Evolutionarily conserved regulation of embryonic fast-twitch skeletal muscle differentiation by Pbx factors

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Summary

Vertebrate skeletal muscles are composed of both slow-twitch and fast-twitch fiber types. How the differentiation of distinct fiber types is activated during embryogenesis is not well characterized. Skeletal muscle differentiation is initiated by the activity of the myogenic basic helix-loop-helix (bHLH) transcription factors Myf5, Myod1, Myf6, and Myog. Myod1 functions as a muscle master regulatory factor and directly activates muscle differentiation genes, including those specific to both slow and fast muscle fibers. Our previous studies showed that Pbx TALE-class homeodomain proteins bind with Myod1 on the promoter of the zebrafish fast muscle gene *mylpfa* and are required for proper activation of *mylpfa* expression and the fast-twitch muscle-specific differentiation program in zebrafish embryos. Pbx proteins have also been shown to bind regulatory regions of muscle differentiation genes in mammalian muscle cells in culture. Here, we use new zebrafish mutant strains to confirm the essential roles of zebrafish Pbx factors in embryonic fast muscle differentiation. Furthermore, we examine the requirements for *Pbx* genes in mouse embryonic skeletal muscle differentiation, an area that has not been investigated in the mammalian embryo. Removing *Pbx1* function from skeletal muscle in *Myf5^{Cre/+};Pbx1^{fl/fl}* mouse embryos has minor effects on embryonic muscle development. However, concomitantly deleting *Pbx2* function in *Myf5^{Cre/+};Pbx1^{fl/fl};Pbx2^{-/-}* mouse embryos causes delayed activation and reduced expression of fast muscle differentiation genes. In the mouse, *Pbx1/Pbx2*-dependent fast muscle genes closely match those that have been previously shown to be dependent on murine *Six1* and *Six4*. This work establishes evolutionarily conserved requirements for Pbx factors in embryonic fast muscle differentiation. Our studies are revealing how Pbx homeodomain proteins help direct specific cellular differentiation pathways.

Keywords: Pbx1, Pbx2, Pbx4, Skeletal muscle, Fiber-type differentiation, Mouse, Zebrafish

Introduction

Skeletal muscle fiber diversity allows for a range of movements, activity and metabolism, but muscle fiber diversity can also affect susceptibility to muscle disease and muscle wasting (reviewed in Schiaffino and Reggiani, 2011; and Talbot and Maves, 2016). There are two main types of skeletal muscle fibers: slow twitch and fast twitch. Understanding how skeletal muscle fiber type differentiation is controlled is important for addressing skeletal muscle defects that occur in muscle aging, metabolic diseases, and muscular dystrophies (reviewed in Ciciliot et al., 2013; Ljubicic et al., 2014; and Talbot and Maves, 2016). Many processes have been described that regulate the plasticity of skeletal muscle fiber-type identities during post-natal periods (reviewed in Bassel-Duby and Olson, 2006; Blaauw et al., 2013; and Schiaffino and Reggiani, 2011). However, the mechanisms that establish muscle fiber-type identities during embryogenesis are less well understood.

Skeletal muscle differentiation is initiated in embryogenesis by the myogenic regulatory factor (MRF) family of basic helix-loop-helix (bHLH) transcription factors: Myf5, Myod1, Myf6, and Myog (reviewed in Hernández-Hernández et al, 2017; Lassar, 2017; and Zammit, 2017). The activity of these factors is highly conserved. In both mice and zebrafish, the MRFs are required for skeletal muscle myogenesis (reviewed in Buckingham and Vincent, 2009; and Rossi and Messina, 2014). In particular, Myod1 functions as a muscle master regulatory factor and controls skeletal muscle differentiation through DNA binding and transcriptional activation

of broad gene expression programs, including genes encoding skeletal muscle contractile proteins (Blais et al., 2005; Cao et al., 2006; Cao et al., 2010; Pliner et al., 2018; Soleimani et al., 2012). In the differentiation of specific muscle fiber types, Myod1 has been associated with promoting fast muscle differentiation. In mouse muscle, Myod1 is expressed more strongly in fast fibers than in slow fibers, and gain- and loss-of-function experiments in mice and zebrafish have shown that Myod1 promotes fast fiber types (Ekmark et al., 2007; Hammond et al., 2007; Hinitz et al., 2011; Hughes et al., 1997; Macharia et al., 2010; Maves et al., 2007; Seward et al., 2001). However, these and other studies have found that Myod1 is also needed for slow fiber differentiation and directly activates both slow and fast muscle differentiation genes (Blais et al., 2005; Cao et al., 2006; Cao et al., 2010; Hinitz et al., 2011; Hughes et al., 1997; Maves et al., 2007; Seward et al., 2001; Soleimani et al., 2012). Myod1's DNA binding and transcriptional activity can be modulated, positively and negatively, by many other transcription factors (reviewed in Berkes and Tapscott, 2005; Fong and Tapscott, 2013; and Talbot and Maves, 2016). Understanding how the activity of MRFs such as Myod1 is regulated during embryogenesis should provide insight into skeletal muscle fiber type specification (Talbot and Maves, 2016).

