expressed genes for PAX3 versus vector and 87 differentially expressed genes for PAX3-FOXO1 versus vector. PAX3 expression specifically affected genes involved in muscle differentiation (10 genes, P = 9 E-42) and muscle development (14 genes, P = 7.2 E–33), whereas PAX3-FOXO1 affected genes involved in cellular catabolism (9 genes, P = 7.5 E-4), cell adhesion (7 genes, P = 1.2 E-3), cell signaling (6 genes, P= 1.5 E-2), and organogenesis (10 genes, P = 1.2 E-2). To select for the highest differentially expressed genes we performed a more stringent analysis applying a threshold of > 8 fold change and a FDR <5% on the PAX3 or PAX3-FOXO1 versus vector probe sets (Table 2 and 3). As expected we found a number of genes that were equally up or down-regulated by both PAX3 and PAX3-FOXO1 relative to the empty vector control. These genes included the down-regulation of *Biglycan*, *Col1a2* (procollagen, type 1, alpha 2), *Glyat* (Glycine-N-acyl-transferase) and *Upp2* (uridine phosphorilase 2).

Several of the genes identified to be up or down-regulated 8-fold or more by PAX3-FOXO1 in primary myoblasts were demonstrated to have cellular functions consistent with the increased cellular proliferation and invasiveness that characterizes ARMS tumor cells. A few genes of note were demonstrated to contribute to cellular proliferation. FGFR4, the receptor for the fibroblast growth factor (FGF) and a gene we found up-regulated nearly 10-fold by

PAX3-FOXO1, was demonstrated by several studies to be a direct transcriptional target of the PAX3-FOXO1 [25,26,27,28,29], thereby validating our analysis. The FGF signaling pathway plays an important role in promoting cell proliferation and differentiation during myogenesis [30]. *FGFR4* is highly expressed in RMS [26], and its expression has been correlated with poor survival in ARMS [31]. Taylor et al. reported that 7.5% of primary RMS tumors possess activating mutations in the FGFR4 gene; and interestingly, FGFR4 knock down in the ARMS cell line RH30 inhibits tumor growth and lung metastasis in a xenograft model [31]. Despite this evidence, our laboratory reported that overexpression of wild-type FGFR4 in PAX3-FOXO1-expressing primary myoblasts does not contribute to oncogenesis [29]. However, the expression of a constitutively active FGFR4 mutant is able to enhance proliferation and cell transformation in primary myoblasts that express PAX3-FOXO1, which is in accordance with the low incidence of constitutively active FGFR4 mutations in 7.5% of primary RMS [31]. Taken together these results suggest that the proposed use of FGFR4 targeting drugs [31] might only be effective in the subset of RMS tumors that contain activating mutations in FGFR4 (7.5%) [29].

Grb10, which we found to be down-regulated over 8-fold, is an adaptor protein known to interact with a number of receptor tyrosine kinases and signaling molecules [32]. In addition, Grb10 has been shown to regulate cell proliferation [33] by interacting with and inhibiting signaling by the insulin receptor (IR) and the insulin-like growth factor receptor 1 (IGF1R) [34,35,36]. Interestingly, IGF1R signaling through up-regulation of IGF2 plays an important role in rhabdomyosarcoma [37,38,39]. Given that Grb10 expression is frequently down-regulated in various cancers [40], we can speculate that the PAX3-FOXO1-dependent reduction in Grb10 expression in primary myoblasts might therefore enhance cellular proliferation.

In addition, several of the genes in this group have biological activities consistent with cellular invasiveness. Specifically, the regulatory peptide cholecystokinin (CCK) (41.8 fold up), normally synthesized in human gut and

brain tissues [41], plays an important role in growth and invasiveness of human pancreatic cancer [42,43] and was found to be frequently up-regulated in various pediatric tumor cell lines including Ewing sarcoma (89%), neuroepitheliomas (100%), leiomyosarcomas (50%) and in rhabdomyosarcoma (18%) [41,44]. Moreover, CCK knockdown by RNA interference impaired the proliferation of a Ewing's sarcoma cell lines [45].

Among several other genes of note, MMP13 (matrix metalloproteinase 13), which we found to be up-regulated over 13 fold, was found to be associated with tumor progression and poor prognoses of invasive breast cancer [46]. Recent studies have shown that MMP13 mediates cell cycle progression in melanocytes and melanoma cells [47] and increased expression of MMP13 promotes invasion in colon cancer [48]. Additionally, Claudin1, which we found to be up-regulated over 10 fold is normally expressed in the tight junctions [49]. Claudin has been reported to be overexpressed in colorectal cancer (CRC) [50,51,52,53], and its expression correlates with increased invasion and aggressive phenotype in oral squamous cell carcinoma [54]. Finally, overexpression of the tyrosine kinase ephrin A4 (EphA4) receptor, which we found to be up-regulated 9.7 fold, can stimulate tumorigenesis and are associated with angiogenesis and metastasis in various human cancers [55,56]. Taken together our gene expression profile analysis on primary myoblasts identified several PAX3-FOXO1 transcriptional targets that, by regulating cellular processes such as proliferation and invasion, might contribute to ARMS formation.

Table 1: List of genes differentially regulated in primary mouse myoblasts overexpressing PAX3. A threshold of >8-fold change plus a False Discovery Rate (FDR) <5% was set to identify significantly changed probe sets. Gene names in bold identify transcriptional targets commonly up-, or down-regulated by PAX3 and PAX3-FOXO1.

Gene	Description	Regulation: PAX3 vs Vector
Pax3	paired box gene 3	104.9
Msln	Mesothelin	33.6
Il15	interleukin 15	19.7
Pramel3	preferentially expressed antigen in melanoma-like 3	18
Ndrg1 /// Ndrl	N-myc downstream regulated gene 1 /// N-myc downstream regulated-like	11.1
Gstk1	glutathione S-transferase kappa 1	10.4
C1r	complement component 1, r subcomponent	9.9
Ddx3y	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	9.6
Usmg2	upregulated during skeletal muscle growth 2	9.4
Mbp	myelin basic protein	9.3
Agr2	anterior gradient 2 (Xenopus laevis)	8.2
Gzme	granzyme E	-8.1
Pla2g1b	Phospholipase A2, group IB, pancreas	-8.1
Cyp3a41	cytochrome P450, family 3, subfamily a, polypeptide 41	-8.6
Foxm1	forkhead box M1	-8.6
Gzme	granzyme E	-8.6
Glyat	glycine-N-acyltransferase	-8.8
Myh1	myosin, heavy polypeptide 1, skeletal muscle, adult	-8.8
Trdn	Triadin	-8.8
Upp2	uridine phosphorylase 2	-8.9
Cd36	CD36 antigen	-9
Cyb561	cytochrome b-561	-9.1
Hist2h3c2	histone 2, H3c2	-9.1
Trdn	Triadin	-9.2
Arx	aristaless related homeobox gene (Drosophila)	-9.4
Wif1	Wnt inhibitory factor 1	-9.7
Clec14a	C-type lectin domain family 14, member a	-11.8
Cmya1	cardiomyopathy associated 1	-13
Fabp4	fatty acid binding protein 4, adipocyte	-13.6
Col1a2	procollagen, type I, alpha 2	-16.3
Bgn	Biglycan	-16.6
Gzmd	granzyme D	-16.8

Table 2: List of genes differentially regulated in primary mouse myoblasts expressing PAX3-FOXO1. A threshold of >8-fold change plus a False Discovery Rate (FDR) <5% was set to identify significantly changed probe sets. Gene names in bold identify transcriptional targets commonly up-, or down-regulated by PAX3 and PAX3-FOXO1.

Gene	Description	Regulation: PAX3-FOXO1 vs Vector
Cck	Cholecystokinin	41.8
Rims2	regulating synaptic membrane exocytosis 2	22.9
Selp	selectin, platelet	13.4
Mmp13	matrix metalloproteinase 13	13.3
Gal	Galanin	12.5
Itgb3bp	integrin beta 3 binding protein (beta3-endonexin)	10.6
Selp	selectin, platelet	10.5
Cldn1	claudin 1	10.2
Dmrt2	doublesex and mab-3 related transcription factor 2	9.7
Epha4	Eph receptor A4	9.7
Fgfr4	fibroblast growth factor receptor 4	9.6
Pramel3	preferentially expressed antigen in melanoma-like 3	9.4
Gpx3	glutathione peroxidase 3	8.9
LOC381251	similar to Nur77 downstream protein 1	8.6
Chi3l3 /// Chi3l4	chitinase 3-like 3 /// chitinase 3-like 4	8.1
Grb10	growth factor receptor bound protein 10	-8.2
Col8a1	procollagen, type VIII, alpha 1	-8.5
Ctsm	cathepsin M	-8.7
Neud4	neuronal d4 domain family member	-9
Upp2	uridine phosphorylase 2	-9
Aspn	Asporin	-9.2
Col1a2	procollagen, type I, alpha 2	-11.3
Ebf1	early B-cell factor 1	-11.8
Gca	Grancalcin	-12.4
Bgn	Biglycan	-15
Glyat	glycine-N-acyltransferase	-15.7

Comparison between differentially expressed genes in PAX3-FOXO1 expressing myoblasts and published data.

Many studies published lists of potential transcriptional targets of PAX3-FOXO1. A summary of genes found differentially expressed by these publications in ARMS primary tumors, ARMS cell lines or heterologous cell lines expressing the fusion protein is illustrated in Table 3 (adapted from Amy Marshall and Gerard Grosveld, unpublished review). We compared this with genes with a 2-fold or greater difference in expression from our PAX3-FOXO1 versus empty vector array data. This comparison produced only 10.6% of similarity (Table 3 last column). Some genes such as the proto-oncogenes c-Met and N-Myc, the anti-apoptotic factor Bcl-2, and the muscle-differentiation regulating proteins MyoD and Myogenin are notably missing from our microarray results. This might be explained if we consider that our primary myoblasts are a less complex model, not affected by additional mutations that occur during rhabdomyosarcomagenesis. Our system might therefore represent a more physiologically relevant cellular system to identify specific PAX3-FOXO1 transcriptional targets. On the other hand, we cannot exclude that some of the observed differences might reflect different transcriptional responses to PAX3-FOXO1 between mouse and human cells. Notwithstanding PAX3-FOXO1-dependent expression of these genes in our system might represent initial steps towards a deregulated myogenic program. For instance, CD9 (up-regulated in our array and in the published data, Table 2) has been found to be expressed in many RMS specimens [57] and was shown to be involved in cell adhesion, proliferation, and cell motility in various metastatic cancers [58,59]. Similarly, Cnr1, also up-regulated in our array and in the published data, was found specifically up-regulated in fusion positive ARMS [25,27,60]. Recently, our laboratory has shown that Cnr1 is specifically up-regulated in ARMS versus ERMS cell lines and in PAX3-FOXO1-expressing primary myoblasts and primary ARMS samples [61]. Cnr1 up-regulation enhances invasiveness of PAX3-FOXO1 expressing primary myoblasts and promotes invasion and metastasis formation of ARMS cells. This makes CNR1

a very interesting potential therapeutic target for the inhibition of metastasis, a major problem of ARMS [61].

Table 3: Summary of genes differentially expressed in ARMS primary tumors, ARMS cell lines or heterologous cell lines expressing PAX3-FOXO1. The table indicates if the gene is up-, or down-regulated and if it is a direct target of PAX3-FOXO1. The last column indicates the gene was found to be differentially expressed in our analysis of PAX3-FOXO1-expressing primary mouse myoblasts (adapted from Amy Marshall and Gerard C. Grosveld, unpublished review). The data presented in this table was compiled from the indicated references.

Gene	Description	Regulation	References	PF target	Regulation in the myoblast array
ABAT	4-aminobutyrate aminotransferase;	Up	[27,60,62]	Yes	-
ACTC	Actin, alpha, cardiac muscle 1	Up	[19,63]	Yes	-
ADAM10	A disintegrin and metalloproteinase domain 10	Up	[62,64]	Yes	-
ADRA2A	Alpha-2 adrenergic receptor subtype C10	Up	[28,60,62]	Yes	-
ADRA2C	Alpha-2 adrenergic receptor subtype C4	Up	[25,28,60,62]	Yes	-
ALK	ALK tyrosine kinase receptor, anaplastic lymphoma kinase Ki-1	Up	[25,28,60,62, 65]	Yes	-
ANK2	Ankyrin 2	Up	[27,28,60]	Yes	-
ASS	Argininosuccinate synthase	Up	[27,60,66]	?	-
ASTN2	astrotactin 2	Up	[28,62]	Yes	-
BIN1	Bridging integrator 1	Down	[28,67]	Yes	-
BMP5	Bone morphogenic protein 5	Up	[27,28,60,62]	Yes	-
C10ORF 6	Family with sequence similarity 178, member A	Up	[28,63]	Yes	-
CCND1	Cyclin D1	Down	[63,67]	?	-
CD9	CD9 molecule/ Tetraspanin-29	Up	[27,28]	Yes	UP
CDH3	Cadherin 3, type 1, P-cadherin (placental)	Up	[28,62]	Yes	UP
CHD7	Chromodomain helicase DNA binding protein 7binding protein 7	Up	[28,68]	Yes	-
CKM	Creatine kinase M chain, muscle	Up	[19,67]	Yes	-
CNR1	Cannabinoid receptor 1	Up	[21,25,60,62, 69]	Yes	UP
COL18A1	Collagen type 18 α 1	Up	[27,60]	?	-
CXCR4	C-X-C chemokine receptor type 4	Up	[28,63,70,71, 72]	Yes	-
CXCR7	C-X-C chemokine receptor type 7	Down	[28,72]	Yes	-
DCX	Neuronal migration protein doublecortin	Up	[27,60,68]	?	UP
DES	Desmin	Down	[62,67]	Yes	-
DUSP4	Dual specificity phosphatase4	Down	[27,68]	?	-

DZIP3	DAZ interacting protein 3, zinc finger	Up	[19,62,68]	Yes	-
ELA1	Elastase-1	Up	[25,60,62]	Yes	-
ENC1	ectodermal-neural cortex 1 (with BTB-like domain)	Up	[28,63]	Yes	-
ENO3	Enolase 3 (beta, muscle)	Up	[60,67]	?	-
EPHA4	Ephrin type-A receptor 4	Up/Down	[21,28,68]	Yes	UP
FGFR2	Fibroblast growth factor receptor 2	Up	[28,62]	Yes	-
FGFR4	Fibroblast growth factor receptor 4	Up	[25,26,27,28]	Yes	UP
FLNB	Filamin B, beta	Down	[21,28]	Yes	-
FNBP1	Formin binding protein 1	Down	[25,63]	?	-
FOXF1	Forkhead box protein F1	Up	[25,28,60,62, 68]	Yes	Down
FOXO1	Forkhead box O1	Up	[28,63]	Yes	-
GADD45 A	Growth arrest and DNA-damage-inducible protein GADD45 alpha	Up	[27,28,68]	Yes	-
GRAF	GTPase regulator associated with FAK	Up	[25,62]	Yes	-
GTF3C1	General transcription factor 3C	Up	[62,68]	Yes	-
H19	imprinted maternally expressed gene, untranslated mRNA	Up	[19,67]	Yes	-
HDAC5	Histone deacetylase 5	Up	[27,28]	Yes	-
HUMMLC 2B	Myosin regulatory light chain 2, skeletal muscle isoform	Down	[27,62]	Yes	-
IGF-II	Insulin like growth factor II	Up	[19,63]	Yes	-
IGFBP3	IGF binding protein 3	Down	[27,68]	Yes	-
IGFBP5	IGF binding protein 5	Up	[19]	Yes	-
IL4R	Interleukin 4 receptor	Up	[27,28,62,63]	Yes	Down
JAKMIP2	Janus kinase and microtubule interacting protein 2	Up	[28,60]	Yes	-
KCNN3	Small conductance calcium-activated potassium channel protein 3	Up	[27,60,68]	Yes	-
KCNS3	Potassium voltage-gated channel subfamily S member 3	Up	[27,60]	?	-
KIAA052 3	WSC domain-containing protein 1	Up	[25,60]	?	-
KIAA055 5	Jak and microtubule interacting protein 2	Up	[27,60]	Yes	-
KIAA077 4	Microtubule associated tumor suppressor candidate 2	Up	[28,62]	Yes	-
LOH11C R2A	Loss of heterozygosity 11 chromosomal region 2 gene A protein	Up	[27,60]	Yes	-
LRRFIP2	Leucine rich repeat (in FLII) interacting protein 2	Up	[60,62]	Yes	-
MARCH3	Membrane-associated RING finger protein 3	Up	[27,68]	Yes	-
MCAM	Melanoma cell adhesion molecule	Up	[27,68]	Yes	-
MEG3	Maternally expressed 3	Up	[27,60,63]	?	-

MN1	Meningioma (disrupted in balanced translocation) 1	Up	[27,28]	Yes	-
MET	Hepatocyte growth factor receptor	Up	[20,27,28,68, 73,74]	Yes	-
MYBPH	Myosin binding protein H	Up/Down	[19,62,63]	Yes	-
MYCN	N-myc proto-oncogene protein	Up	[27,28,62,66, 68]	Yes	-
MYH8	Myosin, heavy chain 8, skeletal muscle, perinatal	Up/Down	[19,62]	Yes	-
MYL1	Myosin, light chain 1, alkali; skeletal, fast	Up/Down	[19,67]	Yes	-
MYL4	Myosin, light chain 4, alkali; atrial, embryonic	Up/Down	[19,62]	Yes	-
MYOD	Myoblast determination protein 1	Up	[19,27,28,68]	Yes	-
MYOG	Myogenin (myogenic factor 4)	Up	[19,73]	Yes	-
NEBL	Nebulette	Up/Down	[27,28,62,68]	Yes	-
NELL1	NEL-like protein 1	Up	[25,27,28,60, 62]	Yes	-
NHLH1	Nescient helix loop helix 1	Up	[25,28]	Yes	-
NPTX2	Neuronal pentraxin II	Down	[28,68]	Yes	-
NRCAM	Neuronal cell adhesion molecule	Up	[27,60]	Yes	-
OLIG2	Oligodendrocyte transcription factor 2	Up	[25,60]	?	-
PALMD	Palmdelphin	Down	[28,62]	Yes	-
PBK	PDZ binding kinase	Up	[27,28]	Yes	-
PCDH7	Protocadherin 7	Up	[21,28]	Yes	-
PDZRN3	PDZ domain containing ring finger 3	Up	[28,60]	Yes	-
PGBD5	PiggyBac transposable element-derived protein 5	Up	[25,60,62]	Yes	-
PHF17	PHD finger protein 17	Up	[28,60]	Yes	UP
PIPOX	pipecolic acid oxidase	Up	[25,28,60,62]	Yes	-
PKP1	Plakophilin 1 (ectodermal dysplasia/skin fragility syndrome)	Up	[28,62]	Yes	-
PLAG1	Pleiomorphic adenoma gene 1 protein	Down	[63,68]	Yes	-
PLK2	Polo-like kinase 2	Down	[27,28]	Yes	-
PODXL	Podocalyxin-like protein 1	Up	[25,28,60]	Yes	-
POU4F1	Brain-specific homeobox/POU domain protein 3A	Up	[27,28,62]	Yes	-
PPARGC 1A	peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	Up	[25,28]	Yes	UP
PRKAR2 B	protein kinase, cAMP-dependent, regulatory, type II, beta	Up	[21,27,68]	Yes	-
PRKCA	Protein kinase C, alpha	Up	[27,28]	Yes	-
PSEN2	presenilin 2 (Alzheimer disease 4)	Up	[27,60]	?	-
PTHLT	Parathyroid hormone-like hormone	Up	[21,28]	Yes	-
QDPR	Quinoid dihydropteridine reductase	Up	[60,68]	Yes	-

RASSF4	Ras association (RalGDS/AF-6) domain family 4	Up	[28,60,62]	Yes	-
RRP22	Ras-like protein family member 10A, on chm 22	Up	[27,60]	?	-
RYR1	Skeletal muscle-type ryanodine receptor	Up	[19,60]	Yes	-
RYR3	Brain-type ryanodine receptor	Up	[28,60,68]	Yes	UP
SLC24A3	Solute carrier family 24 (sodium/potassium/calcium exchanger), member 3	Up	[28,60]	Yes	-
SIX1	SIX homeobox 1	Up	[19,63]	Yes	-
SOX14	SRY (sex determining region Y)-box 14	Up	[25,28]	Yes	-
STX11	Syntaxin 11	Up	[21,28]	Yes	-
SULF1	Sulfatase 1	Up	[27,28]	Yes	-
SVIL	Supervillin	Down	[28,62]	Yes	-
TCF712	Transcription factor 7-like 2 (T-cell specific, HMG-box)	Up	[27,68]	Yes	-
TGFB1	Transforming growth factor, beta 1	Up	[19,28]	Yes	UP
TFAP2β	Transcription factor AP-2 beta	Up	[25,62]	Yes	-
TIAF1	TGF-beta-1-induced antiapoptotic factor 1	Up	[27,60]	?	-
TM4SF10	Transmembrane 4 sUperfamily member 10	Up	[27,68]	Yes	-
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	Up	[27,28]	Yes	-
TNNC2	troponin C type 2 (fast)	Up/Down	[19,27,62,67]	Yes	-
TNNI2	troponin I type 2 (skeletal, fast)	Up/Down	[19,62]	Yes	-
TNNT2	troponin T type 2 (cardiac)	Up	[19,67]	Yes	-
TNNT3	troponin T type 3 (skeletal, fast)	Down	[62,67]	Yes	-
TRAM2	Translocation associated membrane protein 2	Up	[27,28]	Yes	-
TSC22D2	TSC22 domain family, member 2	Up	[28,60]	Yes	-
UBE2G2	Ubiquitin-conjugating enzyme E2G 2 (UBC7 homolog, yeast)	Up	[25,28]	Yes	-

Skp2 is specifically up-regulated in ARMS compared to ERMS cell lines.

The E3 ubiquitin ligase Skp2 regulates the cellular abundance of the cell cycle kinase inhibitor p27Kip1[75]. Low or absent p27Kip1 protein levels have been observed in many tumor types, leading to the conclusion that p27Kip1 may be involved in human tumor progression [76]. It has been shown that PAX3-FOXO1 expression in NIH3T3 fibroblasts promotes the proteasome-dependent degradation of p27Kip1 via increased expression of Skp2 [77]. Moreover, it was reported that PAX3-FOXO1 binds a 220 bp genomic sequence located 49kb downstream to the Skp2 gene, suggesting that this E3 ligase might be a transcriptional target of PAX3-FOXO1 [78]. Although we did not find Skp2 to be differentially expressed in our array analysis or in other gene expression studies, we wished to determine if altered Skp2 expression levels were present in RMS cell lines. Cell lysates from ERMS (JR1, Rh6, Rh2. RD) or ARMS (Rh4, Rh30, Rh41, Rh3, Rh28) cell lines were examined by western blot analysis using Skp2 antibodies (Fig. 1). This analysis showed that although Skp2 is present in low levels in two ERMS cell lines; its expression is significantly up-regulated in PAX3-FOXO1-positive ARMS compared ERMS cell lines. This result suggests that increased expression of Skp2 might be a common characteristic in alveolar rhabdomyosarcoma and may play an active role in PAX3-FOXO1-induced cell transformation.

Figure 1

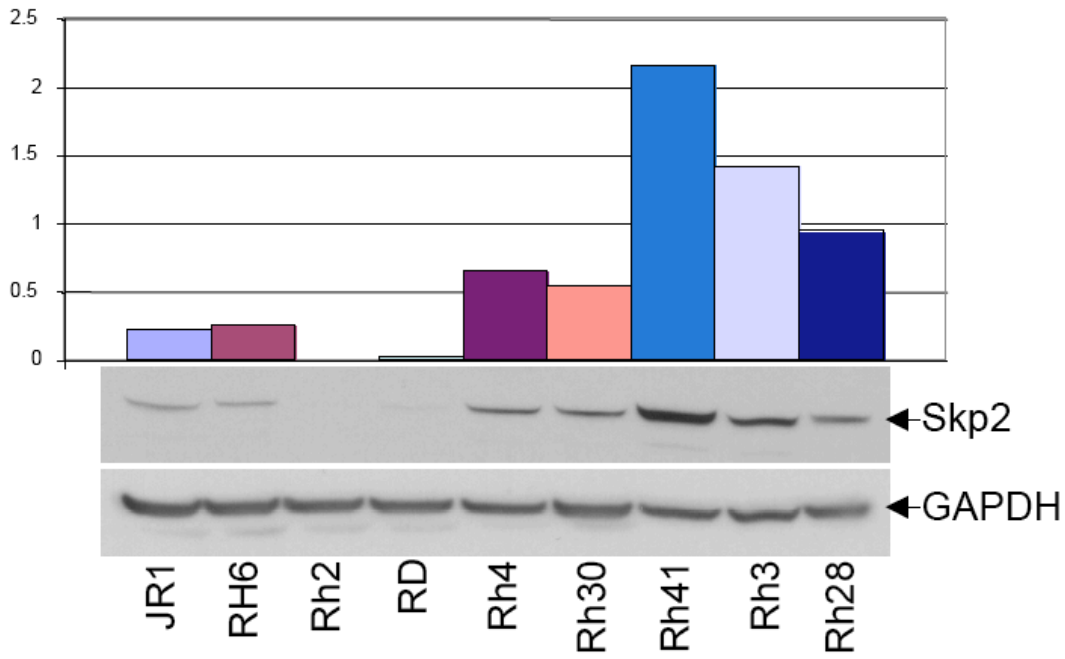

Figure 1. ARMS cell lines express higher amounts of Skp2 compared to ERMS cell lines. Western blot analysis of protein lysates of ERMS (JR1, Rh6, Rh2, RD1) and ARMS (Rh4, Rh30, Rh41, Rh3, Rh28) cell lines using a Skp2 antibody. GAPDH was used as a loading control. Using a Biorad gel imaging system the band intensities were quantified and Skp2 expression was normalized for GAPDH expression in arbitrary units (top panel).

Conclusions.

