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Mapping the Chromatin State Dynamics in Myoblasts

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Abstract

Background—Genome-wide mapping reveals chromatin landscapes unique to cell states. Histone marks of regulatory genes involved in cell specification and organ development provide a powerful tool to map regulatory sequences. H3K4me3 marks promoter regions; H3K27me3 marks repressed regions, and Pol II presence indicates active transcription. The presence of both H3K4me3 and H3K27me3 characterize poised sequences, a common characteristic of genes involved in pattern formation during organogenesis.

Results—We used genome-wide profiling for H3K27me3, H3K4me3, and Pol II to map chromatin states in mouse embryonic day 12 forelimbs in wild type (control) and *Pitx2*-null mutant mice. We compared these data with previous gene expression studies from forelimb Lbx1⁺ migratory myoblasts and correlated *Pitx2*-dependent expression profiles and chromatin states. During forelimb development, several lineages including myoblast, osteoblast, neurons, angioblasts etc., require synchronized growth to form a functional limb. We identified 125 genes in the developing forelimb that are *Pitx2*-dependent. Genes involved in muscle specification and cytoskeleton architecture were positively regulated, while genes involved in axonal path finding were poised.

Conclusion—Our results have established histone modification profiles as a useful tool for identifying gene regulatory states in muscle development, and identified the role of *Pitx2* in extending the time of myoblast progression, promoting formation of sarcomeric structures, and suppressing attachment of neuronal axons.

Keywords

muscle development; myogenesis; homeobox; chromatin

1. Introduction

Muscle formation during development is a tightly regulated process. Non-committed mesoderm-derived cells delaminate, migrate and fuse to form a mature muscle. This process is characterized by a series of developmental stages that are determined by a constellation of sequence specific transcription factors (SSTFs). Limb muscles originate from somites, the anatomical structures of the paraxial mesoderm that form in a rostral-caudal axis. The

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dermomyotome develops as a dorsal epithelium of the early somite and gives rise to skeletal muscles. The dermomyotome is subdivided into hypaxial and epaxial that give rise to the lateral trunk musculature and deep back muscles, respectively. Myogenic precursors in the dermomyotome express the paired homeodomain factors Pax3, Pax7 (Jostes et al., 1990; Goulding et al., 1994) and the basic Helix-Loop-Helix transcription factor Myf5 (Kiefer and Hauschka, 2001). The Pax3⁺ cells maintain their proliferative and undifferentiated status by external cues from the lateral plate mesoderm and surface ectoderm (Amthor et al., 1999). Limb myogenesis is initiated upon positive signaling by Wnt from the dorsal neural tube (Cossu et al., 1996) and Shh from the floor plate and notochord (Gustafsson et al., 2002). BMP signaling from the lateral plate mesoderm inhibits Myod while BMP signaling from the dorsal neural tube inhibits Myf5 (and thus myogenesis). In the limb, many cells co-express Pax3 and Myf5 as the progenitor cells that have entered the myogenic program need a more extended myoblast state before differentiation. Some of the Pax3⁺ cells remain as reserve cells and subsequently express Pax7, Myod, and Myogenin (Shih et al., 2007a). As cells enter the myogenic program, Pax3 regulates *cis*-regulatory modules (CRMs) of Myf5. The transcription of Myf5 is regulated in a spatiotemporal manner by a large number of upstream enhancers distributed over 100kb (Moncaut et al., 2013). It has been shown that Pax3 regulates Myf5 expression from two known CRMs, one in the hypaxial somite at -110kb and another in the limbs at -57.5kb (Carvajal et al., 2008).

Pitx2 is a homeodomain transcription factor expressed in lateral plate mesoderm and muscle anlagen during all stages of myogenic progression (Shih et al., 2007a; 2008). Pitx2 somatic-null mutants die between embryonic day (E) 12.5 and E14.5 due to arrest of organ development (Gage et al., 1999; Kitamura et al., 1999; Lin et al., 1999; Lu et al., 1999). Pitx2 is a competence factor required for the temporally ordered and growth factor-dependent recruitment of a series of specific co-activator complexes that prove necessary for Ccnd2 gene induction (Kioussi et al., 2002). Pitx2 contributes to specification of the anatomical context that surrounds the muscle, including the jaw (Shih et al., 2007b) and the abdominal wall (Eng et al., 2012). Pitx2 regulates the relative amounts and types of cytoskeletal proteins that are produced as muscle cells assemble into muscles and contributes to the higher order muscle assembly (Campbell et al., 2012a).

Protein-DNA interactions comprise the genetic regulatory networks (GRNs) that exist in any cell. These GRNs are responsible for maintaining cellular identity and managing differentiation in response to internal or external stimuli (Davidson et al., 2002). They consist of a multitude of SSTFs interacting directly with DNA and each other in a highly regulated manner throughout the entire genome. Determining these GRNs is required to understand the drivers of biological changes in a cell, and is enabled by sequencing of the chromatin (Chip-seq), which allows for mapping specific protein-DNA interactions across the whole genome (Park, 2009).

Histone modifications control both gene repression and transcription, including CRM activity (Birney, 2012). The chromatin mark Histone H3 lysine 4 trimethylation (H3K4me3) is associated with open chromatin, promoter regions carried out by the Trithorax (trxG) activator protein complex, and frequently found in the presence of RNA polymerase II (Pol II). The modification Histone H3 lysine 27 trimethylation (H3K27me3) is associated with

closed chromatin and gene repression, carried out by the Polycomb (PcG) repressor protein complex (Guenther et al., 2007). However, H3K4me3 and H3K27me3 often co-occur and bivalently mark chromatin, most often in promoter regions (Bernstein et al., 2006). Genes under bivalent promoters tend to show low levels of transcription, but are poised before being activated once the cell commits itself to a lineage (Mikkelsen et al., 2007). Identification of bivalent chromatin is an important factor for decoding gene regulatory networks in development.