We already understand some components of a transcriptional network used to activate skeletal muscle fiber-type specification during embryogenesis (reviewed in Jackson and Ingham, 2013; and Rossi and Messina, 2014). Transcription factors of the Six homeodomain family are needed to promote MRF gene expression and muscle differentiation in both mice and zebrafish embryos (Bessarab et al., 2008; Grifone et al., 2005; Niro et al., 2010; Nord et al., 2013; O'Brien et al., 2014; Richard et al., 2011; Talbot et al., 2019). In mouse embryos with compound loss of *Six1*; *Six4*, and in zebrafish embryos with morpholino knock-down of *six1a* and *six1b*, fast muscle gene expression is reduced in the early myotome, whereas expression of slow muscle

genes is largely not affected (Bessarab et al., 2008; Niro et al., 2010; O'Brien et al., 2014). However, in zebrafish embryos with genetic deletion of all four zebrafish *six1* and *six4* genes, migratory muscle precursor cells do not undergo proper specification or migration, but embryonic fast muscle in the trunk still forms normally (Talbot et al., 2019). The Sox6 transcription factor promotes fast muscle differentiation by repressing slow muscle differentiation in zebrafish embryos and in mouse embryos (Hagiwara et al., 2005; Hagiwara et al., 2007; Jackson et al., 2015; von Hofsten et al., 2008). In zebrafish, the transcription factor *Prdm1a* acts as a critical switch in embryonic muscle fiber-type specification. *Prdm1a* directly represses fast muscle differentiation genes and also represses *sox6* expression, thereby promoting slow muscle differentiation in zebrafish (von Hofsten et al., 2008). However, even though *Prdm1* is expressed in the embryonic mouse myotome, the onset of myogenesis and fast and slow muscle differentiation appear to proceed normally in *Prdm1* mutant mouse embryos (Vincent et al., 2012). Thus, while there are many conserved aspects of muscle fiber-type specification and differentiation, there also appear to be differences between zebrafish and mouse in the regulation of embryonic muscle fiber type.

We previously showed, in zebrafish embryos, that Pbx TALE-class homeodomain proteins are required for the fast muscle differentiation program (Maves et al., 2007; Yao et al., 2013). In zebrafish embryos lacking Pbx proteins through antisense morpholino knockdown, activation of fast muscle differentiation genes, such as *mylpfa* and *atp2a1a*, is delayed and, even upon activation, these genes show reduced expression (Maves et al., 2007; Yao et al., 2013). Studies in zebrafish and mouse embryos, as well as in mammalian cell culture models, have shown that Pbx proteins bind with Myod1 on the promoters of a subset of Myod1 target genes, including *Myog* and *mylpfa* (Berkes et al., 2004; Cho et al., 2015; de la Serna et al., 2005;

Dell'Orso et al., 2016; Heidt et al., 2007; Maves et al., 2007; Pliner et al., 2018). Pbx proteins can bind silent Myod1 target gene promoters prior to Myod1 binding and muscle differentiation, suggesting the potential for Pbx proteins to function as pioneer factors in skeletal muscle differentiation (Berkes et al., 2004; Cho et al., 2015; de la Serna et al., 2005; Dell'Orso et al., 2016; for discussion of Pbx proteins as pioneer factors, see Grebbin and Schulte, 2017 and Selleri et al., 2019). *Pbx* genes are broadly expressed during embryogenesis and are required for many aspects of mouse embryo development (reviewed in Moens and Selleri, 2006; and Selleri et al., 2019). However, these previous studies have not directly addressed the requirements for Pbx proteins in mammalian skeletal muscle development. Considering that the mechanisms underlying muscle fiber-type differentiation can vary between mice and zebrafish, we tested whether Pbx proteins are required for fast muscle differentiation during mouse embryogenesis.

Here we generate new zebrafish mutant strains to confirm that zebrafish embryos lacking Pbx expression show reduced and delayed activation of fast muscle differentiation genes. Furthermore, we investigate the requirements for Pbx factors in early mouse muscle development. We show that both Pbx1 and Pbx2 are broadly expressed in muscle precursors in embryonic mouse somites. We use a conditional knock-out approach to generate mouse embryos lacking both Pbx1 and Pbx2 in *Myf5*-expressing muscle precursors. We uncover that *Myf5^{Cre/+};Pbx1^{fl/fl};Pbx2^{-/-}* embryos show reduced and delayed activation of fast muscle differentiation genes. Our findings demonstrate evolutionary conservation in the requirements for Pbx proteins in promoting activation of the fast muscle differentiation program during vertebrate embryogenesis.

Materials and Methods

Maintenance of animal strains and generation of zebrafish pbx2 mutant strain.

All experiments involving live zebrafish and mice were carried out in compliance with Seattle Children's Research Institute IACUC guidelines.