Since the discovery of the PAX3-FOXO1 fusion protein in ARMS [8], many efforts have been made to understand the oncogenic activity of this aberrant transcription factor. Despite these efforts, a defined mechanism of action for PAX3-FOXO1 still remains elusive. The identification of PAX3-FOXO1 target genes is a crucial step in defining the initial phases of ARMS development. In this paper, we present an expression profile analysis of genes differentially regulated by PAX3-FOXO1 in mouse primary myoblasts. As shown in the literature, PAX3-FOXO1 alone is not a potent oncogene, because additional genetic events are required to induce cellular transformation [79,80,81,82]. Studies performed on fully transformed ARMS tumors, ARMS cell lines or other heterologous cell lines expressing PAX3-FOXO1 might therefore identify genes whose expression might also be influenced by additional genetic events that took place. We believe that our mouse primary myoblast model offers a physiologically relevant mutation-free environment to identify genes whose expression is specifically altered by PAX3-FOXO1. It should be stressed that expression of PAX3-FOXO1 protein in these myoblasts is similar to that in ARMS tumor samples, as it is restricted by induction of the proapoptotic gene *Noxa1.* Our lab has recently demonstrated that in primary myoblasts and in ARMS cell lines PAX3-FOXO1 up-regulates the pro-apoptotic BH3-only factor NOXA in a p53 independent manner. Further up-regulation of NOXA using the cytotoxic drugs bortezomib and γ-secretase inhibitor GS1 [68,69], increases the apoptosis in primary myoblast expressing PAX3-FOXO1 and in 60% of the ARMS cell lines. Moreover, bortezomib treatment *in vivo* reduced the growth of tumors derived from transformed PAX3-FOXO1-expressing myoblasts or from the RH41 ARMS cell line. Taken together these data indicate that this novel PAX3-FOXO1/NOXA pathway could be a potential target for ARMS treatment (Amy Marshall and Gerard Grosveld, personal communication).

Because PAX3-FOXO1 can regulate transcriptional targets that are also regulated by PAX3, we also performed an array analysis on PAX3-expressing myoblasts. The gene expression profile changes in the PAX3 arrays showed a

tendency towards biological processes related to muscle development and muscle contraction, supporting the known function of PAX3 during myogenesis [83] thereby validating our analysis. The same analysis performed on the PAX3-FOXO1 array data revealed a tendency towards cellular catabolism, cellular adhesion, cell signaling, and organogenesis. Interestingly, altered cell adhesion and aberrant signaling play a pivotal role in cancer formation, invasion and metastasis [84,85]. A more close analysis of the genes specifically up- or down regulated by PAX3-FOXO1 indeed showed involvement of genes involved in cell cycle progression and invasion (Table 2). Thus, our data demonstrate that PAX3-FOXO1's effects are pleiotropic contributing to several aspects of tumorigenesis by altering expression of genes essential for these functions. These results are further supported by our findings that Skp2, a protein known to promote cellular proliferation by inhibiting p27Kip1, is specifically up-regulated in ARMS cell lines, potentially defining a role of PAX3-FOXO1 in promoting cell cycle progression. A summary of the possible mechanisms of action of PAX3-FOXO1 in ARMS development is illustrated in Figure 2. Further studies should focus on analyzing the function of the identified genes on normal myogenesis and on testing their involvement in PAX3-FOXO1 mediated cell transformation [79,82]. These studies will help identify the mechanisms involved in ARMS formation and help the development of new, targeted therapies for this catastrophic disease.

Fig.2.

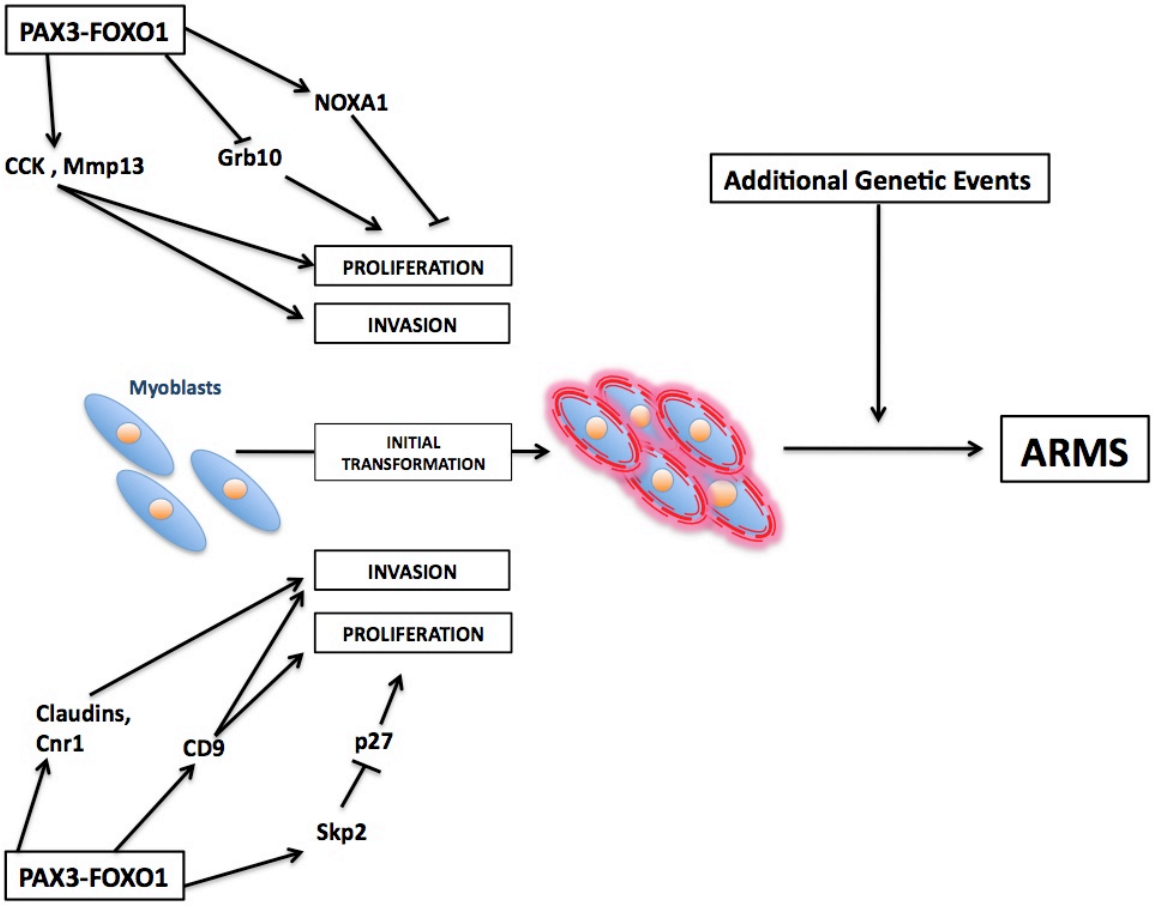

Fig.2 Schematic for the potential mechanisms via which PAX3-FOXO1-mediated changes in gene expression contribute to cellular transformation. PAX3-FOXO1 contributes to several phenotypic aspects of ARMS tumorigenesis by altering genes essential for cell proliferation and invasion. Additional mutations affecting cell cycle progression and survival complement the transformation process initiated by PAX3-FOXO1, leading to the inhibition of myogenic differentiation and ARMS formation.

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Chapter 4

IRIZIO: a Novel Gene Cooperating with PAX3-FOXO1 in Alveolar Rhabdomyosarcoma (ARMS).

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IRIZIO: a novel gene cooperating with PAX3-FOXO1 in alveolar rhabdomyosarcoma (ARMS)

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Rhabdomyosarcoma (RMS) is the most common soft-tissue sarcoma in children with an annual incidence of five new cases per million. Alveolar rhabdomyosarcoma (ARMS) is characterized by the t(2;13) or t(1;13) chromosomal translocations, which generate the *PAX3-FOXO1* or *PAX7-FOXO1* fusion genes, respectively. The oncogenic activity of *PAX3-FOXO1* has been demonstrated *in vitro* and *in vivo*, yet expression of the fusion protein alone in primary myoblasts or a mouse model is insufficient for tumorigenic transformation. To identify genes cooperating with *PAX3-FOXO1* in ARMS tumorigenesis, we generated a retroviral complementary DNA (cDNA) expression library from the Rh30 ARMS cell line. *Arf−/−* myoblasts expressing PAX3-FOXO1 and the retroviral cDNA library rapidly formed tumors after subcutaneous injection into NOD–SCID mice. Tumors formed by *Arf−/−/PAX3-FOXO1/* MarX-library myoblasts contained an unknown cDNA, encoding the C-terminus of the *Homo sapiens* hypothetical protein, FLJ10404, herein named IRIZIO. Expression of full length *IRIZIO* cDNA also cooperated with PAX3-FOXO1 in the transformation of *Arf−/−* myoblasts. Given that IRIZIO is expressed at increased levels in RMS, it might contribute to rhabdomyosarcomagenesis in humans.

Introduction

Rhabdomyosarcomas (RMS) are fast-growing malignant tumors, which account for over half of the soft tissue sarcomas in children (1). RMS is divided into two major histological subtypes, alveolar rhabdomyosarcoma (ARMS) and embryonal rhabdomyosarcoma (ERMS). ARMS may arise from immature skeletal muscle cells that remain partially differentiated, grow in alveoli-like structures and typically affects adolescents. While ERMS does not show any consistent and unique genetic alterations, ARMS is characterized by chromosomal translocations fusing the *PAX3* [t(2;13)] or *PAX7* [t(1;13)] gene to *FKHR* (*FOXO1*) (2). The *PAX3-FOXO1* gene plays a key role in 55–75% of ARMS, whereas the *PAX7-FOXO1* fusion gene is present in the remaining 10–22% of cases (3). The PAX3-FOXO1 protein possesses greater transcriptional activity than wild-type PAX3 protein (4), which plays a role during embryonic myogenesis (5). The presence of PAX3-FOXO1 might initiate a deregulated muscle developmental program in affected cells. Moreover, PAX3-FOXO1 has been linked to increased incidence of metastases and unfavorable outcome (3). Despite its oncogenic activity (6,7), *PAX3-FOXO1* requires additional genetic events to cause ARMS (8,9). Disruption of the pRb and p53 pathways are implicated in ARMS tumorigenesis in humans and mice (10–13). Our experiments demonstrate that PAX3-FOXO1 in combination with a defective p53 pathway (*Arf−/−*) alone is insufficient to transform primary mouse myoblasts and needs a compromised pRB pathway for full transformation. Using an expression complementary DNA (cDNA) library from an ARMS cell line, we identified a gene that cooperates in the tumorigenic transformation of *PAX3-FOXO1*-expressing *Arf−/−* myoblasts. This gene, *IRIZIO*, is upregulated in ARMS and may therefore contribute to rhabdomyosarcomagenesis in humans.

Abbreviations: ARMS, alveolar rhabdomyosarcoma; BSA, bovine serum albumin; cDNA, complementary DNA; DAB, 3,3′diaminobenzidine; ERMS, embryonal rhabdomyosarcoma; HA, hemagglutinin; NES, nuclear export signal; ORF, open reading frame; PCR, polymerase chain reaction; RMS; rhabdomyosarcoma.

Materials and methods

Constructs

The MARX vector was a gift from Dr David Beach. *IRIZIO* (FLJ10404) *Δ7* and *Δ7/8* cDNA were cloned into the MSCV-I-GFP vector using standard cloning techniques: the 5′ half of the cDNA sequence was excised from the plasmid pOBT-FLJ10404 (Open Biosystems, Thermo Fisher Scientific, Waltham, MA), starting from the unique StuI restriction site present in exon 9; the rest of the splice-specific sequences were polymerase chain reaction (PCR)-amplified from cDNA prepared from the ARMS cell line RH2, using primers specific for the region upstream of the StuI site. PCR fragments were generated with Phusion High-Fidelity PCR Kit (Finnzymes; Fisher Scientific, Pittsburgh, PA) using the following primers: forward 5′-ATGCCAAAGCTC-GTCAAGAATC-3′ and reverse 5′-GGCTGCTCAGGAAGCTGTTGAC-3′. Plasmids pSM2-Non-Targeting shRNA and IRIZIO-specific shRNA were purchased from Open Biosystems.

Generation of the ARMS cDNA library and its use in an in vivo complementation transformation screen

We generated a cDNA expression library from the RH30 ARMS poly(A)+ RNA in a MarX retroviral vector modified by the introduction of two SfiI sites harboring dissimilar internal sequences allowing directional cloning of the cDNAs (Figure 1) using a BD Biosciences smart kit for the synthesis of full-length cDNA (complexity = 2×10^7 clones). Freshly isolated p*16/Arf−/−* myoblasts (passage 4) were transduced with MSCV-SV40-Puro, MSCV-*PAX3*-SV40-Puro or MSCV-*PAX3-FOXO1*-SV40-Puro retroviruses and selected with 1.5 μg ml^{-1}puromycin (Sigma, St Louis, MO). Cells (2×10^4) were plated in soft agar in 6 cm dishes and colonies were scored 12–14 days later. *Arf−/−* mouse primary myoblasts expressing MSCV-SV40-Puro, MSCV-*PAX3*-SV40-Puro and MSCV-*PAX3-FOXO1*-SV40-Puro were transduced with the RH30 MarX-cDNA library and 48 h after transduction, plates were split 1:2 and cultured in the presence of 200 μg ml^{-1} hygromycin B (50 mg/ml; Cellgro, Manassas, VA) for 7–9 days. Hygromycin B resistant myoblasts (2×10^6) from each pool were subcutaneously injected into the hind and fore right flanks of NOD/SCID mice. cDNA inserts were PCR amplified from tumor genomic DNA with oligonucleotide primers flanking the SfiI sites in the MarX vector (forward primer: 5′-TTTATCCAGCCCTCACTCC-3′ and reverse primer: 5′-CGCTCACAATTCCACACTC-3′). Inserts were identified by DNA sequence analysis.

Isolation of mouse primary myoblast and tumor cells

Primary myoblasts were isolated from 1- to 3-day-old *Arf−/−* or wild-type littermates as described previously (14). Myoblasts were cultured in Ham's F10 medium supplemented with 20% of Cosmic Calf Serum (Hyclone, Logan, UT), human basic fibroblasts growth factor 1 ng/ml (Promega, Madison, WI), penicillin/streptomycin (100 μ/ml and 100 μg/ml, respectively) (Gibco-Invitrogen Corporation, Grand Island, NY) and plated on gelatin coated dishes at 37°C, 5% CO_2 and 6% O_2.

Tumor bearing mice were sacrificed using carbon dioxide asphyxiation and tumors were excised using sterile scissors. Tumor cells were isolated in the same way we isolate myoblasts (14). Part of the tumor cell suspension was used for genomic DNA isolation and the rest was cultured.

Cell culture, retroviral transduction and DNA transfection

ERMS (RD, JR1, RH6 and RH2), ARMS (RH30, RH4, RH41 RH3 and RH28) and 293T (human embryonic kidney) cells lines were grown in Dulbecco's Modified Eagle's Medium, 10% Cosmic Serum (Hyclone) and penicillin/streptomycin (100 μ/ml and 100 μg/ml, respectively; Gibco-Invitrogen Corporation). Human myoblasts (Lonza, Allendale, NJ) were cultured following the supplier's protocol. Retrovirus supernatants were generated by transfecting 293T cells using calcium phosphate as described previously (15). GFP+ cells were sorted by fluorescence activated cell sorting 48 h after transduction or were selected chemically depending on the selectable marker. Puromycin at

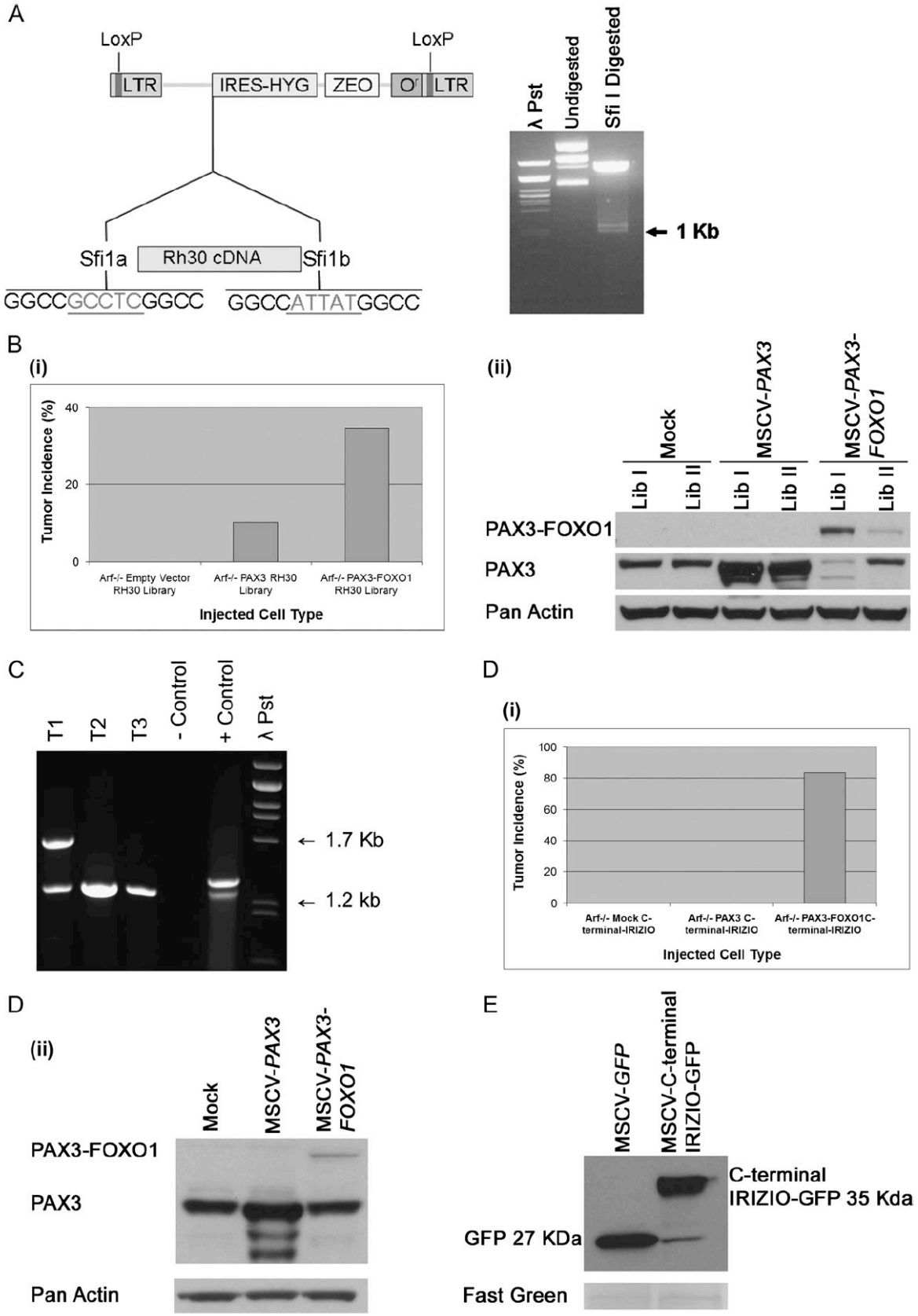

Fig. 1 *In vivo* tumorigenicity of *Arf−/−* myoblasts expressing *PAX3-FOXO1* and a RH30 Marx retroviral expression library. (**A**) Diagram showing the RH30 cDNA library inserted into the modified MarX retroviral vector. Two dissimilar SfiI sites (Sfi1a and Sfi1b) were cloned upstream the IRES-Hygro gene, which

2–4 μg ml^{-1} for RH4; Hygromycin B (Cellgro) was used at a concentration of 200 μg ml^{-1}. Leptomycin B (Sigma) was used at a concentration of 400 nM for 2 h at 37°C.

Histology and immunohistochemistry

Slides of 4–6 μm sections cut from formalin-fixed paraffin-embedded tissues were deparaffinized and rehydrated. For Myogenin and Ki67 stainings, heat-induced epitope retrieval was performed by heating slides in a BioCare Medical Decloaking Chamber at 125°C for 30 s in Target Retrieval buffer (pH 9.0; DAKO, Carpinteria, CA) followed by a 30 min cooldown period. For Desmin, antigen retrieval was performed by heating slides in a BioCare Medical Decloaking Chamber at 125°C for 30 s in citrate buffer (pH 6.0; Invitrogen, Carlsbad, CA) followed by a 30 min cooldown period. Slides were placed in tris buffer saline tween20 buffer (ThermoShandon, Freemont, CA) prior to assay. The following steps were performed on an autostainer at room temperature with tris buffer saline tween20 (ThermoShandon, Freemont, CA) buffer rinses between steps. Endogenous peroxidases were blocked by incubating slides for 5 min with 3% aqueous hydrogen peroxide. For Myogenin, non-specific protein binding was blocked with Background Sniper (30 min; BioCare Medical, Concord, CA). Slides were incubated with mouse anti-human myogenin (clone F5D; DAKO) used at 1:200 for 30 min, then mouse on mouse polymer (BioCare Medical; 30 min) followed by the chromagen 3,3′ diaminobenzidine (DAB, 5 min; ThermoShandon). For Desmin, slides were incubated with mouse anti-Desmin (clone D33; DAKO) used at 1:20 for 30 min, then sequentially with horseradish peroxidase-labeled streptavidin (10 min; ThermoShandon) and the chromagen DAB+ (10 min; DAKO). For Ki67, non-specific protein binding was blocked with Background Sniper (30 min; BioCare Medical). Slides were incubated with anti-human Ki67 (ThermoShandon) used at 1:200 for 30 min, then with Rabbit on Rodent polymer (30 min; BioCare Medical) followed by the chromagen DAB (5 min; ThermoShandon). Slides were counterstained with hematoxylin (ThermoShandon). For PAX3 and hemagglutinin (HA) staining, all assay steps, including deparaffinization, rehydration and any epitope retrieval, were performed on the Bond Max with Bond wash buffer (Leica, Bannockburn, IL) rinses between steps. Heat-induced epitope retrieval was performed by heating slides in ER1 (Leica) for 20 min. The Refine (Leica) detection system was used. Slides were incubated sequentially with hydrogen peroxide (5 min), primary antibody (15 min), post primary (8 min), anti-rabbit or anti-mouse horseradish peroxidase-conjugated polymer (8 min), DAB (10 min) and hematoxylin (5 min). The primary polyclonal anti-PAX3 antibodies (1:500) were purified as described (7). The secondary rabbit anti-goat (Jackson ImmunoResearch, West Grove, PA) was used at 1:600. The mouse anti-HA-Tag (1:1000) was obtained from Cell Signaling Technology (Beverly, MA).

Immunofluorescence

Wild-type and *Arf−/−* primary myoblasts (30 × 10^5 per well) plated on BD BioCoat collagen type I 4-well culture slides were fixed in 4% paraformaldehyde in phosphate-buffered saline for 15 min on ice. After permeabilization for 10 min in 0.1% Triton in phosphate-buffered saline, cells were blocked in 10% normal goat serum in phosphate-buffered saline and stained with HA antibody (Cell Signaling) and GRP94 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) following the manufacturer's protocol. Bound antibodies were visualized with Alexa 488 goat anti-rabbit (Invitrogen) and Cy3 donkey anti-rat (Jackson Laboratory, Bar Harbor, ME) secondary antibodies. Confocal microscopy was performed on a Nikon TE2000E2 microscope equipped with a Nikon C1Si confocal imaging system using Nikon EZC1 software. Excitation was done with 404, 488 and 561 nm dIode-pumped solid state lasers. Images were acquired with a Nikon ×60 1.45 numerical aperture objective.

Generation of cell/tissue lysates and western blotting

Cells were lysed in Cell Signaling Buffer (Cell Signaling, Beverly, MA) with 1 mM phenylmethylsulfonyl fluoride (1 mM final concentration) or RIPA buffer with Protease (Roche, Indianapolis, IN) and Phosphatase (Sigma) inhibitor cocktails. Available western blots of ARMS primary tumor biopsies were blocked in 5% non-fat milk. Polyclonal anti PAX3 antibodies were diluted 1:1000 in 5% milk; anti-MyoD and anti-myogenin were purchased from Pharmigen (BD Biosciences Pharmingen, San Diego, CA) and used at a concentration of 1.5 μg ml−1 in 5% milk; anti-Myf5 (Santa Cruz Biotechnology) was diluted 1:500 in 5% milk and anti-Myf6 (Aviva, San Diego, CA) 1:1000 in 5% bovine serum albumin (BSA); anti-HA and anti-E2F1 (Cell Signaling) were diluted in 5% BSA following the manufacturer's protocol. Subcellular fractionation was performed using the Qproteome Kit (Qiagen, Alameda, CA). The purity of the cytoplasmic and membrane fractions was determined by western blotting, using glyceraldehyde-3-phosphate dehydrogenase (Chemicon-Millipore, Billerica, MA) and N-Caderin (BD Biosciences, San Jose, CA) antibodies, respectively, both diluted 1:2000 in 5% BSA. The nuclear fraction was stained with Histone H3 antibody (Upstate Biotechnology, Lake Placid, NY) diluted 1:1000 in 5% BSA. Antibodies used for the signaling pathway analysis (supplementary Figure 4 is available at *Carcinogenesis* Online) included anti-total- and phospho-IGFIR, anti-total-Akt and phospho-Akt (Ser 473 and Ser 308), anti-total- and phospho-S6, anti-phospho-Erk1/2 (Thr202/Tyr204) and anti-phospho-p90RSK (Thr359/Ser363), purchased from Cell Signaling Technology and diluted 1:1000 in 5% BSA. Anti-phospho-cJun (Active Motif, Carlsbad, CA) was diluted 1:1000 in 5% BSA. The optical density of IRIZIO (HA) and Pan Actin bands (Figure 3C) was measured using the ImageJ software (http://rsbweb.nih.gov/ij/index.html).

RNA isolation and reverse transcription–PCR

RNA was isolated from myoblasts (2 × 10^6 cells/10 cm dish) or from ERMS and ARMS cell lines from cultures that were 80% confluent using the MirVANA RNA extraction kit (Applied Biosystems/Ambion, Austin, TX). RNA (1 μg) was used for first strand cDNA synthesis using the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen) following the manufacturer's instructions.

