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The expression and function of PAX3 in development and disease

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Abstract

The *PAX3* gene encodes a member of the PAX family of transcription factors that is characterized by a highly conserved paired box motif. The PAX3 protein is a transcription factor consisting of an N-terminal DNA binding domain (containing a paired box and homeodomain) and a C-terminal transcriptional activation domain. This protein is expressed during development of skeletal muscle, central nervous system and neural crest derivatives, and regulates expression of target genes that impact on proliferation, survival, differentiation and motility in these lineages. Germline mutations of the murine *Pax3* and human *PAX3* genes cause deficiencies in these developmental lineages and result in the Splotch phenotype and Waardenburg syndrome, respectively. Somatic genetic rearrangements that juxtapose the PAX3 DNA binding domain to the transcriptional activation domain of other transcription factors deregulate PAX3 function and contribute to the pathogenesis of the soft tissue cancers alveolar rhabdomyosarcoma and biphenotypic sinonasal sarcoma. The wild-type PAX3 protein is also expressed in other cancers related to developmental lineages that normally express this protein and exerts phenotypic effects related to its normal developmental role.

Keywords

Transcription factor; Paired box; Homeodomain; Alternative splicing; Target gene; Skeletal muscle development; Central nervous system development; Neural crest; Melanocyte; Waardenburg syndrome; Splotch; Fusion oncoprotein; Rhabdomyosarcoma; Soft tissue sarcoma; Melanoma

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1. Introduction

The *PAX3* gene (paired box gene 3; also known as WS1, WS3, CDHS and HUP2) encodes a member of the paired box or PAX family of transcription factors. This family is characterized by a highly conserved paired DNA binding domain first identified in *Drosophila* segmentation genes (Tremblay and Gruss, 1994). There are nine human members (*PAX1-PAX9*) and nine homologous murine members (*Pax1-Pax9*) that are grouped into four subfamilies. *PAX3* and *PAX7* (along with *Pax3* and *Pax7*) comprise a subfamily termed group III, based on a high degree of sequence homology and similar genomic and functional organization (Stuart et al., 1994). The nine human PAX genes are located at non-contiguous loci throughout the human genome, and most are located on separate chromosomes.

1.1. *PAX3* gene location and structure

The human *PAX3* gene is present in the q36.1 region on the long arm of chromosome 2 (Table 1). The *PAX3* gene consists of 10 exons (Barber et al., 1999) within a 100 kb region, spanning from location 222,199,888 bp to 222,298,996 bp (Ensembl assembly release GRCh38.p10) (Fig. 1). Exons 2, 3 and 4 encode the conserved paired domain, whereas exons 5 and 6 encode the conserved octapeptide and homeodomain, and exons 7 and 8 encode the transactivation domain (Baldwin et al., 1995).

1.2. *PAX3* mRNA structure and splicing

As a result of alternative processing and splicing, at least ten distinct *PAX3* isoforms have been detected at the mRNA level (Table 1, Fig. 2). The longest isoform is *PAX3e*, which comprises 10 exons (Barber et al., 1999) and encodes for a 505 amino acid protein. The consensus GT donor at the 5' end of intron 9 is not found in species other than human, and thus the longest isoforms produced by other species, such as mouse, correspond to *PAX3c* and *PAX3d* (Barber et al., 1999; Parker et al., 2004). These latter two isoforms consist of 8 or 9 exons, respectively, and are formed by events that suppress the exon 8 to exon 9 splice or the exon 9 to exon 10 splice and thereby result in extension of the transcripts into intron 8 or intron 9, respectively. The three long isoforms (*PAX3c*, *PAX3d*, and *PAX3e*) contain all known characterized functional domains and differ at the extreme C-terminal end. *PAX3c* and *PAX3d* appear to be the predominant isoforms expressed in melanoma (Matsuzaki et al., 2005), and *PAX3d* is expressed at higher levels than *PAX3c* in a series of cancer cell lines (Parker et al., 2004). In functional studies, *PAX3c* and *PAX3d* similarly stimulated endpoints such as cell growth in melanocytes whereas *PAX3e* inhibited these activities (Wang et al., 2006). Furthermore, a comparison of *PAX3c* and *PAX3d* in assays of transcriptional function revealed that both isoforms have stimulatory activity, with *PAX3d* having greater activity than *PAX3c* (Barber et al., 1999; Barr et al., 1999).

Several shorter *PAX3* isoforms have also been detected (Fig. 2). The *PAX3g* and *PAX3h* isoforms include exon 9 or exons 9 and 10, respectively, but differ from the *PAX3e* or *PAX3d* isoforms due to a splicing event that joins exon 7 to exon 9 and thereby skips exon 8. In addition to removing exon 8, which encodes a portion of the transactivation domain, the translational reading frame is altered in the transition from exon 7 to exon 9, and thus novel

protein sequence is introduced at the C-terminal end of these isoforms. A dominant negative function for Pax3g is suggested by the absence of transcriptional activity and the ability to inhibit activity of the full-length protein (Pritchard et al., 2003). Though PAX3g may inhibit several biological endpoints, such as growth and migration of melanocytes, PAX3h appears to stimulate these endpoints (Wang et al., 2006). The two shortest isoforms, PAX3a and PAX3b, consist of 4 or 5 exons, respectively, and are formed by alternative splicing events within intron 4. These splicing events result in extension of the mRNA into intron 4 or splicing to a novel cryptic exon within intron 4. These two alternative PAX3 transcripts lack the 3' exons encoding the homeodomain and the transactivation domain, and thus form truncated proteins, which functionally demonstrate either no activity or only inhibition of biological endpoints such as melanocyte growth and migration (Wang et al., 2006).

Studies of human PAX3 and murine Pax3 transcripts identified a frequent alternative splicing event that either includes or excludes the codon CAG as the 3' splice acceptor at the 5' end of exon 3 (Vogan et al., 1996). This alternative splicing event results in the presence or absence of a glutamine residue within the paired box motif. The two isoforms (Q+ and Q-) resulting from this alternative splicing are co-expressed at all embryonic stages at which Pax3 is expressed. This alternative splice is also found in several tumors in which PAX3 is expressed, including in the PAX3-FOXO1 fusion transcripts expressed in alveolar rhabdomyosarcoma. The level of the Q+ isoform is approximately two-fold higher than that of the Q- isoform, and there is no significant variation in this ratio in various developmental time points and tumor types. In assays of transcriptional function with several PAX3 DNA binding sites, the Q- isoform has similar or greater DNA binding and transactivation function than the corresponding Q+ isoform (Du et al., 2005). Though most studies of this splicing event did not establish which PAX3 isoforms contain this alternative splice, limited sequencing studies of full-length human cDNAs identified this splicing event as a variant of the PAX3d isoform, and this spliced form has been separately termed the PAX3i isoform. However, more comprehensive full-length sequencing studies are needed to determine whether this alternative splicing event occurs in other PAX3 isoforms.