The chromatin states of mouse E12 forelimbs from control and *Pitx2* somatic-null mice were analyzed for H3K4me3, H3K27me3 and Pol II signatures. Correlation studies were performed for the sequences marked with different signatures. Differential enrichment of histone marks and Pol II were observed around a number of *Pitx2* target genes identified in *Lbx1^{EGFP/+}* migratory myoblasts (Campbell et al., 2012b). Quantitative real-time PCR assays (qPCR) assessed gene expression and revealed that the H3K27me3 chromatin mark best predicted relative gene expression. Collectively, these studies suggest that *Pitx2* dynamically regulates the chromatin state of genes involved in the myoblast proliferative state and axonal path finding in the developing forelimb.

2. Materials and Methods

2.1. Mice

All research was conducted according to the protocols reviewed and approved by the Oregon State University Institutional Animal Care and Use Committee. The *Pitx2^{+/-Z}* mouse line was maintained on an outcrossed ICR background. Noon on the day of a vaginal plug was considered embryonic day (E) 0.5. Yolk sacs of embryos were used for genotyping.

2.2. Gene Expression Arrays in Mouse Forelimb Migratory Myoblasts

Pitx2 target genes in mouse *Lbx1⁺* migratory muscle cells from E12.5 *Lbx1^{EGFP/+}/Pitx2^{+/+}* (WT) [27] and *Lbx1^{EGFP/+}/Pitx2^{Z/Z}* (MT) mice (Campbell et al., 2012a) forelimbs, the dataset GSE31945 NCBI Gene Expression Omnibus and subsets of GSM791677, GSM791678, GSM791683, and GSM791684 were used (Table 1S). All data analysis was done in R using the Bioconductor package and its components (Gentleman et al., 2004). GEO data was first imported into R using the GEOquery package (Davis and Meltzer, 2007). The Simpleaffy package was used to normalize all datasets based on the RMA algorithm. Subsequent analysis and comparison was performed using the Limma Packageactive (Smyth, 2005). Relevant *Pitx2* target sequences were identified as coding sequences with a significant fold-change difference between WT and MT of an adjusted P value < 0.1 (Benjamini-Hochberg FDR).

2.3. Chip-Seq Data Analysis

ChIP seq data was analyzed from dataset GSE49010 representing day E12.5 forelimbs from *Pitx2^{+/+}* (WT) and *Pitx2^{Z/Z}* (MT) mouse as described previously (Eng et al., 2014). WT data was obtained from our previous GSE49010 while MT data was newly generated and deposited in NCBI GEO under GSE71128 (Campbell et al., 2012). Fastq files along with their inputs were aligned to the mouse genome (mm10/NCBI38) reference assembly using

Bowtie2 version 2.2.3, with default parameters (Langmead and Salzberg, 2012). Samtools version 1.0 was used to convert the aligned.sam files into sorted.bam files (Li et al., 2009). The bedtools version 2.12.0 command bamToBed was used to convert the sorted.bam files into.bed files. The peak-calling algorithm MACS version 2.1.0 was used to identify regions of the mouse genome significantly enriched in the ChIP-seq samples over the controls (Zhang et al., 2008). MACS2 was run with the following parameters: “-f BAM -B --SPMR --broad --broad-cutoff 0.1 -g 1.87e9 --shiftsize 80.” The function annotatepeaks.pl from Homer version 4.7 was used for annotation and comparison of called peaks (Heinz et al., 2010). Peaks were assigned to genes if the gene nearest to the peak was less than 2,000 bp away from the gene’s TSS. The function mergepeaks.pl with “-d given” was used to determine overlapping peaks of different chromatin marks within each sample, and to identify genes with different chromatin states between WT and MT. The annotatepeaks.pl function was used separately with the -hist option to generate bins of ChIP fragment density centered around the transcription start sites of known transcripts. The output bed and bedgraph files were converted to bigbed and bigwig, respectfully. The resulting files were visualized with the UCSC genome browser (Karolchik et al., 2003). Raw sequences from MT forelimbs was deposited in the NCBI Gene Expression Omnibus GSE71128.

3. Results

3.1. Pitx2-dependent chromatin state of mouse forelimbs

Muscle forelimbs are distorted and fail to form higher order muscle assembly in the absence of Pitx2 (Campbell et al., 2012b). The altered expression profile of numerous gene families is the result of changes in the chromatin state due to the absence of Pitx2 functional protein in muscle cells. To correlate gene expression and chromatin state, ChIP-seq analyses for H3K4me3, H3K27me3 and Pol II in WT and MT E12 forelimbs were performed and their signatures compared. Mouse E12 forelimb harbors several muscle lineages and, to maximize fidelity, the current analysis is focused on the Pitx2 target sequences in the Lbx1 lineage (Campbell et al., 2012b; 2012a).