For the exon junction analysis, probe/primer sets overlapping splice junctions of *IRIZIO* messenger RNA between exons 6 and 9 were designed using the Primer Express software from Applied Biosystems (Carlsbad, CA). *IRIZIO full-length* splice variant: forward 5′-TGACTGCTGCTACTGTGAGTTCTTC-3′; reverse 5′-GCTTTCTCTCTGCCATTTCCTTT-3′ and probe 5′-ACAATGCGGCAAAAG-3′. *IRIZIOΔ7* variant: forward 5′-GG AAGTTCTGTGACTGCTGCTA-3′; reverse 5′-AGCGGGTGGCGCATT-3′ and probe 5′-TGTGAGTTCTTCGGCC-3′. *IRIZIOΔ7/8* variant: forward 5′-TGACTGCTGCTACTGTGAGTTCTTC-3′; reverse 5′-TGCCAACTGGGCCTTCT-3′ and probe 5′-CAATGC GGAAA-3′. PCRswere performed using the TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) supplemented with primers, probes and cDNA. Quantification of each splice variant was achieved using a standard curve (expressed in copy number) constructed by amplifying known amounts of target DNA. For *IRIZIOΔ7* and Δ7/8, we used the MSCV constructs harboring the cDNA of the two splice variants. For *IRIZIO full-length* including exon 7, we synthesized a specific DNA oligonucleotide corresponding to the exon junction region (exons 6–7): 5′-GGGCTGGGTGAGGAAGAGGATAGCAGCTCTGAGCGAAGCTCCTGCACCTCATCCTCCACCCACCAGAGAGATGGGAAGTTCTGTGACTGCTGCTACTGTGAGTTCTTCGGCCACAATGCGGCAAAAGGAAAGGAAATGGCAGAGAGAAAGCTATGATTCTGATGAGTATGTATACGTGTGTAATCCCAGAGAAGTGAACGCTTGGGAGTGATGAAGGCAGAGTGGAAGCAAAAAGGCTCTC-3′. Expression of target genes was normalized for *GAPDH*. To analyze *IRIZIO* expression after shRNA knockdown, we used a primer/probe set targeting exon 9: forward 5′-GAGCCAAGCTCAAAGGAAGTTC-3′; reverse 5′-GCTTCCCACCTGAGGACACA-3′ and probe 5′-AGGAGCTGCCTGAGC-3′. Standard curve was calculated using serial dilution of MSCV-HA*IRIZIOΔ7* plasmid. *GAPDH* was used as internal control.

allows directional cloning of the cDNAs. The agarose gel on the right shows DNA of the RH30 MarX-cDNA library digested with SfiI. (**B**) (i) Tumor incidence in NOD/SCID mice injected with two independent pools of *Arf−/−* primary myoblasts transduced with empty vector, MSCV-*PAX3* or MSCV-*PAX3-FOXO1* together with the RH30 cDNA library. LibI and LibII indicate the two pools of myoblasts independently transduced with the same library. Tumors generated from MSCV-*PAX3* injected cells developed later those from MSCV-*PAX3-FOXO1* injected cells. (ii) Western blot showing PAX3 and PAX3-FOXO1 expression in *Arf−/−*/MSCV-Puro, *Arf−/−*/MSCV-*PAX3* and *Arf−/−*/MSCV-*PAX3-FOXO1* primary myoblasts also expressing the RH30 MarX library (see supplementary Table I, available at *Carcinogenesis* Online) prior to injection into NOD/SCID mice. (**C**) Agarose gel electrophoresis showing the PCR-amplified products of MarX cDNA proviruses present in the DNA of A*rf−/−*/MSCV-*PAX3-FOXO1/RH30-MarX*-derived tumor cells. T1–T3 represent different tumors from which the cDNAs were amplified. Two specific bands where identified in tumor one: the identity of the upper band will be described elsewhere. The lower band represents the 3′ region of the *FLJ10404/IRIZIO* gene in all three tumors. Genomic DNA extracted from *Arf−/−*/MSCV-*PAX3-FOXO1* cells expressing the RH30 library prior to injection was used as a positive control. Water in the PCR mix was used as a negative control. (**D**) (i) NOD/SCID mice were injected with *Arf−/−* myoblasts expressing empty vector, MSCV-*PAX3* or MSCV-*PAX3-FOXO1* also expressing the Marx-*IRIZIO*-Fragment. Only *Arf−/−*/MSCV-PAX3-FOXO1/MarX-C-terminal-IRIZIO cells produced tumors. (ii) Western blot showing PAX3 and PAX3-FOXO1 expression in MarX-C-terminal-IRIZIO-transduced *Arf−/−/MSCV-PAX3* or A*rf−/−/MSCV-PAX3-FOXO1* primary myoblasts (supplementary Table I is available at *Carcinogenesis* Online). (**E**) Western blot of NIH3T3 cells transiently transfected with expression plasmids encoding GFP or C-terminal-IRIZIO-GFP. Bands were visualized using anti-GFP antibody. Fast green staining of the blot was used as a protein loading control.

Animals

NOD/SCID mice were purchased from the Jackson Laboratory. p*16/Arf*–/– were obtained from Dr David Beach. Mice were maintained at St Jude Children's Research Hospital following the Institutional Animal Care and Use Committee guidelines.

Results

Transformation of primary myoblasts by PAX3-FOXO1 depends upon inactivation of the pRb and p53 pathways

The t(2;13) generating the PAX3-FOXO1 fusion protein is considered the main initiating event in ARMS formation. It has been reported that transformation of muscle progenitors by this oncogene in mice depends on inactivation of both the p53 and pRb tumor suppressor pathways (13). To ascertain that this is also true for cultured low-passage primary mouse myoblasts, we transduced wild-type, *Arf*–/– and p*16/Arf*–/– myoblasts (passage 4) with retroviruses encoding *GFP*, *PAX3* or *PAX3-FOXO1* and tested them for tumor formation by injection (2×10^6 cells per site) into the fore and hind right flanks of –five to six NOD-SCID mice (16). As shown in Table I wild-type or *Arf*–/– myoblasts transduced with these retroviruses did not form tumors during the 6 months observation period, whereas p*16/Arf*–/–/*PAX3-FOXO1* myoblasts produced five tumors within 4–6 weeks. In comparison, p*16/Arf*–/–/*GFP* myoblasts produced one tumor after 4 months and p*16/Arf*–/–/*PAX3* myoblasts produced four tumors after 2–6 months, appearing considerable later than p*16/Arf*–/–/*PAX3-FOXO1* tumors. This experiment showed that transformation of myoblasts by PAX3-FOXO1 is dependent on combined compromised p53/pRb pathways.

C-terminal IRIZIO cooperates with PAX3-FOXO1 to transform Arf–/– primary mouse myoblasts

Based on these results, we set out to identify novel genes that might cooperate with *PAX3-FOXO1* in the transformation of *Arf*–/– mouse primary myoblasts. We generated a cDNA library from the RH30 ARMS cell line (12) in a modified Marx retroviral vector (17) (Figure 1A). Two independent pools of *Arf*–/– myoblasts stably expressing MSCV-SV40-Puro, MSCV-*PAX3*-SV40-Puro or MSCV-*PAX3-FOXO1*-SV40-Puro were transduced with the RH30 retroviral cDNA library (LibI and LibII, Figure 1B, panel ii) and the resulting hygromycin resistant cell pools were subcutaneously injected into the right fore- and hind flanks of NOD/SCID mice (supplementary Table I, upper panel, is available at *Carcinogenesis* Online). Tumor formation was monitored for up to 5 months post injection. Within this observation period, 7 of 13 mice injected with both *Arf*–/–/*PAX3-FOXO1*/MarX-RH30 cell pools developed tumors within 4–8 weeks, generating a total of nine tumors resulting in a tumor incidence of 34.6% (Figure 1B, panel i). Two mice injected with *Arf*–/–/*PAX3*/MarX-RH30 cells also developed tumors but only after 10 and 14 weeks post injection, respectively, resulting in a tumor incidence of 10%. *Arf*–/–/*MSCV-Puro*/MarX-RH30 injected mice did not develop tumors. The cooperating cDNAs were recovered from the genomic DNA of the PAX3-FOXO1 tumors by PCR (Figure 1C), subcloned and sequenced. Three of six analyzed tumors contained a cDNA encoding part of the hypothetical protein FLJ10404, which we dubbed IRIZIO. In all cases, an identical partial cDNA sequence was found, all starting with a GTG codon, which when used as a start codon would result in a C-terminal IRIZIO open reading frame (ORF) of 227 amino acids. An in-frame ATG codon was also present downstream of the GTG, potentially encoding a further truncated C-terminal IRIZIO peptide of 49 amino acids. We next determined which portion of C-terminal IRIZIO was encoded by the cDNA fragment by fusing it in-frame to the 5′ end of the GFP ORF in the MSCV retroviral vector. Lysates of NIH3T3 cells transiently transfected with this IRIZIO-GFP construct were immunoblotted using GFP antibodies (Figure 1E), showing a fusion protein of ~34 kDa. Thus, the IRIZIO peptide measured a mere 7 kDa given that the size of GFP protein is 27 kDa, indicating that translation starts at the first in-frame ATG, which matches the consensus Kozak translation initiation sequence (12).

To verify cooperation between the isolated cDNA and *PAX3-FOXO1*, we reinserted the partial *IRIZIO* cDNA into the MarX vector and transduced a second pool of *Arf*–/– myoblasts expressing MSCV-SV40-Puro, MSCV-*PAX3*-SV40-Puro or MSCV-*PAX3-FOXO1*-SV40-Puro. Only NOD/SCID mice (Figure 1D; supplementary Table I is available at *Carcinogenesis* Online, lower panel) injected with *Arf*–/–/*PAX3-FOXO1*/MarX-*IRIZIO* myoblasts developed tumors 3–4 weeks after the injection. This confirmed that the 3′ *IRIZIO* retrovirus cooperated with PAX3-FOXO1 in the neoplastic transformation of primary Arf–/– myoblasts.

Arf–/–/*PAX3-FOXO1*/*C-terminal-IRIZIO tumors resemble ARMS tumors at the morphological and molecular level*

To determine the morphology and molecular characteristics of the *Arf*–/–/*PAX3-FOXO1*/C-terminal-IRIZIO tumors in NOD/SCID mice, we prepared paraffin sections and isolated hygromycin and puromycin resistant cell lines from these tumors. Tumors were diagnosed by histopathology and immunohistochemistry to be the solid variant of ARMS (Figure 2A). The tumors were highly infiltrative into the surrounding muscle, and residual host muscle fibers were scattered within the mass, predominately along the margins. Although there were no prominent fibrous septae as are typical for ARMS, the cells were arranged in ill-defined aggregates with central loss of cellular cohesiveness. The skeletal muscle origin of the tumors was confirmed by scattered diffuse positive staining for myogenin and multifocal areas of positive desmin expression. Affinity-purified PAX3 antibody showed expression of endogenous PAX3 and exogenous PAX3-FOXO1. Sections were also stained with Ki67 antibody, which stains proliferating cells in all phases of the cell cycle (supplementary Figure 1 is available at *Carcinogenesis* Online), confirming the neoplastic nature of *Arf*–/–/*PAX3-FOXO1*/C-terminal-*IRIZIO* tumors. To further investigate whether the *Arf*–/–/*PAX3-FOXO1*/C-terminal-*IRIZIO* fragment tumors resembled primary ARMS at the molecular level, we performed western blot analysis using cell lysates from *Arf*–/– injected myoblasts, tumor cells and primary ARMS tumor biopsies using antibodies detecting muscle cell-specific differentiation markers (Figure 2B). Except for Myf5, the murine *IRIZIO*

Table I. NOD/SCID mice were subcutaneously injected in the fore and hind right flanks with WT, *Arf*–/– or p*16/Arf*–/– primary mouse myoblasts transduced with MSCV-GFP, MSCV-*PAX3*-GFP or MSCV-*PAX3-FOXO1*-GFP retrovirus

Primary myoblast	Construct	Number of injected mice	Number of injections per mouse	Mice with tumors	Total number of tumors	Time of tumor formation
WT	MSCV-GFP	2	2	0	0	—
WT	MSCV-*PAX3*	2	2	0	0	—
WT	MSCV-*PAX3-FOXO1*	2	2	0	0	—
Arf–/–	MSCV-GFP	2	2	0	0	—
Arf–/–	MSCV-*PAX3*	2	2	0	0	—
Arf–/–	MSCV-*PAX3-FOXO1*	4	2	0	0	—
Arf–/–/p16–/–	MSCV-GFP	5	2	1	1	4 months
Arf–/–/p16–/–	MSCV-*PAX3*	5	2	4	4	2–6 months
Arf–/–/p16–/–	MSCV-*PAX3-FOXO1*	6	2	4	5	4–6 weeks

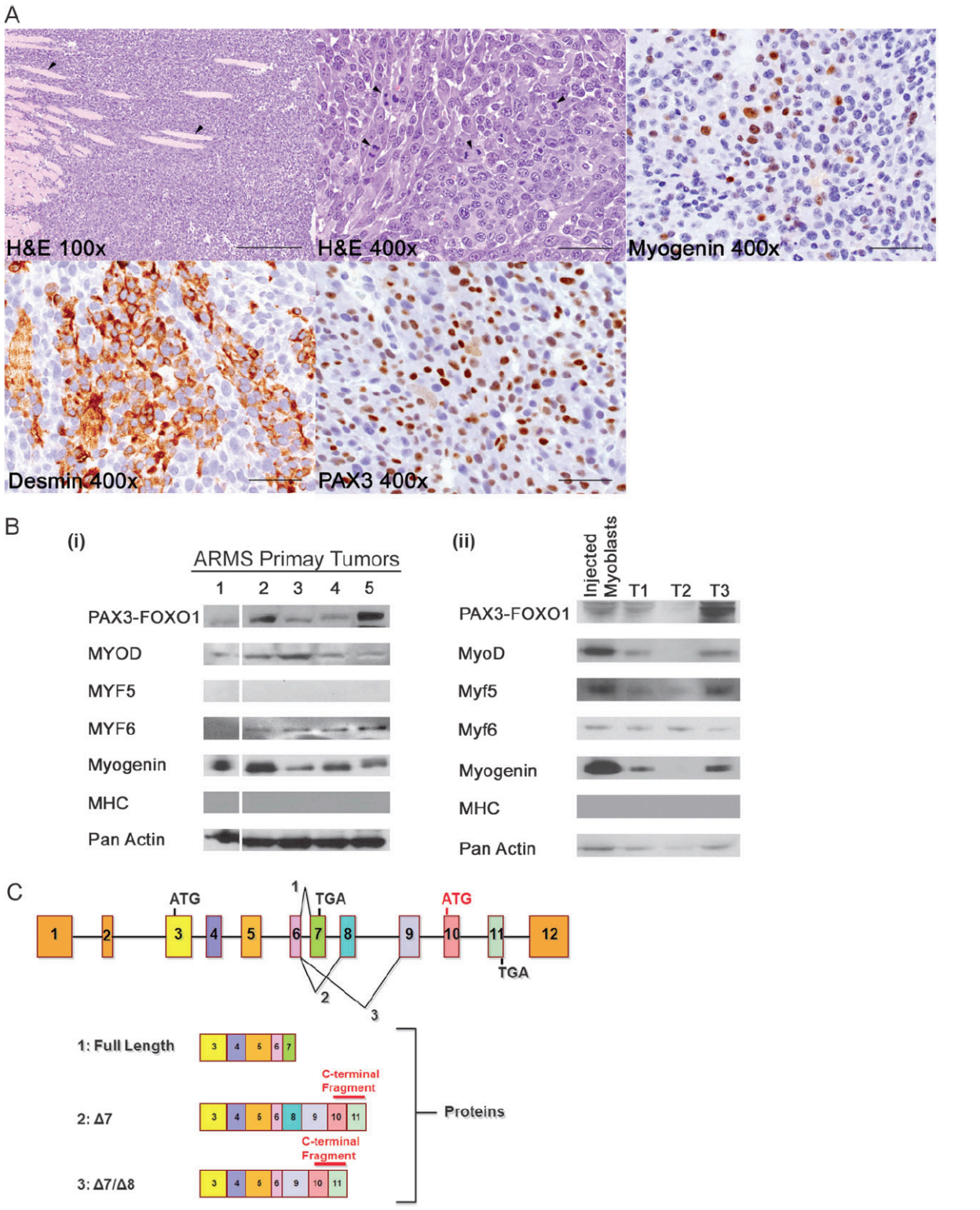

Fig. 2 C-terminal-IRIZIO-derived tumors express similar molecular markers as ARMS primary tumors. (**A**) Tumor sections derived from mice injected with *Arf−/−/PAX3-FOXO1/C-terminal-IRIZIO* myoblasts were stained with hematoxylin and eosin, with Myogenin, Desmin or PAX3 antibodies. Scale bars in images: ×100 magnification scale bar, 250 μm; ×400 magnification scale bar, 50 μm. The arrowheads in the ×100 hematoxylin and eosin image indicate muscle fibers, which are being separated by the tumor. In the ×400, they indicate mitotic figures. (**B**) Western blot showing expression of the muscle cell-specific markers MyoD, Myf5, Myf6 and Myogenin in five primary ARMS tumors (i) and *Arf−/−/PAX3-FOXO1/C-terminal-IRIZIO* tumors (ii). Pre-injection *Arf−/−/PAX3-FOXO1/C-terminal-IRIZIO* cells were used as control. (**C**) The *IRIZIO* gene is located on chromosome 5 and is composed of 12 exons. The splice variant 1 (full length) contains exon 7 and generates a truncated protein due to the presence of a stop codon in the ORF. The variants Δ7 and Δ7/8 encode larger proteins. The portion of the protein encoded by the C-terminal IRIZIO cDNA is indicated by the red line above the Δ7 and Δ7/8 proteins.

fragment-derived tumors and the human ARMS tumors expressed similar differentiation markers (Figure 2B). Therefore, tumors generated by cells expressing the *Arf−/−/PAX3-FOXO1*/C-terminal-*IRIZIO* fragment showed both morphological and molecular similarity with primary ARMS.

IRIZIO encodes at least three differentially spliced messenger RNAs but only two transform *PAX3-FOXO1* expressing *Arf−/−* myoblasts.

To establish that also full-length IRIZIO transformed *Arf−/−*/PAX3-FOXO1 myoblasts, we queried the Ensembl database and found that three alternatively spliced transcripts are known for human IRIZIO (Figure 2C). Although more splice variants are listed for the human gene, only one is listed for the mouse. Because of this, we focused our attention to the splice form that is present in both mouse and human. IRIZIO shows high sequence conservancy among mammals, especially at the 3′ end of the ORF. Human *IRIZIO* is located on chromosome 5 and is composed of 12 exons with an ORF starting in exon 3. Sequence alignment between human and mouse full-length transcripts showed an overall homology of 80%. Alternative splicing involves exons 7 and 8 (Figure 2C). Exon 7 contains a stop codon in the ORF, resulting in a truncated protein lacking the carboxy-terminal region. Splicing out exon 7 (*Δ7*) or exons 7 and 8 (*Δ7/8*) would generate larger proteins that both include the C-terminal IRIZIO peptide described above. DNA and protein sequences of IRIZIO *Δ7* and *Δ7/8* are reported in supplementary Figure 2 (available at *Carcinogenesis* Online). The existence of the *Δ7* and *Δ7/8* splice variants was confirmed by semi-quantitative reverse transcription–PCR using RNA from ARMS and ERMS cell lines, several human tissues and primers specific for exons 5 and 9. The identity of each cDNA was verified by sequence analysis (data not shown). The reverse transcription–PCR did not detect the splice variant including exon 7. Therefore, we only cloned cDNAs for *the Δ7* and *Δ7/8* splice variants into the MSCV-I-GFP retroviral vector. Because there are no suitable commercial antibodies available for IRIZIO, an N-terminal HA tag was added to each construct. We repeated the *in vivo* transformation experiments by expressing the HA-*Δ7* and HA-*Δ7/8 IRIZIO* variants in *Arf−/−*/vector, Arf−/−/PAX3 and *Arf−/−/PAX3-FOXO1* myoblasts. Lysates obtained from cells prior to injection were immunoblotted with PAX3 and HA antibodies to confirm expression of HA-PAX3-FOXO1 and IRIZIO (Figure 3B). All mice injected with IRIZIOΔ7 expressing myoblasts developed a subcutaneous tumor within 8 weeks postinjection resulting in a higher tumor incidence (50%) than in mice injected with IRIZIOΔ7/8 expressing cells, which formed two independent tumors in one animal, 13 weeks postinjection (tumor incidence of 20%; Table II and Figure 3A). Sections from paraffin-embedded *PAX3-FOXO1/HA*-IRIZIO*Δ7* tumors were immunostained with HA, PAX3, Myogenin, Desmin and Ki67 antibodies, confirming expression of all these proteins (Figure 3D; supplementary Figure 1 is available at *Carcinogenesis* Online). Histologically, the PAX3-FOXO1/HA-IRIZIOΔ7 tumors showed the classic pattern for ARMS, including distinct fibrous septae and central loss of cellular cohesiveness. Many cells had positive staining for myogenin and nearly all cells stained positively for Desmin. Lysates of cultured cells isolated from the excised tumors were immunoblotted for the expression of PAX3, HA–IRIZIOΔ7 and muscle-specific differentiation markers, showing an expression pattern similar to that of primary ARMS tumors (Figures 2B and 3C). Analysis of tumors derived from *Arf−/−/PAX3-FOXO1/HA*-IRIZIO*Δ7/8* myoblasts showed identical results (data not shown) suggesting that the IRIZIO*Δ7/8* splice variant operates similar to IRIZIO*Δ7*, albeit with reduced efficiency. To determine if the C-terminal region of IRIZIO is necessary for transformation, we also tested the *in vivo* tumorigenicity of two HA-IRIZIO*Δ7* deletion mutants also lacking part of exon 10 and the entire exon 11 (HA-*IRIZIOΔ7/Δ10-11*) or exons 9–11 (HA-*IRIZIOΔ7/Δ*9-11). We compared tumor formation in mice injected with *Arf−/−/PAX3-FOXO1* myoblasts expressing vector, *HA-IRIZIOΔ7*, HA-*IRIZIOΔ7/Δ10-11* or HA-*IRIZIOΔ7/Δ*9-11 over a period of 5 months (Table II). All mice injected with myoblasts expressing the HA-IRIZIO*Δ7* splice variant readily developed tumors during this time period (within 4–8 weeks, tumor incidence 80%) but also two mice injected with HA-*IRIZIOΔ7/Δ10-11* developed a tumor (within 8–9 weeks, tumor incidence 20%), whereas HA-*IRIZIOΔ7/Δ*9-11 injected mice remained healthy (Figure 3E). Because the level of expression of all IRIZIO constructs was similar (Figure 3B and E, panel ii), this suggested that *IRIZIO Δ7* transformed *Arf−/−/PAX3-FOXO1* myoblasts more efficiently than splice variant HA-*IRIZIOΔ7/8* or the deletion mutant HA-*IRIZIOΔ7/Δ10-11*. Moreover, the C-terminal region encoded by exons 9–11 of *IRIZIO Δ7* is essential for transformation.

The IRIZIOΔ7 splice variant is overexpressed in ERMS and ARMS cell lines

Alternative splicing events affect the functional diversity of a large number of genes (18). Alternative splicing is crucial for normal development and physiology of numerous tissues and organs and deregulation has been associated with human disease (19, 20). To determine the abundance of each *IRIZIO* splice variant in ARMS cell lines, we performed a quantitative exon junction real-time PCR using specific primers/probe sets in the region between exons 6 and 9. We used cDNAs from several ERMS and ARMS cell lines and cDNA from proliferating and differentiating primary human myoblasts was used as a control (Figure 4A). This showed that the full-length 12 exon variant is least abundant in both ERMS and ARMS cell lines. Conversely, the *Δ7* variant appeared to be upregulated in both ERMS and ARMS cell lines compared with the primary human myoblasts at any stage of differentiation, whereas there was no significant change in the expression level of *Δ7/8 RNA*. This is in agreement with our observation that the *Δ7* splice variant cooperated with *PAX3-FOXO1* in the transformation of Arf−/− myoblasts. However, upregulation of *IRIZIOΔ7* also

Table II. NOD/SCID mice were subcutaneously injected in the fore and hind flanks (right side) with *Arf-/-*/MSCV-Puro, *Arf-/-*/MSCV-*PAX3-Puro* or Arf−/−/MSCV-*PAX3-FOXO1-Puro* primary myoblasts also transduced with MSCV-HAIRIZIO*Δ7*–GFP or MSCV-HAIRIZIO*Δ7/8*–GFP retrovirus

Primary myoblast background	Cooperating genes	Construct	Number of injected mice	Number of injections per mouse	Mice with tumors	Total number of tumors	Time of tumor formation
Arf−/−	MSCV-HA *IRIZIOΔ7*-GFP	MSCV-SV40-Puro	5	2	0	0	—
Arf−/−	MSCV-HA *IRIZIOΔ7*-GFP	MSCV-*PAX3*-SV40-Puro	5	2	0	0	—
Arf−/−	MSCV-HA *IRIZIOΔ7*-GFP	MSCV-*PAX3-FOXO1*-SV40-Puro	5	2	5	5	8–10 weeks
Arf−/−	MSCV-HA *IRIZIOΔ7/8*-GFP	MSCV-SV40-Puro	5	2	0	0	—
Arf−/−	MSCV-HA *IRIZIOΔ7/8*-GFP	MSCV-*PAX3*-SV40-Puro	5	2	0	0	—
Arf−/−	MSCV-HA *IRIZIOΔ7/8*-GFP	MSCV-*PAX3-FOXO1*-SV40-Puro	5	2	1	2	13 weeks
Arf−/−	GFP	MSCV-*PAX3-FOXO1*-SV40-Puro	5	2	0	0	
Arf−/−	MSCV-HA *IRIZIOΔ7*-GFP	MSCV-*PAX3-FOXO1*-SV40-Puro	5	2	5	6	5–8 weeks
Arf−/−	MSCV-HA *IRIZIOΔ7/Δ10-11*-GFP	MSCV-*PAX3-FOXO1*-SV40-Puro	5	2	2	2	7–12 weeks
Arf−/−	MSCV-HA *IRIZIOΔ7/Δ9-11*-GFP	MSCV-*PAX3-FOXO1*-SV40-Puro	5	2	0	0	—

The relative tumorigenicity of the *IRIZIO* deletion mutants was evaluated by injecting NOD/SCID mice with Arf−/−/*PAX3-FOXO1* myoblasts expressing HA-*IRIZIOΔ7/Δ10-11* or HA-*IRIZIOΔ7/Δ9-11*. Cells expressing HA-*IRIZIOΔ7* were used as a positive control.

