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Pax3 regulation of FGF signaling affects the progression of embryonic progenitor cells into the myogenic program

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Pax3/7-dependent stem cells play an essential role in skeletal muscle development. We now show that Fgfr4 lies genetically downstream from Pax3 and is a direct target. In chromatin immunoprecipitation (ChIP)-on-chip experiments, Pax3 binds to a sequence of the Fgfr4 gene that directs Pax3-dependent expression at sites of myogenesis in transgenic mouse embryos. The activity of this regulatory element is also partially dependent on E-boxes, targets of the myogenic regulatory factors, which are expressed as progenitor cells enter the myogenic program. Other FGF signaling components, notably Sprouty1, are also regulated by Pax3. In vivo manipulation of Sprouty expression reveals that FGF signaling affects the balance between Pax-positive progenitor cells and committed myoblasts. These results provide new insight into the Pax-initiated regulatory network that modulates stem cell maintenance versus tissue differentiation.

[Keywords: Pax3; Fgfr4; Sprouty1; myogenesis; embryonic ChIP-on-chip; somite; skeletal muscle progenitor cells]

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The balance between stem cell self-renewal and progression into a differentiation program is of critical importance for tissue growth and regeneration. During skeletal muscle development, each muscle contains a pool of resident muscle stem cells that can either differentiate into muscle fibers or remain as proliferating progenitors. These cells express two related paired-homeobox transcription factors, Pax3 and Pax7, that are essential for ensuring the myogenic potential and survival of the progenitors (Buckingham and Relaix 2007). In the embryo, skeletal muscle is derived from transitory segmented structures called somites. Pax3/Pax7-positive muscle progenitor cells are located in the dermomyotome, which constitutes the dorsal epithelial layer of each somite. These cells enter the myotome, the first skeletal muscle to form, in the central compartment of the somite, as the dermomyotome disaggregates, from embryonic day 10.5 [E10.5] in the mouse embryo [Relaix et al. 2005]. Pax3 is more extensively expressed than Pax7, initially, and Pax3 mutant embryos display somite defects with loss of the epaxial and hypaxial extremities of the dermomyotome. Muscles such as those of the limb, which normally form after delamination and migration of muscle progenitor cells from the hypaxial dermomyotome, are absent in the Pax3 mutant. Progenitor cell delamination and migration depends on c-Met [Bladt et al. 1995], which is a Pax3 target [Epstein et al. 1996; Relaix et al. 2003]. As they enter myogenesis, muscle progenitor cells down-regulate Pax3 and Pax7 and activate genes for the myogenic determination factors Myf5, Mrf4, and MyoD. Subsequent muscle differentiation depends on MyoD, Mrf4, or myogenin [Buckingham 2006]. During development, each forming muscle mass therefore contains a heterogeneous population of cells reflecting the multiple steps of myogenesis. The decision to enter the myogenic program or to stay undifferentiated is a crucial choice that remains poorly understood. One way to approach this process is to identify relevant Pax3 target genes. Recently it was shown that the myogenic determination gene Myf5 is a direct Pax3 target [Bajard et al. 2006]. However, entry into the myogenic program and skeletal muscle differentiation must be modulated to retain the progenitor cell pool.

Signaling pathways have been largely implicated in stem cell behavior. The FGF signaling pathway has been...
reported to act on stem cell fate in different developing organs such as liver (Calmont et al. 2006), the auditory sensory epithelium (Shim et al. 2005), or the tooth (Klein et al. 2008). FGF signaling has been implicated in myogenesis in vivo, both in promoting progenitor cell proliferation (Buckingham 2006; Hammond et al. 2007). In the embryo, Fgfr4 is transcribed at sites of myogenesis (Stark et al. 1991), in replicating myoblasts, but is not detected in avian muscle fibers (Marcelle et al. 1995). More recently, colocalization of Fgfr4 transcripts with Pax7 in muscle progenitor cells has been reported in the chick embryo (Ben-Yair and Kalcheim 2005). Misexpression of Fgf4 in the chick limb inhibits myogenesis by down-regulating Fgfr4 (Edom-Vovard et al. 2001), and indeed electroporation of a dominant-negative form of Fgfr4 prevents expression of the myogenic regulatory factors, whereas Pax3 remains unchanged (Marics et al. 2002). Despite the evidence in the chick embryo that Fgfr4 is important for myogenesis, Fgfr4-null mice develop normally (Weinstein et al. 1998), which may reflect overlapping function with other FGF receptors.