2. PAX3 Protein

2.1. PAX3 functional domains

The PAX3 protein is a transcription factor characterized by an N-terminal DNA binding domain and a C-terminal transactivation domain (Fig. 1). The DNA binding domain consists of a paired box (PB), octapeptide and homeodomain (HD), and the transactivation domain contains a proline-, serine- and threonine (PST)-rich region.

The PB consists of a stretch of 128 amino acids that is well-conserved in evolution (Czerny et al., 1993; Bopp et al., 1989). The PB is formed by N-terminal (PAI) and C terminal (Kim et al.) subdomains (PAI+RED=PAIRED) (Fig. 1), each consisting of a three-helix fold that includes a helix-turn-helix (HTH) motif, with the C-terminal helix of each HTH making base-specific contacts in the major groove of DNA. The PB recognizes highly related DNA sequences (TCACGC/G, with minor variability seen for each PAX orthologue) (Apuzzo and Gros, 2007).

The HD is typically 60 amino acids long and contains three alpha-helices. PAX3 and three other PAX members (PAX4, PAX6, and PAX7) possess this DNA binding domain, while PAX2, PAX5 and PAX8 contain a rudimentary domain consisting of the first alpha-helix (Tremblay and Gruss, 1994). Most HDs recognize sequences containing a TAAT core motif. HDs found in PAX proteins cooperatively dimerize on palindromic sites of the type 5'-TAAT (N)₂₋₃ ATTA-3', either as a homodimer or as a heterodimer with another homeodomain (Wilson et al., 1993; Buckingham and Relaix, 2007). Cooperative interactions are present between the HD and PB domains, and are responsible for the functional interdependence of these two domains in DNA binding (Underhill and Gros, 1997; Apuzzo and Gros, 2007).

The C-terminus of PAX3 contains a 78 amino acid long PST-rich region reminiscent of transcriptional activation domains of other transcription factors, such as OCT-2 and CTF-1 (Tanaka and Herr, 1990; Chalepakis et al., 1994b). This C-terminal PAX3 region confers transcriptional activation in reporter assays. Though the C-terminal PAX7 region also has transactivating activity, there are subtle functional differences between these PAX3 and PAX7 regions (Bennicelli et al., 1999). These C-terminal transactivation domains appear to be potentially inhibited by transcriptional repression domains located in both the HD and the N-terminus (including the first half of the PB) of PAX3 and PAX7.

2.2. DNA binding properties of PAX3

Early studies demonstrated that PAX3 binds in vitro to the e5 sequence present upstream of the *Drosophila* even-skipped gene. This sequence contains PB and HD binding sites that include GTTCC and ATTA motifs, respectively (Chalepakis et al., 1994a). Efficient binding of PAX3 to the e5 sequence requires the presence of both PB and HD binding sites. Furthermore, an intervening region of 2-10 bp between the PB and HD binding sites is essential for efficient PAX3 binding (Chalepakis et al., 1994c).

Cyclic amplification and selection of targets (CASTing) methodology revealed that optimal consensus DNA sequences recognized by the PAX3 PB domain include TCGTCAC(G/A)C(T/C/A)(T/C)(C/A/T)A and CGTTCACG(G/C)TT (Chalepakis and Gruss, 1995; Epstein et al., 1996). These sequences are similar to consensus DNA-binding sequences for the PAX2 and PAX6 PB (Epstein et al., 1994a), and the closely related *Drosophila* paired PB. A search for optimal binding sequences for the combined PB and HD domains of PAX3 identified ATTA and GTNNN motifs in the majority of isolated binding sites, and the sequence ATTA-(N)_n-GTTAT in 20% of the binding sites (Phelan and Loeken, 1998). Comparison of PAX3 and PAX7 binding sites suggested that these proteins bind identical DNA motifs. However, while PAX7 can activate target gene expression from combined PB/HD or HD alone, PAX3 is not effective in inducing transcription from genes with only HD motifs (Soleimani et al., 2012).

2.3. PAX3 interacting proteins

The role of PAX3 as a transcriptional regulator is clarified by the identification of other proteins that interact with PAX3 and modulate its transcriptional activity. First, PAX3 is capable of forming homodimers (PAX3/PAX3) and heterodimers (PAX3/PAX7) that can

influence DNA binding to a palindromic HD binding site (P2-TAATCAATTA) (Schafer et al., 1994). Furthermore, PAX3 can bind to other transcription factors such as SOX10, ETS1, GLI2, and ZIC2, thereby resulting in synergistic activation of downstream genes (Lang and Epstein, 2003; Himeda et al., 2013; Kubic et al., 2015a). In addition, the Hippo pathway effectors TAZ and YAP65 bind and function as coactivators of PAX3 to regulate expression of PAX3 target genes, such as *MITF* (Murakami et al., 2006; Manderfield et al., 2014). To extend these findings to chromatin structure, PAX3 also interacts with PAX3/7BP, an essential adaptor linking PAX3 and PAX7 with the H3K4 methyltransferase complex, which activates target gene expression and proliferation in muscle precursor cells (Diao et al., 2012).

PAX3 can also function as a transcriptional inhibitory factor by binding co-repressors. Putative co-repressors interacting with PAX3 and inhibiting PAX3-dependent transcriptional activation include DAXX, RB1, HIRA, Calmyrin (EF-hand calcium-binding protein), KAP1 and Grg4 (Groucho co-repressor) (Magnaghi et al., 1998; Wiggan et al., 1998; Hollenbach et al., 1999; Hollenbach et al., 2002; Lang et al., 2005; Hsieh et al., 2006). DAXX, HIRA and RB1 interact with the PAX3 HD, while Calmyrin and KAP1 interact with the PAX3 PB. DAXX and Calmyrin interactions with PAX3 appear to also involve the octapeptide motif. These co-repressors may alter chromatin configuration at a subset of PAX3 target genes (e.g., HIRA), inhibit the ability of PAX3 to bind to its DNA recognition sequences (e.g., Calmyrin), compete with heterochromatin protein 1 for binding with PAX3 on target promoters (e.g., KAP1) and modulate PAX3 transcription activity through direct interaction (e.g., DAXX).