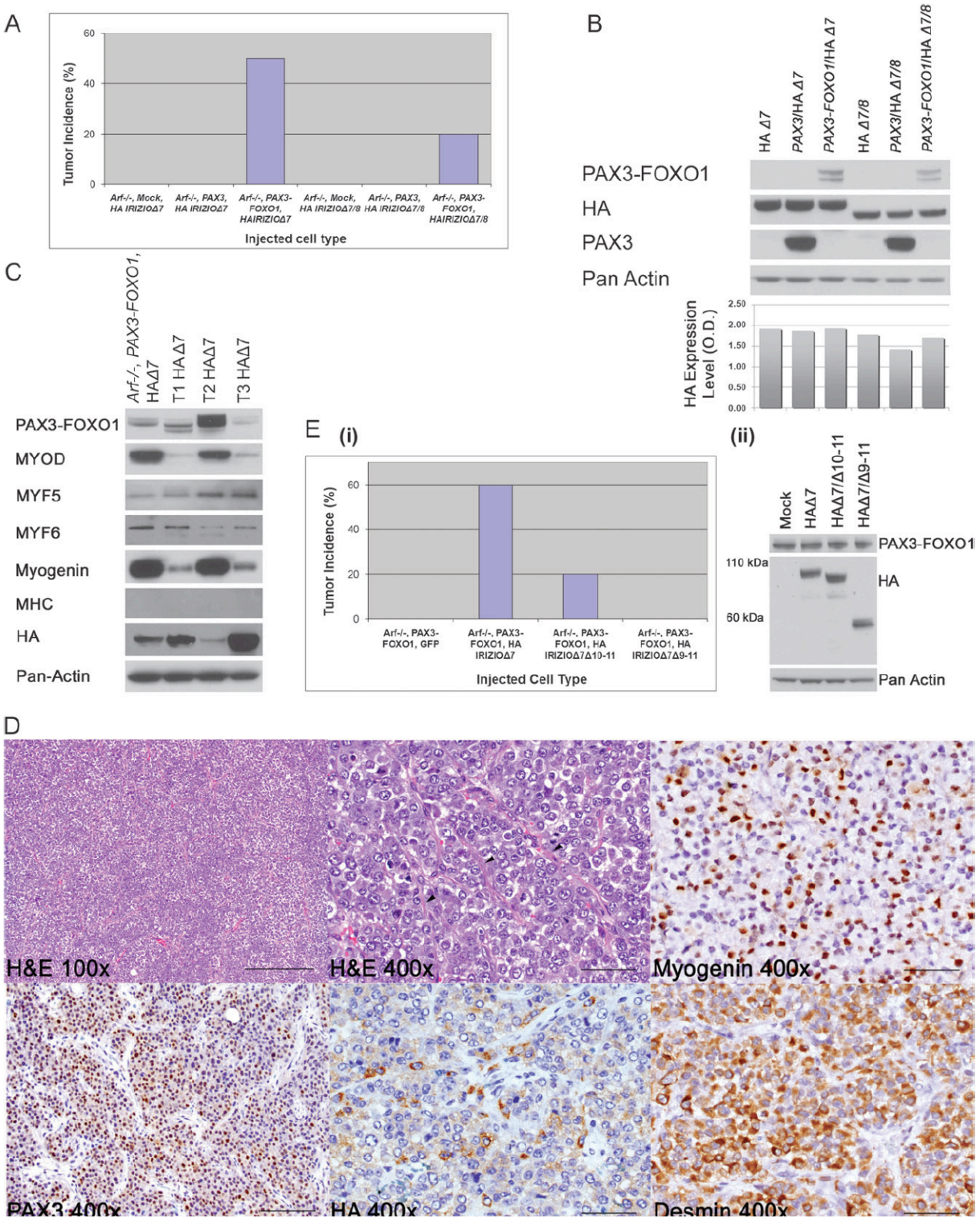

Fig. 3 *IRIZIO Δ7 and Δ7/8* splice variants cooperate with *PAX3-FOXO1A* to transform Arf−/− primary myoblasts. (**A**) Tumor incidence in NOD/SCID mice injected with Arf−/− primary myoblasts transduced with empty vector, MSCV-*PAX3* or MSCV-*PAX3-FOXO1* and also transduced with MSCV-HA-*IRIZIOΔ7*–GFP or MSCV-HA-*IRIZIOΔ7/8*–GFP retrovirus. Tumor formation in HA-*IRIZIOΔ7* injected mice occurred within 8–10 weeks post injection, whereas mice injected with HA-*IRIZIOΔ7/8* developed tumors 13 weeks after injection (Table II). Results were obtained from two independent experiments. (**B**) Western blots of Arf−/− myoblast expressing MSCV-HA-*IRIZIOΔ7*–GFP or MSCV-*HA-IRIZIOΔ7/8*–GFP injected into NOD/SCID mice (Table II). PAX3 and HA antibodies were used for the detection of PAX3 or PAX3-FOXO1 and IRIZIO splice variants, respectively. Pan Actin antibody was used as a control for equal protein loading. The histogram underneath the blot shows the relative intensity of IRIZIOΔ7 and Δ7/8 bands as calculated by normalizing the optical density (O.D.) of the HA-IRIZIO bands with that of the Pan-Actin bands. (**C**) Western blot analysis of *IRIZIOΔ7*–GFP tumor cells isolated from tumors listed in Table II using HA, PAX3, MyoD, Myf5, Myf6 or Myogenin antibodies. Pan-actin staining was used as a loading control. But for Myf5, the expression pattern of myogenic markers in

occurs in ERMS tumor cell lines thereby excluding exclusive cooperation with *PAX3-FOXO1*. Thus, *IRIZIOΔ7* overexpression might be an additional event contributing to transformation of myogenic progenitor cells, suggesting that overexpression of the Δ7 splice variant might play a causative role in human rhabdomyosarcomagenesis.

We next addressed if knockdown of *IRIZIO* in *Arf–/–/PAX3-FOXO1/IRIZIOΔ7* cells isolated from a tumor in NOD/SCID mice would suppress their *in vivo* tumorigenicity. After subcutaneous injection into mice, cells transduced with IRIZIO shRNA retrovirus or control non-targeting shRNA virus, showing 80% and no knockdown of IRIZIO protein, respectively (supplementary Figure 3A is available at *Carcinogenesis* Online), developed tumors equally efficiently (supplementary Figure 3B is available at *Carcinogenesis* Online). A repeat experiment with the ARMS RH4 cell line, comparing non-targeting shRNA cells with cells that showed a 75% knockdown of IRIZIO RNA, resulted in equally efficient tumor formation (data not shown) in NOD-SCID mice. This suggested that IRIZIO expression is important during tumor formation, but once established IRIZIO expression is dispensable for maintaining the transformed state.

Subcellular localization of IRIZIO variants

To determine the subcellular localization of HA-IRIZIOΔ7 and HA-IRIZIOΔ7/8, we performed immunostaining with an HA antibody of primary *Arf–/–* myoblasts expressing these proteins. Confocal microscopy showed that both splice variants localized in the cytoplasm with increased fluorescence signal in the perinuclear region. To further define this staining pattern, we performed a co-immunostaining with an antibody specific for the endoplasmatic reticulum protein GRP94 (Figure 4B, panels i and ii). This showed partial colocalization with the IRIZIOΔ7 and IRIZIOΔ7/8 signals. Because the C-terminal region of IRIZIO stimulates PAX3-FOXO1's transforming activity, we repeated the experiment with *Arf–/–* myoblasts expressing the HA-*IRIZIOΔ7/Δ10-11* or HA-*IRIZIOΔ7/Δ9-11* mutants to determine whether this region affected protein localization. HA-IRIZIOΔ7/Δ10-11 showed a staining pattern identical to that of the other splice variants (Figure 4B, panel iii) suggesting that the region encoded by exons 10 and 11 does not affect protein localization. However, the mutant lacking the sequences encoded by exon 9 to exon 11 showed a mostly nuclear staining. Hence, the sequences encoded by exon 9 maintain the protein in the cytoplasm, indicating that IRIZIO probably shuttles between the cytoplasm and nucleus with the equilibrium of transport strongly toward the cytoplasmic side (Figure 4B, panel iv). To exclude that PAX3-FOXO1 protein had any effect on IRIZIO localization, we repeated the same immunostaining experiments with *Arf–/–/PAX3-FOXO1* myoblasts, which produced identical results (data not shown). Sequence analysis of the IRIZIO C-terminal region identified a putative bipartite NLS (21) –KRARHKLKKK– at the 3′ end of exon 8 and a putative leucine-rich nuclear export signal (NES) (22) –LDLSPLTL– in exon 9 (Supplementary Figure 2 is available at *Carcinogenesis* Online). To test if IRIZIO indeed shuttles, we transduced primary *Arf–/–* myoblasts with MSCV-HA-*IRIZIOΔ7*-GFP, MSCV-HA-*IRIZIOΔ7/8*-GFP, MSCV-HA-*IRIZIOΔ7/Δ9-11*-GFP or MSCV-HA-*IRIZIOΔ7/Δ10-11*-GFP retrovirus and plated GFP$^+$-sorted cells on collagen-coated glass slides and treated the cells for 2 h with vehicle or Leptomycin B, which blocks CRM1-mediated nuclear export. Immunostaining with HA antibody (supplementary Figure 4 is available at *Carcinogenesis* Online) showed that HA-IRIZIO*Δ*7, HA-IRIZIO*Δ*7/8 and HA-IRIZIO*Δ*7/*Δ*10-11 localized in the nucleus of Leptomycin B-treated cells, whereas in vehicle-treated myoblasts, these proteins resided in the cytoplasm. No difference in localization was observed in cells expressing the HA-IRIZIO*Δ*7/*Δ*9-11 mutant lacking the NES sequence. Our results therefore confirmed the nuclear-cytoplasmic shuttling nature of IRIZIO. We also analyzed IRIZIO localization by western blotting using subcellular fractions of *Arf–/–* myoblasts overexpressing HA-*IRIZIOΔ7*, HA-*IRIZIO*Δ7/8, HA-*IRIZIOΔ7/Δ9-11* or HA-*IRIZIOΔ7/Δ10-11* (Figure 4C). This confirmed the cytoplasmic localization of the first three proteins but paradoxically the *IRIZIOΔ7/Δ*9-11 also appeared in the cytoplasmic fraction. We believe this to be an artifact of the subcellular fractionation procedure given that immunostaining showed nuclear localization. Together, these results indicated that IRIZIOΔ7 and IRIZIOΔ7/8 mainly localize in the cytoplasm of primary mouse myoblasts and they partially colocalized with the endoplasmic reticulum.

Discussion

Cancer is a multistep process involving several cooperating mutations in oncogenes and tumor suppressor genes (23). In this paper, we utilized an *in vivo* genetic screen to identify novel cooperating partners for PAX3-FOXO1, the pathognomonic sign of ARMS. The screen was based on the observation that PAX3-FOXO1 transforms mouse primary myoblasts that harbor a compromised p53 and pRb pathway (11, 13). We transduced primary mouse myoblasts with a compromised p53 pathway (*Arf–/–*) stably expressing *PAX3-FOXO1* with a retroviral cDNA expression library generated from the fully transformed ARMS cell line RH30. We expected that cooperating cDNAs would interfere directly or indirectly with the pRb pathway. After injection of these cells into NOD-SCID mice, three independent tumors carried a cDNA representing the 3′ half of exon 9 and exons 10 and 11 of *IRIZIO*, encoding a 49 amino acid C-terminal polypeptide. Despiting using a full-length cDNA cloning strategy, the IRIZIO clone isolated from the library was a partial cDNA because full-length IRIZIO cDNA does not contain Sfi1 restriction sites. A multispecies alignment of the *IRIZIO* transcript (Ensembl gene ID: ENSG00000146067) revealed that the coding exons are highly conserved among mammals. We identified three messenger RNA species generated by differential splicing for human *IRIZIO*; the longest coding isoform (full length) contained a stop codon in exon 7 resulting in a truncated protein lacking the 49 amino acid C-terminal region. Because of this and the *Δ7* transcript listed for the mouse in the Ensembl database, we focused our attention on the other two splice variants Δ7 and Δ7/8, which produced proteins that contain the 49 amino acid C-terminus. In silico analysis of the IRIZIO Δ7 and Δ7/8 secondary structures using the conserved domain database on the National Center for Biotechnology Information website revealed no obvious homology to any known domain present in the database. IRIZIO belongs to the FAM193 gene family together with the Cr8Orf gene. The official name for those genes is 'family with sequence similarity 193, member B (FAM193B) or member A (FAM193A)', respectively. Protein sequence alignment between IRIZIOΔ7 and FAM193A using the National Center for Biotechnology Information Basic Local Alignment Search Tool revealed an overall homology of 53%. Interestingly, the highest similarity is in the last 76 amino acids (89% homology) of both proteins, which includes the library-isolated C-terminal IRIZIOΔ7 sequence. This sequence was also analyzed using the SWISS-MODEL and InterProScan databases (24, 25). This secondary structure analysis showed that IRIZIO is composed of random coil regions (77.4%), alpha-helices (19.5%) and extended beta sheets (3.1%), suggesting that IRIZIO is largely an unfolded polypeptide lacking any fixed three-dimensional structure.

Mice injected with *Arf–/–IRIZIOΔ7/PAX3-FOXO1* myoblasts produced tumors in 50% of the injection sites after 8 weeks, whereas in mice injected with *Arf–/–/IRIZIOΔ7/8/PAX3-FOXO1* myoblasts,

IRIZIOΔ7 tumors was similar to that in primary ARMS tumors (Figure 2). (**D**) Hematoxylin and eosin, Myogenin, Desmin, PAX3 or HA staining of tumor sections derived from mice injected with *Arf–/–/PAX3-FOXO1*/HA-*IRIZIOΔ7*–GFP myoblasts. Scale bars in images: ×100 magnification scale bar, 250 μm; ×400 magnification scale bar, 50 μm. The arrowheads in ×400 hematoxylin and eosin image indicate the fibrous septae. (**E**) (i) NOD/SCID mice, subcutaneously injected with 2 × 10^6 *PAX3-FOXO1/Arf–/–* myoblasts expressing HA-*IRIZIOΔ7* or the HA-*IRIZIOΔ7/Δ10-11* or HA-*IRIZIOΔ7/Δ9-11* mutants, developed tumors at 5–8 weeks, 7–12 weeks or no tumors within the 6 months observation period, respectively (Table II). (ii) Western blot showing expression of HA-IRIZIOΔ7, HA-IRIZIOΔ7/Δ10-11 or HA-IRIZIOΔ7/Δ9-11 in *Arf–/–*/PAX3-FOXO1 cells using an HA antibody prior to injection into NOD/SCID mice. Pan actin staining was used as a loading control.

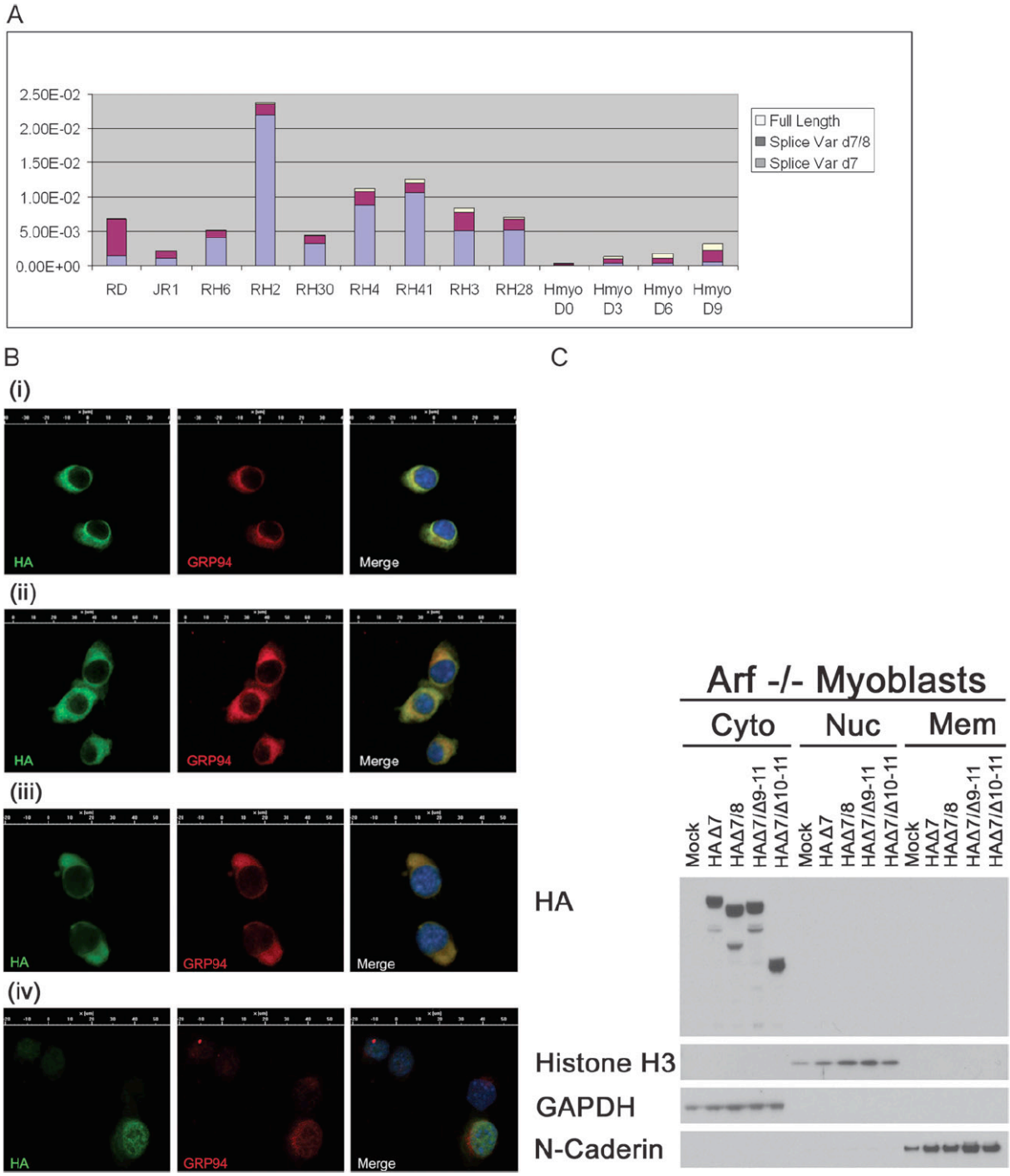

Fig. 4 1*IRIZIO Δ7* is overexpressed in ERMS and ARMS cell lines. (**A**) Graph showing the expression pattern of *IRIZIO* splice variants in ERMS (RD, JR1, RH6 and RH2) and ARMS (RH30, RH4, RH41, RH3 and RH28) cell lines determined by quantitative real-time reverse transcription–PCR analysis. Expression level of the differentially spliced *IRIZIO* variants was obtained using primer/probe sets specific for the exon junction region of each variant, between exon 6 and exon 9. Proliferating (D 0) and differentiating (D3–D9) primary human myoblasts were used as a control for wt *IRIZIO* expression. (**B**) Immunohistochemistry of *Arf−/−* primary myoblasts expressing HA-*IRIZIOΔ7*-GFP (i), HA-*IRIZIOΔ7/8*-GFP (ii), HA-*IRIZIOΔ7/Δ10-11*-GFP (iii) HA-*IRIZIOΔ7/Δ9-11* (iv). Proliferating myoblasts grown on collagen-coated slides were stained with antibodies against the ER protein GRP94 and HA. Confocal images were taken using a Nikon (TE2000E2) microscope at ×600 magnification. (**C**) Western blot analysis performed on subcellular fractions of *Arf−/−* primary myoblasts expressing HA-*IRIZIOΔ7*-GFP, HA-*IRIZIOΔ7/8*-GFP, HA-*IRIZIOΔ7/Δ10-11*-GFP or HA-*IRIZIOΔ7/Δ9-11-GFP*. To determine the purity of each fraction, the blot was probed using antibodies for glyceraldehyde-3-phosphate dehydrogenase (Cytosol), Histone H3 (Nucleus) and N-Cadherin (Membranes). IRIZIO proteins were visualized using HA antibodies.

only 20% of the injection sites produced tumors after 12 weeks. This suggested that the region encoded by exon 8 affected *IRIZIO's* transforming activity. It is noteworthy that we identified a putative bipartite NLS in exon 8 and a putative leucine-rich NES in exon 9. The localization experiments using the combination of the IRIZIO*Δ7/Δ*9-11 mutant and the Leptomycin B nuclear export inhibitor strongly

suggest that IRIZIOΔ7 is shuttling through the nucleus and that the potentially resulting posttranslational modifications of the protein may be important for its transforming activity in combination with PAX3-FOXO1. Additional experiment will have to show the functionality of the putative NLS and NES.

Tumor formation by *Arf−/−/IRIZIOΔ7/PAX3-FOXO1* myoblasts was delayed compared with tumors generated by *Arf−/−/PAX3-FOXO1* myoblasts expressing the *IRIZIO* C-terminal fragment. Also, tumors generated with IRIZIOΔ7 seemed more differentiated than those with the C-terminal fragment, as evidenced by the higher level of myogenin staining and the diffuse expression of Desmin. Because this fragment measures 49 amino acids, it is reasonable to speculate that it will bind to another protein(s), thereby altering the activity of this target rather than having a direct enzymatic or structural function of its own. Currently, we have not yet identified potential interaction partners of the IRIZIO peptide. We also determined if presence of the C-terminal peptide was essential for the transforming activity of IRIZIOΔ7 and found that the protein missing the peptide (*IRIZIOΔ10-11*) displayed reduced transforming activity. This suggests that the peptide is important but not essential for IRIZIOΔ7's transforming activity, whereas a mutant missing a larger part of the C-terminus (*IRIZIOΔ9-11*) locating to the nucleus had no transforming activity. Thus, although the 49 amino acid peptide alone is sufficient to cooperate with PAX3-FOXO1 in transformation, the entire domain mediating cooperation may extend further N-terminal although we have not mapped the boundary of this extension.

An exon junction real time reverse transcription–PCR showed that compared with primary human myoblasts the IRIZIO*Δ7* transcript is overexpressed in ERMS and ARMS cell lines, whereas the full-length variant appeared to be the least abundant in all samples. Our IRIZIO knockdown experiments suggest that the protein plays a role in tumor formation but not in tumor maintenance. Both in mouse Arf−/−/PAX3-FOXO1/IRIZIO*Δ7* tumor cells and the ARMS cell line RH4, knockdown of IRIZIO*Δ7* did not affect the *in vivo* tumorigenicity. To understand whether IRIZIO*Δ7* or IRIZIO*Δ7/Δ8* expression affects signaling pathways that might feed into the Rb pathway, we checked the phosphorylation status of Akt, Erk1/2, p90, cJun and S6 in Arf−/− myoblasts expressing PAX3-FOXO1 in combination with GFP, IRIZIO*Δ7* or IRIZIO*Δ7/Δ8*, but no obvious differences were noted (supplementary Figure 5 is available at *Carcinogenesis* Online). We also looked for changes in the cellular localization of E2F1, but again, E2F1 remained in the cytoplasm of the different myoblast cell lines (supplementary Figure 6 is available at *Carcinogenesis* Online).

Despite the lack of an obvious link with the pRb pathway, our *IRIZIOΔ7* data are consistent with the observation that overexpression of IRIZIO messenger RNA is associated with recurrent lung tumor formation in a specific subgroup of patients (26), confirming that IRIZIO can function as an oncogene in other tumors also. This is in agreement with our data that IRIZIO RNA is upregulated both in ARMS and ERMS cell lines excluding a unique cooperation between PAX3-FOXO1 and IRIZIO. Additional studies are required to determine which IRIZIO splice variant is overexpressed in those lung cancer patients to better understand the function of this novel oncogene in human tumorigenesis.

Supplementary material

Supplementary Figures 1–6 and Table I can be found at http://carcin.oxfordjournals.org/

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Chapter 5

Plod1 (Lysyl Hydroxylase 1) Cooperates with *PAX3-FOXO1* in Transforming *Arf-/-* Primary Mouse Myoblasts.

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Manuscript in preparation

Abstract

Alveolar rhabdomyosarcoma (ARMS) is a pediatric solid tumor related to the skeletal muscle lineage. 70% of ARMS cases carry the t(2;13)(q35;q14) chromosomal translocation, which produces a fusion transcription factor with the DNA binding domains of PAX3 and the transactivation domain of FOXO1[1]. Expression of PAX3-FOXO1 in muscle progenitor cells is thought to be the initiating event in the development of ARMS, but alone is insufficient to cause tumor formation. To identify genes that synergize with PAX3-FOXO1 in ARMS development, we generated a cDNA expression library from the RH30 ARMS cell line. Co-expression of this library with PAX3-FOXO1 in *Arf-/-* primary mouse myoblasts, followed by subcutaneous injection into NOD/SCID mice resulted in tumor formation. Using this approach, we indentified *PLOD1* (*LH1*) as a *PAX3-FOXO1* cooperating gene in transformation of *Arf-/-* myoblasts.

Keywords: Plod1, PAX3-FOXO1, ARMS, Alveolar Rhabdomyosarcoma

Introduction

Rhabdomyosarcoma (RMS) belongs to a family of mesenchymal-derived soft tissue tumors arising from the skeletal muscle lineage [1]. RMS is divided into two main subtypes: embryonal (ERMS) and alveolar (ARMS), displaying distinctive genetic and histological characteristics. While ERMS represents the majority of the RMS cases, ARMS is less frequent (20-30% of rhabdomyosarcomas) but is generally associated with frequent metastasis and a less favorable clinical outcome. ARMS is associated with recurring chromosomal translocations, t(2;13)(q35;q14) and t(1;13)(p36;q14), which fuse the DNA binding domains of PAX3 or PAX7 to the transactivation domain of FOXO1, respectively [2,3,4]. This results in the expression of the PAX3/7-FOXO1 fusion proteins that possess greater transcriptional activity than wild-type PAX3/7 [5]. Hence, PAX3/7-FOXO1 fusion proteins are believed to initiate a deregulated muscle developmental program in the affected cells, which leads to their tumorigenic transformation. If expressed exogenously, PAX3-FOXO1 is able to induce transformation in both chicken and mouse fibroblasts [6,7]. In conditional *PAX3-FOXO1* knock-in mice, homozygosity of this fusion oncogene accompanied by *Ink4a/Arf* loss-of-function resulted in tumor formation [8], showing that PAX3-FOXO1 expression alone is not sufficient to transform myogenic progenitor cells [8,9,10].

To identify genes involved in PAX3-FOXO1 tumor progression we performed a genetic complementation screen using a cDNA expression library derived from the ARMS cell line, RH30. Co-expression of PAX3-FOXO1 together with the RH30 cDNA library in *Arf-/-* mouse myoblasts resulted in clonal tumor formation and subsequent identification of *PLOD1* (procollagen-lysine 1, 2-oxoglutarate 5-dioxygenase 1) as a PAX3-FOXO1 cooperating gene. *PLOD1* encodes the enzyme lysyl hydroxylase 1 (*LH1*). This enzyme is localized to the lumen of the endoplasmic reticulum (ER) and is present in three isoforms (*LH1-3*) in human and mouse [11,12,13,14,15]. PLOD1 hydroxylates lysine residues in collagen and collagen-like proteins harboring the X-Lys-Gly repeat sequence [16]. The hydroxylysine residues serve as attachment sites for

carbohydrates, important for the stabilization of the modified proteins through intermolecular cross links [17]. Null mutations in PLOD1 in humans causes type VIA Ehlers-Danlos syndrome, a connective tissue disorder characterized by joint hypermobility, skin fragility and hyperextensibility [18]. *PLOD1* is expressed in a wide variety of tissues [19,20,21], and in the mouse is highly expressed in the liver, kidney, lung, heart as well as skeletal muscle [22]. In this study, we show a novel and surprising role for *PLOD1* in PAX3-FOXO1-driven tumorigenesis in *Arf-/-* mouse myoblasts.