Zebrafish were raised and staged as previously described (Westerfield, 2000). Time (hpf) refers to hours post-fertilization at 28.5°C. In some cases, embryos were raised for periods at 24°C. For studies prior to 24 hpf, somite (s) number was used for staging, and mutant and control embryos were somite-stage matched. The wild-type stock and genetic background used was AB. The *pbx4*^{b557} mutant strain was previously described and is likely a null allele (Pöpperl et al., 2000). *pbx4*^{b557} genotyping was performed as previously described (Kao et al., 2015). The *pbx2*^{scm10} mutant strain was generated with CRISPR/Cas9, using procedures we have previously described (Supplemental Figure 1; Farr et al., 2018). The *pbx2*-targeting single-guide construct was made by annealing the following oligonucleotides and ligating the resulting fragment into BsaI-digested pDR274 (Hwang et al., 2013): 5' – [phos]TAGGTGTAGCCGGGCCAGAGAA – 3' and 5' – [phos]AAACTTCTCTGGCCCGGCTACA – 3'. To genotype the *pbx2*^{scm10} strain, a dCAPS assay was designed (Neff et al., 1998) such that amplification of the mutant allele generated an XcmI site. The sequence of the *pbx2* CRISPR target site is shown in Supplemental Figure 1, along with the sequence of the *pbx2*^{scm10} allele and its conceptual translation. Genotyping primers are provided in Table 1.

The mouse strains used were previously described: the *Pbx1* knock-out allele (Selleri et al., 2001; hereafter called *Pbx1*^{-/-}), the *Pbx1* conditional allele (*Pbx1*^{Δneo;Δex3}; Koss et al., 2012; hereafter called *Pbx1*^{fl}), the *Pbx2* knock-out allele (Selleri et al., 2004; hereafter called *Pbx2*^{-/-}),

and the *Myf5 Cre* allele (*Myf5^{tm3(cre)Sor}*; Tallquist et al., 2000; hereafter called *Myf5^{Cre}*; kindly provided by P. Soriano). Intercrosses were used to combine strains, which were maintained on a largely C57BL/6 background. Mouse embryos were obtained through timed matings, designating E0.5 as the morning on the day a vaginal plug was observed. Genotyping was performed by PCR using genomic DNA isolated from embryonic sacs or tail tissue. Protocols for *Pbx1*, *Pbx1^{fl}*, *Pbx2*, and *Myf5^{Cre}* genotyping were previously described (Koss et al., 2012; Selleri et al., 2001; Tallquist et al., 2000). Genotyping primers are provided in Table 1.

Immunocytochemistry and RNA in situ hybridization

Zebrafish whole-mount RNA in situ hybridizations were performed on somite-stage-matched embryos as previously described (Maves et al., 2007; Talbot et al., 2010). The following cDNA probes were used: *krox-20* (*egr2b*-Zebrafish Information Network, Ruzicka et al., 2019; Oxtoby and Jowett, 1993); *myog* (Weinberg et al., 1996); *smyhcl* (Bryson-Richardson et al., 2005); *atp2a1* (Maves et al., 2007); *myl1* (Yao et al., 2013); *mylpfa* (formerly *mylz2*; Xu et al., 2000). Zebrafish embryos were imaged using a Leica TCS SP5 confocal microscope. Images were assembled using Adobe Photoshop.

For anti-Pbx1/Myf5 staining in mouse embryos, E11.5 embryos were fixed in 4% PFA/1X PBS overnight at 4°C and then dehydrated and stored in methanol at -20°C. Embryos were rehydrated using a methanol series performed at room temperature. Rehydrated embryos were washed in 1X PBST (1X PBS + 0.1% Triton X-100), followed by a puncture of the dorsal end of the neural tube to facilitate antibody diffusion. Embryos were rinsed in 1X PBSDT (1X PBS, 1% DMSO, 0.1% Triton X-100) followed by permeabilization in 1X PBS + 2% TritonX-100 for 30 minutes at room temperature. Permeabilized embryos were then rinsed in 1X PBSDT

and blocked overnight in 10% normal donkey serum in 1X PBSDT. 1:100 rabbit polyclonal anti-Myf5 (IgG_{H+L}, Santa Cruz, sc-302) and 1:100 monoclonal mouse anti-Pbx1b (IgG₁, Santa Cruz, 41.1, Jacobs et al., 1999) were incubated in 2% normal donkey serum in 1X PBSDT for 2 days at 4°C. Embryos were then washed for five hours at room temperature in 1X PBSDT. For secondary antibodies, Alexa 488 donkey anti-mouse IgG₁ and Alexa 594 donkey anti-rabbit IgG_{H+L} were used at 1:500 dilution in 2% normal donkey serum in 1X PBSDT overnight at 4°C. After secondary antibody staining, embryos were washed in 1X PBSDT. After post-fixation, embryos were pre-incubated in 30% glycerol + 0.1% (v/v) βScale2 (4M urea, 50% glycerol in distilled water) for 45 minutes; then *Clear*^{T2} in 25% PEG/10% formamide was used for 1 hour at room temperature, followed by overnight in 50% PEG/20% formamide. Cleared embryos were mounted in 0.5% agarose gel in water and settled until gel polymerized; then *Clear*^{T2} in 50% PEG/20% formamide was applied and the specimen was overlain with a coverslip. Optical stacks were collected using an upright Olympus single photon confocal microscope (20x water dipping objective, NA=0.95). Image datasets were analyzed using Fiji/ImageJ software.