Pax3 may also exert other functions through protein-protein interactions independent of its role as a transcription factor. In particular, Pax3 associates with the p53 and Mdm2 proteins, and stimulates Mdm2-mediated ubiquitination and degradation of the p53 protein (Wang et al., 2011). These effects can be generated by the isolated Pax3 PB or HD, and do not require the Pax3 transactivation domain.

2.4. PAX3 target genes

PAX3 binding sites are located in a variety of genomic locations. Some are present in or near target genes, such as the 5' proximal promoter (e.g., *MET* and *MITF*), the first intron (e.g., *CXCR4* and *NRCAM*), and the 3' untranslated region (UTR) (e.g., *NFI*) (Epstein et al., 1994b; Watanabe et al., 1998; Galibert et al., 1999; Lagha et al., 2008; Kubic et al., 2015b). PAX3 binding sites were also identified in further upstream and downstream regions. *DMRT2*, *MYOD1*, and *MYF5* are regulated by PAX3 via binding sites located 18 kb, 20 kb, and 57.5 kb, respectively, upstream of the corresponding transcription start sites (Kucharczuk et al., 1999; Bajard et al., 2006; Cao et al., 2010).

In contrast, PAX3 regulates FGFR4 expression by binding to a site located 19.2 kb downstream from the FGFR4 translation start site and 6.5 kb downstream of the last exon (Lagha et al., 2008). Though PAX3 functions as a transcriptional activator for most target genes, PAX3 may act as a transcriptional repressor of some targets, such as MBP. In addition, some genes with PAX3 binding sites (e.g., *NFI*) do not show altered expression in

PAX3-deficient cells, suggesting that not all PAX3 binding sites are functional or other changes compensate for lack of PAX3 binding (Lakkis et al., 1999).

In addition to the above-described candidate gene approaches, genome-wide screening of genomic DNA with a CASTing strategy identified PAX3 binding sites in genes including *ITM2A*, *FATH*, *FLT1*, *TGFA*, *BVES*, and *EN2* (Barber et al., 2002). Subsequent analysis of selected genes revealed that these binding sites confer PAX3-dependent regulation. In addition, array-based expression profiling of PAX3- and control-transfected medulloblastoma cells identified additional putative PAX3 downstream targets such as *STX*, *TIMP3*, *MARCKS*, *RELA*, *BMP5*, *PTPRJ*, *GOS1*, *SLAP*, *IL6R*, and *ID4* (Mayanil et al., 2001). Similar microarray studies in melanocytes revealed that different PAX3 isoforms (PAX3c, PAX3e, and PAX3g) regulate distinct but overlapping sets of genes associated with differentiation, proliferation, migration, adhesion, apoptosis or angiogenesis (Wang et al., 2007).

PAX3 target genes can be divided into various groups based on their biological function (Table 2). One group of genes is associated with muscle development (e.g., *MET*, *MYF5*, *MYOD1*, *WNT1*, *DIRT2* and *FGFR4*). A second group of genes is associated with neural and melanocyte development (e.g., *MITE*, *TYRP1*, *RET*, *TBX2*, *NGN2*, *HES1*, *NRCAM*, and *TGFB2*). In addition, PAX3 also targets alpha-satellite repeats of centromeres and mediates the formation of heterochromatin (Bulut-Karslioglu et al., 2012), extending the function of PAX3 beyond regulation of protein coding genes to the regulation of chromatin structure (Wu et al., 2015).

2.5. Phosphorylation, ubiquitination and acetylation of PAX3 protein

PAX3 contains three major sites of phosphorylation: serines 201, 205 and 209 (Dietz et al., 2009; Dietz et al., 2011) (Fig. 2). The kinases that phosphorylate PAX3 includes GSK3 β for serine 201 and CK2 for serines 205 and 209 (Dietz et al., 2011; Iyengar et al., 2012). Variation of phosphorylation states appears to occur in an ordered fashion during myogenesis. In melanoma cells, phosphorylation by GSK3 β increases the stability of the PAX3 protein (Kubic et al., 2012). Furthermore, pharmacologic studies revealed that phosphorylation of the N-terminal PAX3 region regulates the transcriptional function of the PAX3-FOXO1 fusion protein in rhabdomyosarcoma (see Section 4.2) (Amstutz et al., 2008). The phosphorylation status of these serine residues affects the oncogenic function of the wild-type PAX3 protein in melanoma and the PAX3-FOXO1 fusion protein in rhabdomyosarcoma, and pharmacologic or genetic manipulation of the phosphorylation status results in changes in phenotypes such as proliferation, transformation and invasion (Iyengar et al., 2014; Loupe et al., 2015).

In addition to phosphorylation, the PAX3 protein is also modified by ubiquitination and acetylation. Lysines 437 and 475 in the C-terminal region of PAX3 are important for monoubiquitination and subsequent degradation of the PAX3 protein (Boutet et al., 2007). Both TAF1 (a major subunit of the TFIID transcriptional initiation complex) and anaphase-promoting complex/cyclosome E3 ligase participate in this degradation process (Boutet et al., 2010; Cao et al., 2015). These same two lysines (437 and 475) in the PAX3 protein can

be acetylated. Acetylated PAX3 is a substrate of SIRT1 deacetylase and may contribute to decreased stem cell proliferation and increased neurogenic activity (Ichi et al., 2011).

3. Expression and function of PAX3 during development

3.1. Pax3 expression in myogenic precursors

Pax3 is a major regulator of embryonic myogenesis (Buckingham and Relaix, 2007). Pax3 expression is first seen at embryonic day 8.5 in the pre-somitic paraxial mesoderm before segmentation occurs to give rise to the somites (Bober et al., 1994; Goulding et al., 1994; Williams and Ordahl, 1994). As somites form, Pax3 is initially expressed throughout the somites, and later is confined to the dorsal epithelial portion, which is the dermomyotome (Goulding et al., 1991). Pax3 expression becomes concentrated in the epaxial and hypaxial regions of the dermomyotome (Tajbakhsh and Buckingham, 2000; Buckingham and Relaix, 2007). When muscle precursor cells detach from the dermomyotome to form the underlying differentiated muscle of the myotome, Pax3 expression is downregulated and myogenic transcription factors are activated (Myf5 and Myod1) (Tajbakhsh and Buckingham, 2000). Another population of Pax3-expressing cells detach from the dermomyotome and migrate to distant myogenic sites, such as the limbs, diaphragm, and hypoglossal cord (Franz et al., 1993; Bober et al., 1994; Goulding et al., 1994; Tajbakhsh et al., 1997; Tremblay et al., 1998). Finally, cells expressing Pax3 and Pax7 derived from the central dermomyotome provide a pool of myogenic progenitors for muscle growth during fetal development.