In this study, we explore FGF signaling in the myogenic context in the mouse embryo. We establish that Fgfr4 lies genetically downstream from Pax3 at sites of myogenesis and show by chromatin immunoprecipitation (ChIP)-on-chip experiments that Fgfr4 is directly activated by Pax3 in vivo through a 3′ regulatory element. This Pax3-dependent element also contains E-boxes, allowing subsequent regulation by myogenic determination factors, such as MyoD, in cells that have entered the myogenic program. We also show that other components of the FGF signaling pathway, notably Sprouty1, are regulated by Pax3 during myogenesis. These observations, together with in vivo manipulation of Sprouty, leads us to propose that Pax3, through regulation of FGF signaling, modulates muscle progenitor cell progression into the myogenic program.

Results

Fgfr4 lies genetically downstream from Pax3 during myogenesis

We examined Fgfr4 expression at different stages of development from E9.5 to E12.5 in different Pax3 genetic backgrounds (Fig. 1). In normal mouse embryos, Fgfr4 transcripts are detected at sites of myogenesis, notably in the somites at E9.5–E10.5 (Fig. 1A,C) and at later stages also in the limb buds and some head muscles (Fig. 1E,G).

In the presence of Pax3-FKHR, in which the DNA-binding domain of Pax3 is fused to the strong transcriptional activation domain of FKHR (FOXO1A) (Relaix et al. 2003), Fgfr4 is overexpressed in the somites at E9.5–E10.5 (Fig. 1B,D). Conversely, in embryos that express a dominant-negative form of Pax3, Pax3-En, in which the DNA-binding domain of Pax3 is fused to the repression domain of Engrailed (Bajard et al. 2006), Fgfr4 transcripts are undetectable.

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are down-regulated in somites and limb buds as shown at E11.5 (Fig. 1F). In Pax3 mutants, Fgf4 expression is only detectable at some sites, notably in anterior muscles (Fig. 1H) and at sites in the trunk where Pax7 is also directing myogenesis at E12.5 (Relaix et al. 2004, 2005). When Pax3 function is impaired, apoptosis occurs. The presence of an nlacZ reporter in the Pax3Pax3-EnIRESnlacZ (Pax3Pax3-En) allele (Fig. 1F) and in the Pax3Pax3-En-IRESnlacZ mutant (Fig. 1H) indicates the extent to which cells are lost, notably in the hypaxial extremity of the somites and limb buds. Nevertheless, labeled cells are present in Pax3Pax3-En/Embryos, with Fgf4 transcripts are barely detectable (Fig. 1F). This effect is more severe than in the Pax3 mutant (Fig. 1H), probably because Pax3-En interferes with Pax7 as well as Pax3 activity, preventing Fgf4 activation by either Pax factor Fgf4 is not expressed at other non-myogenic sites of Pax3 expression, such as the neural tube (Fig. 1E, E′, G, G′). We therefore conclude that expression of Fgf4 in the myogenic lineage depends on Pax3.

**Identification of a distal element in the Fgf4 locus that binds Pax3 in vivo**

To test if Pax3 directly regulates Fgf4 transcription, we performed an in vivo ChIP-on-chip experiment. To develop conditions for this assay, we used a transgenic line (P34) where five Pax3 consensus binding sites are placed upstream of a tk-nlacZ transgene (Relaix et al. 2004), which provides a readout of Pax3/Pax7 transcriptional activity and therefore a positive control of Pax3 binding to DNA. Somites and limb buds were pooled from P34 transgenic embryos at E11.5 (Fig. 2A). ChIP with Pax3 antibodies showed robust binding of Pax3 to the P34 element (Fig. 2B). We also detected Pax3 binding to a ~57.5-kb element of the Myf5 gene that we had previously shown to be directly bound by Pax3 (Bajard et al. 2006). These results demonstrate that our ChIP protocol is efficient with small quantities of material.