For anti-Pbx2/Myod1 staining in mouse embryos, E11.5 embryos were fixed in pre-chilled 4% PFA/1X PBS for one hour. Fixed embryos were incubated in 30% sucrose/1X PBS and embedded in OCT frozen blocks. 30-micron-thick sagittal sections were taken on a Leica cryostat machine. Cryosections were blocked with 3% BSA/1% normal donkey serum in a humidified slide box and subsequently incubated with anti-Pbx2 (1:100, polyclonal rabbit, Santa Cruz, G-20) and anti-Myod1 (5.8A, mouse monoclonal IgG₁) diluted in block solution. After rinses in 1X PBS with 0.25% Triton X-100, sections were then incubated with 1:500 Alexa 488 mouse IgG₁ and Alexa 594 rabbit secondary antibodies in block solution for 1.5 hours at room temperature. Fluorescently labeled specimens were mounted in 2% n-propyl-gallate in 100%

glycerol. Images were collected using a Leica SP5 inverted fluorescent confocal microscope with 20x air objective (NA=0.7) or 40x oil objective (NA=1.4). Fiji/ImageJ software was used for image analysis.

Mouse whole-mount RNA in situ hybridizations were performed on somite-stage-matched embryos as previously described (Di Giacomo et al., 2006). *Myog* probe was synthesized from a full-length mouse *Myog* cDNA cloned into the BamHI site of pCS2+. *Tnnc2* probe was synthesized from a 600bp cDNA fragment cloned into pCRII-TOPO. *Atp2a1* probe was synthesized from a 1236bp cDNA fragment cloned into pCRII-TOPO. *Myh6* probe was synthesized from a 1243bp cDNA fragment cloned into pCRII-TOPO. Additional cDNA probes for *Myh3*, *Myl1*, *Myh7*, *Myh4*, and *Myh2* were generated by adding T7 promoters to PCR-amplified cDNA products. Primers used to amplify cDNA probes are provided in Table 1.

Quantitative (q) RT-PCR

For mouse embryo qRT-PCR, total RNA samples were prepared from whole, individual, genotyped mouse embryos by disrupting the embryos in TRIzol (Ambion; Thermo Fisher Scientific) by trituration through a 16G needle followed by a 26G needle, after which the standard TRIzol protocol was followed. The isopropanol-precipitated RNA was solubilized in water and then further purified using an RNeasy Kit (Qiagen). 100 ng of RNA samples plus random hexamers were used in a reverse-transcriptase reaction with SuperScriptII Reverse Transcriptase (Invitrogen) or with the iScript cDNA Synthesis Kit (Bio-Rad). Real-time PCR was performed using SYBR Green (Bio-Rad) and an Applied Biosystems 7900HT System, or using the KAPA SYBR FAST kit (KAPA Biosystems) on a Bio-Rad CFX96 machine, according

to the manufacturer's instructions. Expression levels of the genes of interest were normalized to *Ornithine decarboxylase 1 (Odc1)*. For primers, see Table 1.

For each gene tested, a $\Delta\Delta C_t$ value was determined for each of the control/mutant pairs of sibling embryos. Two-tailed, unpaired Student's t-tests with Welch's correction were used to determine if the observed values differed significantly from the null hypothesis of $\Delta\Delta C_t$ values of 0. The resulting P values are shown in the graphs in Figures 2 and 4. To construct the graphs, the tables of $\Delta\Delta C_t$ values were log transformed to give fold differences ($2^{-\Delta\Delta C_t}$). The boxes for each gene in the graphs extend from the 25th to 75th percentiles, the whiskers are at the minimum and maximum, the bar within the box represents the median, and the "+" symbol represents the mean. Statistical analysis was performed and the plots were made in GraphPad Prism7.

RNA-seq library preparation and sequencing

Total RNA samples were obtained, using TRIzol Reagent (Life Technologies) and then cleaned using RNeasy Kit (Qiagen), from three *Myf5^{Cre/+};Pbx1^{fl/fl};Pbx2^{-/-}* mutants and three *Pbx1^{fl/+};Pbx2^{-/-}* control sibling whole mouse embryos at E11.5. Library preparation and sequencing was carried out by the FHCRC Genomics Shared Resource. Sequencing libraries were prepared from total RNA using the TruSeq RNA Sample Prep Kit (Illumina) according to the manufacturer's instructions. Library size distributions were validated using an Agilent 2100 Bioanalyzer. Additional library quality control, blending of pooled indexed libraries, and cluster optimization were performed using the QPCR NGS Library Quantization Kit (Agilent Technologies). RNA-seq libraries were pooled (4-plex) and clustered onto a flow cell lane using

an Illumina cBot. Sequencing occurred on an Illumina HiSeq 2000 using a single-read, 100 base read length (SR100) sequencing strategy.

RNA-seq data processing and analysis

Image analysis and base calling were performed with Illumina's Real Time Analysis v1.12 software. Files were demultiplexed of indexed reads and generated in FASTQ format using Illumina's CASAVA v1.8. software. Reads were removed that did not pass Illumina's base call quality threshold. Reads were aligned to mouse genome build mm9, using TopHat 1.4 (Trapnell et al., 2009). SAMtools v0.1.18 (Li et al., 2009) was used to sort and index the TopHat alignments. The gene expression profiles of embryos were compared using the Bioconductor package edgeR. Sequencing data will be available at NCBI GEO.

Functional annotation enrichment analysis was performed by submitting Ensemble Gene IDs from Table S1 and Table S2 to DAVID analysis (Huang da et al., 2009).