The R Bioconductor package and components were applied as described in the materials and methods section. The Pitx2-regulated 125 protein-coding transcripts identified by microarray (Campbell et al., 2012b), will be referred to as Pitx2 targets from herein. However, chromatin marks based on the peak-calling algorithm were exhibited only in the promoter region (0–2,000bp) or gene body domains (exons, introns, and 5’ and 3’ un-transcribed sequences), called loci from herein, of 101 Pitx2 target transcripts in WT and 103 in MT (Table 1). Pitx2 targets were grouped based on the combinations of H3K4me3, H3K27me3, and Pol II present at loci using Homer tools mergepeaks. No loci were marked with only Pol II, or with both H3K27me3 and Pol II. Summing loci for each mark resulted in 35% occupied by Pol II in WT (35/101) and 28% in MT (29/103). Likewise, 18% of the loci were occupied by only H3K4me3 in WT (18/101) and 22% were occupied in MT (23/103), while only 13% (13/101) and 12% (12/103) were occupied only by H3K27me3 in WT and MT respectively. Pol II promoter occupancy is correlated with active gene expression in the presence of H3K4me3 (Guenther et al., 2007). The majority of Pitx2 targets were occupied by both H3K4me3 and H3K27me3, 36% in WT (36/101) and in 37% in MT (38/103), while

the trivalent state, H3K4me3, H3K27me3 and Pol II occupancy, represented 13% of the targets in WT (13/101) and 7% in MT (7/103). Venn diagrams illustrate the overlap of chromatin marks (Pol II, H3K27me3, and H3K4me3) in WT and MT (Fig 1). Bivalently marked loci are found at higher frequency in embryonic stem cells (Bernstein et al., 2006) than in committed postmitotic cells (Mikkelsen et al., 2007). The presence of numerous poised, inactive (bivalent) loci at E12 forelimbs, in both WT and MT mice, suggests that genes involved in patterning and organ development are stalled to allow the next set of genes to be activated and establish the next developmental state of the mitotic cells. The strong increase of solely H3K4me3 (22%) activated loci in combination with the decreased poised trivalent loci (54%) in MT suggests that Pitx2 negatively regulates the transcription of its targets.

3.2. Mapped read distribution around Refseq TSSs

The confirmed changes in chromatin state in Pitx2 target transcripts was followed by analysis of the patterning changes in the chromatin distribution. Homer tools v4.7 was used to generate tag density plots (Fig 2A–D) in different sequence sets. The detected tag density for all three marks were in accord with all sequences in the genome (Fig 2A), (Barski et al., 2007). The overall tag density was higher in the WT biopsies relative to the MT. This follows for all marks and persists despite the normalization of tag density for mapped reads. Examination of tag densities around the Pitx2 target transcripts (Fig 2B) showed a similar pattern. All curves appeared slightly less sharp due to the smaller sampling size (20K total transcripts vs. 125 Pitx2 target transcripts). The density of H3K27me3 tags became increasingly noisy, but the two-peak distribution remained visible. The tag density of H3K4me3 and Pol II was decreased, while tag density of H3K27me3 was increased in both WT and MT. These data imply that chromatin was more condensed around identified Pitx2 target transcripts, relative to all transcripts in both WT and MT. When compared to all identified Pitx2-target, non-SSTF transcripts (Fig 2C), an almost identical pattern emerged (Fig 2C). This pattern remained the same in the Pitx2 target SSTFs but was noisy due to the smaller transcript number (Fig 2D). In summary, embryonic forelimb chromatin for all genomic sequences was marked for H3K4me3 and Pol II, with dynamic changes between WT and MT. Chromatin was rich for H3K4me3 marks, and for H3K4me3 and H3K27me3 for SSTF targets, suggesting that SSTF chromatin harbors active and inactive domains to determine distinct cell lineages as myogenesis progresses.

3.3. Chromatin state of Pitx2 target genes

To investigate the link between chromatin state and gene expression, the chromatin state around Pitx2 target genes in the Lbx1 lineage was visualized in detail (Table 1). Chromatin profiling for H3K4me3, H3K27me3 and Pol II in coding, and 5' and 3' non-translated sequences identified active and repressed domains (Fig 3). Analysis indicated that 23 transcripts (Table 1, indicated as bold) including Pitx2 exhibited differential chromatin marks. These genes are involved in organogenesis (Pitx2, Rfx8, Ebf1, Acta2), neurogenesis and axon path-finding (Atn1, Mab21l1, Tubb3, Dcx, Nrcam, Cadps, Lin7, Zswim5), transport (Ddah1, Slco3a1, Atp1b1), cell cycle (Csrnp3), cytoskeleton and organelle organization (Hook1, Cntn2, Crmp, Stmn2, Nol4, Lamp5), myogenesis (Fstl5) and osteogenesis (Skor1).

Genes were clustered in six groups based on the chromatin signature of the WT myoblasts. Genes that exhibited H3K4me3, H3K27me3 and Pol II presence, trivalent state (Fig 3A; Pitx2, Ebf1, Nl4, Slco3a1, Zswim5, Ddah1). Genes that exhibited H3K4me3 and Pol II presence, activated state (Fig 3B; Atp1b1, Mab2111, Astn1, Csrnp3, Hook1). Genes that exhibited H3K4me3 and H3K27me3 presence, bivalent state (Fig 3C; Lin7). Genes that exhibited only H3K4me3 presence, open chromatin (Fig 3D; Hook1). Genes that exhibited only H3K27me3 presence, closed chromatin (Fig 3E; Scor1). Genes that did not exhibit any chromatin signature in the WT (Fig 3F; Fstl5).