The amplified ChIP product was hybridized to a custom-made NimbleGen array that probes sequences from genomic loci related to signaling and development. In transgenic mouse embryos, X-Gal staining showed β-galactosidase (β-Gal) activity in the myotome and limb buds (Fig. 3C,D), resembling the endogenous Fgf4 expression, shown at E11.5 and E12.5 (Fig. 3A,B). These results indicate that the 559-bp element is sufficient to direct myogenic expression of Fgf4 in the embryo. We therefore investigated whether this is Pax3-dependent. When all six Pax sites were mutated, the transgene was no longer expressed at sites of Fgf4 transcription (Fig. 3E,F), demonstrating that they are essential for the myogenic activity of the Fgf4 distal element. From these results, we conclude that myogenic expression of Fgf4 is directly regulated by Pax3/Pax7 acting through the 559-bp element.

We then looked more closely at expression in the somite and limbs. Fgf4 is transcribed in the myotome (Supplemental Fig. S1), not in the dermomyotome, except for a weak expression in the extremities (data not shown), where Pax3 is also expressed. Immunohistochemistry experiments show colocalization of nuclear Pax3 staining in cells that have surrounding Fgf4 staining (green) (Fig. 3G,H). Pax3+, Fgf4+ cells are present, notably dorsally, consistent with the absence of Fgf4 expression in the Pax3-negative cells of the dermomyotome, which is beginning to disaggregate at E10.5 (Relaix et al. 2005). When we looked at β-galactosidase versus Fgf4 expression on sections of transgenic embryos expressing the Fgf4(559bp)tk-nlacZ transgene, we detected colocalization in cells in the myotome at E11.5 (Fig. 3I,J), demonstrating that the 559-bp sequence reflects Fgf4 expression. Comparison of Pax3 and β-Gal labeling of transgenic embryos in muscle masses of the trunk shows colocalization at E11.5 (Fig. 3K) in 18% of β-Gal-positive cells, estimated across several sections, whereas at E10.5, this percentage is higher (43%) (Fig. 3L). A similar developmental difference was seen for the forelimbs (Fig. 3M,N). This correlates with the arrival of Pax3+/positive cells in the somite or limbs, which subsequently express myogenic factors and differentiate, and might suggest that this transgene is also dependent on factors other than Pax3.

The 559-bp element also contains E-box consensus sites (Fig. 2D, E1–E4) for the myogenic factor family of transcription factors. To investigate their potential involvement, we examined genetically the relationship be-
between MyoD, Myf5, and Fgfr4. Expression of Fgfr4 in Myf5nlacZ/nlacZ mutant embryos is absent, until MyoD is activated later and the myotome begins to form (Supplemental Fig. S2), consistent with Fgfr4 expression in cells of the myotome. This might also suggest direct regulation by MyoD, although in the absence of MyoD, Myf5 may act on Fgfr4. The primary importance of Pax3 in Fgfr4 expression is supported by the Pax3Pax3+/- mouse. When Pax3 is compromised, Fgfr4 expression is down-regulated (Fig. 1; Supplemental Fig. S3D), whereas Myf5/MyoD continue to be expressed in most of the myotome (Supplemental Fig. S3E,F).

We mutated the four E-boxes in the 559-bp element. Since E2 is close to P2 (Fig. 2D), we took care not to interfere with this Pax-binding site, and similarly when P2 was mutated, the E2 site was kept intact. These E-box mutations result in reduced expression of the transgene in the trunk, whereas expression in more anterior muscles and in the limbs is not so notably affected (Fig. 4C,D). Expression is not detected in myotomes of imma-
ture posterior somites, whereas variable partial expression is seen in more mature muscle masses in the trunk. This suggests that Fgf4 expression in the myotome, which is Pax3-dependent [Fig. 3C–F], is also E-box-dependent. Immunostaining on sections, at E11.5, shows partial colocalization of MyoD with reporter β-Gal from the transgene in myogenic cells in both trunk and forelimbs (~50%) [Fig. 4E,F].