Results

*Zebrafish *mzpbx2*;*pbx4* mutant embryos show impaired activation of fast muscle differentiation*

In zebrafish, the main *pbx* genes expressed during early embryonic myogenesis stages are *pbx2* and *pbx4* (Farr et al., 2018; Waskiewicz et al., 2002). Our previous studies of Pbx functions in zebrafish skeletal muscle development used antisense morpholinos to knock down both *pbx2* and *pbx4* expression, and we showed that these *pbx2-MO*;*pbx4-MO* embryos have delayed

activation and reduced expression of fast muscle genes (Maves et al., 2007; Yao et al., 2013). Here, we have used CRISPR to generate a loss of function allele of zebrafish *pbx2* (Materials and Methods; Supplemental Figure 1). We find that *pbx2*^{-/-} fish are homozygous viable as adults, similar to *Pbx2*^{-/-} mice (Selleri et al., 2004). We crossed *pbx2* mutant fish with the established *pbx4* mutant strain (Pöpperl et al., 2000) to generate a *pbx2*^{-/-};*pbx4*^{+/-} viable strain. Incrossing these fish generated *mzpbx2*^{-/-};*pbx4*^{-/-} mutant embryos, thereby removing the maternal contribution of *pbx2* expression (Waskiewicz et al., 2002) in addition to the zygotic functions of *pbx2* and *pbx4*. We find that *mzpbx2*^{-/-};*pbx4*^{-/-} embryos almost completely lack *krox20* expression in the hindbrain (Fig. 1), as expected for zebrafish embryos lacking Pbx proteins (Waskiewicz et al., 2002). We examined fast and slow muscle development in *mzpbx2*^{-/-};*pbx4*^{-/-} embryos using RNA in situ hybridization (Fig. 1). As controls, we simultaneously collected embryos from incrosses of *pbx4*^{+/-} fish. We observe that the fast muscle genes *atp2a1*, *myl1*, and *mylpfa*, as well as *myog*, show reduced expression in *mzpbx2*^{-/-};*pbx4*^{-/-} embryos compared to somite-stage matched controls (Fig. 1). Trunk skeletal muscle expression of the slow muscle gene *smyhc1* appears largely unaffected in *mzpbx2*^{-/-};*pbx4*^{-/-} embryos (Fig. 1). These results corroborate our previously described findings in *pbx2*-MO;*pbx4*-MO embryos (Maves et al., 2007; Yao et al., 2013). We observed no detectable effect on muscle development in *mzpbx2*^{-/-} embryos and only subtle, variable effects on fast muscle gene expression in *pbx4*^{-/-} embryos (data not shown). These results confirm that Pbx proteins are needed for the proper activation of fast muscle differentiation genes in zebrafish embryogenesis.

Loss of Pbx1 has little effect on early mouse muscle differentiation

In mouse, previous studies have shown that *Pbx1* is expressed broadly in embryonic somites and is required for multiple developmental programs (Capellini et al., 2006; Selleri et al., 2001; Selleri et al., 2019). To investigate the requirements for Pbx factors in mouse muscle differentiation, we examined mouse embryos lacking Pbx1 (*Pbx1*^{-/-}; Selleri et al., 2001). Using qRT-PCR, we find no significant effects on muscle fiber-type markers in E9.5 *Pbx1*^{-/-} embryos (Fig. 2A). In E11.5 *Pbx1*^{-/-} embryos, we find downregulation of *Myog* expression and upregulation of the slow muscle gene *Myh7*, but no significant changes in the other muscle differentiation genes (Fig. 2B). Using RNA in situ hybridization, we observe some downregulation of *Myog* expression in *Pbx1*^{-/-} embryos at E11.5, consistent with our qRT-PCR results (Fig. 2C-D). These results suggest that loss of Pbx1 alone has subtle effects on early mouse muscle differentiation.

Mouse Pbx1 and Pbx2 are both expressed in embryonic skeletal muscle precursors

Previous studies have shown that both *Pbx1* and *Pbx2* mRNAs are expressed broadly in mouse somites at E8.5 (Capellini et al., 2006). We thus wanted to determine whether Pbx1 and Pbx2 proteins are expressed in mouse embryo somites, which contain the primary myotome, the precursors of early mouse muscle (Biressi et al., 2007). Using antibody staining, we observe broad somitic expression of Pbx1 and Pbx2 at E11.5, (Fig. 3A,C), and they both show nuclear localization (Fig. 3B,D). Pbx1 and Pbx2 show overlap with Myf5 and Myod1 (Fig. 3A-D), which both mark myogenic cells in the somites (Comai et al., 2014; Relaix et al., 2005). Thus, both Pbx1 and Pbx2 are expressed in embryonic mouse myogenic progenitors.