During the later stages of fetal development, myogenic progenitors expressing Pax3 and Pax7 form the satellite cells embedded under the basal lamina of muscle fibers. These cells contribute to muscle growth during the postnatal period and muscle regeneration thereafter. Though Pax7 is expressed by most adult satellite cells, which remain in a quiescent state until activated by injury, a subset expresses Pax3, often with coexpression of Pax7. The proportion of satellite cells that expresses Pax3 varies in different muscles; for example, Pax3-expressing satellite cells are frequent in the forelimb and diaphragm but not in the hind limb (Buckingham et al., 2003; Relaix et al., 2006).

3.2. Pax3 expression in developing nervous system

Pax3 expression is first observed during formation of the neural tube at embryonic day 8.5. In particular, Pax3 is expressed in the dorsal region of the neural groove and, as the neural groove deepens and closes, Pax3 continues to be expressed in the dorsal region of the neural tube (Goulding et al., 1991). This expression extends along the neural tube from the forebrain to the posterior neurospore. The localization of Pax3 expression to the dorsal half of the neural tube gives rise to a sharp ventral border between the basal and alar plates visible up through embryonic day 13 (Goulding et al., 1991). As the neural tube thickens, Pax3 expression is confined to proliferative cells in the inner ventricular zone and is downregulated as these cells migrate superficially. In addition, Pax3 expression is turned off in a rostral to caudal direction as neural tube development proceeds. Similarly, though Pax3 is initially expressed in neuroepithelium of the dorsolateral walls of the forebrain, midbrain and hindbrain, this expression becomes progressively restricted to more caudal regions during embryonic days 12-13 (Goulding et al., 1991).

3.3. Pax3 expression in neural crest and derivatives

The neural crest is a multipotent population of cells that arises at the border between neural and non-neural ectoderm and gives rise to variety of cells and tissues in the developing vertebrate (Knecht and Bronner-Fraser, 2002; Milet and Monsoro-Burq, 2012). During neural induction, Pax3 is expressed at the lateral and posterior edges of the neural plate (and then the closed neural tube), the region from which the neural crest cells emerge and migrate (Monsoro-Burq et al., 2005). Pax3 is subsequently found to be expressed by a variety of cell types derived from the neural crest, such as melanoblasts, dorsal root ganglia, and Schwann cell precursors. For melanoblasts, Pax3 expression is present during migration to the skin and persists in the developing hair follicles (Hornyak et al., 2001; Blake and Ziman, 2005). In addition, Pax3-expressing cells related to the melanoblast lineage also contribute to the development of multiple inner ear components (Freyer et al., 2011; Kim et al., 2014). In Schwann cell precursors, Pax3 is restricted to proliferating cells and functions to regulate differentiation and associated myelination (Kioussi et al., 1995). Finally, Pax3 is also expressed in a subpopulation of cranial neural crest cells and in structures derived from these cells, such as mesenchyme of the mandible and maxilla.

3.4. Pax3 mutation during development

The significance of Pax3 in development is further evidenced by analyses of germline mutations involving the *Pax3* gene. The *spotch* phenotype is found in several spontaneous mutant mouse strains as well as in chemical- or radiation-induced strains, and is characterized by white patches in the belly, tail and feet in the heterozygous state and more significant phenotypic changes in the homozygous state (Russell, 1947; Moase and Trasler, 1992). In addition, as described below, there is a related human disease termed Waardenburg syndrome. Point mutations and deletions affecting functional domains of the mouse *Pax3* gene were identified in the *spotch* mouse. Functional studies showed that these mutations alter or abolish Pax3 transcriptional activity, and result in a loss of biological function. Different *Spotch* alleles (such as *Spotch*^{4H}, *Spotch*^{2H}, *Spotch*-delayed) are caused by different *Pax3* mutations, which combine with differences in genetic background and environmental factors to result in varying phenotypic severity (Epstein et al., 1991; Vogan et al., 1996).

In heterozygous *spotch* mice, the characteristic white spots are attributed to defects in neural crest cells resulting in localized deficiencies in pigment-forming melanocytes. Mice homozygous for these *Pax3* mutations demonstrate embryonic lethality around embryonic day 15. There are prominent neural tube closure defects, such as spina bifida and exencephaly. Multiple neural crest-derived structures are absent or abnormal, including dorsal root ganglia, enteric ganglia, and melanocytes. Though Pax3 may not be specifically required for neural crest migration, loss of function contributes to defects in neural crest specification, proliferation, survival and/or differentiation. The severity of neural crest defects increases in posterior regions of the body, resulting in a progressive loss of cardiac, trunk, and caudal neural crest (Relaix et al., 2004). Loss of the cardiac neural crest cells results in heart malformations, due to the contributions of these cells to heart innervation and structures of the cardiac outflow tract. Some of these malformations, such as neural tube defects, can be rescued by crosses with p53-deficient mice, thus revealing that p53

inactivation and corresponding inhibition of p53-mediated apoptosis is a major function of Pax3 during early neural development (Pani et al., 2002; Wang et al., 2011). Finally, there are significant defects in muscle development in homozygous Splotch mice. The limb musculature fails to develop whereas the axial musculature shows varying degrees of abnormality. This hypoplasia in the limb musculature is associated with increased cell death in myogenic precursors in the lateral dermomyotome (Borycki et al., 1999) and diminished migration of any surviving precursors into limb buds (Bober et al., 1994; Goulding et al., 1994; Daston et al., 1996). This muscle defect is attributed to reduced expression of *Met*, a Pax3 target gene (Bladt et al., 1995; Epstein et al., 1995; Daston et al., 1996; Yang et al., 1996).

3.5. Regulation of Pax3 expression

Deletion studies in transgenic mice revealed that the region located 1.6 kb upstream of the *Pax3* transcription start site contains regulatory sequences sufficient for induction of Pax3 expression and restriction to the hindbrain and trunk (Natoli et al., 1997; Degenhardt et al., 2010). In this sequence, two evolutionarily conserved regions named NCE1 and NCE2 were found to be important for Pax3 expression in the neural tube and neural crest cells (Fig. 3) (Degenhardt et al., 2010). Anterior and posterior expression of Pax3 are supported by NCE1 and NCE2, respectively (Sanchez-Ferras et al., 2012). NCE1 contains binding sites for Pbx factors and is required for hindbrain expression (Phelan et al., 1995; Naini et al., 2008). NCE2 is recognized by Cdx factors (Sanchez-Ferras et al., 2012) and is thus indirectly activated by the Wnt signaling pathway (Sanchez-Ferras et al., 2012). In addition, NCE2 contains response elements for the sonic Hedgehog pathway, and is responsible for restriction of Pax3 expression to the lateral neural plate and dorsal neural tube and repression in the ventral neural tube (Goulding et al., 1993; Sanchez-Ferras et al., 2012). Both NCE1 and NCE2 contain binding sites for Tead2 and the POU class III factors Brn1 and Brn2 (Pruitt et al., 2004), which are proposed to maintain rather than induce Pax3 expression.