Pax3 regulation of the FGF signaling pathway and its impact on myogenesis

Other components of the FGF signaling pathway are also potential Pax3 targets. One such sequence is Sprouty1, which is up-regulated in Pax3PAX3-FKHR/GFP embryos [data not shown] and down-regulated in the somites of Pax3Pax3-En/+ embryos [Fig. 5A,B]. As in Figure 1, X-Gal staining of the somites confirms that the cells that normally express Pax3 are present [Fig. 5A’,B’]. Sprouty1 transcripts are detected in Pax3-positive progenitor cells at the edges of the dermomyotome, as well as in the myotome [Fig. 5A; Supplemental Fig. S4].

Sprouty1, which modulates the FGF signaling pathway [Hacohen et al. 1998], is reduced in Pax3-GFP-positive cells isolated by flow cytometry from interlimb somites of Pax3GFP/nlacZ null embryos [Fig. 5C]. This is also the case for the FGF signaling intermediates, phospho-AKT and phospho-p38, whereas phospho-ERK is up-regulated [Fig. 5C], showing the in vivo impact of Pax3 on the FGF pathway. Up-regulation of phospho-ERK in the Pax3+/− somitic cells probably reflects the down-regulation of Sprouty1 that is known to negatively regulate phospho-ERK [Hanafusa et al. 2002].

In order to look at FGF function during myogenesis in the mouse embryo, we perturbed FGF signaling by manipulating Sprouty expression. This strategy avoids potential problems due to the presence of other FGF receptors that may compensate for loss of Fgfr4 function in the Fgfr4 mutant. A transgenic line that expresses Sprouty2 under the control of a CAG promoter [Sprouty2GOF] [Calmont et al. 2006; Basson et al. 2008] was targeted to Pax3-expressing cells and their derivatives by crossing with a Pax3Cre line [Engleka et al. 2005]. In this experiment, because of transgene availability, Sprouty2 was manipulated; however, Sprouty1 and Sprouty2 are thought to have equivalent functions [Kim and Bar-Sagi 2004; Taketomi et al. 2005]. Expression of the transgene reduces phospho-ERK levels, as normalized to β-tubulin [Fig. 5D]. Sprouty2 was clearly expressed at sites of myogenesis, as indicated by alkaline phosphatase staining from the transgene reporter [Calmont et al. 2006; data not shown]. We analyzed the number of Pax7- versus myogenin (Myog)-positive cells on serial sections of trunk and limb muscles at E13.5 [Fig. 5E,F]. At this stage, Pax7 is coexpressed with Pax3 in trunk and limb muscle progenitor cells. This ratio varies across each muscle mass, but we observed a consistent bias toward Pax7 in the Sprouty gain of function embryos as shown quantitatively for back muscles in Figure 5G.
indeed, the 559-bp element also contains a Six-binding site, and its activation in these cells may also be regulated by Pax3/7, rather than exerting its effects indirectly via the myogenic regulatory factors. In keeping with this, when Pax3 activity is compromised in Pax3Pax3-En/+ embryos, the endogenous Fgfr4 gene is down-regulated despite the continuing expression of Myf5 and MyoD in most of the myotome. It is notably in the limbs, where Myf5 is directly regulated by Pax3 [Bajard et al. 2006], that mutation of the E-boxes has the least effect. In Fgfr4(559bp)-tknlacZ transgenic embryos, the progressive maturation of somites and their muscle derivatives along the anterior/posterior axis permits visualization of the role of the E-boxes at different developmental stages within the same embryo (see Fig. 4). Expression of the transgene at the onset of myogenesis in the most posterior somites is severely affected in the absence of E-boxes, indicating that this requires myogenic factors as well as Pax3, present in the cells that delaminate from the edges of dermomyotome to form the early myotome [Buckingham and Relaix 2007]. In more mature anterior somites, where a Pax3/7-positive population of progenitor cells has invaded the myotome as a result of de-epithelialization of the central dermomyotome, the E-box requirement is rather less striking. In interlimb somites at E10.5, when this invasion is initiated, many Pax3/7-positive cells express the transgene, whereas this number is lower at E11.5. During this later phase of myogenesis [Buckingham and Relaix 2007], transgene expression may therefore be initiated in Pax3/7-positive cells and only subsequently come under myogenic factor regulation, which provides a means of maintaining Fgfr4 expression in differentiating muscle cells in which Pax3/7 are down-regulated. Although the transgene is expressed in MyoD-positive cells in the limbs, E-box dependence at this site is much less evident, providing another example of differences in the regulatory circuits operating in limb versus trunk myogenesis [Relaix et al. 2004].