Pitx2 mutants were generated by ablation of the homeodomain located between exon 4 and 5. ChIP-seq analysis indicated the expected absence of signature in the ablated sequences that was used as a technical control (Fig 3A). The Pitx2 locus was trivalent in WT while the Pol II signature was missing in MT due to the truncated protein. Ebf1, a helix-loop-helix transcription factor, is involved in B-cell lymphopoiesis (Hagman et al., 1993) in positioning the mesenchyme-derived ulna and radius and connective tissues surrounding tendons (Mella et al., 2004) and adipogenesis (Jimenez et al., 2007). While Elf1 was in the trivalent state in WT forelimbs, it was in an active state in the MT, suggesting that Pitx2 represses its activity towards the adipogenic and cartilage formation pathways.

The solute carrier organic anion transporter family member 3a1 (Slco3a1), associated with pathways that transport glucose, bile salts, organic acids, metal ions and amino acids, was inhibited by Pitx2 (Fig 3A). The dimethyl arginine dimethylaminohydrolase (Ddah1) plays a role in nitric oxide generation by regulating cellular concentrations of methylarginines, which in turn inhibit nitric oxide synthase activity, and is highly expressed during forelimb development (Breckenridge et al., 2010). While Ddah1 is in a trivalent state in WT, it is activated in the MT suggesting that Pitx2 acts as a repressor at this locus (Fig 3A).

The sodium/potassium-transporting ATPase subunit beta-1 (Atp1b1), an integral membrane protein responsible for establishing and maintaining the electrochemical gradients of Na and K ions across the plasma membrane, was active in WT with open chromatin in MT (Fig 3B), suggesting that Pitx2 regulates Na transport during limb development. Mab2111 is a downstream target of transforming growth factor beta (TGF β) signaling (Mariani et al., 1999) and is involved in development of several organs including limb, neuronal and vascular systems (Wong and Chow, 2002; Heanue and Pachnis, 2006). Mab2111 exhibited domains with a trivalent signature in MT while the H3K27me3 inactivation signature was missing in WT (Fig 3B). Mab2111 is expressed in similar tissues with Pitx2, and Mab2111 mutants are characterized by axial turning and abdominal malformations similar to Pitx2. The chromatin signature in the forelimb suggests that Pitx2 promotes Mab2111 activation. Astrotactin, Astn1, is a neuronal adhesion molecule that acts as a ligand for glial-guided migration of neuroblasts (Adams et al., 2002). Astn1 domains enriched for H3K4me3 and Pol II were located close to the TSS (Fig 3B). The absence of the Pol II signature in MT suggests that its active transcription is Pitx2-regulated. Cysteine and Glycine-Rich Protein 3 (Csrnp3) promotes myogenic differentiation by acting as a cofactor of Myod, myogenin and MRF4 to increase their interactions with specific DNA regulatory elements (Kong et al., 1997). Csrnp3 also acts as a scaffold protein that promotes the assembly of macromolecular complexes along sarcomeres and cytoskeleton (Arber et al., 1997). The Csrnp3 locus was

active in MT with H3K4me3 and Pol II occupancies, while in WT the Pol II signature was missing (Fig 3B). The *Csrnp3* locus harbored active regulatory regions just downstream of the TSS suggesting that *Pitx2* regulates *Csrnp3* active regulatory regions and extends the myoblast state before their commitment to specified lineages.

Lin7, plays a role in establishing and maintaining the asymmetric distribution of channels and receptors at the plasma membrane of polarized cells, and in stabilizing the cell junctions (Bohl et al., 2006). *Lin7* was characterized by a bivalent chromatin state in WT and open chromatin in MT, suggesting that *Pitx2* regulates cell polarization during limb development.

Hook homologue 1, *Hook 1*, is expressed in tubular endosomes and facilitates the directed recycling of clathrin-independent endocytosis cargo proteins, such as CD98 and CD147, through its interaction with microtubules and their cytoplasmic sequences on sorting endosomes (Maldonado-Báez et al., 2013). This sorting provides a means to monitor protein quality control of cell surface proteins. The *Hook1* locus was active to transcription in MT, occupied by H3K4me3 and Pol II, while the Pol II mark was missing in WT (Fig 3B). This chromatin change suggests that *Pitx2* influences the activity of this locus and the ability of myoblasts to segregate and rapidly recycle proteins, an activity that is carefully regulated in tissues during development.

The Ski family transcriptional corepressor 1, *Skor1*, interacts with *Lbx1* and cooperatively represses transcription and acts as a transcriptional corepressor for *Lbx1* in regulating cell fate determination in the spinal cord (Mizuhara et al., 2005). *Skor1* interacts with general transcriptional corepressors, such as *Hdac1*, *Ctbp* and *Grg1*. *Skor1* represses *TGFβ* signaling through inhibition of transcriptional activity of *Smad* proteins and negative regulation of the bone morphogenetic proteins (BMPs) (Arndt et al., 2007). BMPs serve multiple functions in many cell and tissue types including proliferation, apoptosis, differentiation, chemotaxis, angiogenesis and matrix production during embryogenesis (Luo, 2004). The *Skor1* locus was poised in MT and repressed in WT (Fig 3A) suggesting that *Pitx2* represses the *Skor1*-mediated repression cascade involved in specification of the osteogenic lineage during development.

Foliatin like 5, *Fstl5*, a calcium binding protein, is involved in axonal guidance of DRG neurons during development (Masuda et al., 2009). The H3K4me3 occupancy in the *Fstl5* locus was exhibited only in the MT (Fig 3F), suggesting that *Pitx2* represses *Fstl5* activity in myoblasts as they migrate along with peripheral neuronal axons.