The finding that deletion of NCE1 and NCE2 does not significantly alter Pax3 expression in mice indicates that additional regulatory sequences support Pax3 expression. An additional region regulating neural tube and neural crest expression is present in the fourth intron of the *Pax3* gene (Degenhardt et al., 2010; Moore et al., 2013) and contains two conserved non-coding elements, CNE1 and CNE3 (or ECR2), which serve as enhancers of Pax3 expression in the developing central nervous system. This latter element contains TCF/LEF binding sites, supporting an additional direct regulation of *Pax3* by the Wnt signaling pathway (Degenhardt et al., 2010). CNE3 also binds to Nkx6.1, which serves as a repressor and restricts Pax3 expression to the dorsal region of the neural tube (Moore et al., 2013). In addition to this intronic element, an enhancer located 6 kb upstream of the *Pax3* transcription start site mediates Pax3 expression in the hypaxial somite (Fig. 3) (Brown et al., 2005). Taken together, the regulatory elements adjacent to the *Pax3* promoter, in the fourth intron and in the far upstream region provide evidence for the complexity and a time- and site-specific fine tuning of Pax3 expression during development.

In addition to these elements regulating Pax3 expression at the transcriptional level, there are several microRNAs that regulate Pax3 expression at the post-transcriptional level. There are binding sites for miR-1, miR-27b, miR-128a, and miR-206 within the 3' UTR of the Pax3 mRNA (Crist et al., 2009; Goljanek-Whysall et al., 2011; Motohashi et al., 2012). Expression of one or more of these microRNA's will decrease Pax3 mRNA stability and/or translation, resulting in decreased Pax3 expression (Boutet et al., 2012). The muscle-specific microRNAs are induced by Myodl in the somites (Hirai et al., 2010), and miR-27b is inhibited by Pitx2c in early myogenic precursors and then later expressed in differentiating embryonic myotome and activated satellite cells (Lozano-Velasco et al., 2011). Whereas Pax3 is expressed in the absence of these microRNAs to promote proliferation and migration and to inhibit differentiation, upregulation of one or more of these microRNAs leads to Pax3 downregulation, thereby inducing differentiation and further inhibiting proliferation and migration. In subsequent myogenic stem cell populations, miR-206 and miR-128a help maintain the quiescent state by inhibiting expression of *Pax3* and other developmental genes (Goljanek-Whysall et al., 2011; Motohashi et al., 2012). In a subpopulation of quiescent stem cells, Pax3 is expressed despite the presence of miR-206 by generating "resistant" transcripts with alternative polyadenylation sites, which remove the miR-206 binding sites from the Pax3 3' UTR (Boutet et al., 2012).

4. PAX3 in human disease

4.1. PAX3 germline mutations in Waardenburg syndrome

The importance of PAX3 in neural tube, neural crest and muscle development is also confirmed by the molecular genetic findings in Waardenburg syndrome. This syndrome is a group of four autosomal dominant genetic disorders (WS1, WS2, WS3 and WS4), which are characterized by variable features in affected individuals including hearing loss, eye abnormalities and pigmentation disorders (Waardenburg, 1951). WS1 is also frequently characterized by a midfacial alteration called dystopia canthorum (Farrer et al., 1994), and WS3 (Klein-Waardenburg syndrome) is comparable to WS1 with additional musculoskeletal abnormalities affecting the upper limbs. WS1 and WS3 are frequently caused by mutations in PAX3, whereas WS2 is caused by mutations in *MITF* and WS4 is caused by mutations in *EDN3*, *EDNRB*, or *SOX10*. Though these mutations were initially detected by directed Sanger sequencing of polymerase chain reaction (PCR)-amplified exons from selected genes, more recent next generation sequencing approaches can screen larger panels of genes related to Waardenburg syndrome and other genetic causes of hearing loss (Xiao et al., 2016).

Heterozygous mutations in the *PAX3* gene are observed in nearly 80% of WS1 cases (Fig. 4), whereas partial or total deletion of *PAX3* and contiguous genes are often observed in WS3 cases. Smaller mutations can also occur in WS3 and could be either heterozygous or homozygous (Hoth et al., 1993; Tassabehji et al., 1995). Approximately 100 sequence changes in the *PAX3* gene have been reported in these disorders, and very few of these changes are recurrent. These reported sequence changes include missense mutations (38%), small deletions (20%), nonsense mutations (15%), gross deletion (11%), splicing mutations (8%), and small insertions (8%) (Jalilian et al., 2015). The majority of these *PAX3* mutations

are localized to exons 2 through 6 with the highest frequency in exon 2 (Pingault et al., 2010). These frequently mutated regions alter the structure of the paired domain or homeodomain, and thus affect DNA binding function. Mutations were not found in exons 9 and 10, and only a few mutations were reported in exons 1, 7, and 8, including mutations that affect the transactivation domain (Baldwin et al., 1995; Carey et al., 1998; Jalilian et al., 2015). There is no correlation between the mutation type, location and severity of the phenotype (Baldwin et al., 1995; Tassabehji et al., 1995). In a subset of WS1 cases, such as those recently reported in the Chinese and Korean populations, the proband's *PAX3* mutation was not detected in either parent, suggesting the occurrence of a de novo mutational event (Wang et al., 2010; Jang et al., 2015). However, the report of two WS1-affected siblings in which the shared *PAX3* mutation was not present in either parent also points to the possibility of germinal mosaicism in rare cases (Chen et al., 2018).

PAX3 mutations have been also identified in rare disorders related to Waardenburg syndrome. Craniofacial-deafness-hand syndrome (CDHS) has been described in one family with a mutation in *PAX3* exon 2 (Asher et al., 1996). This autosomal dominant syndrome is considered to be an allelic variant of Waardenburg syndrome and is characterized by craniofacial anomalies, profound sensorineural deafness, and ulnar deviation of the hand with contractures of the fingers (Sommer et al., 1983). A rare case has also been described in which a child had heterozygous mutations for both *PAX3* and *MITF* (Glass, 1990). One parent had WS1 caused by a mutation in the splicing site of *PAX3* intron 4 and the other parent had WS2 caused by a pathogenic variant in *MITF*. The child showed significantly more pigmentary defects than his parents, suggesting an additive effect of these two gene alterations.