Interference with FGF signaling, through artificially increased levels of Sprouty, tends to prevent Pax3/7-positive myogenic progenitor cells from entering the skeletal muscle differentiation program. This is consistent with observations in the zebrafish embryo, where interference with Fgf8 [a potential ligand of Fgfr4] [MacArthur et al. 1995] leads to an increase in Pax3/7-positive progenitor cells in somites [Hammond et al. 2007]. Results obtained in chick head muscles also suggest that the FGF pathway, mediated via Fgfr8, can influence the ratio between proliferative progenitors [in this case, MyoR+] and differentiated cells [Myf5+] [von Scheven et al. 2006]. Pax3 activation of Fgfr4 will promote myogenesis, and, indeed, down-regulation of this receptor was found to adversely affect muscle differentiation in the chick embryo [Edom-Vovard et al. 2001; Marics et al. 2002]. However,
we show that Sprouty1 also lies genetically downstream from Pax3, and preliminary results suggest that it may also be a direct Pax3 target. In the mouse embryo, the transcription of Sprouty1 in Pax3-positive progenitor cells at the edges of the dermomyotome, as well as in the myotome, suggests a model in which Sprouty1 dampens premature myogenesis. Such an interplay between Sprouty1 and Fgfr4 in myogenic progenitors is consistent with a recent transcriptome analysis on quiescent and activated satellite cells in adult muscle (Fukada et al. 2007), which shows that Sprouty1 transcripts are restricted to the former, whereas Fgfr4 transcripts are also present in the activated cells, which now express MyoD (Kastner et al. 2000). Fgfr4 expression has also been reported in regenerating muscle after injury (Zhao and Hoffman 2004), and in Fgfr4-null mice, muscle regeneration is affected (Zhao et al. 2006). In this model, it has been suggested that MyoD affects Fgfr4 expression through Tead2 acting on the promoter. It remains to be seen whether the 559-bp element is also active in Pax7-positive satellite cells of adult muscle.

During myogenesis in the embryo, we propose that Pax3, acting directly on Fgfr4 and on Sprouty1, orchestrates the potential effects of FGF signaling on the formation of skeletal muscle. We showed previously that Pax3 directly activates the myogenic determination gene Myf5 (Bajard et al. 2006). Pax3 regulation of FGF signaling now provides a further level of myogenic control by governing the balance between stem cell maintenance and tissue differentiation.

Material and methods

Preparation of embryos

Embryos were collected after natural overnight matings, and dated taking E0.5 as the day after the vaginal plug.

X-Gal staining, immunohistochemistry, and whole-mount in situ hybridization

X-Gal staining was performed as described in Bajard et al. (2006). Whole-mount in situ hybridization with digoxigenin-labeled probes was performed as described in Tajbakhsh and Cossu (1997). The mouse Fgfr4 probe was a gift from Christophe Marcell.

The Sprouty1 probe was cloned by PCR using the following primers: Spry1A (5’-AGAATTCATTTAACATGATTCCTGAG-3’) and Spry1B (5’-AAAGGCCCAGAGGATCAAGGCCACCTTATAGGAGCTG-3’).