MATERIALS AND METHODS

Generation of the ARMS cDNA library and constructs.

Generation of the cDNA expression library from RH30 ARMS cell line cloned in the MarX retroviral vector and the functional genetic screen has been described previously [23]. Human full-length *PLOD1* cDNA (Open Biosystems-Thermo Scientific, Waltham, MA) was cloned into the MSCV-IRES-YFP vector. The PLOD1ΔFe mutant was generated by PCR-mediated mutagenesis to change the Fe^{2+} cofactor binding amino acids H656A, D658A and H708A within the lysyl hydroxylase active site. An additional mutant lacking the last 33 C-terminal amino acids (PLOD1-33C) was also generated. C-terminally HA-tagged full length *PLOD1* was generated by PCR using the following primers: forward (specific to the MSCV plasmid) 5'-AAGCCCTTTGTACAC CCTAAGC-3'; reverse (including HA tag) 5'-GGGAATTCTTAAGCGTAATCTGGAA CATCGTATGGGTAGGGATCGACGAAGGAGACTGGG-3', and cloned into MSCV-IRES-GFP. An HA-tagged C-terminal *PLOD1* fragment was generated from the full-length *PLOD1*-HA cDNA by removing the N-terminal portion by SacII and BglII restriction enzyme digest.

In vivo functional genetic screen and mouse primary myoblast cell isolation.

Isolation of *Arf-/-* primary myoblasts has been described previously [24]. The *In vivo* functional genetic screen using *Arf -/-* myoblasts expressing MSCV-SV40-Puro, MSCV-*PAX3*-SV40-Puro, or MSCV-*PAX3-FOXO1*-SV40-Puro together with the RH30 MarX-cDNA library has been described previously [23]. MarX vector cDNA inserts were PCR amplified form tumor genomic DNA using the following oligonucleotide primers (forward primer: 5'-TTTATCCAGCCCT CACTCC-3'; reverse primer: 5'-CGCTCACAATTCCACACTC-3'). Inserts were identified by DNA sequence analysis.

Culture of cell lines.

ERMS (RD, JR1, RH6, RH2) and ARMS (RH30, RH4, RH41 RH3, RH28) cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (Cosmic Serum from HyCLONE, Logan, UT) and penicillin/streptomycin solution (100U/ml and 100µg/ml, respectively, Gibco-Invitrogen Corporation, Grand island, NY). Human myoblasts were purchased from Lonza (Allendale, NJ) and cultured according to the manufacturer's instructions.

Immunofluorescence and immunohistochemistry.

Myoblasts growing on collagen type I 4-well culture slides (BD Biosciences, San Jose CA) were fixed and stained as described previously [23]. Cells were stained using anti-HA antibody (1:100, Cell Signaling, Beverly, MA) and anti-GRP94 antibody (1:400, Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies Alexa 488 goat anti-rat (Invitrogen, Carlsbad, CA) and Cy3 donkey anti-mouse (Jackson Laboratory, Bar Harbor, ME) were diluted 1:400 in 10% normal goat serum in PBS. Confocal microscopy was performed on a Nikon TE2000E2 microscope equipped with a Nikon C1Si confocal imaging system using Nikon EZC1 software. Histology and

immunohistochemistry were performed on formalin-fixed paraffin-embedded tissues as described previously [23]

Western blotting.

Cells were lysed in 1x lysis buffer (Cell Signaling, Beverly, MA) or RIPA buffer as described [23]. Proteins were separated on 4-12% gradient (SDS)-polyacrylamide gels (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose, blocked in 5% non-fat milk in TBST (w/v) and probed with affinity purified anti-PAX3 antibody [7] (1:1000), anti-MyoD and anti-Myogenin (1.5 μg ml^{-1}, BD Pharmingen San Diego, CA), anti-Myf5 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Myf6 (1:1000, Aviva, San Diego, Ca), anti-Desmin (Santa Cruz Biotechnology) and anti-HA (1:1000, Cell Signaling, Beverly, MA). PLOD1 expression was detected using anti-PLOD1 antibody (1:500, Abnova, Taipei, Taiwan). All antibodies were diluted in 5% non-fat milk in TBST (w/v) with the exception of anti-HA, anti-Myf6 and anti-PLOD1 antibodies, which were diluted in 5% BSA in TBST (w/v).

Real-Time PCR.

Quantitative Real-Time PCRs (QRT-PCRs) were performed using the TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Probe and primers specific for human *PLOD1-3* were designed using Primer Express software (Applied Biosystems). For *PLOD1*: probe (5'-FAM-ACATGCGACTTTTCA-BHQ1-3'); primers (forward 5'-GGCTCCACTACCCC CAGAAA-3'; reverse 5'-GGTGCTGCTCGTGGTTGTG-3'). For PLOD2: probe (5'-FAM-TTCTAAGGTACAATTGCTC-BHQ1-3'); primers (forward 5'-TCAGGGAGGTGGTTGCAAA-3'; reverse 5'-AGCCTTTTCGTGACTCAA-3'). For PLOD3: probe (5'-FAM-CACCAAATCGTGCGCC-BHQ1-3'); primers (forward 5'-CGGTTTTGCCACCACCAT-3'; reverse 5'-TCGTCATCATCTTG TACTTCC-3'). cDNA used for the QRT-PCR analysis was synthesized using RNA isolated from ERMS and ARMS cell lines (collected at 80% confluency) and human myoblasts ($2x10^6$ cells plated on a 10 cm dish) following a

published protocol [23]. The standard amplification curve was generated by amplifying known amounts of MSCV-*PLOD1*-IRES-YFP plasmid or, in the case of PLOD2 and PLOD3, by amplification of five-fold dilutions of a known amount of purified cDNA. Expression analysis was normalized using a GAPDH internal control (Human GAPDH Endogenous Control VIC/MGB Probe, primer Limited Applied Biosystems, Foster City, CA). A standard curve for the internal control was generated using a plasmid containing human *GAPDH* cDNA.

NOD/SCID mice.

NOD/SCID (NOD.CB17-*Prkdc*^{scid}/J) or NOD-*scid* IL2Rγ^{null} mice [25] mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were maintained at St. Jude Children's Research Hospital following the Institutional Animal Care and Use Committee guidelines.

RESULTS

A C-terminal PLOD1 fragment cooperates with PAX3-FOXO1A to transform *Arf-/-* myoblasts.

To identify novel oncogenes that cooperate with PAX3-FOXO1 in the transformation of primary mouse myoblasts, we developed an *in vivo* complementation assay based on co-expression of PAX3-FOXO1 together with a cDNA expression library (complexity $2x10^7$) generated from the RH30 ARMS cell line in *Arf-/-* myoblasts [23]. Freshly isolated primary *Arf-/-* myoblasts were transduced with the RH30 cDNA library together with empty-, *PAX3*-Puro-, or *PAX3-FOXO1*-Puro MarX retroviruses. *Arf-/-* myoblasts from each pool were injected subcutaneously into the right fore- and hind-flanks of NOD/SCID mice and tumor formation was monitored over a five-month period. Mice injected with *Arf-/-/PAX3-FOXO1*/MarX-RH30-library myoblasts developed tumors quicker and with higher frequency than those injected with *Arf-/-/PAX3*/MarX-RH30-library myoblasts while those injected with *Arf-/-/*empty vector/MarX-RH30-library myoblasts did not develop tumors [23] (Supplementary Table 1). The identity of the cDNA in the retroviral insert of PAX3-FOXO1 derived tumors was determined by PCR amplification followed by DNA sequence analysis using MarX vector specific primers (Fig. 1A). Three out of six tumors scored for a single insert encoding the 3' region of the *PLOD1* gene representing about one third of the open reading frame. Another tumor contained the same *PLOD1* cDNA and a lower molecular weight band whose identity is described elsewhere [23]. The partial *PLOD1* sequence contains an in-frame ATG codon resulting in a C-terminal-PLOD1 protein of 247 amino acids. The isolation of the same cDNAs from independent tumors argues for a cooperative role of the encoded protein in the tumorigenic transformation of *Arf-/-* myoblasts expressing PAX3-FOXO1, suggesting that the C-terminal 247 amino acids of PLOD1 (C-ter PLOD) are sufficient for this effect.

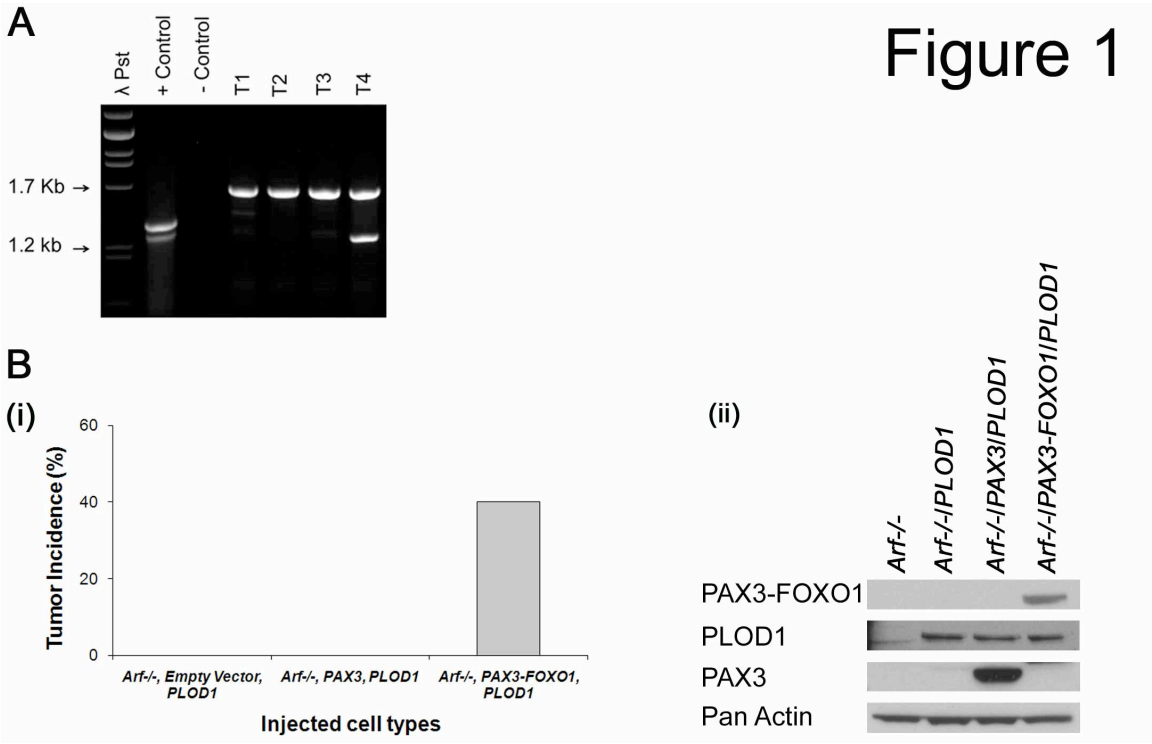

Figure 1

Figure 1. PLOD1 cooperates with PAX3-FOXO1 in the transformation of *Arf-/-* myoblasts. (A) Agarose gel showing PCR products of cDNAs recovered from *Arf-/-/PAX3-FOXO1*/MarX-RH30-library-derived tumors (Table 1). The 1.7 Kb band, was identified by DNA sequencing as the 3' region of the *PLOD1* gene. A total number of seven tumors were analyzed of which 4 contained the same 3' *PLOD1* retrovirus (T1-T4). The additional band in T4 represents a partial cDNA of a non-related gene. Genomic DNA extracted from *Arf-/-/PAX3-FOXO1/*MarX-RH30-library cells prior to injection was used as a positive control (+Control). **(Bi)** Tumor incidence within a 5-month period in NOD/SCID mice, subcutaneously injected with *Arf-/-* myoblasts expressing MSCV-empty vector, *PAX3* or *PAX3-FOXO1,* together with full length *PLOD1* (Table 2). **(Bii)** Immunoblot performed on retrovirus-transduced myoblasts prior to injection. An anti-PAX3 antibody was used for PAX3-FOXO1 and PAX3 detection. Over expressed PLOD1 was detected with an anti-PLOD1 antibody.

Full-length PLOD1 also transforms PAX3-FOXO1 expressing myoblasts.

Given that C-ter *PLOD1* cDNA isolated from the RH30 library represented only the C-terminal third of the full length protein (727-amino acids), we inserted the cDNA encoding full-length PLOD1 in the MSCV-IRES-YFP retroviral vector and transduced *Arf-/-* myoblasts expressing PAX3-FOXO1, PAX3 or empty vector. Lysates of these cells were immunoblotted using anti-PAX3 and PLOD1 antibodies to confirm expression of both proteins (Fig. 1B(ii)). We injected $2x10^6$ YFP^+ cells from each pool subcutaneously into NOD/SCID mice. Only mice injected with *PAX3-FOXO1/PLOD1* expressing cells developed tumors within 14 weeks post-injection (Fig. 1B(i) and Table 1).

No tumors were formed with control *Arf-/-* myoblasts expressing empty vector, PAX3 or PAX3-FOXO1 in the absence of PLOD1 (data not shown). *Arf-/-/PAX3-FOXO1*/*PLOD1*-derived tumors were diagnosed by histopathology and immunohistochemistry to be the solid variant of alveolar rhabdomyosarcoma. The skeletal muscle origin of the tumors was confirmed by scattered diffuse positive staining for myogenin and multifocal areas of positive desmin expression (Fig. 2A,B). Tumor-derived sections were also stained for PAX3 and GFP/YFP expression to confirm the cellular origin of these tumors and Ki67 to show tumor cell proliferation (Fig. 2A and Supplementary Fig.1). To further characterize these tumors at the molecular level we immunoblotted tumor-derived cell lysates with muscle-specific differentiation markers (Fig. 2B). With the exception of MyoD, the expression pattern of these markers in the *Arf-/-/PAX3-FOXO1*/*PLOD1*-derived tumor cells was similar to that of ARMS primary tumor cells [23]. Overall, these results indicated that both a C-terminal portion and full length PLOD1 cooperate with PAX3-FOXO1 in the transformation of *Arf-/-* myoblasts and the resulting tumors show histological and gene expression similarities with ARMS.

***PLOD* family members are over expressed in ARMS cell lines.**

The commercially available anti-PLOD1 antibody proved to be inadequate to visualize endogenous PLOD1 expression. Therefore we used quantitative real-

time PCR to determine the expression level of *PLOD1* in ARMS cells lines compared to that in ERMS cell lines [26] (Fig. 2D) and proliferating and differentiating human skeletal muscle myoblasts and mature skeletal muscle. Figure 2C shows that after 3 days of differentiation human myoblasts activated the expression of myogenic differentiation markers that are similar to that of the RH30 ARMS line. This makes these cells a suitable control for the normal gene expression profile at this stage of myoblast differentiation. The real-time PCR analysis showed *PLOD1* expression in 2/4 ERMS (JR1 and RH6) and 2/5 ARMS (RH41, RH30) cell lines exceeded that of day 3 differentiating myoblasts. Given that there are three lysyl hydroxylases isoforms expressed in humans [16], we also analyzed *PLOD2* and *PLOD3* expression levels in the RMS cell lines using QRT-PCR (Fig. 2 E, F). Although no significant difference was detected in the PLOD2 levels between ARMS samples and day 3 differentiating myoblasts, *PLOD2* expression was significantly elevated in ARMS cell lines compared to that in ERMS cell lines. Similarly, *PLOD3* was over expressed in ARMS lines compared to ERMS lines and significant overexpression was also detected in 3/5 ARMS (RH41, RH3, RH28) cell lines compared to day 3 differentiating myoblasts. Overall our expression analysis shows that, with the exception of RH4, at least one PLOD family member is up regulated in ARMS cell lines, consistent with PLOD family members playing a role in alveolar rhabdomyosarcomagenesis.

Figure 2

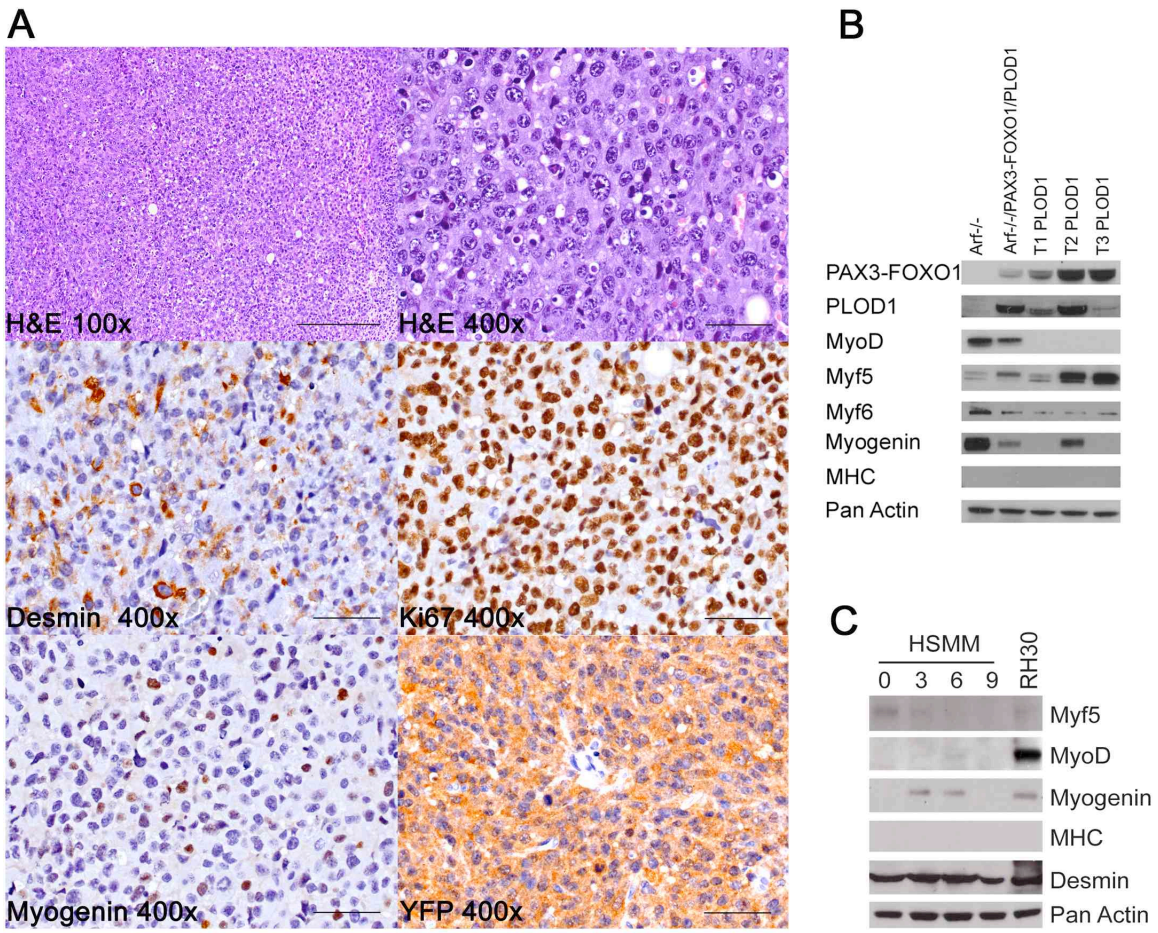

Figure 2. Histopathologic analysis of *Arf-/-/PAX3-FOXO1/PLOD1*-derived tumors and *PLOD1* expression ARMS cell lines. (A) Paraffin sections from a representative *Arf-/-/PAX3-FOXO1/PLOD1* tumor (T2) stained with hematoxylin/eosin (H&E), Myogenin, Desmin, Ki67 or GFP (YFP) antibodies. Scale bars in images: 100X magnification scale bar = 250um; 400X magnification scale bar = 50um. **(B)** Immunoblot of lysates of cells isolated from the tumors reported in Table 2 to detect the myogenic differentiation markers MYOD, Myf5, Myf6, Myogenin, and Myosin Heavy Chain (MHC). PAX3-FOXO1 and PLOD1 were detected using anti-PAX3 and anti-PLOD1 antibodies, respectively. **(C)** Immunoblot of proliferating and differentiating (day 0-9) human myoblasts and the RH30 ARMS cell line showing the expression of myogenic and differentiation markers.

Figure 2

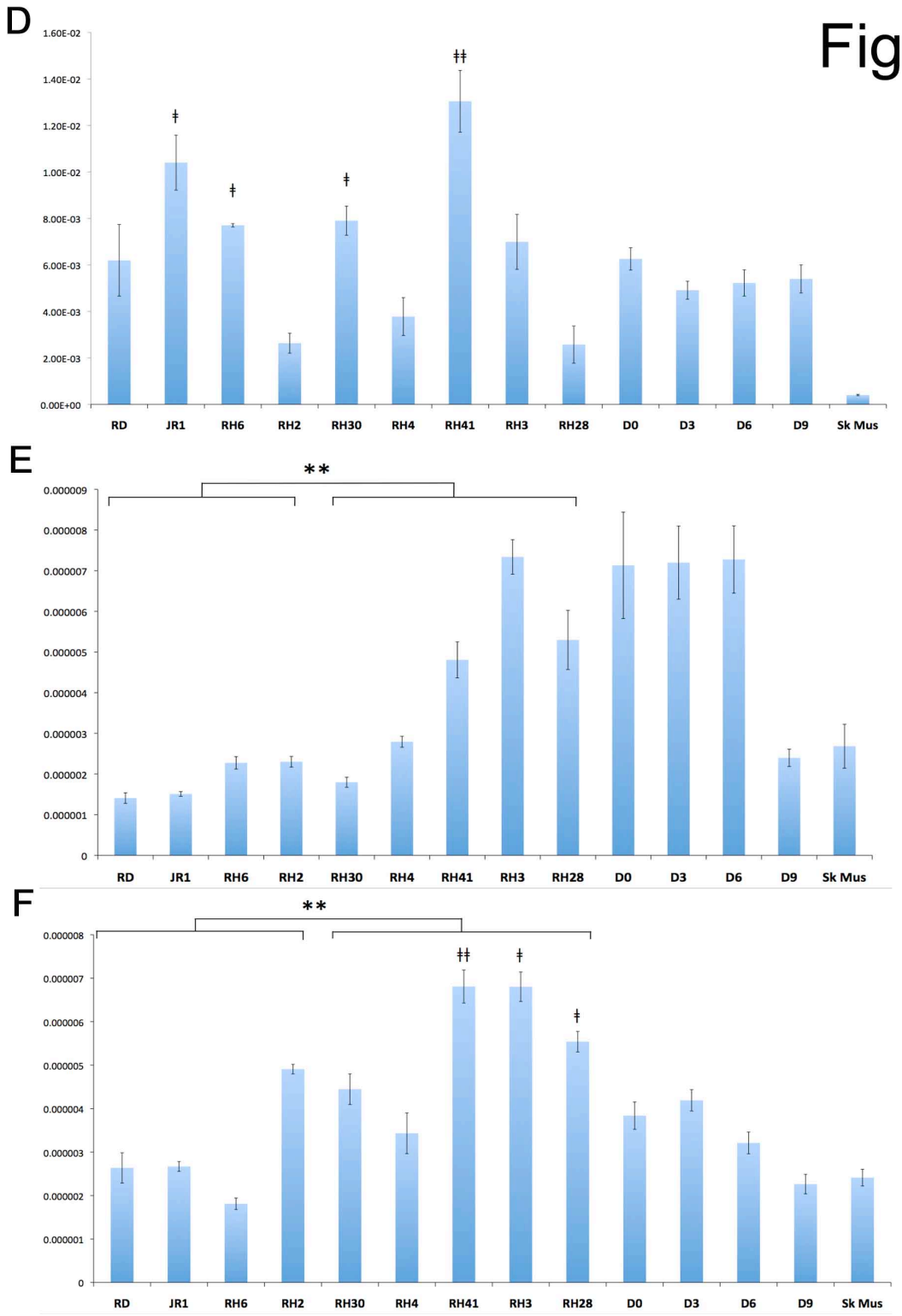

Figure 2 (cont'd). Histopathologic analysis of *Arf-/-/PAX3-FOXO1*/*PLOD1*-derived tumors and *PLOD1* expression ARMS cell lines. (D, E. F) Quantitative real time PCR analysis of *PLOD1, PLOD2* and *PLOD3* expression normalized to GAPDH in ERMS (RD, JR1, RH6, RH2), ARMS (RH30, RH4, RH41, RH3, RH28), proliferating (Hmyo D0) and differentiating (Hmyo D3-D9) human myoblasts and mature skeletal muscle (Sk muscle). Symbol (**) indicates the significance (P value <

0.01) of PLOD2 and PLOD3 mRNA overexpression levels in the ARMS cell lines compared to that in ERMS cell lines. Symbols (ǂ) and (ǂǂ) indicate the significance (P value < 0. 05 and < 0.01 respectively) of PLOD1 and PLOD3 mRNA overexpression in individual ERMS and ARMS cell lines compared to that in day 3 differentiating myoblasts.

Full-length PLOD1 and C-term PLOD1 localize within the ER of primary mouse myoblasts.

In an attempt to better understand the role of PLOD1 in the PAX3-FOXO1 driven tumorigenesis, we investigated the subcellular localization of full-length PLOD1 or C-term PLOD1 in primary mouse myoblasts. Newly synthesized PLOD1 possesses a N-terminal signal peptide that mediates entry into the endoplasmic reticulum [16]. In contrast C–term PLOD1 lacks the signal peptide but contains the last 40 amino acid portion, which mediates its retention in the lumen of the ER [27,28]. Both full-length PLOD1 and the C-term PLOD1 cDNAs were 3' HA tagged and ectopically expressed in *Arf-/-* myoblasts. The expression of full-length and the C-term PLOD1 was confirmed by western blot analysis using an anti-HA antibody (Fig. 3B). Sorted GFP^+ cells were co-immunostained with anti-HA and anti-GRP94 antibodies (GRP94 is an ER marker), and were imaged using confocal microscopy (Fig. 3A). As expected, full-length PLOD1 localized exclusively to the ER of primary myoblasts, whereas C-term PLOD1 partially localized in the ER with some diffuse staining throughout the cell. This result showed that both full length and C-term PLOD1 were ER localized in mouse primary myoblasts.