4.2. Gene fusions in alveolar rhabdomyosarcoma

Alveolar rhabdomyosarcoma (ARMS) is an aggressive pediatric soft tissue sarcoma that occurs mainly in the extremities and trunk (Wexler LH, 1997). In most ARMS cases, there is a recurrent t(2;13)(q35;q14) chromosomal translocation that breaks *PAX3* and joins it to *FOXO1* (Fig. 5A), which encodes a member of the forkhead (or FOX) transcription factor family (Seidal et al., 1982; Turc-Carel et al., 1986; Douglass et al., 1987; Wang-Wuu et al., 1988). The t(2; 13) breakpoint always disrupts the seventh intron of *PAX3* and the first intron of *FOXO1* (Davis et al., 1995; Barr et al., 1998). By swapping portions of the two genes, the 2;13 translocation thereby generates a *PAX3-FOXO1* fusion gene that is transcribed into a *PAX3-FOXO1* fusion transcript (Barr et al., 1993; Galili et al., 1993; Shapiro et al., 1993). In this fusion transcript, the 5' end of the *PAX3* coding sequence fuses in frame with the 3' end of the *FOXO1* coding sequence to yield a *PAX3-FOXO1* fusion protein, which is 836 amino acids in length. The t(2; 13) breakpoint is positioned such that the N-terminal *PAX3* DNA binding domain is preserved, and separated from most of the *PAX3* transactivation domain. In addition, the translocation breakpoint disrupts the forkhead DNA binding domain, but the *FOXO1* transcriptional activation domain remains intact. This arrangement results in fusion of regions encoding the N-terminal *PAX3* DNA-binding domain and the C-terminal *FOXO1* transactivation domain to create a novel fusion transcription factor.

The *PAX3-FOXO1* fusion results in increased activity at both the functional and expression levels. Transcriptional studies revealed that, compared to the wild-type PAX3 protein, the PAX3-FOXO1 fusion protein is a stronger activator of genes with PAX3 binding sites (Bennicelli et al., 1995; Fredericks et al., 1995; Bennicelli et al., 1996). This enhanced transcriptional activity is related to decreased sensitivity of the FOXO1 activation domain to inhibitory effects of N-terminal PAX3 domains when compared to the effects of these N-terminal domains on the C-terminal PAX3 activation domain. In addition to generating a potent transcription factor, the translocation also results in high-level expression of this fusion protein, which is associated with higher steady-state transcript levels of PAX3-FOXO1 mRNA relative to wild-type PAX3 transcript levels (Davis and Barr, 1997). This overexpression is usually not attributable to changes in *PAX3-FOXO1* copy number. Nuclear runoff analysis confirmed that *PAX3-FOXO1* is more actively transcribed than wild type *PAX3*, and thus PAX3-FOXO1 overexpression is due to increased copy number-independent transcription.

PAX3-FOXO1 and related gene fusions can be detected in tumors by various methodologies, including reverse transcription-PCR, fluorescence in situ hybridization or next generation sequencing (Duan et al., 2012; Shern et al., 2014). Whereas 60% of ARMS tumors have a *PAX3-FOXO1* fusion, an additional 20% have a 1;13 translocation that fuses the closely related *PAX7* gene to *FOXO1* and the remaining 20% do not express either of these two fusions (Barr et al., 2002). The far majority of this latter ARMS group is truly fusion-negative, but a small subset has variant fusions of PAX3 to other proteins. In one rare variant, PAX3 is fused to the activation domain of FOXO4, another forkhead transcription factor that is similar in structure and function to FOXO1. In two other rare variants, PAX3 fuses with activation domains from NCOA1 or NCOA2, two members of the nuclear receptor transcriptional coactivator family (Sumegi et al., 2010) (Fig. 5B). Finally, whole genome sequencing studies identified a single ARMS case in which the PAX3 DNA binding domain is fused with the C-terminal region of INO80D, which is a subunit of the ATP dependent chromatin remodeling complex (Shern et al., 2014).

The PAX3-FOXO1 fusion protein contributes to tumorigenesis by binding to PAX3 binding sites in target genes, and causing an aberrant change in expression of these downstream targets. In ARMS cells, PAX3-FOXO1 generally functions as a transcriptional activator and increases expression of target genes. Many of these target genes were identified by microarray-based expression profiling following introduction of a PAX3-FOXO1 expression construct into non-ARMS cells or introduction of 5' PAX3-directed siRNA into ARMS cells (Tomescu et al., 2004; Nabarro et al., 2005; Davicioni et al., 2006; Ebauer et al., 2007). More recently, ChIP-Seq approaches were used to identify genomic regions that bind to the PAX3-FOXO1 protein in ARMS cells (Cao et al., 2010; Gryder et al., 2017). This latter approach revealed that PAX3 and E-box DNA binding motifs were highly enriched in genomic regions bound by PAX3-FOXO1. Furthermore, the vast majority of these bound regions were located >2.5 kb distal to the nearest transcriptional start site. Of note, numerous PAX3-FOXO1-associated regions previously defined by standard promoter-based analyses were not confirmed by these ChIP studies. Integration of PAX3-FOXO1 binding studies with additional ChIP studies showed that PAX3-FOXO1 often binds along with MYOD1, MYOG and MYCN to set up super enhancers in the vicinity of target genes in ARMS cells. In

addition, PAX3-FOXO1 interacts with proteins, such as CHD4 and BRD4, involved in defining chromatin structure; these interactions are critical to PAX3-FOXO1-mediated downstream effects as indicated by the high sensitivity of PAX3-FOXO1-positive ARMS cells to small molecules or siRNAs directed against these proteins.