Coimmunofluorescence on sections was carried out as previously described (Relaix et al. 2003) except for Pax7 and Fgfr4 antibodies, where an antigen retrieval procedure was used (Gyraud-Morel et al. 2007). The following antibodies were used: anti-Pax3 (monoclonal, DSHB, 1/250), anti-Pax7 (monoclonal, DSHB, 1/200), anti-MyoD (monoclonal, DAKO, 1/200), anti-myogenin (polyclonal, Santa Cruz, 1/200), anti-β-galactosidase (polyclonal, provided by J.-F. Nicolas, 1/300), and anti-Fgfr4

**Figure 5.** Components of the FGF signaling pathway are regulated by Pax3. Sprouty modulates myogenesis in vivo. (A,B) Whole-mount in situ hybridization for Sprouty1 transcripts in control (A) and Pax3Pax3-En−/− (B) embryos at E10.5. (A’,B’) X-Gal staining of Pax3lacZ/+ (A’) and Pax3Pax3-En−/− (B’) embryos at E10.5. The red arrows indicate somites. Black arrowheads point to Pax3-independent Sprouty1 expression in the distal forelimb bud (FL). (C) Western blot of the same number of GFP-positive cells isolated by flow cytometry, from somites of Pax3Pax3+/+ (A) and Pax3Pax3-En−/− (B) embryos at E11.5, using the antibodies indicated; (p) phosphorylated. (D) Western blots on extracts (100 µg, 50 µg, and 25 µg of total protein) from limbs of control and Sprouty2 gain of function (Spry2 GOF) transgenic embryos at E13.5, using the antibodies indicated. (E,F) Coimmunohistochemistry on DAPI-stained transverse sections of equivalent muscles in the interlimb region of control (E) and Spry2 GOF (F) embryos at E13.5 using antibodies to Pax7 (red) and myogenin (Myog) (green). (G) Quantitative analysis of the ratio of Pax7 to myogenin-positive cells on sections, counted using Metamorph software. Each bar represents the differential ratio [(Pax7/Myog)Spry2GOF − (Pax7/Myog)control] between Spry2GOF and control embryos quantified for equivalent sections from the same deep back muscles, with a minimum of 500 total cells counted per section. The ratio [Pax7/Myog] is significantly higher in Spry2GOF samples compared to the control (P < 0.05, with the two-sided Wilcoxon signed rank test).
antibody (Geneka) or 2 µL of Normal Rabbit Serum (Chemicon). The forward and reverse primers used were: fwd [5’-TTAACCCCTTAATCCCAACCCAG-3’] and rev [5’-AACGGTGAATCTGAGGTCCCGAG-3’]). After sequence verification, the PCR product was cloned into a pGEM-T easy vector (Promega), and the 559 bp Notl–Notl fragment was then cloned into the Notl site of the tk-nlacZ plasmid (Hachdouel et al. 2000). Mutagenesis was performed using the QuickChange Multi Site-Directed Mutagenesis kit (Stratagene) using as a matrix Fgfr4(559bp) subcloned into pGEM-T easy plasmid (Promega). The TGAC core sequence of the Pax3 consensus binding sites was replaced by gct (Bajard et al. 2006) except for sites 2 and 5, in which the consensus was mutated into agt to avoid recreating a potential Pax site. The CA[nn]TG core sequence of the E-box consensus was replaced by CgmnAG except for E-box2, in which the consensus has been replaced by CA[nn]Ta to avoid affecting the Pax3 site, p2. Mutation of the Pax3 site, p2, has no effect on the E2 sequence.

Plasmid constructions used for transgenesis

To generate the Fgfr4(559bp)-tk-nlacZ transgene, the 559-bp Fgfr4 element (EU697430) was synthesized by PCR using an Expand High Fidelity kit (Roche). The equivalent of 150 µg (Wiebe et al. 2007) with the following modifications. Chromatin was sheared to a range of 0.1 to 1 kb. The equivalent of 150 µg of chromatin was used in each immunoprecipitation experiment and immunoprecipitated with 2 µL of Pax3 whole-serum antibody (Geneka) or 2 µL of Normal Rabbit Serum (Chemicon).