Conditional loss of Pbx1 and Pbx2 reveals requirements for mouse Pbx proteins in early fast muscle differentiation

Because several studies have shown that Pbx1 and Pbx2 have compensatory and overlapping functions (Capellini et al., 2006; Ferretti et al., 2011; Koss et al., 2012), we next wanted to address requirements for both Pbx1 and Pbx2 in early mouse muscle differentiation. *Pbx2*^{-/-} mice are viable (Selleri et al., 2004). *Pbx1*^{-/-};*Pbx2*^{-/-} compound constitutive mutant embryos have multiple organogenesis defects and die by E10.5 (Capellini et al., 2006), precluding an examination of early muscle differentiation. Therefore, we decided to examine conditional loss of *Pbx1* on a *Pbx2*^{-/-} background (Ferretti et al., 2011; Koss et al., 2012). We used the *Myf5*-*Cre* deleter strain (Gensch et al., 2008; Tallquist et al., 2000) in combination with the conditional *Pbx1* allele (*Pbx1*^{fl/fl}; Koss et al., 2012) to remove Pbx1 from early myogenic cells. In *Myf5*^{Cre/+};*Pbx1*^{fl/fl};*Pbx2*^{-/-} embryos, we used antibody staining to show loss of Pbx1 in Myf5-expressing cells in the somites (Fig. 3E-G), confirming efficient Cre-mediated inactivation of Pbx1. We find that *Myf5*^{Cre/+};*Pbx1*^{fl/fl} mice are viable and exhibit kyphosis as adults (data not shown), indicating a possible muscle weakness phenotype. We crossed *Myf5*^{Cre/+};*Pbx1*^{fl/fl} mice with the *Pbx2*^{-/-} line to remove both Pbx1 and Pbx2 from muscle precursors. In crosses of *Pbx1*^{fl/fl};*Pbx2*^{-/-} mice with *Myf5*^{Cre/+};*Pbx1*^{fl/+};*Pbx2*^{-/-} mice, we observe expected Mendelian ratios for conditional double mutant *Myf5*^{Cre/+};*Pbx1*^{fl/fl};*Pbx2*^{-/-} embryos at E11.5 and E17.5, but we obtain negligible numbers of *Myf5*^{Cre/+};*Pbx1*^{fl/fl};*Pbx2*^{-/-} pups at postnatal day 7 (Table 2). From multiple litters, we were able to identify 3 dead P0-P1 pups in the cage bedding, that, upon genotyping were *Myf5*^{Cre/+};*Pbx1*^{fl/fl};*Pbx2*^{-/-}. These results indicate that *Myf5*^{Cre/+};*Pbx1*^{fl/fl};*Pbx2*^{-/-} mutant animals likely die at or just after birth.

To determine the effects of conditional loss of Pbx1 and Pbx2 on early muscle differentiation, we collected RNA from whole *Myf5^{Cre/+};Pbx1^{fl/fl};Pbx2^{-/-}* embryos and their sibling controls at E11.5 and performed RNA-seq. Using the cut-offs of 1.2 fold change (FC) and $P < 0.05$, we identified 229 genes down-regulated and 144 genes up-regulated in *Myf5^{Cre/+};Pbx1^{fl/fl};Pbx2^{-/-}* mutants (Supplementary Tables S1-S2). DAVID bioinformatics analysis (Huang et al., 2009) revealed significant enrichment of multiple functional annotation terms related to muscle development and differentiation in the genes down-regulated in *Myf5^{Cre/+};Pbx1^{fl/fl};Pbx2^{-/-}* mutant embryos (Fig. 4A). For the up-regulated gene set, DAVID analysis did not identify any functional annotation terms with significant enrichment following multiple testing correction (not shown). Within the gene set showing downregulation in *Myf5^{Cre/+};Pbx1^{fl/fl};Pbx2^{-/-}* embryos (Table S1), we noticed the presence of genes associated with fast-twitch muscle differentiation, including genes that we showed to be Pbx-dependent in zebrafish, such as *Mylpf* (Fig. 1; Maves et al., 2007; Yao et al., 2013). We directly examined muscle fiber-type-associated genes (Schiaffino and Reggiani, 2011) in the RNA-seq data and found 11/13 fast muscle genes with significant down-regulation ($FC > 1.2$, $P > 0.05$) in *Myf5^{Cre/+};Pbx1^{fl/fl};Pbx2^{-/-}* embryos, whereas 9/14 slow muscle genes showed either up-regulation or no significant change (Fig. 4B). We used qRT-PCR to validate some of these genes on an additional set of *Myf5^{Cre/+};Pbx1^{fl/fl};Pbx2^{-/-}* embryos and their control siblings and found that, indeed, fast muscle genes showed significant down-regulation, whereas slow muscle genes generally are unaffected (Fig. 4C).

We next validated the results from our RNA-seq and qRT-PCR analyses by performing RNA in situ hybridization experiments. We tested *Myog* and some of the top down-regulated fast muscle genes (*Tnnc2*, *Myh3*, *Atp2a1*, *Myf1*, and *Mylpf*) by RNA in situ at E11.5 (Fig. 5). We

observe that these fast muscle genes show reduced expression in *Myf5^{Cre/+};Pbx1^{fl/fl};Pbx2^{-/-}* embryos compared to somite-stage matched control siblings (Fig. 5). Slow muscle genes *Myl4*, *Myh7*, and *Myh6* appear largely unaffected in *Myf5^{Cre/+};Pbx1^{fl/fl};Pbx2^{-/-}* embryos at E11.5 (Fig. 5). In addition, *Myh3*, *Myl4*, *Myh7*, and *Myh6* have strong heart expression that appears unaffected in *Myf5^{Cre/+};Pbx1^{fl/fl};Pbx2^{-/-}* embryos (Fig. 5). These findings are consistent with our RNA-seq and qRT-PCR analyses and establish that Pbx proteins are needed for the proper activation of a program of fast muscle differentiation genes in mouse embryogenesis. Taken together, these results confirm that Pbx proteins are needed for the proper activation of a program of fast muscle differentiation genes in both mouse and zebrafish embryogenesis.