Activation of target genes by PAX3-FOXO1 alters multiple biological signaling pathways, including proliferation, cell death, myogenic differentiation, and migration. Introduction of PAX3-FOXO1 expression constructs into a number of cell lines results in transforming behavior in culture and tumorigenesis in vivo (Lam et al., 1999; Anderson et al., 2001). This deregulated proliferative activity is associated with increased expression of multiple target genes such as *MYCN* and *MET*, and generally requires additional collaborating events to ensure optimal high-level MYCN expression and to inhibit the TP53 and RB1 pathways (Naini et al., 2008; Xia et al., 2009; Cao et al., 2010; Pandey et al., 2017). In addition to these proliferative effects, experiments with siRNA or shRNA expression constructs that decrease PAX3-FOXO1 expression indicate that PAX3-FOXO1 also inhibits cell death and myogenic differentiation (Ebauer et al., 2007; Kikuchi et al., 2008; Xia et al., 2009). The apoptotic function is mediated by the downstream targets including *TFAP2B* and *FGFR4*. PAX3-FOXO1 induces expression of genes such as *MYOD1* that stimulate myogenesis as well as target genes such as *JARID2* that inhibit differentiation, resulting in a complicated scenario in which PAX3-FOXO1 facilitates entry into myogenesis and variably blocks the final steps (Graf Finckenstein et al., 2008; Cao et al., 2010; Calhabeu et al., 2013; Walters et al., 2014). Finally, a role in local and distal dissemination is indicated by the stimulation of basement membrane invasion in vitro and lung colonization in vivo when PAX3-FOXO1 is introduced into murine myoblasts. These invasive and metastatic effects require expression of the target gene *CNR1* (Marshall et al., 2011) and are associated with a larger program of pro-invasive mRNA expression changes (Loupe et al., 2016a). An additional PAX3-FOXO1 target that contributes to metastasis is *CXCR4*, which encodes a chemokine receptor that modulates homing to the bone marrow (Libura et al., 2002). Finally, the fusion protein promotes aneuploidy by inducing gene expression changes that override regulatory cell cycle checkpoints and dysregulate maintenance of chromosome number and structure (Loupe et al., 2016b). In addition to protein-encoding target genes, PAX3-FOXO1 also regulates expression of a series of microRNAs that impact on the above-described cellular functions, such as differentiation (Loupe et al., 2016a; Loupe et al., 2016b; Loupe et al., 2017; Hanna et al., 2018).

A mouse model that develops ARMS tumors was developed using a conditional knock-in approach that generates a *Pax3-Foxo1* fusion in specific myogenic lineages (Keller et al., 2004). Of the lineages studied, the fetal myoblast, a prenatal myogenic progenitor defined by *Myf6* regulatory elements, showed the highest susceptibility to ARMS formation (Abraham et al., 2014). Though ARMS tumorigenicity in the original mice was low, tumor frequency is substantially increased in *Pax3-Foxo1* mice homozygous for *Cdkn2a* or *Trp53* deletion, confirming the need for additional collaborating events (Keller et al., 2004). Homozygosity of *Pax3-Foxo1* was also necessary to achieve enhanced tumor formation suggesting the need for increased *Pax3-Foxo1* and/or decreased wild-type *Pax3* dosage in tumorigenesis.

4.3. Gene fusions in biphenotypic sinonasal sarcoma

Biphenotypic sinonasal sarcoma (BSNS) is a low-grade malignancy occurring in adults that is defined by both myogenic and neural differentiation (Lewis et al., 2012). This cancer is associated with a recurrent t(2;4)(q35;q31.1) chromosomal translocation that fuses *PAX3* to the *MAML3* gene (Fig. 5C), which encodes a member of the mastermind-like family of transcriptional coactivators for the Notch signaling pathway (Wang et al., 2014). In a smaller subset of BSNS cases, *PAX3* is rearranged without *MAML3* involvement, and some of these variant cases contain a *PAX3-NCOA1* or *PAX3-FOXO1* fusion (Fritchie et al., 2016; Huang et al., 2016; Wong et al., 2016). In the characteristic *PAX3-MAML3* fusion, the N-terminal *PAX3* DNA binding domain is fused with the C-terminal *MAML3* transactivation domain to generate a powerful activator of target genes with *PAX3* binding sites. The transactivation potential of *PAX3-MAML3* is similar to that of *PAX3-FOXO1*, but multiple characteristic *PAX3-FOXO1* targets (such as *FGFR4* and *MYCN*) are not comparably expressed in BSNS. Though overlapping fusions are found in both ARMS and BSNS, the biological differences suggest that the target cell environment significantly modulates the transcriptional output.

4.4. Wild-type PAX3 in other cancers

Wild-type *PAX3* is highly expressed in multiple tumor types, which are often related to developmental lineages in which wild-type *PAX3* is normally expressed. In particular, *PAX3* is expressed in tumors related to neural tube-derived lineages, such as medulloblastoma and glioblastoma, and tumors related to neural crest-derived lineages, such as melanoma, malignant nerve sheath tumor, neurofibroma and Ewing's sarcoma (Schulte et al., 1997; Scholl et al., 2001; Gershon et al., 2005; He et al., 2010). *PAX3* is also expressed in several tumors associated with myogenic differentiation, including embryonal rhabdomyosarcoma and Wilms tumor with a myogenic component (Frascella et al., 1998; Barr et al., 1999; Hueber et al., 2009). Finally, *PAX3* expression has also been noted in several other cancer types, such as breast carcinoma, gastric carcinoma and osteosarcoma, without a clear connection to *PAX3*-expressing developmental lineages (Tan et al., 2014; Zhang et al., 2015; Liu et al., 2017). Clinical correlative studies have further suggested that *PAX3* expression may be associated with a worse prognosis in a subset of these cancers, such as glioma and gastric carcinoma (Chen et al., 2012; Zhang et al., 2015).

The oncogenic role of wild-type *PAX3* has been studied in several cancer model systems, including glioma and melanoma cell lines. In these studies, *PAX3* expression was downregulated by RNA interference or antisense approaches or upregulated by cDNA complementation (Mayanil et al., 2001; He et al., 2005; Xia et al., 2013). In cell culture experiments, these manipulations of *PAX3* expression reveal that *PAX3* contributes to the control of proliferation, apoptosis, differentiation and motility. Complementary in vivo xenograft studies demonstrate that *PAX3* contributes to tumorigenicity and metastasis. Downstream transcriptional targets of *PAX3* involved in these phenotypic effects include *MET*, which encodes a cell surface receptor involved in multiple oncogenic signaling pathways (Mascarenhas et al., 2010). These findings thus suggest that *PAX3* has a major regulatory role in the development, maintenance and progression of these tumors, which may be related to its role in normal development.

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Abbreviations:

ARMS	alveolar rhabdomyosarcoma
BSNS	biphenotypic sinonasal sarcoma
CASTing	cyclic amplification and selection of targets
ChIP-Seq	chromatin immunoprecipitation sequencing
HD	homeodomain
HTH	helix-turn-helix
PAI	N-terminal paired box subdomain
PB	paired box
PCR	polymerase chain reaction
PST	proline, serine and threonine
Q+	glutamine-including
Q–	glutamine-excluding
RED	C-terminal paired box subdomain
UTR	untranslated region
WS	Waardenburg syndrome

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Figure 1. Schematic representation of *PAX3* gene, mRNA and protein organization.