Figure 3

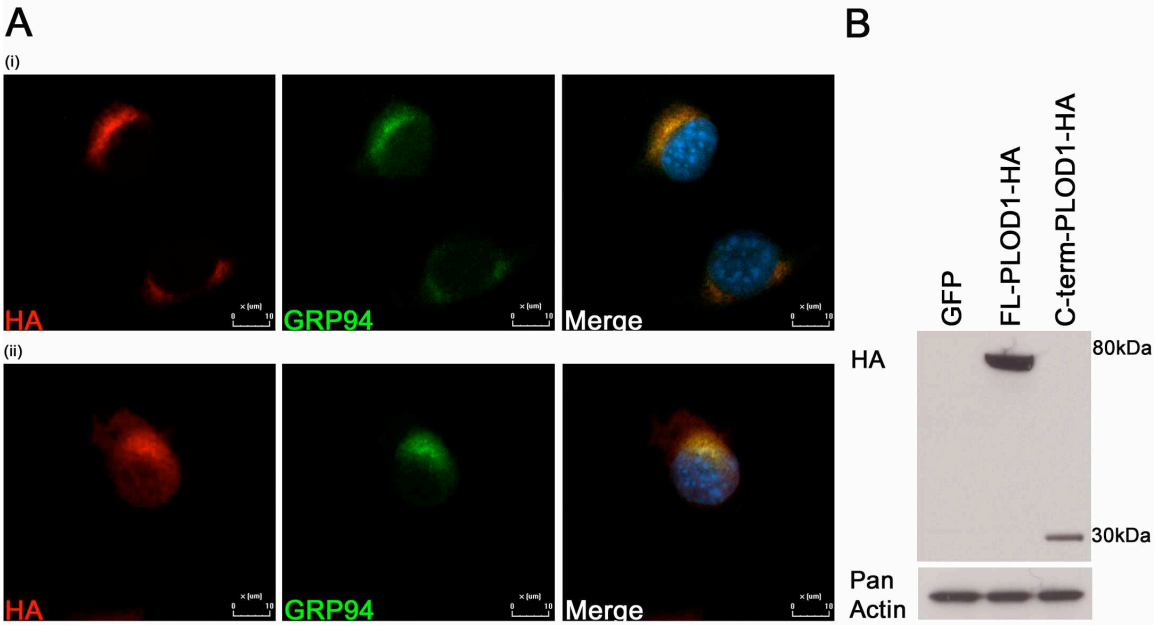

Figure 3. **Subcellular localization of full-length and C-terminal Plod1. (A)** Confocal images of freshly isolated primary *Arf-/-* myoblasts expressing retrovirally transduced full length PLOD1-HA (upper panel) and C-terminal PLOD1-HA (lower panel). Co-immunostaining for ER localization was performed using anti-HA and GRP94 antibodies. **(B)** anti-HA Immunoblot showing PLOD1 (FL-PLOD1-HA) and C-terminal PLOD1 fragment (C-Term-PLOD1-HA) expression in *Arf-/-* primary myoblasts. Pan-Actin antibody was used as loading control.

Tumorigenicity of PLOD1 mutants lacking lysyl hydroxylase activity.

To address if the lysyl hydroxylase activity of PLOD1 is important for PAX3-FOXO1 driven tumorigenesis, we generated a PLOD1 mutant (H656A, D658A, H708A; PLOD1ΔFe) lacking the ability to bind Fe^{2+}, which is a necessary cofactor for this activity [29]. To further characterize domains important for PLOD1's transforming activity, we also generated a second mutant lacking the last 33 amino acids of its 40 amino acid tail, which mediates retention in the lumen of the ER [27,28]. Deletion of this region included H708 thereby also abrogating the lysyl hydroxylase activity, as well as amino acids, R718 and F724, which are involved in substrate binding [29]. All constructs including the wild-type Full-length PLOD1 were 3' HA tagged and ectopically expressed in *Arf-/-* myoblasts together with PAX3-FOXO1. NOD/SCID mice have been injected with the different pools of *Arf-/-* myoblasts but it is too early to conclude if the different mutations abrogate PLOD1's transforming activity or not. We hope to have the result of this experiment in the final version of the thesis.

Discussion

By using an *in vivo* genetic screen with a retroviral cDNA expression library we report the identification of *PLOD1* as a gene that cooperates with PAX3-FOXO1 in the transformation of mouse primary *Arf-/-* myoblasts. Others and we have shown previously that PAX3-FOXO1 expression must occur in combination with secondary genetic alterations impairing both the p53 and pRb pathways in order for tumorigenesis to occur [8]. Based on this observation, we designed a screen in which *Arf-/-* primary myoblast expressing *PAX3-FOXO1* were transduced with an RH30 cDNA expression library and assayed their ability to form subcutaneous tumors in NOD/SCID mice. The design of the screen predicts identification of cDNAs encoding proteins that directly or indirectly affect pRb-mediated cell cycle control. The screen resulted in identification of the same partial cDNA clone in four independent tumors, encoding the C-terminal 247 amino acids of PLOD1. Subsequently the full length PLOD1 was also found to co-operate with PAX3-FOXO1 in the transformation of primary myoblasts. Histological analysis of these tumors showed myogenic gene expression signatures similar to that of the solid variant of ARMS. Together, these results indicate that over expression of PLOD1 cooperates in ARMS tumor formation. Moreover, due to the design of the assay these results imply that PLOD1 over expression can influence the pRb pathway, in order to synergize with PAX3-FOXO1. However, we were unable to show any contribution *in vitro* of PLOD1 to primary myoblast proliferation (data not shown). Though it is important to note that these primary myoblasts were cultured on gelatin coated plates, and we cannot exclude the possibility that the effect of PLOD1 on this matrix is different from its effect on tumor growth in vivo and therefore undetectable in our culture system.

The PLOD1 C-terminal region is sufficient for transformation and is important for the localization and retention of PLOD1 in the endoplasmic reticulum. ER-resident proteins are generally characterized by the presence of a C-terminal tetra peptide Lys-Asp-Glu-Leu (KDEL) [30] or a signaling motif consisting of two C-terminal lysines (KKXX or KXKX) [31]. PLOD1 contains neither of these

motifs [32] but its luminal association with and retention in the ER lumen is mediated by its C-terminal 40 amino acids [27,28]. Our immunostaining experiments with primary myoblasts showed that the C-terminal 247 amino acid PLOD1 fragment was sufficent to confer at least partial ER localization even in the absence of a signal peptide. The characteristic of certain enzymes to translocate into the ER in the absence of a signal sequence has been reported. An internal sequence of the human UDP-glucuronosyltransferase isoform UGT1A6 (amino acids 140-240) is responsible for this enzymes ER localization [33]. However, PLOD1 does not show significant sequence homology with this region of UGT1A6. Several papers reported that the C-terminal region of PLOD1 contains the iron-dependent oxygenase domain which constitutes the lysyl hydroxylase active site [15,27,28,34], thus the truncated 247 amino acid C-terminal PLOD1 contains the entire iron-dependent oxygenase domain and therefore likely retains lysyl hydroxylase activity. This catalyzes the post-translational hydroxylation of X-Lys-Gly repeats in collagen and collagen-like proteins. Altered post-translational modification mediated by PLOD1 hydroxylation might therefore influence protein-protein interaction of collagens or other uncharacterized secreted proteins, influencing the transformation state of cells.

Nonetheless, over expression of PLOD1 does occur in cancer as analysis of cancer array data available in Oncomine showed up regulation of PLOD1 in several human cancers (Supplementary Figure 2). Using the UCSC cancer genomics browser (https://genome-cancer.soe.ucsc.edu/), we also found up regulation of PLOD1 in estrogen receptor negative breast cancer (Supplementary Figure 3), which is associated with invasive disease and poor outcome [35]. Moreover Oncomine analysis of PLOD2 and PLOD3 expression, two highly homologous family member of PLOD1, showed over expression in multiple malignancies (Supplementary Figure 4, 5). In addition, our real-time PCR analysis performed on ARMS cell lines showed that 4 out 5 lines (RH30, RH41, RH3, RH28) up regulated at least one endogenous *PLOD* family member in comparison to control 3 day differentiated normal human skeletal

muscle myoblasts or ERMS cell lines. Despite tha fact that one study reported down regulation of PLOD1 in rhabdoid tumors [36], the larger picture supports the view that up regulation of PLOD family members is associated with human cancer. It will be important to identify the targets of lysyl hydroxylation that mediate the tumorigenic activity of this newly identified oncogenic gene family. Extensive additional studies will be required to fully understand the role of PLOD family members in human cancer.

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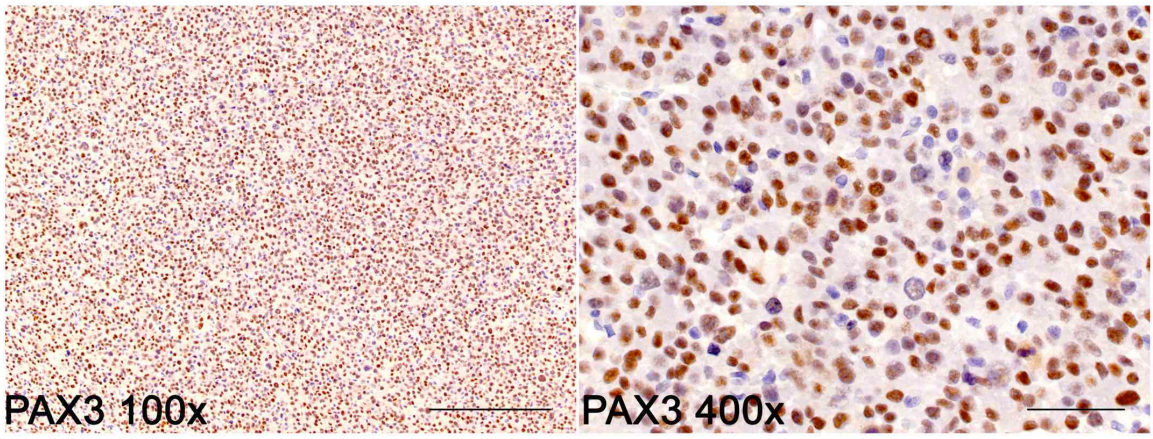
Supplementary Table and Supplementary Figures

Primary myoblast background	Cooperating genes		N° of mice injected	N° of injections per mouse	Mice with tumors	Total N° of tumors	Time of tumor formation
Arf-/-	RH30 library	MSCV-SV40-Puro	10	2	0	0	
Arf-/-	RH30 library	MSCV-*PAX3*-SV40-Puro	10	2	2	2	10 and 14 weeks
Arf-/-	RH30 library	MSCV-*PAX3-FOXO1*-SV40-Puro	13	2	7	9	4-8 weeks

Supplementary Table 1: RH30 cDNA library complementation screen performed using NOD/SCID mice. *Arf-/-* primary myoblasts overexpressing MSCV-empty vector, MSCV-*PAX3* or MSCV-*PAX3-FOXO1* were transduced with the RH30 cDNA retroviral library and subcutaneously injected into the right fore- and hind flanks of NOD/SCID mice. Tumor formation was monitored for up to five moths post injection.

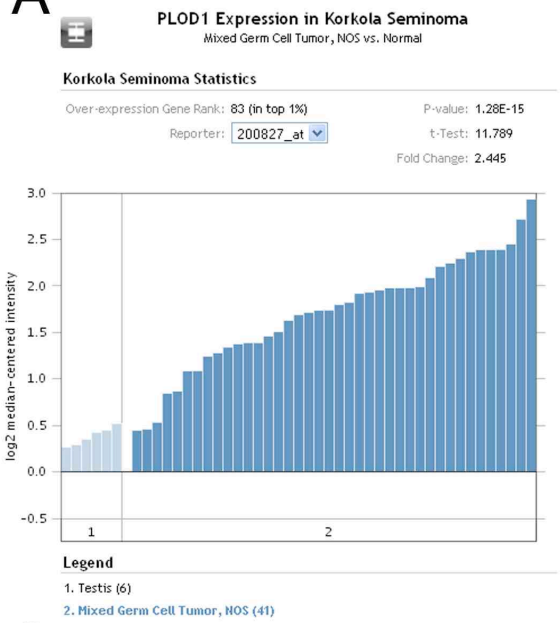
Supplementary Figure 1



Sup. Fig. 1. PAX3 immunostaining of PLOD1-derived tumor. Formalin-fixed and paraffin embedded sections from *Arf-/-/PAX3-FOXO1*/*PLOD1*-derived tumor (T2) stained using PAX3 antibody. Scale bars: 100X magnification scale bar = 250um; 400X magnification scale bar = 50um.

Supplementary Figure 2

A



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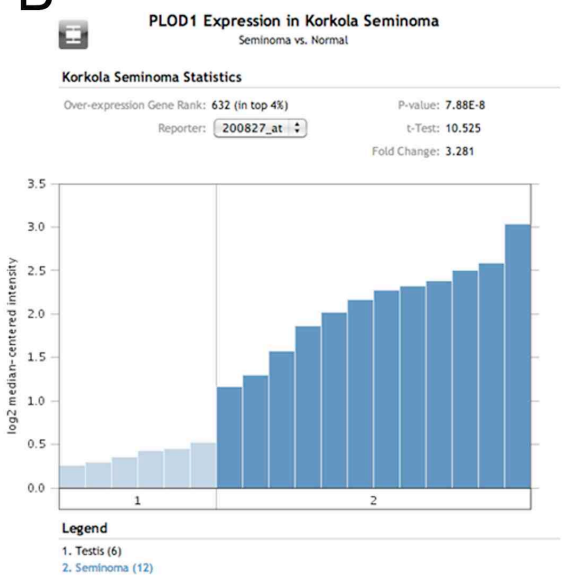

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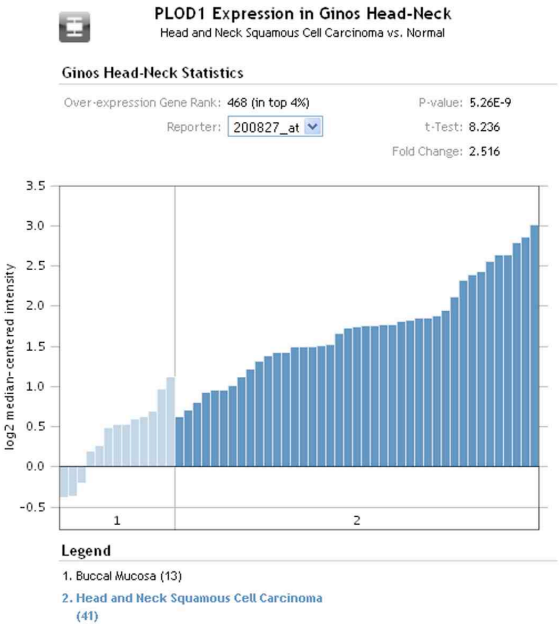

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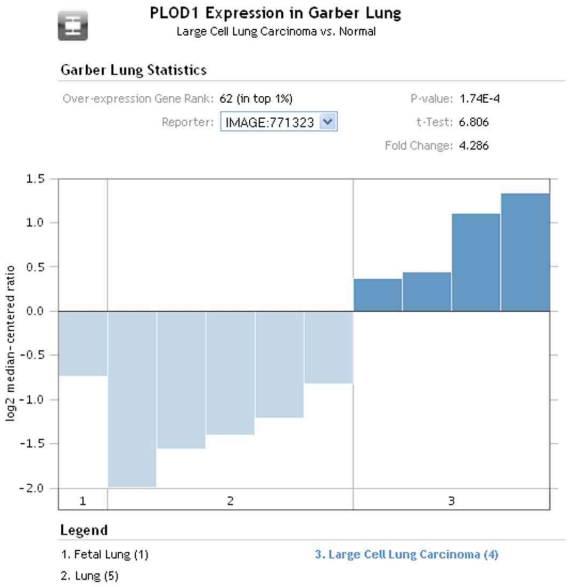

Supplementary Figure 2

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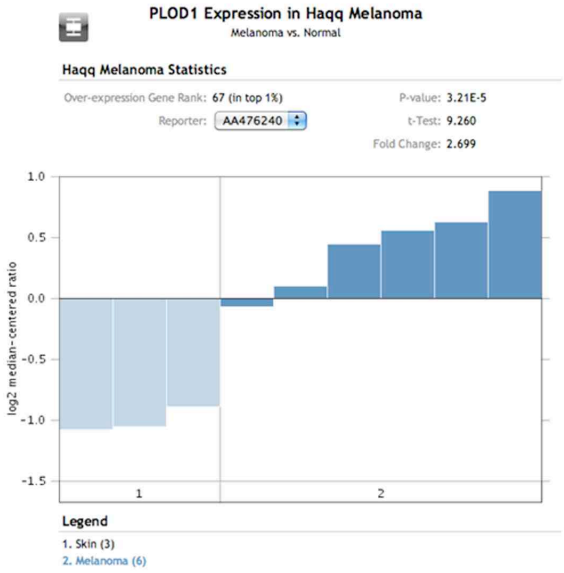

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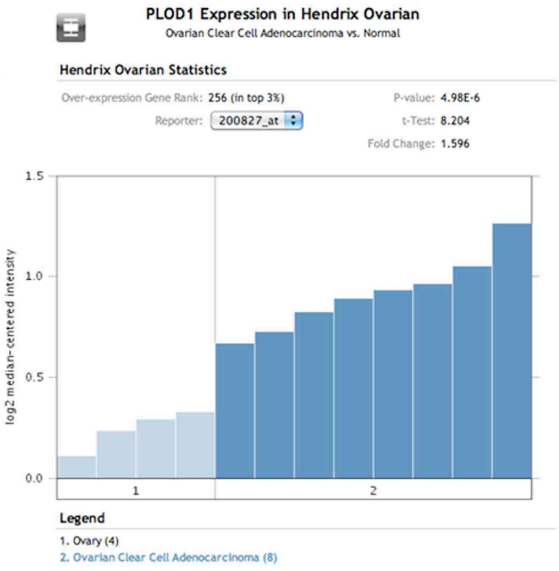

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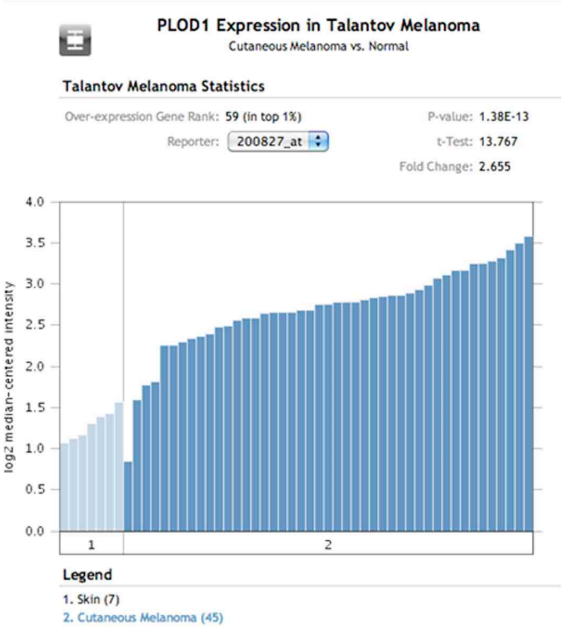
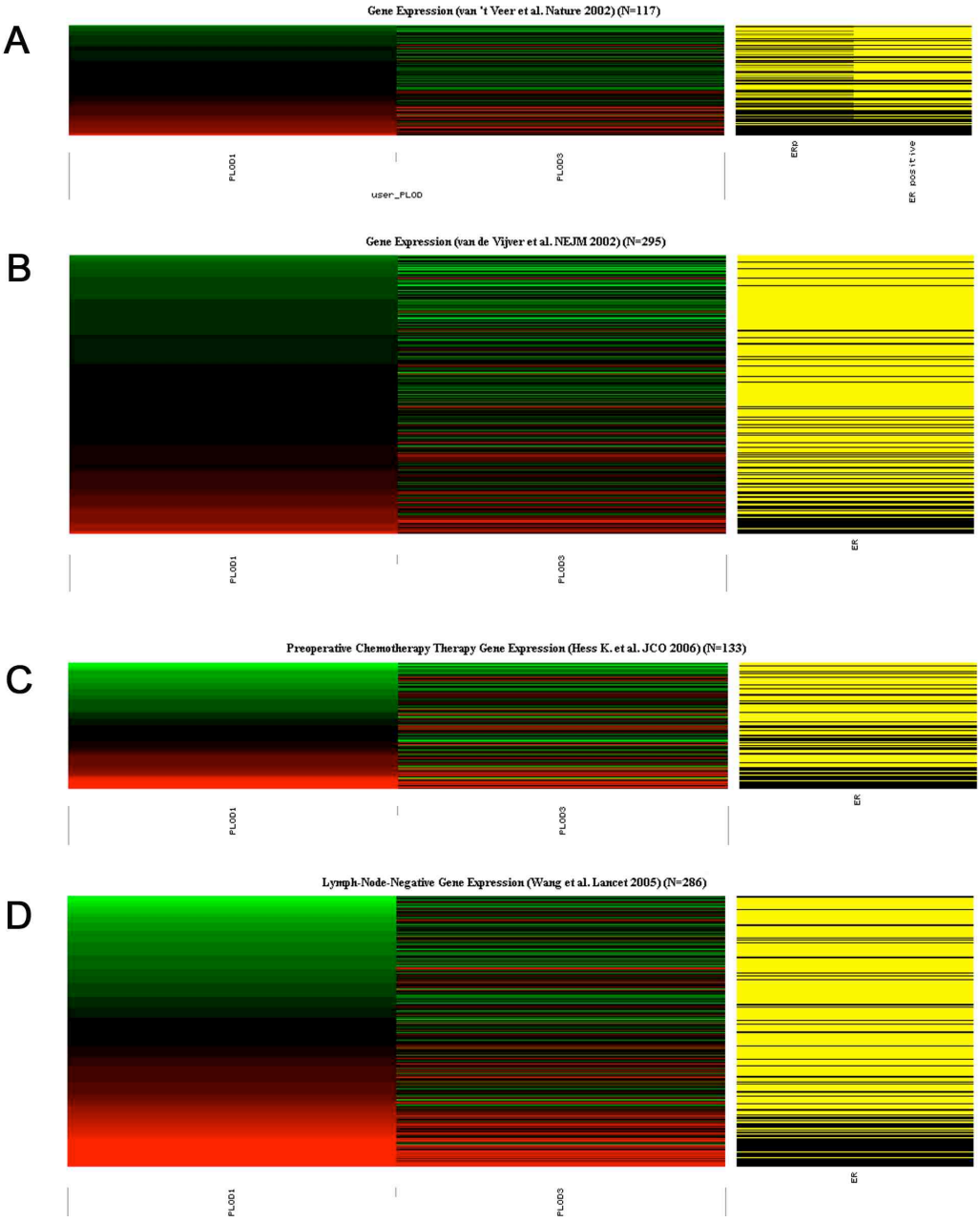
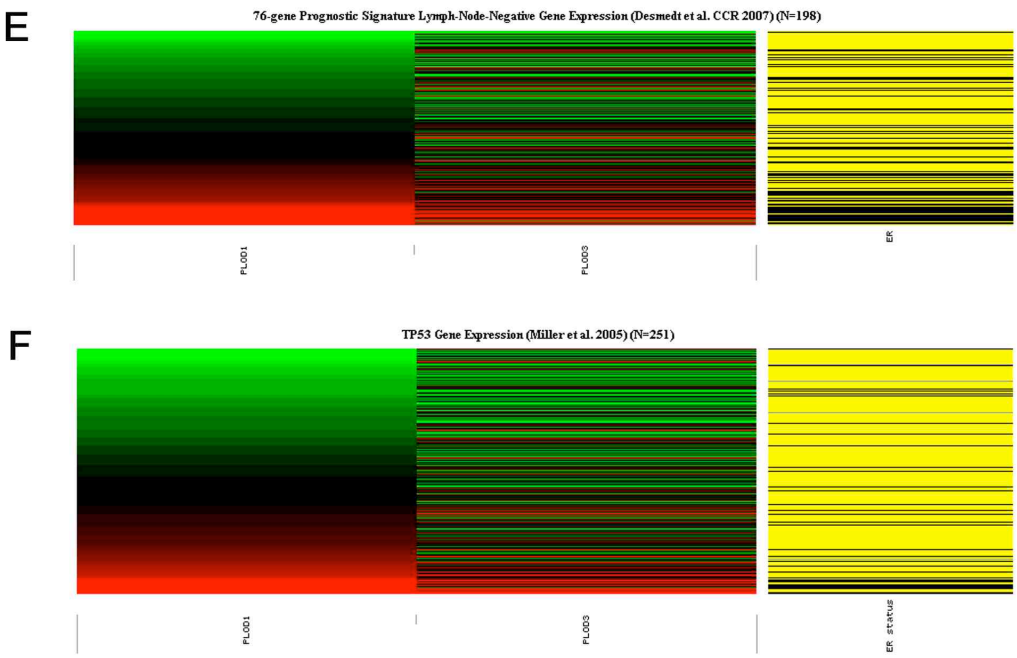


Sup. Fig. 2. Tumor versus normal tissue gene expression analysis of *PLOD1* using the Oncomine database. PLOD1 is significantly overexpressed in seminoma (A and B) [1], head/neck squamous cell carcinoma (C) [2], lung carcinoma (D) [3], melanoma (E,G) [4,5], and ovarian cancer (F) [6].

Supplementary Figure 3



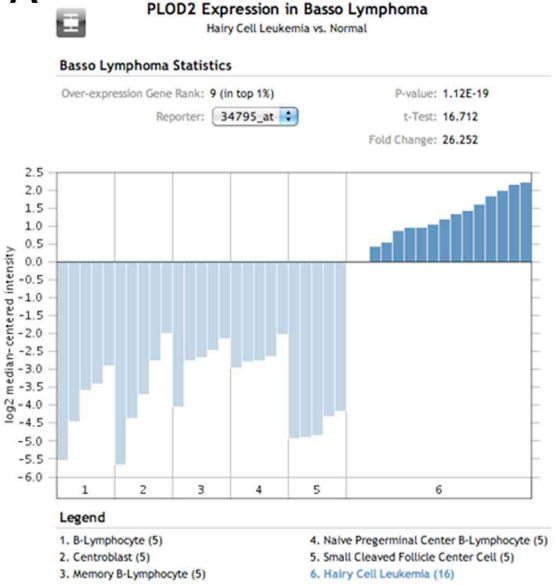
Supplementary Figure 3



Sup. Fig. 3. Ranked expression values of *PLOD1* compared to *PLOD3* and clinical characteristics of breast cancer samples from several studies (A)[7], (B)[8], (C)[9], (D)[10], (E)[11], (F)[12]. Analysis performed using (https://genome-cancer.soe.ucsc.edu/). In heat map: green, low relative expression; red, high relative expression.