Discussion

Here we show evolutionary conservation of requirements for Pbx proteins in mammalian and zebrafish embryonic fast-twitch muscle differentiation. These results establish that Pbx homeodomain proteins have conserved roles in the control of the activation of fast muscle differentiation genes in different species.

Because of the redundancy of, and early requirements for, Pbx proteins in mouse embryogenesis (reviewed in Selleri et al. 2019), we needed to make use of conditional loss-of-function in *Myf5^{Cre/+};Pbx1^{fl/fl};Pbx2^{-/-}* compound mutants. A similar approach, using different conditional *Cre* lines, has previously been taken to study Pbx functions in craniofacial, spleen, and spinal cord motor neuron development and in adult neurogenesis (Ferretti et al., 2011; Grebbin et al., 2016; Hanley et al., 2016; Koss et al., 2012; Losa et al., 2018; Welsh et al., 2018). Based on their expression patterns, *Pbx1* and *Pbx2* are the *Pbx* genes most likely to be involved

in mouse embryonic skeletal muscle development (Capellini et al., 2006; Di Giacomo et al., 2006; Wagner et al., 2001). Future studies could additionally include loss of *Pbx3* (Rhee et al., 2004) to determine whether this Pbx family member also functions in muscle development and fiber-type differentiation.

We chose the *Myf5-Cre* line to conditionally knock out *Pbx1* in early skeletal muscle development because *Myf5* is expressed early in developing somites in the presumptive primary myotome, prior to *Myod1* expression (Kassar-Duchossoy et al., 2004; Ott et al., 1991). The *Myf5-Cre* line we employed has been previously used to examine satellite cells in limb and extraocular muscles, to ablate embryonic myogenic cells, and to conditionally ablate genes in skeletal muscle (Comai et al., 2014; Gensch et al., 2008; Greschik et al., 2017; Kuang et al., 2007; Stuelsatz et al., 2014). We note that the *Myf5-Cre* allele may not be effecting a full deletion of *Pbx1* in all myoblasts (Fig. 3; Comai et al., 2014), possibly explaining our finding that slow muscle gene *Myh7* is upregulated in *Pbx1*^{-/-} mouse embryos (Fig. 2B) but does not appear affected in *Myf5*^{Cre/+}; *Pbx1*^{fl/fl}; *Pbx2*^{-/-} embryos (Fig. 4C). However, we observe similar defects in the activation of fast muscle differentiation following both conditional inactivation of *Pbx1* and *Pbx2* in mouse embryos and whole-animal *mzpbx2;pbx4* loss-of-function in zebrafish embryos. These results indicate that the *Myf5-Cre* line is enabling adequate *Pbx* gene inactivation, leading to a deeper understanding of the function of this family of homeodomain proteins in muscle development in different species.

The defects in activation of fast muscle differentiation genes that we observe in *Myf5*^{Cre/+}; *Pbx1*^{fl/fl}; *Pbx2*^{-/-} mouse embryos are similar to those observed in *Six1*; *Six4* double mutant mouse embryos (Niro et al., 2010). In *Six1*^{-/-}; *Six4*^{-/-} mouse embryos, activation of fast muscle genes, including *Atp2a1*, *Myll*, and *Tnnc2*, is inhibited, while slow muscle genes, such as

Myl4, are still expressed (Niro et al., 2010). Six1 and Six4 proteins directly bind regulatory regions of fast muscle genes (Niro et al., 2010). In future studies, it will be of interest to examine potential genetic and biochemical interactions between Pbx proteins and Six proteins during muscle development. In addition, such further studies, using both zebrafish and mice, could help advance our understanding of the conserved and non-conserved roles for Pbx and Six factors in muscle development and fiber-type differentiation during vertebrate evolution.

In conclusion, we provide evidence for evolutionarily conserved regulation of embryonic fast muscle differentiation by Pbx factors. In future studies, we plan to determine the requirements for Pbx factors in later stages of muscle development and fiber-type differentiation in both zebrafish and mice. In addition, we plan to take advantage of our zebrafish and mouse mutant strains to examine, in developing embryos, the Pbx-dependent chromatin events in myogenesis and muscle differentiation. The goal will be to determine whether Pbx proteins are needed during skeletal muscle development to open chromatin, or to promote Myod1 binding or Myod1 activity. Ultimately, these studies will help us understand how broadly-expressed factors like Pbx help master regulatory factors like Myod1 activate their transcriptional targets in a temporally and spatially controlled manner to drive cell-type-specific differentiation programs in different species.