The *PAX3* gene structure is depicted based on transcript *PAX3-205*. The exons of *PAX3* are named using Arabic numerals, and the horizontal arrows indicate the direction of transcription. The locations of start and stop codons are indicated by the dashed lines and vertical arrows. Representative sizes are shown by the thin bracketed horizontal line segments beneath the gene, mRNA and protein diagrams. Conserved domains are shown as open boxes within the protein diagram, and functional domains are shown as thick horizontal lines above and below the protein diagram. The red dots and brown triangle beneath the protein diagram corresponds to phosphorylation and monoubiquitination sites, respectively. Abbreviations: PB, paired box domain; HD, homeodomain; PST, proline-, serine- and threonine-rich region; PAI, N-terminal subdomain; RED, C-terminal subdomain; DBD, DNA binding domain; TAD, transcription activation domain; TID, transcription inhibition domain.

Isoform**PAX3a (PAX3-208)** **PAX3b (PAX3-201)** **PAX3c (PAX3-204)** **PAX3d (PAX3-206)** **PAX3e (PAX3-205)** **PAX3g (PAX3-203)** **PAX3h (PAX3-202)** **PAX3i (PAX3-207)** **Figure 2. Exons organization in PAX3 mRNA isoforms.**

The exons are represented with colored boxes. For each transcript, the exon number is indicated inside the box, and the exon size (in base pairs) is showed below the box. The vertical yellow line in exon 1 indicates the start codon, whereas the vertical red line in the last exon indicates the stop codon. The dashed line indicates regions where exon 8 is spliced out, and the out of frame exons that follow are shown in a different color. For each variant, the 3' UTR in the last exon is represented by stippling. The asterisk in exon 3 of the PAX3i (PAX3-207) isoform indicates the alternative splice that removes three base pairs.

Figure 3. Elements regulating PAX3 expression.

The cis-acting elements, their position relative to the PAX3 gene and relevant transcription factors involved in the regulation of PAX3 expression are shown (see section 3.5 for details).

Figure 4. Distribution of PAX3 mutations in Waardenburg syndrome.

The displayed genetic changes include missense mutations, truncating mutations (nonsense and frameshift deletion) and others (frameshift duplications, frameshift mutation, insertions, and deletions). The diagram was generated using Mutation Mapper software (Vohra and Biggin, 2013) and data were partially collected from (Pingault et al., 2010).

Figure 5. Comparison of wild-type and fusion products in ARMS and SNS.

A. *PAX3-FOXO1* fusion generated by 2;13 translocation in ARMS (and rarely in SNS). **B.** *PAX3-NCOA1* and *PAX3-NCOA2* fusions generated by 2;2 and 2;8 translocations in ARMS (and rarely in SNS). **C.** *PAX3-MAML3* fusion generated by 2;4 translocation in SNS. Conserved domains are shown as opened boxes, while functional domains are indicated as horizontal lines. The vertical dashed lines indicate translocation fusion points. Abbreviations: DBD, DNA binding domain; TAD, transactivation domain; HDD, heterodimerization domain; NRID, nuclear receptor interaction domain; PB, paired box; HD, homeodomain; FH, forkhead domain; bHLH/PAS, basic helix-loop-helix/Per/ARNT/Sim homologous domain; LXXLL, typical LXXLL α -helix motifs; HAT, histone acetyltransferase; NBD, Notch-binding domain.

Table 1.Human *PAX3* and mouse *Pax3* - chromosome location, transcripts and proteins

Species	Ensembl Gene	Chrom	Transcript				Protein		
			Name	Ensembl ID	Pre-spliced (nt)	Exons	Spliced (nt)	UniProt	Length (aa) Mass (Da)
Human	ENSG00000135903	2q36.1	PAX3-201 (PAX3b)	ENST00000258387.5	5,230	5	958	P23760	206 22,743
			PAX3-202 (PAX3h)	ENST00000336840.10	98,209	9	2038		407 45,217
			PAX3-203 (PAX3g)	ENST00000344493.8	99,109	8	3109		403 44,822
			PAX3-204 (PAX3c)	ENST00000350526.8	98,862	8	3610		479 52,968
			PAX3-205 (PAX3e)	ENST00000392069.6	99,094	10	3170		505 55,975
			PAX3-206 (PAX3d)	ENST00000392070.6	98,011	9	2258		484 53,464
			PAX3-207 (PAX3i)	ENST00000409551.7	97,538	9	1782		483 53,336
			PAX3-208 (PAX3a)	ENST00000409828.7	5,117	4	1254		215 25,136
Mouse	ENSMUSG000000004872	1						P24610 & Q8BRF1	
			Pax3-201 (PAX3b)	ENSMUST000000004994.15	95,572	9	3078		484 53,445
			Pax3-202 (PAX3a)	ENSMUST0000000087086.6	95,865	8	3863		479 52,948

Table 2.

Representative PAX3 target genes.

Symbol	Biochemical Category	Representative Functions	PAX3 binding location
<i>CXCR4</i>	Chemokine receptor	Motility	1st intron
<i>DMRT2</i>	Transcription Factor	Differentiation	5' enhancer
<i>FGFR4</i>	Receptor tyrosine kinase	Proliferation, differentiation, migration, metabolism	3' enhancer
<i>HES1</i>	Transcription Factor	Proliferation, differentiation	5' promoter
<i>MET</i>	Receptor Tyrosine Kinase	Proliferation, invasion, survival	5' promoter
<i>MITF</i>	Transcription Factor	Differentiation, proliferation, survival, melanogenesis	5' promoter
<i>MYF5</i>	Transcription Factor	Muscle differentiation	5' enhancer
<i>MYOD1</i>	Transcription Factor	Muscle differentiation	5' enhancer
<i>NF1</i>	GTPase-activating protein	Differentiation, control of cell growth, melanogenesis	3' UTR
<i>NGN2</i>	Transcription Factor	Differentiation	5' promoter
<i>NRCAM</i>	Cell adhesion molecule	Intercellular adhesion	1st Intron
<i>RET</i>	Receptor Tyrosine Kinase	Proliferation, migration, differentiation	5' promoter
<i>TGFB2</i>	Ligand	Growth suppression	5' promoter
<i>TYRP1</i>	Enzyme	Cell growth, melanogenesis	5' promoter
<i>WNT1</i>	Ligand	Proliferation, differentiation	5' promoter