Supplementary Figure 4

A



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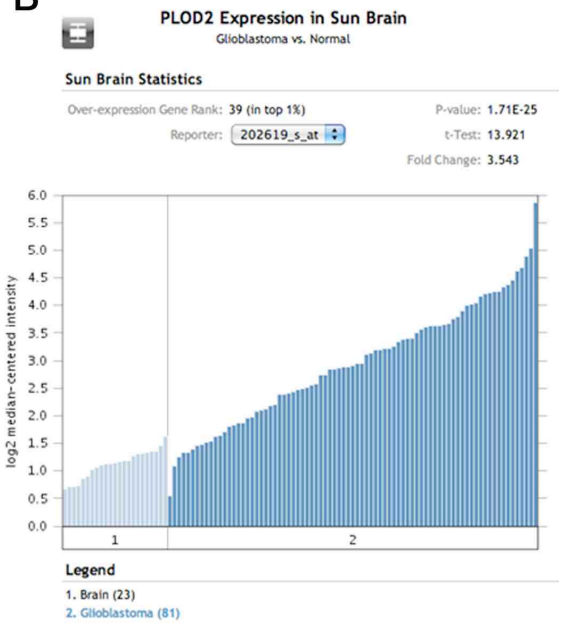

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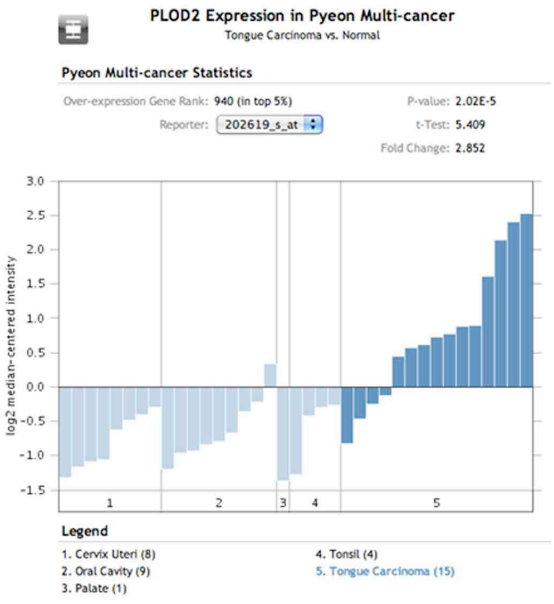

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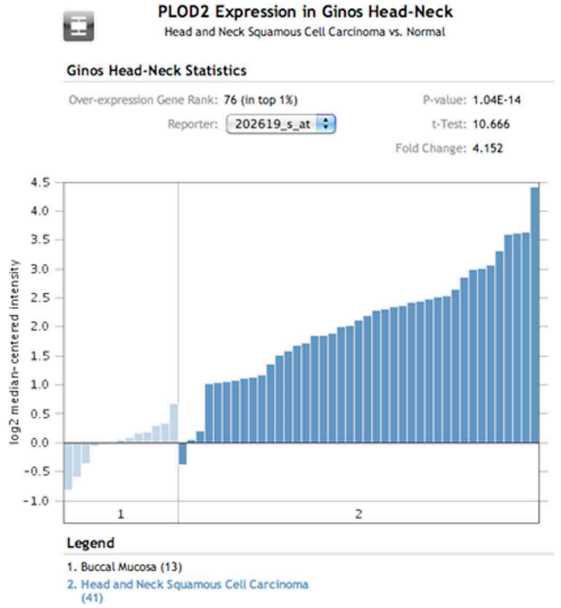

Supplementary Figure 4

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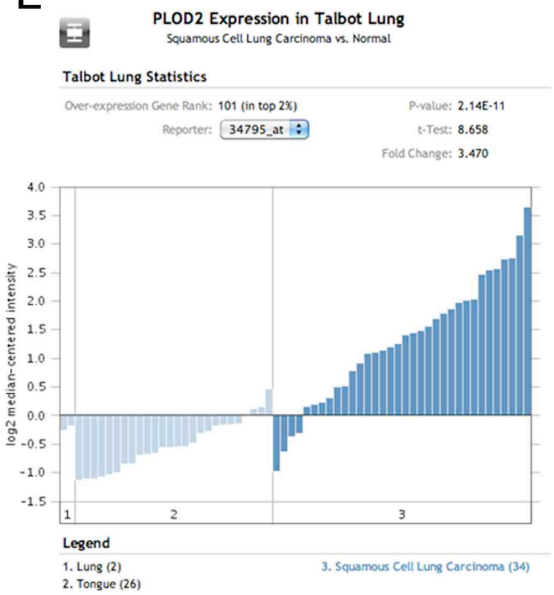

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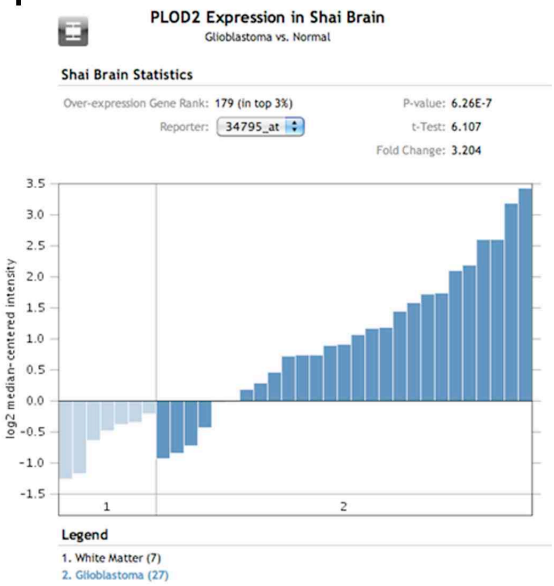

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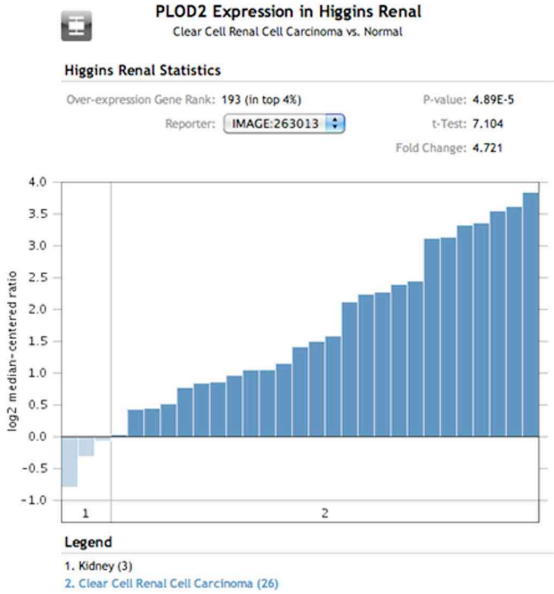

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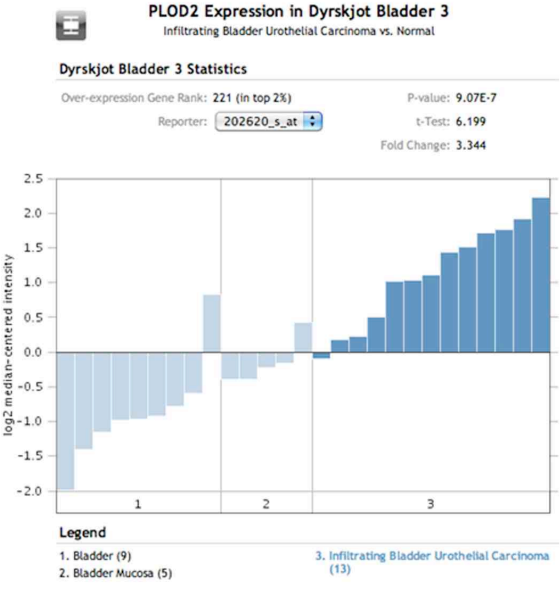
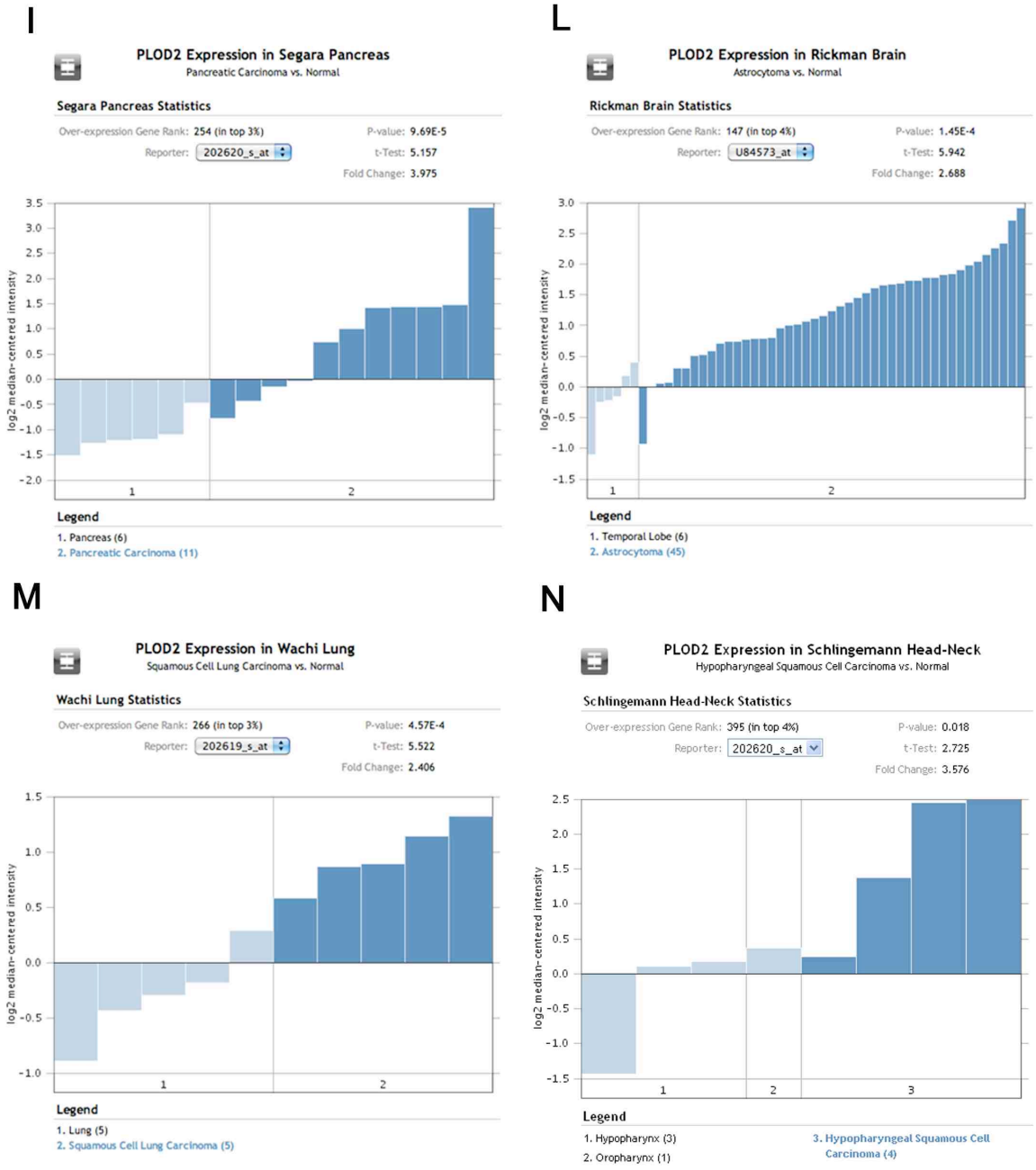


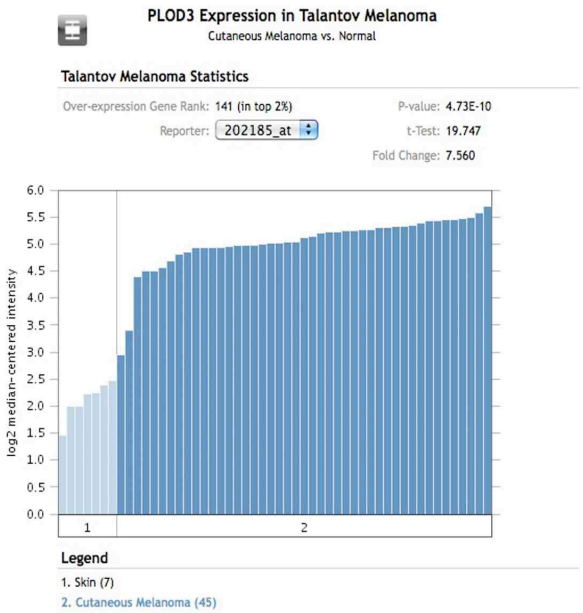
Supplementary Figure 4



Sup. Fig. 4. Oncomine *PLOD2* gene expression analysis in multiple human cancers compared with normal tissues. PLOD2 is significantly overexpressed in lymphoma (A) [13], glioblastoma (B) [14], tongue carcinoma (C) [15], head and neck squamous cell carcinoma (D) [2], squamous cell lung carcinoma (E and M) [16,17], glioblastoma (F) [18], clear cell renal cell carcinoma (G) [19], bladder carcinoma (H) [20], pancreatic carcinoma (I) [21], astrocytoma (L) [22], hypopharyngeal squamous cell carcinoma (N) [23].

Supplementary Figure 5

A



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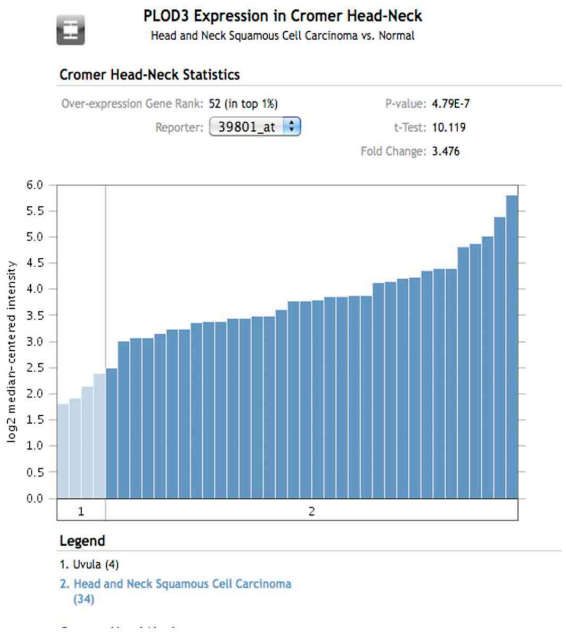

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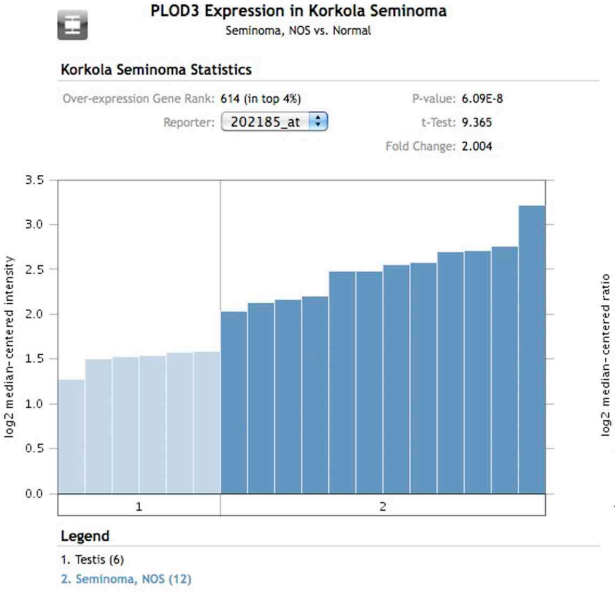

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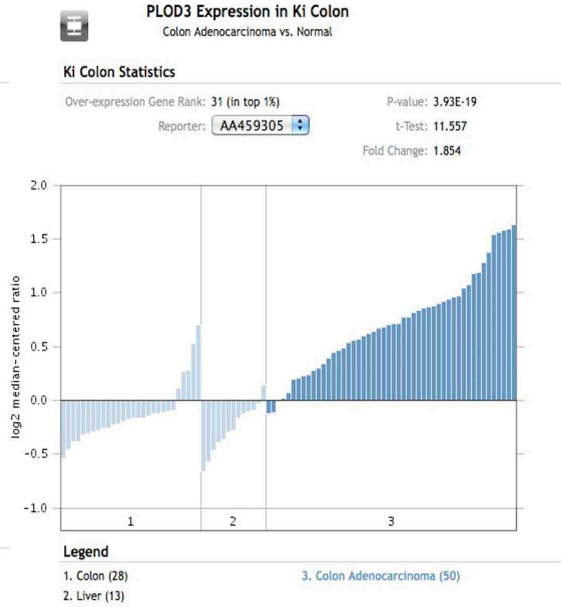
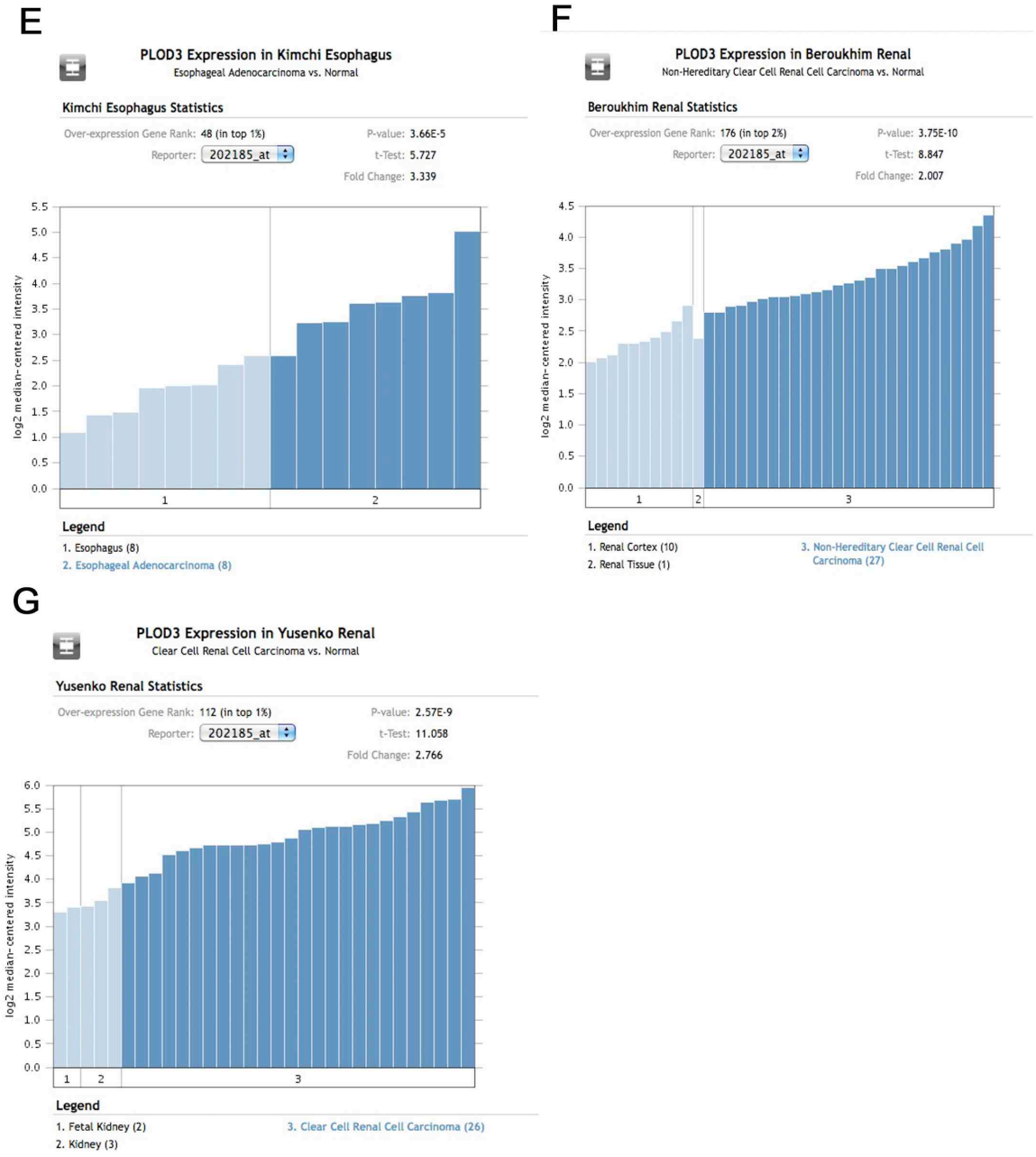


Supplementary Figure 5



Sup. Fig. 5. Oncomine *PLOD3* gene expression analysis in multiple human cancers compared with normal tissues. PLOD3 is significantly overexpressed in melanoma (A) [4], head and neck squamous cell carcinoma (B) [24], seminoma (C)[1], colon adenocarcinoma (D)[25], esophageal adenocarcinoma (E) [26], non-hereditary clear cell renal cell carcinoma and clear cell renal cell carcinoma (F,G) [27,28].

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Chapter 6

Conclusions and Future Directions

6.1. Summary and conclusion.

Malignant transformation results from the gradual acquisition of mutations in two categories of genes: proto-oncogenes and tumor suppressor genes, which are normally involved in positive and negative control of cell proliferation, respectively [1,2]. Deregulation of a single oncogene alone is often insufficient to induce cellular transformation. For example, *in vitro* studies over expressing the oncogenic RAS protein in mouse fibroblasts induces growth arrest and senescence and is not sufficient to transform mouse fibroblasts [3,4], but inactivation of the p53 pathway in addition to RAS overexpression can fully transform primary fibroblasts [5,6]. Oncogenic Ras signaling activates the Arf-p53 pathway thereby halting proliferation [7]. Similarly to the RAS oncogene, the TEL1:AML1 fusion protein, which is the most common translocation in childhood leukemia, is not highly oncogenic when expressed in mouse models, but becomes tumorigenic in the presence of other synergistic mutations [8,9][Quintana and Grosveld 2011]. TEL1:AML1 is an example of a recurrent chromosomal translocation; recurrent chromosomal translocations are frequently associated with a variety of cancers, especially hematologic malignancies and childhood sarcomas [10].

The chimeric PAX3-FOXO1 fusion gene is the result of the t(2;13)(q35;q14) translocation [11,12]. Expression of PAX3-FOXO1 is characteristic of the pediatric cancer, alveolar rhabdomyosarcoma (ARMS) [13]. Many studies have associated PAX3-FOXO1 expression with increased cell proliferation,

cell survival and inhibition of cell differentiation [10,11,12,13,14,15,16]. Section 2.4 in chapter 2 summarizes the different genetic mechanisms by which PAX3-FOXO1 contributes to cellular transformation.

The first aim of this thesis was to identify PAX3-FOXO1 target genes that might contribute to tumor pathogenesis. Chapter 3 describes the results obtained from a microarray analysis performed on wild type mouse primary myoblasts stably expressing PAX3-FOXO1, PAX3 or empty vector, showing that PAX3-FOXO1 specifically regulates genes involved in cell cycle progression, cellular invasiveness, and tumor cell metastasis. We also compared our data to the results obtained by others and performed in primary ARMS samples, ARMS cell lines or various cell types expressing the fusion protein. The low similarity uncovered by this comparison (10.6%) shows that the primary mouse myoblast system, which is independent of the influence of secondary genetic events that take place during tumorigenesis, might represent a physiologically relevant model to study the primary consequences of PAX3-FOXO1-mediated transcriptional alterations, representing initial steps of PAX3-FOXO1-mediated cell transformation.

Despite the fate-changing effects of PAX3-FOXO1 on normal cell function several publications [14,15] as well as results described in this thesis (Chapter 4) have shown that expression of PAX3-FOXO1 alone is insufficient to induce the transformation of myogenic cells; additional genetic events involving the inactivation of the pRb and p53 pathways are required for tumorigenic

transformation to occur. However, the individual mutations that cooperate with PAX3-FOXO1 in ARMS tumorigenesis remain poorly defined.

The second aim of this thesis was to screen an Rh30 cDNA library for genes cooperating with PAX3-FOXO1 in ARMS tumorigenesis. Primary Arf-/- myoblasts transduced with PAX3-FOXO1 and the Rh30 cDNA library were injected subcutaneously into NOD/SCID mice. Tumor formation was used to select for Rh30 cDNA library derived clones that cooperate with *PAX3-FOXO1* in the transformation of primary myoblasts. Histopathology and immunohistochemistry analysis of the tumors derived from this screen showed that the tumors resembled the solid variant of alveolar rhabdomyosarcoma. Moreover, immunoblots of tumor-derived cell lysates probed with antibodies against muscle-specific differentiation markers revealed a similar gene expression pattern to that of ARMS primary tumors. Therefore transformation of primary mouse myoblasts with PAX3-FOXO1 and certain clones of this Rh30 cDNA library closely recapitulate ARMS tumors. Although we identified genes that cooperate with PAX3-FOXO1 in transformation, there are obvious drawbacks to our approach. We aimed at creating an ARMS cell cDNA library containing full length inserts, but despite testing random inserts that suggested the presence of full length cDNAs, both cooperating cDNAs encoded C-terminal protein fragments rather than full-length proteins. Partial cDNA clones encoding proteins that lost their function will be missing in this screen. Another point of critique is the use of an ARMS cell line rather than a primary tumor as the source of the cDNA library. The cell line will contain mutations that are not

present in primary tumors and could therefore score for genes that are cell line specific rather than tumor specific. Notwithstanding, two genes were identified using this approach, neither of which had a prior association with cancer. The first cDNA described in this thesis (Chapter 4) is an unknown gene identified by the accession number FLJ10404 (FAM193B) and herein named *IRIZIO*. Characterization of the *IRIZIO* gene revealed the existence of at least three splice variants: the longest variant included exon 7 (Full Length) and due to the presence of a stop-codon in this exon resulted in a truncated protein lacking the C-terminus, while the other 2 isoforms lacking exon 7 (Δ7) or exons 7 and 8 (Δ7/8) encoded longer polypeptides. Real time PCR analysis performed on RMS cell lines compared to human myoblasts showed over expression of IRIZIOΔ7 in all the RMS cell lines suggesting a role for IRIZIO in rhabdomyosarcomagenesis. The second isolated cDNA (Chapter 5) encoded *PLOD1* (*procollagen-lysine 1, 2-oxoglutarate 5-dioxygenase 1*), an enzyme localized to the endoplasmic reticulum (ER) lumen. The PLOD family consists of three proteins (PLOD1-3) encoded by distinct loci in the human and mouse genomes [16,17,18,19,20]. All three enzymes catalyze the hydroxylation of lysine residues of collagen and collagen-like proteins harboring the X-Lys-Gly repeat sequence [21]. We show that the PLOD family members are overexpressed in several human cancer types including rhabdomyosarcoma cell lines, suggesting that PLOD genes also potentially have an active role in cancer.