Purified immunoprecipitated DNA was resuspended into 200 µL of distilled water. The equivalent of 10% of input chromatin was DNA purified in parallel and resuspended in an equal volume.

Real-time PCR

Four microliters of ChIP product were used in quantitative PCR experiments. A serial 1/10 dilution series of the 10% input chromatin was used to develop a standard curve for calculating the percent input for each ChIP sample. The following primers were used: Pax3-binding sites of the P34 transgene, P34fwd [5’-AGGAATTCGATCCCTCTG CACC-3’], P34rev [5’-AGCCTTGATGGCATTCTCCAAGG-3’], the −57.5-kb Myf5 element, −57.5fwd (5’-TGCTCCCCCATATTA CTTCCAAGCT-3’), −57.5rev (5’-TTCCCGTGATTTTTGGA GAAGG-3’), Albumin enhancer, Albfwd (5’-GGAGCGAGA TGGTACTTITGTTG-3’), Albrev (5’-ACATGCAGTCCAAATC TCTTCTG-3’). For the Myf5 200-bk-flanking sequence, primers previously described were used (Bajard et al. 2006). The Fgfr4 −50 mer showing the strongest hybridization signal in ChIP-chip is located at +19.2 kb and encompasses sites P4/P5, Fgfr4 +19.2kFwd (5’-CACACAGTGTGCCACACT-3’), Fgfr4 +19.2kvw (5’-GGAGCGAGGGAAGAGTTTG-3’).

Ligation-mediated PCR and ChIP-on-chip

Fifty microliters of the ChIP product DNA (i.e., 25% of the original material) were first purified using a QIAquick PCR purification column following the manufacturer’s instructions (Qiagen). Ligation-mediated PCR of ChIP and input material was performed as in the NimbleGen protocol. The amplified material was analyzed using a 2100 Bioanalyzer with a dsDNA 12,000 laboratory chip (Agilent Technologies), and displayed an average size of 300 bp with a range of 150 to 900 bp. Four micrograms of each sample were sent to Nimblegen for labeling and hybridization to custom microarrays. The microarrays had 210 loci, tiled at a density of one 50-nucleotide probe per 24 bp. Each locus on the array was repeat-masked and contained the entire coding region as well as 30 kb upstream and 10 kb downstream. Further details of the design of the NimbleGen custom array will be published elsewhere. Pax3 antibody ChIP were hybridized with input chromatin, and control antibody ChIP products were hybridized with input chromatin from each of the biological replicates A and B.

Fgfr4 distal element identification and Pax3 site selection

The Fgfr4 transcript and coding sequence was based on the Ensemble ENMUST00000005452 transcript and ENSMUG0000005320 gene sequence. The University of California at Santa Cruz Genome Browser was used to predict a 559-bp region surrounding the Pax3-binding sites that was highly conserved in mammals, and sequences from the mouse, human, rat, and cow were aligned with ClustalW. Bases that were conserved between the species were shaded, with a minimum of three out of four base matches required for shading (Fig. 2D).

Electrophoretic mobility shift assay (EMSA)

Pax3 synthesis with a reticulocyte lysate and EMSA were carried out as described previously in Bajard et al. [2006]. Probe sequences were as follows, with Pax3 sites underlined: the Pax3-binding site of P34 [5’-TCTGATCCCTCGTCACG CTTCGAATGTTCCCAT-3’], Fgfr4P4/P5 [5’-AACAGAGGTTTGGCCGTTAATCAACAAAGCTGGTCACCAGC-3’].
TGCCCCACATC-3''), Fgfr4P4/P5M (5'-AACAGAGGTTGGGGCTGGAATACCAACAGCCTGGACATCCACGCTTTGCCCCACCACATC-3'), Fgfr4P4M/P5M, where both Pax3 sites have been mutated (5'-AACAGAGGTTGGGGCTGGAATACCAACAGCCTGGACATCCACGCTTTGCCCCACCACATC-3').

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References