Genes involved in cell growth, organogenesis, lineage specification and cytoskeleton organization were characterized with inactive chromatin domains in the absence of *Pitx2*. Genes involved in axonal path finding and muscle differentiation were characterized with active regulatory chromatin domains in the absence of *Pitx2*. *Pitx2* might regulate chromatin regions involved in different areas of myoblast integrity, specification and muscle formation. Chromatin signatures indicated a complex gene network required at each developmental stage as several lineages intermingle to form a functional organ.

4. Discussion

Genome wide association studies like chromatin profiling are useful tools to map regulatory activity in the genome, and when combined with gene expression data, a much more precise model of regulatory networks can be developed. In this study, we combined gene expression data from lineage-specific *Lbx1*^{EGFP/+} E12 mouse forelimb myoblasts with ChIP-seq data of histone modifications in E12 forelimbs to identify regulatory sequences influenced by Pitx2. Several transcripts were differentially expressed in WT and MT migratory myoblasts using new packages in R to re-analyze our previous micro-array data (Campbell et al., 2012b). Many of the identified transcripts with known functions play a role in the development of nervous system and/or cytoskeletal organization of the myofibers, further supporting previous observations (Campbell et al., 2012b; 2012a) that Pitx2 regulates higher order architectural assembly of the developing muscle.

The chromatin states around *Pitx2*-regulated transcripts exhibited a two-fold greater frequency of trivalent poised target transcripts in MT relative to WT (13% vs. 7%). This increase in inactive but poised target transcripts could imply that ablation of *Pitx2* at this stage in the embryonic forelimb created a time-shift, “stalled cells”, during development. Transcripts that are normally “repressed” were switched to “poised” due to deposition of excessive H3K4me3, thus priming them for later activation. The temporal shift is further supported by the 22% increase in transcripts marked solely with H3K4me3 in MT.

Even though the Pitx2 targeted transcripts were identified from *Lbx1*⁺ lineage myoblasts, changes in the chromatin state of many target transcripts in the whole forelimb were also identified by MACS2. The whole forelimbs contain vascular, bone, and other cell types, which generate noise in the data sets, likely to be responsible for disagreements between the expression profiling data and chromatin state. Additionally, Homer tools was used to create tag-density plots around different subsets of transcripts in the E12 mouse genome. The average H3K4me3 signal was decreased in both WT and MT around all target transcripts relative to all transcripts, especially the SSTFs, suggesting that at this developmental stage the target transcripts are less active than all other transcripts, on average. This observation was supported by the exact same trend in the Pol II occupancy data, and the large increase in H3K27me3 signal density. In general, the WT tag density was greater than the MT tag density despite normalization per million reads. This persisted with the target transcripts for both the SSTFs and non-SSTFs data. An increase in H3K4me3 tag density around target transcripts in MT was expected, but the opposite was observed. Because this pattern is seen in every case, it is most likely an artifact of the normalization method. Homer tools does not have the option to generate tag-density plots relative to the input controls, which further exacerbates the difference. Increased WT signal strength is not significantly shown in the bigwig visualization with the UCSC genome browser, which takes the fold enrichment over input controls into account.

5. Conclusion

Genes regulated by Pitx2 in mouse embryonic forelimb are involved in neuronal, muscular and bone development. Pitx2 promotes myogenesis by extending the myoblast state while

maintaining the availability of genes involved in axon path finding and osteogenesis in a poised state.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Chromatin state changes in Pitx2 Mutants

Venn diagrams illustrating the co-occurrence of enriched regions around identified Pitx2 target sequences in WT and *Pitx2* MT forelimbs, respectively. The MT data are represented by darker shades. Green represents H3K4me3; red represents H3K27me3; and gray represents Pol II in each sample. Homer v4.7 was used to match each identified target sequence to its nearest peaks.

Figure 2. Constant tag density in Wild-Type and Pitx2 Mutants

(A) Normalized tag densities around each known Refseq TSS for all three marks in WT and MT. Homer v4.7 annotatePeaks.pl was used to count the number of aligned reads around Refseq TSSs by scanning in 5bp bins. The average number of reads per bin per sequence is plotted on the y axis against the distance of the bin from the TSS. (B) Normalized tag densities around only the 125 Pitx2 target sequences identified. (C) Normalized tag densities around only the subset of SSTFs from (B). (D) Tag densities around all sequences not in (C).

Figure 3. Pitx2-dependent chromatin state of mouse embryonic forelimbs

UCSC genome browser visualization of selected gene loci. Genes were selected based on strong H3K27me3 marked chromatin, and/or high relative fold changes determined from the GSE31945 array data. WT and MT tracks of the same mark are overlaid. H3K4me3 is represented by green, H3K27me3 red, and Pol II gray. MT tracks are illustrated by the darker shades. (A) Trivalent, poised genes (H3K4me3/H3K27me3/Pol II). (B) Activated genes (H3K4me3/Pol II). (C) Bivalent genes (H3K4me3/H3K27me3). (D) Genes with open chromatin (H3K4me3). (E) Genes with close chromatin (H3K27me3). (F) Genes with no significant chromatin signature.