The Rh30 cDNA screen was specifically designed to identify proteins that directly or indirectly affect Rb-mediated cell cycle control. Numerous experiments were performed in order to link IRIZIO or PLOD1 to the pRb pathway. However, we were unable to show any contribution *in vitro* of IRIZIO or PLOD1 to primary myoblast proliferation or to signaling pathways linked to the pRb pathway (E2F1, Akt, Erk1/2, p90, cJun and S6). Even though there was no measurable effect on the pRb pathway, it is clear from this work that IRIZIO and the PLOD family members promote PAX3-FOXO1 transformation of myoblasts and may therefore have a role in ARMS tumor development as well as in other cancer types. Additional studies will be required to fully understand the role of these proteins in human cancer.

This thesis demonstrates a successful method by which to identify direct target genes downstream of PAX3-FOXO1 and genes that cooperate with the fusion protein in driving ARMS tumorigenesis. We were able to identify transcriptional targets whose effects on cellular behavior might initiate cell transformation. Moreover we isolated two genes that can enhance ARMS tumorigenesis that had not been previously identified to play a role in cancer development. Identification of such ARMS associated genes, and their subsequent characterization, may lead to new avenues of ARMS research, and the genes identified may represent possible novel therapeutic targets for the treatment of this aggressive, and often deadly, pediatric disease.

6.2. Future directions.

6.2.1. Characterization of endogenous *IRIZIO.*

As discussed in chapter 4, overexpression of *IRIZIO* is able to transform Arf-/-/PAX3-FOXO1 myoblasts. Moreover, we found that IRIZIO is overexpressed in RMS cell lines suggesting that, when deregulated, this gene possesses oncogenic activity and might contribute to rhabdomyosarcoma formation. Besides these results, the function of endogenous IRIZIO remains to be elucidated. To better understand the function of IRIZIO, we took advantage of the mouse ES cell line (RRN280), obtained from BayGenomics laboratories, which contains a trapping cassette within the first intron of mouse *Irizio* (FAM193B). The vector used to gene trap *Irizio* contains a splice acceptor and a polyadenylation signal flanking a β-galactosidase/neomycin-resistance fusion gene (βgeo), which is driven by the endogenous *Irizio* promoter. After obtaining a chimeric mouse from this cell line we generated mice heterozygous and homozygous for the targeted *Irizio* gene. Homozygous mice appear to be *Irizio* hypomorphs given that they express only 10% of Irizio RNA and protein, compared to wild type mice. As yet we were unable to identify any obvious phenotype associated with severely reduced *Irizio* expression but the β-Glactosidase reporter gene driven by the *Irizio* promoter will allow us to precisely define the expression pattern of *Irizio* in embryonic and adult tissues. Crossing *Irizio-/-* mice with *p16-Arf-/-* mice will also determine if the strongly reduced Irizio levels will delay tumor formation in the absence of these genes, which predispose mice to tumor development at an

early age [22,23]. A previous report has found *IRIZO* mRNA overexpressed in a specific subgroup of patients with recurrent lung tumor formation [24]. Thus, another valuable approach to verify the oncogenic characteristic of Irizio will be to by cross *Irizio-/-* mice with the 129S4-*Kras*^{tm4Tyj}/J mice. This strain carries a *Kras* gene with an activating point mutation, whose expression is blocked by the presence of a stop codon flanked by loxP sites (http://jaxmice.jax.org/strain/008180.html). Intranasal infection with an adenovirus encoding Cre results in the excision of the stop codon and expression of mutant *Kras*, leading to time-controlled lung tumor formation in the infected mice. Using this approach we can determine if the reduced expression of Irizio can prevent or delay lung tumor formation in *Kras*^{tm4Ty}*/ Irizio-/-* double mutant mice.

6.2.2. Determination of the link between IRIZIO and cell cycle progression.

As stated earlier in this chapter and in chapter 4, our cDNA screen was based on the observation that PAX3-FOXO1 transforms mouse primary myoblasts that harbor a compromised p53 and pRb pathway [25,26]. By transducing Arf-/- myoblasts with PAX3-FOXO1 we expected that the cooperating cDNAs would interfere directly or indirectly with pRb pathway control. Despite numerous attempts, we were unable to identify a direct link between *IRIZIO* and the pRB pathway (see the discussion section in chapter 3). We recently performed gene expression profiling using publicly available array data

(http://www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc=GSE7578) [27], indicating that IRIZIO is upregulated in three medulloblastoma cell lines that underwent Bmi-1 knockdown [27]. This resulted in inhibition of proliferation, loss of anchorage-independent growth, and suppression of tumor formation in *nude* mice [27]. Bmi-1 is a transcriptional repressor that belongs to the Polycomb group (PcG) of transcriptional regulators [27]. PcG proteins epigenetically regulate gene expression by altering chromatin states at specific promoters [28]. Several studies have shown that Bmi-1 act as an oncogene; it promotes cell growth by silencing the *INK4a/ARF* tumor suppressor locus [27,28,29,30], which encodes the cell cycle inhibitors $p16^{ink4a}$ and $p19^{arf}$, and by repressing $p21^{waf1/cip1}$ [31]. We can speculate that IRIZIO up-regulation due to Bmi-1 knockdown might be a compensatory mechanism to overcome the growth inhibition seen in the medulloblastoma cell lines [27]. Interestingly, a recent publication reported that Bmi-1 is expressed in postnatal satellite cells and plays an important role in the maintenance of the satellite cells and muscle regeneration [32]. It will be important to assess if Bmi-1-dependend *IRIZIO* regulation has an effect on myogenic cells, in which *IRIZIO* expression might play a role in maintaining the correct balance between cell division and cell differentiation. In this respect, it is conceivable that overexpression of IRIZIO in a p53 compromised background (Arf-/-) and in the presence of an oncogene such as PAX3-FOXO1 can lead to transformation of primary myoblasts. Understanding the role of IRIZIO as a Bmi-1 regulated gene might therefore shed light on the function of this novel gene.

6.2.3. Is the lysyl hydroxylase activity of PLOD1 essential for cell transformation?

As mentioned in chapter 4, PLOD1 overexpression contributes to tumorigenic transformation of *Arf-/-* myoblasts expressing PAX3-FOXO1. Moreover, we showed that all PLOD family members (PLOD1-3) are overexpressed in ARMS cell lines and in many other tumors, thereby indicating that the PLOD family of lysyl hydroxylases contributes to tumorigenesis in humans. It will therefore be important to determine if their lysyl hydroxylase activity proper is necessary for cellular transformation or some other as yet unknown function of this group of enzymes. It is possible that incorrectly hydroxylated collagen might alter the composition of the extra cellular matrix, which in turn can affect cell growth. To address this question, we generated a PLOD1 mutant incapable of binding Fe^{2+}, an essential cofactor for lysyl hydroxylase activity [33]. In addition, we generated a truncated mutant lacking 33 aminoacids of its C-terminus. This region mediates both PLOD1's retention to the lumen of the endoplasmic reticulum (ER) and its substrate binding capacity. Expression of these mutants in *Arf-/-*/PAX3-FOXO1 myoblasts and subsequent injection in NOD/SCID mice will determine if the lysyl hydroxylase activity of PLOD1 is necessary for tumorigenesis. To further verify the oncogenic potential of PLOD1, we are also planning on breeding *p16-Arf-/-* mice with *Plod1* deficient mice [16]. Comparing the tumor incidence of *p16-Arf-/-/ Plod1-/-* versus *p16-Arf-/-/ Plod1+/-, or p16-Arf-/-/ Plod1+/+* will determine if the lack of PLOD1 is

able to reduce or perhaps abrogate tumor formation in *p16-Arf-/-* mice [22,23]. Similarly, we can isolate primary myoblasts from *Arf-/-/ Plod1-/-* or *Arf-/-/ Plod1+/+* animals and transduce them with PAX3-FOXO1 retrovirus. If after injection in NOD/SCID mice *Plod1*$^{+/+}$ cells show delayed or abrogated tumor formation compared to *Plod1-null* cells it would further confirm PLOD1's contribution to tumorigenic transformation.

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Samenvatting en conclusies.

Transformatie van cellen tot maligne cellen komt tot stand door een opeenvolgende accumulatie van mutaties in (proto-) oncogenen of tumorsuppressorgenen. Deze genen hebben respectievelijk een positief en negatief effect op celdeling.

Ontregeling van één enkel oncogen is vaak onvoldoende om celtransformatie te induceren. Een voorbeeld hiervan is een *in vitro* studie, waarbij overexpressie van het oncogene RAS eiwit de groei van muizen fibroblasten stopt en veroudering induceert en onvoldoende is de muizen fibroblasten te transformeren, terwijl additionele inactivatie van de p53 cascade de fibroblasten volledig transformeert. De oncogene signaaltransductie van Ras aktiveert de Arf-p53 cascade wat de proliferatie stopt. Vergelijkbaar met oncogeen RAS is het fusie-eiwit TEL1-AML1, het product van de meest voorkomende chromosomale translocatie in leukemie bij kinderen, is op zichzelf niet erg oncogeen als het tot expressie gebracht wordt in muizen, maar induceert transformatie samen met andere mutaties. De TEL1-AML1 translocatie is een voorbeeld van een niet-willekeurige chromosomale translocatie; Dit soort translocaties is vaak geassocieerd met uiteenlopende soorten van kanker, vooral bij hematologische maligniteiten en sarcoma's bij kinderen.

Het chimere PAX3-FOXO1 eiwit is het resultaat van de chromosomale translocatie t(2;13)(q35;q14). Expressie van het PAX3-FOXO1 eiwit is karakteristiek voor de bij kinderen voorkomende kanker Alveolaire Rhabdomyosarcoma (ARMS). Vele studies hebben PAX3-FOXO1 expressie in verband gebracht met verhoogde cel proliferatie, overleving en remming van de differentiatie. Sectie 2.4 in hoofdstuk 2 vat de verschillende genetische mechanismen samen waarmee PAX3-FOXO1 bijdraagt aan cellulaire transformatie.

Het eerste doel van dit proefschrift was om PAX3-FOXO1 gereguleerde genen te identificeren die mogelijkerwijs bijdragen aan de pathogenese van de tumor.

Hoofdstuk 3 beschrijft de resultaten die verkregen werden door middel van een microarray analyse uitgevoerd met wild type primaire muizen myoblasten die PAX3-FOXO1, PAX3, of lege vector expresseerden, waaruit bleek dat PAX3-FOXO1 specifiek genen reguleert die betrokken zijn bij progressie van de cel cyclus, de cellulaire invasiviteit en tumor cel metastase. We vergeleken onze data ook met resultaten van anderen, verkregen met primaire tumor monsters, ARMS cellijnen, of een variëteit aan cel types die het fusie-eiwit expresseren. De lage mate van overeenkomst die door deze vergelijking aan het licht kwam (10,6%) laat zien dat het primaire myoblasten systeem, wat vrij is van secundaire genetische veranderingen die plaatsvinden tijdens de tumorigenese, wellicht een fysiologisch relevant model is om de primaire consequenties van de PAX3-FOXO1-gestuurde transcriptionele veranderingen te bestuderen. Deze vertegenwoordigen de initiële stappen van cel transformatie.

Ondanks de effecten van PAX3-FOXO1 op de normale celfunktie hebben verscheidene publicaties en resultaten beschreven in dit proefschrift (Hoofdstuk 4) laten zien dat expressie van PAX3-FOXO1 alleen onvoldoende is om transformatie van myogene cellen te veroorzaken; additionele genetische veranderingen zoals inaktivatie van de pRb en p53 cascades zijn nodig om tumorigene transformatie te bewerkstelligen. Echter de individuele mutaties die met PAX3-FOXO1 samenwerken bij het veroorzaken van ARMS zijn slecht bekend.

Het tweede doel van dit proefschrift was om uit een Rh30 cDNA bibliotheek genen te selecteren die mogelijkerwijs samenwerken met PAX3-FOXO1 bij de formatie van ARMS. Primaire *Arf-/-* myoblasten werden getranduceerd met PAX3-FOXO1 en de Rh30 cDNA bibliotheek en werden vervolgens subcutaan geïnjecteerd in NOD/SCID muizen. Tumorvorming werd gebruikt als maatstaf voor de selectie van klonen uit deze Rh30 cDNA bibliotheek die samenwerken met PAX3-FOXO1 bij de transformatie van primaire myoblasten. Histopathologische en immuunhistochemische analyse van tumoren van dit experiment liet zien dat dit overeen kwam met de “solide” variant van

alveolaire rhabdomysarcoma. Aanvullende immunoblots met cellysaten afkomstig van deze tumoren, geïncubeerd met antilichamen die specifiek zijn voor gedifferentieerde spiercellen, lieten een eiwitexpressie patroon zien dat vergelijkbaar was met dat van primaire ARMS tumoren. Hieruit volgt dat transformatie van primaire muizen myoblasten, met behulp van PAX3-FOXO1 en klonen uit de bovengenoemde Rh30 cDNA bibliotheek, nauwgezet de eigenschappen van ARMS tumoren recapituleren. Hoewel we genen geïdentificeerd hebben die samenwerken met PAX3-FOXO1 in transformatie zijn er duidelijke nadelen verbonden aan deze aanpak. We streefden ernaar om een ARMS cDNA bibliotheek te maken die volle-lengte inserties bevatte, maar ondanks het testen van willekeurige inserties die suggereerden dat er volle-lengte cDNAs aanwezig waren, kodeerden de samenwerkende cDNAs voor C-terminale fragmenten in plaats van volle-lengte cDNAs. Ook cDNA klonen die koderen voor eiwitten die niet funkioneel zijn gaan verloren in deze test. Een ander punt van kritiek is het gebruik van een ARMS cellijn in plaats van een primaire tumor als de bron voor de cDNA bibliotheek. De cellijn zal mutaties bevatten die niet aanwezig zijn in primaire tumoren en kan daardoor genen vinden die cellijn-specifiek maar niet tumor-specifiek zijn. Ondanks dat, werden er twee genen gevonden die geen van beiden eerder met kanker in verband gebracht waren.

De eerste cDNA kloon, die in dit proefschrift beschreven wordt (Hoofdstuk 3) representeert een onbekend gen dat als *FLJ10404* (FAM193B) te boek staat maar dat we hier *IRIZIO* noemen. Karakterisering van het *IRIZIO* gen bracht het bestaan van tenminste drie splice varianten aan het licht: De langste variant bevatte exon 7 (genaamd: “full length”), dat een stopcodon bevat wat resulteert in een verkort eiwit waarvan de C-terminus ontbreekt, terwijl de twee andere varianten die respectievelijk exon 7 (D7) en exonen 7 en 8 (D7/8) missen, voor langere polypeptiden koderen. Real time PCR analyse waarbij rhabdomyosarcoma (RMS) cellijnen vergeleken werden met human myoblasten gaf een overexpressie van *IRIZIOD7* mRNA in alle RMS cellijnen te zien, wat suggereert dat *IRIZIO* een rol speelt bij

rhabdomyosarcomagenese. Het tweede cDNA dat geïsoleerd werd (Hoofdstuk 4), codeert voor PLOD1 (procollageen-lysine 1, 2-oxoglutaraat 5-dioxygenase), een enzym dat zich in het lumen van het endoplasmatisch reticulum (ER) bevindt. De familie van PLOD eiwitten bestaat uit 3 eiwitten (PLOD1-3) die gecodeerd worden door verschillende loci in het humane en muizen genoom. Alle drie deze enzymen katalyseren de hydroxylatie van lysine residuen in collageen of collageen-achtige eiwitten, die de gerepeteerde X-Lys-Gly sequentie bevatten. Wij laten zien dat de PLOD enzymen overgeëxpresseerd worden in verscheidene vormen van kanker, waaronder rhabdomyosarcoma cellijnen, wat suggereert dat de PLOD genen mogelijkerwijs een actieve rol bij kanker vervullen.
De analyse van de Rh30 cDNA bibliotheek was specifiek ontworpen om eiwitten te identificeren die direkt of indirekt de controle van Rb op de cel cyclus beïnvloeden. Vele experimenten werden gedaan om verband aan te tonen tussen IRIZIO of PLOD1 en de Rb signaalcascade. We waren echter niet in staat om *in vitro* enige bijdrage te signaleren van IRIZIO of PLOD1 aan de proliferatie van primaire myoblasten of aan de signaalcascades geassocieerd met het pRb eiwit. (E2F1, Akt, Erk 1/2, p90, cJun en S6) Ondanks dat er geen meetbare effecten waren op de pRb cascade, kunnen we uit deze experimenten wel concluderen dat IRIZIO of de PLOD enzymen de transformatie van myoblasten door PAX3-FOXO1 stimuleren en een funktie vervullen bij de ontwikkeling van ARMS. Ze zijn daardoor mogelijkerwijs betrokken bij zowel de ontwikkeling van ARMS als andere vormen van kanker. Additionele studies zijn nodig om de rol van deze eiwitten bij kanker te kunnen begrijpen.

Dit proefschrift etaleert succesvolle methoden om genen te identificeren die zowel door PAX3-FOXO1 gereguleerd worden als genen die coöpereren met PAX3-FOXO1 bij het ontstaan van ARMS tumoren. We waren in staat om transcriptioneel gereguleerde targetgenen te vinden wiens effecten op het gedrag van de cel, transformatie kunnen veroorzaken. Bovendien waren wij in

staat twee genen te isoleren die ARMS tumorgenese kunnen versnellen, waarvan een rol bij kanker ontwikkeling niet eerder beschreven was. De identificatie van zulke ARMS geassocieerde genen, en de karakterisering daarvan, zouden misschien als nieuwe therapeutische doelwitten kunnen dienen om deze agressieve, vaak dodelijk ziekte bij kinderen succesvol te behandelen.

6.2. Toekomst plannen.

Zoals besproken in hoofdstuk 4 is overexpressie van IRIZIO in staat *Arf-/-/PAX3-FOXO1* myoblasten te transformeren. Bovendien vonden we dat IRIZIO overgeëxpresseerd wordt in RMS cellijnen, wat doet veronderstellen dat deze deregulatie oncogene activiteit heeft en daarmee bijdraagt aan de ontwikkeling van rhabdomyosarcoma. Ondanks deze resultaten moet de funktie van endogeen IRIZIO nog ontrafeld worden. Om IRIZIO's funktie beter te begrijpen hebben we gebruik gemaakt van de muizen ES cellijn (RRN280) van BayGenomics Laboratories, die een "trapping cassette" in het eerste intron van muizen *Irizio* (FAM193B) bevat. De gene trap vector in *Irizio* bevat een β-galactosidase/neomycine fusiegen (βgeo) geflankeerd door een splice acceptor en polyadenylatie signaal dat door de endogene Irizio promoter geëxpresseerd wordt. Na het verkrijgen van een chimere muis met deze cellijn genereerden wij muizen die heterozygoot and homozygoot waren voor het gemuteerde *Irizio* gen. Homozygote muizen bleken *Irizio* hypomorf te zijn, omdat ze vergeleken met wild type muizen nog steeds 10% van het Irizio RNA en eiwit expresseerden. We waren tot nu toe niet in staat om een duidelijk fenotype te identificeren dat geassociëerd is met de sterk gereduceerde expressie van *Irizio* maar het *β-Glactosidase* reporter gen, onder de controle van de *Irizio* promoter, stelt ons in staat om heel precies het expressie patroon van *Irizio* in embyonale en adulte weefsels te bepalen. Door *Irizio-/-* muizen te kruisen met *p16/Arf-/-* muizen kunnen we ook bepalen of de sterk verminderde *Irizio* niveaus het optreden van tumoren zal vertragen in deze muizen die normaal al op jonge leeftijd tumoren ontwikkelen. Een eerder rapport meldde

dat *IRIZIO* RNA overgeëxpresseerd wordt in een specifieke subgroep van patienten met recidive longkanker. Dus, een andere waardevolle benadering om de oncogene karakteristieken van Irizio te verifiëren is om Irizio-/- muizen te kruisen met 129S4-*Kras*^{tm4Tyj}/J muizen. Deze lijn bevat een geactiveerd *Kras* gen, waarvan de expressie geblokkeerd wordt door een stopcodon geflankeerd door Lox-P recombinatie sequenties (http://jaxmice.jax.org/strain/008180.html). Infectie via de neus met een Cre expresserend adenovirus heeft verwijdering van het stopcodon en expressie van mutant *Kras* tot gevolg, wat een tijds-afhankelijke ontwikkeling van longtumoren in de geinfecteerde muizen induceert. Door gebruik te maken van dit model kunnen we bepalen of verminderde expressie van Irizio de vorming van longtumoren voorkomt of vertraagd in *Kras*^{t4Ty}*/ Irizio-/-* dubbel mutante muizen.

6.2.2 Bepaling van de relatie tussen IRIZIO en progressie van de celcyclus.

Zoals eerder beschreven in dit hoofdstuk en hoofdstuk 4, was onze screening gebaseerd op de observatie dat PAX3-FOXO1 muizen myoblasten transformeert waarvan de p53 en pRb cascades geinaktiveerd waren. Door Arf-/- myoblasten te transformeren met PAX3-FOXO1 verwachtten we dat coöpererende cDNAs direct of indirect zouden interfereren met de controle van de Rb cascade. Ondanks talrijke pogingen waren we niet in staat een direct verband te leggen tussen Irizio expressie en de pRb cascade (zie de discussie sektie in hoofdstuk 3). Kort geleden hebben we gen-expressie profilering gedaan gebruik makend van publiek toegankelijke arraydata (http://www.ncbi.nlm.nih.gov/projects/geo/
query/acc.cgi?acc=GSE7578), wat liet zien dat *IRIZIO* opgereguleerd wordt in drie medulloblastoma cellijnen waarin Bmi-1 expressie via knockdown verminderd was. Dit resulteerde in remming van proliferatie, verlies van cel contact onafhankelijke groei en remming van tumorvorming in naakte muizen. Bmi-1 is een transcriptionele repressor die behoord tot de Polycomb (PcG) groep van eiwitten die transcriptie reguleren. PcG eiwitten reguleren

genexpressie epigenetisch via verandering in het chromatine van specifieke promoters. Meerdere studies hebben aangetoond dat Bmi-1 kan functioneren als een oncogen; het stimuleert celgroei door volledige repressie van het *INK4a/ARF* tumorsuppressor locus, dat codeert voor de celcyclus remmers $p16^{ink4a}$ **en** $p19^{arf}$ en van het $p21^{waf1/cip1}$ *locus*. We speculeren dat in deze medulloblastoma cellijnen verhoogde expressie van IRIZIO wellicht een mechanisme is om te compenseren voor de verminderde celgroei veroorzaakt door Bmi-1 knockdown. Het is daarbij interessant dat een recente publicatie meldde dat Bmi-1 in post-natale satellietcellen tot expressie komt en daarmee een belangrijke rol vervult bij het in stand houden van de satellietcel popula*tie en spiercel regeneratie*. Het is daarom belangrijk te bepalen of Bmi-1-afhankelijke regulatie van *IRIZIO* een effect heeft op myogene cellen, zodat IRIZIO een rol zou kunnen spelen bij het handhaven van de balans tussen celdeling en differentiatie. Het is in dit opzicht voorstelbaar dat overexpressie van IRIZIO de transformatie van primaire fibroblasten kan bewerkstelligen als die de p53 cascade hebben verloren (Arf-/-) en een oncogen zoals PAX3-FOXO1 expresseren. Begrip omtrend de rol van *IRIZIO* als een gen dat gereguleerd wordt door Bmi-1 zou daarmee inzicht over de funktie van dit nieuwe gen kunnen verschaffen.

6.2.3 Is de lysyl hydroxylatie aktiviteit van PLOD1 essentiëel voor celtransformatie?

Zoals genoemd in hoofdstuk 4, draagt PLOD1 expressie bij aan de tumorigene transformatie van *Arf-/-* myoblasten die PAX3-FOXO1 expresseren. Bovendien lieten we zien dat alle PLOD familieleden (PLOD1-3) overgeëxpresseerd worden in ARMS cellijnen en in vele andere tumoren, er daarmee op duidend dat de familie van PLOD lysyl hydroxylases bijdraagt aan humane tumorgenese. Het is daarom belangrijk te bepalen of de lysyl hydroxylase activity zelf noodzakelijk is voor cellulaire transformatie of dat dit veroorzaakt wordt door een andere, vooralsnog onbekende funktie van deze groep van enzymen. Het is mogelijk dat incorrect gehydroxyleerd collageen wellicht de

compositie van de extracellulaire matrix verandert wat veranderde celgroei tot gevolg kan hebben. Om deze vraag te kunnen beantwoorden, hebben we een PLOD1 mutant gemaakt die niet in staat is Fe^{2+} te binden, een essentiële cofactor voor de lysyl hydroxylase aktiviteit. Bovendien hebben we een mutant gemaakt die 33 aminozuren van de C-terminus mist. Deze regio is betrokken bij zowel retentie in het endoplasmatisch reticulum (ER) als bij binding van het substraat. Expressie van deze mutanten in *Arf-/-/PAX3-FOXO1* myoblasten en injectie in NOD/SCID muizen zal aan het licht brengen of de lysyl hydroxylase aktiviteit van PLOD1 nodig is voor tumorigenese. Om verder de oncogene aktiviteit van PLOD1 te verifiëren, zullen we *p16/Arf-/-* muizen kruisen met *Plod1*-deficiënte muizen. Door de tumor incidentie in *p16/Arf-/-/Plod-/-* muizen te vergelijken met die in *p16/Arf-/-/Plod+/-* of *p16/Arf-/-/Plod+/+* muizen zullen we zien of strek verminderd PLOD1 in *p16/Arf-/-* muizen het voorkomen van tumoren zal verminderen of misschien verhelpen (22,23). In eenzelfde lijn van onderzoek kunnen we ook primaire myoblasten van *p16/Arf-/-/Plod-/-* of *p16/Arf-/-/Plod+/-* dieren met *PAX3-FOXO1* retrovirus transduceren. Als na injectie in NOD/SCID muizen *Plod1+/+* cellen in vergelijking met *Plod1-nul* cellen een vertraagde of geen tumor formatie laten zien zou dat verder PLOD1's bijdrage aan tumorigene transformatie bevestigen.

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