Table 1

Pitx2 Target Genes in Migratory Myoblasts

Gene name	Gene Symbol	Adjusted P. Val	FC MT/WT	Function	Chr	Chromatin State WT	Chromatin State MT
Paired-like homeodomain transcription factor 2	Pitx2	6.21E-105	-29.06	organogenesis, cell cycle	3	K4 K27 Pol II	K4 K27
T-box 1	Tbx1	1.72E-04	-2.17	organogenesis	16	K4 K27	K4 K27
LSM12 homolog (S. cerevisiae)	Lsm12	3.96E-04	-2.11	RNA processing	11	K4 Pol II	K4 Pol II
Fibroblast growth factor 15	Fgf15	3.92E-03	-1.94	organogenesis	7	K4 K27	K4 K27
Phosphatidylinositol 4-kinase, catalytic, beta polypeptide	Pi4kb	3.76E-03	-1.94	phospholipid metabolism	3	K4 Pol II	K4 Pol II
SRY-box containing gene 6	Sox6	3.92E-03	-1.94	cartilage development	7	NA	NA
Zinc finger protein 64	Zip64	4.88E-03	-1.92	promotes osteogenesis	2	K4 Pol II	K4 Pol II
TRAF3 interacting protein 3	Traf3ip3	4.65E-03	-1.92	autophagy	1	NA	NA
DPH5 homolog (S. cerevisiae)	Dph5	5.00E-03	-1.91		3	K4 Pol II	K4 Pol II
Golgi transport 1 homolog B (S. cerevisiae)	Golt1b	1.12E-02	-1.85	vesicle transport	6	K4 Pol II	K4 Pol II
Polyadenylate binding protein-interacting protein 1	Paip1	1.12E-02	-1.85		13	K4 Pol II	K4 Pol II
Rho GTPase activating protein 29	Arhgap29	1.12E-02	-1.85	tubulogenesis	3	K4 Pol II	K4 Pol II
Potassium large conductance calcium-activated Channel, subfamily M, alpha member 1	Kcma1	1.46E-02	-1.83	synaptic transmission,	14	K4 K27	K4 K27
Coiled-coil domain containing 141	Ccdc141	1.88E-02	-1.81	neural induction	2	K4	K4
Kelch-like 4 (Drosophila)	Klhl4	2.03E-02	-1.81		X	NA	NA
Cathepsin S	Ctss	3.05E-02	-1.77	endopeptidase activity	3	NA	NA
Endonuclease domain containing 1	Endod1	4.03E-02	-1.75	apoptosis – KEGG	9	K4 Pol II	K4 Pol II
Sushi-repeat-containing protein, X-linked 2	Srpx2	3.98E-02	-1.75	synaptogenesis	X	NA	NA
Actin, alpha 2	Acta2	4.79E-02	-1.73	vasculogenesis	19	K4 Pol II	NA
Regulatory factor X 8	Rfx8	5.16E-02	-1.72	pancreas development	1	K4 Pol II	K4
Reprimo	Rprm	5.68E-02	-1.71	cell cycle	2	K4	K4
Actinin alpha 3	Actn3	5.97E-02	-1.71	muscle contraction	19	K4 Pol II	K4 Pol II
Lymphatic vessel endothelial hyaluronan receptor 1	Lyve1	6.30E-02	-1.71	polysaccharide catabolism	7	NA	NA
Hydrolethalus syndrome 1	Hyls1	7.32E-02	-1.69	ciliogenesis	9	K4 Pol II	K4 Pol II
Complement component 3a receptor 1	C3ar1	8.99E-02	-1.67	locomotion	6	NA	NA
Myomesin 2	Myom2	9.36E-02	-1.67	muscle contraction	8	NA	NA

Gene name	Gene Symbol	Adjusted P. Val	FC MT/WT	Function	Chr	Chromatin State WT	Chromatin State MT
Zinc finger, SWIM domain containing 5	Zswim5	9.68E-02	1.67	CNS	4	K4 K27 PolII	K4
Lectin, galactoside binding-like	Lgalsl	9.02E-02	1.67	carbohydrate binding	11	K4 Pol II	K4 Pol II
Astrotactin 1	Astn1	8.15E-02	1.68	neuron migration/adhesion	1	K4 Pol II	K4
Homeo box D4	Hoxd4	8.57E-02	1.68	pattern specification	2	K4 K27	K4 K27
Lipid Phosphate Phosphatase-Related Protein Type 1	Lppr1	7.43E-02	1.69	neurite outgrowth	4	K4	K4
Bruno-like 5, RNA binding protein (Drosophila)	Celf5	7.43E-02	1.69	myotonic dystrophy	10	K4 K27	K4 K27
Dimethylarginine dimethylaminohydrolase 1	Ddah1	7.48E-02	1.69	amino acid transport	3	K4 K27 PolII	K4 PolII
RIKEN cDNA 4933400N17 gene	Rbfox1	7.72E-02	1.69	myotonic dystrophy	16	K4	K4
Fatty acid binding protein 7, brain	Fabp7	7.32E-02	1.69	Neuromuscular process	10	NA	NA
Family with sequence similarity 57, member B	Fam57b	7.32E-02	1.69	inhibition of adipogenesis	7	NA	NA
Transcription factor AP-2, alpha	Tfap2a	6.96E-02	1.7	direct regulator of Bmp2, 4	13	K4 K27 PolII	K4 K27 Pol II
Solute carrier family 32 member 1	Slc32a1	5.94E-02	1.71	neurotransmitter transport	2	K27	K27
Myosin, heavy polypeptide 7, cardiac muscle, beta	Myh7	5.97E-02	1.71	muscle contraction	14	NA	NA
Coxsackie virus and adenovirus receptor	Cxadr	4.79E-02	1.73	cell junction, myogenesis	16	K4 Pol II	K4 Pol II
Fasciculation and elongation protein zeta 1 (zyglin I)	Fez1	4.77E-02	1.73	microtubule organizing center	9	NA	NA
Peroxisome proliferative activated receptor, gamma, Coactivator 1 alpha	Ppargc1a	4.35E-02	1.74	insulin signaling – KEGG	5	K4	K4
CUGBP, Elav-like family member 4	Celf4	4.51E-02	1.74	spliceosome assembly	18	K4 K27	K4 K27
Hook homolog 1 (Drosophila)	Hook1	4.13E-02	1.74	cytoskeleton	4	K4	K4 Pol II
Microtubule-associated protein 2	Map2	4.35E-02	1.75	axonogenesis	1	K4	K4
RAS oncogene family member 3C	Rab3c	3.67E-02	1.75	exocytosis	13	K4	K4
Follistatin-like 5	Fstl5	3.45E-02	1.76	muscle growth	3	NA	K4
Homeo box D3 Opposite Strand	Hoxd3os1	3.36E-02	1.76		2	K4 K27	K4 K27
Early B-cell factor 1	Ebfl	3.52E-02	1.76	adipogenesis	11	K4 K27 Pol II	K4 Pol II
Calcium channel, voltage-dependent, alpha 2/delta Subunit 2; similar to Cacna2d2 protein	Cacna2d2	3.13E-02	1.77	muscle synaptic transmission	9	K4 K27	K4 K27
Transcription factor 21	Tcf21	3.11E-02	1.77	organogenesis	10	K27	K27
Homeo box B5 Opposite Strand	Hoxb5os	3.10E-02	1.77		11	K4 K27	K4 K27
Neural proliferation, differentiation and control gene 1	Npdc1	3.05E-02	1.77		2	K4 K27	K4 K27
Homeo box B2	Hoxb2	3.05E-02	1.77	pattern specification	11	K4 K27 PolII	K4 K27 PolII

Gene name	Gene Symbol	Adjusted P. Val	FC MT/WT	Function	Chr	Chromatin State WT	Chromatin State MT
Cysteine-serine-rich nuclear protein 3	Csrp3	3.09E-02	1.77	apoptosis	2	K4	K4 Pol II
Solute carrier organic anion transporter 3a1	Slco3a1	2.82E-02	1.78	organic anion transport	7	K4 K27 PolII	K4
Runt-related transcription factor 1	Runx1l1	2.86E-02	1.78	fat cell differentiation	4	K4 Pol II	K4 Pol II
Basonuclin 1	Bnc1	2.41E-02	1.79		7	K4 K27	K4 K27
Meis homeobox 2	Meis2	2.58E-02	1.79	organogenesis	2	K4 K27 PolII	K4 K27 PolII
Mab-21-like 1 (C. elegans)	Mab21l1	1.89E-02	1.81	neurons	3	K4 Pol II	K4 K27 PolII
Protocadherin 8	Pcdh8	1.52E-02	1.83	cell adhesion, muscle	14	K4 K27	K4 K27
Neurexin III	Nrxn3	1.26E-02	1.84	synapse organization	12	NA	NA
lin-7 homolog A (C. elegans)	Lin7a	1.24E-02	1.84	synaptic transmission	10	K4 K27	K4
Synaptosomal-associated protein 91	Snap91	1.12E-02	1.85	immune system	9	K4 K27	K4 K27
Mab-21-like 2 (C. elegans)	Mab21l2	1.17E-02	1.85	neurons	3	K4 K27 Pol II	K4 K27 Pol II
Nucleolar protein 4	Nol4	1.12E-02	1.85	organelle	18	K4 K27 Pol II	K4 K27
Guanine nucleotide binding protein, gamma 3	Gng3	1.21E-02	1.85		19	NA	NA
Paired box gene 3	Pax3	2.23E-02	1.86		1	K4 K27	K4 K27
Doublecortin	Dcx	3.45E-02	1.88	CNS development	X	K4	NA
Synaptosomal-associated protein 25	Snap25	7.82E-03	1.88	neurotransmitter secretion	2	NA	K4
Neuron-glia-CAM-related cell adhesion molecule	Nrcam	8.46E-03	1.88	axon guidance, adhesion	12	K4	K4 K27
RNA binding protein, fox-1 homolog 3	Rbfox3	8.00E-03	1.88		11	K4 K27	K4 K27
neurexin I	Nrxn1	8.46E-03	1.88	synaptogenesis	17	NA	NA
Ca2+-dependent secretion activator	Cadps	6.36E-03	1.9	catecholamine secretion	14	K4 K27	K4
Inter-alpha (globulin) inhibitor H5	Itih5	4.65E-03	1.92	polysaccharide metabolism	2	K4	K4
Solute carrier family 1 (glial high affinity glutamate transporter), member 2	Slc1a2	4.00E-03	1.93	glucose transport	2	K4 K27	K4 K27
SOGA family member 3	Soga3	3.76E-03	1.94		10	K4 K27	K4 K27
ATPase, Na+/K+ transporting, beta 1 polypeptide	Atplb1	3.92E-03	1.94	ATP biosynthesis	1	K4 K27 Pol II	K4
RIKEN cDNA C130071C03 gene	C130071C03Rik	1.48E-02	1.95		13	K27	K27
Contactin 2	Cntn2	3.42E-03	1.95	neurons, cytoskeleton	1	K27	K4
Cerebellin 2 precursor protein	Cbln2	2.87E-03	1.96		18	K4 K27	K4 K27
Contactin 1	Cntn1	2.74E-03	1.97	cell adhesion	15	K4	K4
Prior incubation determinant 1	Ptd1	2.56E-03	1.97		1	K4	K4

Gene name	Gene Symbol	Adjusted P. Val	FC MT/WT	Function	Chr	Chromatin State WT	Chromatin State MT
Homeo box C8	Hoxc8	2.63E-03	1.97	pattern specification process	15	K4 K27	K4 K27
Claudin 9	Cldn9	2.28E-03	1.98	cell adhesion	17	NA	NA
UNC homeobox	Unx	2.18E-03	1.98	cartilage condensation	5	K27	K27
Solute carrier family 17, member 6	Slc17a6	1.18E-03	2.02	synaptic transmission	7	K27	K27
Suppression of tumorigenicity 18	St18	1.18E-03	2.02		1	NA	NA
Kinesin family member 5C	Kif5c	1.18E-03	2.03	axonogenesis, guidance	2	K4 Pol II	K4 Pol II
Myocardial infarction associated transcript	Miat	1.05E-03	2.04	neuron differentiation	5	K4 K27	K4 K27
Myelin transcription factor 1	Myt1	9.91E-04	2.04	cell cycle	2	NA	NA
Secretogranin III	Seg3	8.78E-04	2.05		9	NA	NA
Homeo box B3	Hoxb3	1.87E-02	2.06	pattern specification	11	K4 K27	K4 K27
Fibroblast growth factor 5	Fgf5	7.94E-04	2.06	organogenesis	5	K4 K27	K4 K27
RUN and FYVE domain containing 3	Rufy3	1.20E-02	2.06	chromosome partitioning,	5	K4 Pol II	K4 Pol II
Hydroxyprostaglandin dehydrogenase 15 (NAD)	Hpgd	5.01E-04	2.09	fatty acid metabolic process	8	K4	K4
Neurogenic differentiation 6	Neurod6	4.83E-04	2.09	neurogenesis	6	NA	NA
Sorbin and SH3 domain containing 2	Sorbs2	4.34E-04	2.1		8	K4	K4
ELAV-like 2 (Hu antigen B)	Elavl2	1.44E-03	2.12		4	K4 K27 Pol II	K4 K27 Pol II/K27
Glutamic acid decarboxylase 1	Gad1	2.25E-04	2.15	synaptic transmission	2	K27	K27
Homeo box D3	Hoxd3	8.17E-05	2.21	pattern specification	2	K4 K27	K4 K27
Zinc finger protein of the cerebellum 1	Zic1	4.00E-04	2.22	pattern specification	9	K4 K27	K4 K27
ELAV-like 4 (Hu antigen D)	Elavl4	3.37E-05	2.31		4	K4 Pol II	K4 Pol II
Neuron navigator 2	Nav2	2.84E-06	2.43		7	K4 K27 PolII	K4 K27 PolII
Inhibitor of DNA binding 4	Id4	3.32E-02	2.44	neuroblast proliferation	13	K4 Pol II	K4 Pol II
LIM homeobox protein 1	Lhx1	2.85E-02	2.5	neuron specification	11	K4 K27	K4 K27
Stathmin-like 3	Stmn3	5.10E-04	2.52	cytoskeleton organization	2	K27	K27
Lysosomal-associated membrane protein family, 5	Lamp5	7.03E-07	2.52		2	K4 K27	K27
Collapsin response mediator protein 1	Crmpl	7.03E-07	2.52	neuron, cytoskeleton	5	K4 K27	K4 K27
Glycoprotein m6a	Gpm6a	1.54E-04	2.61		8	K4	K4
Cell adhesion molecule with homology to L1CAM	Chl1	5.46E-08	2.68	axonogenesis, guidance	6	K4 K27	K4 K27
Thrombospondin, type I, domain containing 7A	Thsd7a	1.35E-09	2.92		6	K4 Pol II	K4 Pol II

Gene name	Gene Symbol	Adjusted P. Val	FC MT/WT	Function	Chr	Chromatin State WT	Chromatin State MT
Roundabout homolog 3 (Drosophila)	Robo3	8.22E-10	2.96	neuron migration	9	K27	K27
Neurocan	Ncan	2.15E-06	3.06	adhesion, synapsis	8	K27	K27
Tubulin, beta 3; tubulin, beta 3, pseudogene 1	Tubb3	9.40E-12	3.25	axon guidance	8	K4	K4 K27
Reticulon 1	Rtn1	3.52E-12	3.32	inhibition of axonal growth	12	K4 K27	K4 K27
Nescent helix loop helix 2	Nhlh2	1.75E-12	3.36	physical activity behavior	3	K4 K27	K4 K27
Insulin-like growth factor binding protein-like 1	Igfbpl1	1.82E-13	3.52	regulation of cell growth	4	K4 K27	K4 K27
Homeo box B6	Hoxb6	3.63E-14	3.63	pattern specification process	11	K4 K27	K4 K27
Homeo box B9	Hoxb9	1.73E-19	4.45	pattern specification process	11	K27	K27
Stathmin-like 2	Stmn2	2.12E-13	4.49	cytoskeleton organization	3	K27	NA
Microtubule-associated protein tau	Mapt	1.12E-19	4.49	microtubule organization	11	K4 K27 Pol II	K4 K27 Pol II
Internexin neuronal intermediate filament protein, alpha	Ina	9.15E-14	4.93	cytoskeleton organization	19	K4 K27	K4 K27
Deleted in colorectal carcinoma	Dcc	8.20E-21	5.7	neuron migration, apoptosis	18	K4 K27	K4 K27
SKI family transcriptional co-repressor 1	Skor1	3.77E-52	10.96	BMP inhibitor	9	K27	K4 K27

Bold genes represent the ones with different chromatin marks in WT and MT biopsies; K4, H3K4me3; K27, H3K27me3