Acknowledgements

We thank Peter Currie, Zhiyuan Gong, Phil Ingham, Heike Pöpperl, and Phil Soriano for generously providing reagents. The Zebrafish International Resource Center (supported by grants RR12546 and RR15402-01 from the NIH) provided cDNA clones. We thank the FHCRC

Genomics Shared Resource for excellent technical assistance with the RNA-seq experiments. We thank the SCRI Aquatics Facility for zebrafish care. This work was supported by NIH 1R03AR057477 (L.M.), funded in part by the American Recovery and Reinvestment Act, NIH 1R03AR065760 (L.M.), the Seattle Children’s Myocardial Regeneration Initiative, by NIH 1R01AR45113 (S.J.T.), and by NIH RO1 DE024745 (L.S.).

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Tables

Table 1 Primers used

Gene (Z-zebrafish; M-mouse)	Primer Sequence (Forward 5'-3'; Reverse 5'-3')
Animal genotyping	
<i>Pbx2^{scm10}</i> (Z)	CCATGAGAAATGTTTCAGGAGGAG; TCGCAGCAGATACTGCCGCTGCCGCTCCGCCACCTTT
<i>Pbx4^{b557}</i> (Z)	ACTCGGCGGACTCTCGCAAGC; GGCTCTCGTCGGTGATGGCCATGATCT
<i>Pbx1</i> (M)	TGAGTATTCGGGGAGCCCAAGAA; CATGATGCCCAGTCTGTAGGGGT
<i>Pbx1</i> neo (M)	CTATCAGGACATAGCGTTGG
<i>Pbx1^{fl}</i> (M)	ATCAGTGCCATTTCTCCAGCC; TGGGTATCCATTCCAAAGGTGG
<i>Pbx2</i> (M)	TCCTTAAGTCTGTTTGCCTCGCTG; CTGCTCATACTTCTCCAGCTCTGA
<i>Pbx2</i> neo (M)	CTATCAGGACATAGCGTTGG
<i>Myf5^{Cre}</i> (M)	CGTAGACGCCTGAAGAAGGTCAAC; CACATTAGAAAACCTGCCAACACC
<i>Myf5^{Cre}</i> lox (M)	ACGAAGTTATTAGGTCCCTCGAC
RNA in situ probes	
<i>Tnnc2</i> (M)	CAACCATGACGGACCAACAG; TCCAGTCACAGAGCCCAAAG
<i>Atp2a1</i> (M)	TGGTGTGTGGGCATGTTGG; ACACGGTTCAAAGACATGGAGG
<i>Myh6</i> (M)	AGCTGGAGAAGATCCGCAA; AGAGGTTATTCTCGTCGTGC
<i>Myh3</i> (M)	AGCCACTGGGGACCTTGCCA; GCAGGCTGCCAGGTCCATCC
<i>Myl1</i> (M)	GGGTCCACCCTCCAGACGCT; GCCAGTCTTCCCCAACATTGCGT
<i>Mylpf</i> (M)	GGAGATCTAAGACATGGCAC; GTTTTATTGAGGGCCAGGGT
<i>Myl4</i> (M)	GTCCACCTCCACTGGAGAGC; AGCTGGGGCTCTTTATTTCC
<i>Myh7</i> (M)	CAGGCCCAGTCCCAGAGGTGT; TCCCGGGACAGGTCCAGAGCG
qRT-PCR	
<i>Odc1</i> (M)	TTGCCACTGATGATTCCAAA; CATGGAAGCTCACACCAATG
<i>Pbx1</i> (M)	CTCATGCGACTGGACAACAT; TCGGAATGCTCCACTGAGTT
<i>Myog</i> (M)	ATCTCCGCTACAGAGGCGGG; GGTGAGCCGCGAGCAAATGA
<i>Myh3</i> (M)	GGATGCCTGCAGGGACGCAA; GCGCTGGTGTCTGCTCCTT
<i>Mylpf</i> (M)	GGCTGCCGGGGCAGGACTAT; CGCTCCTGCCCTTCTCTTGGC
<i>Myl1</i> (M)	GCAACAGGAGGACTTCAAGGAGGC; CCCGGAGGACGTCTCCCACC
<i>Myl4</i> (M)	AGCGAGGCAGAGGTGGAGCA; TCTCACGCAGGGCCAAGCAC
<i>Myh7</i> (M)	GAGCAAGGCCGAGGAGACGC; TGCCGGGACAGCTCCCCATT
<i>Tnnc2</i> (M)	GGAGTGCGGAGGAGACAACC; ACCGCCATCAGCATCGAACA
<i>Atp2a1</i> (M)	CCAATGAGCTCCCTGCTGAGG; GCAGTGACGGTTTCTTCGCC

Table 2 Offspring of *Pbx1^{fl/fl};Pbx2^{-/-}* X *Myf5^{Cre/+};Pbx1^{fl/+};Pbx2^{-/-}* Mice

Genotype	Frequencies at E11.5			Frequencies at E17.5			Frequencies at P7		
	Observed	Expected	P	Observed	Expected	P	Observed	Expected	P
<i>Myf5Cre/+;</i> <i>Pbx1fl/fl;Pbx2-/-</i>	25	22.25	0.902	7	12	0.227	2	20.25	4.335 E-05
<i>Myf5Cre/+;</i> <i>Pbx1fl/+;Pbx2-/-</i>	20	22.25		13	12		29	20.25	
<i>Pbx1fl/fl;Pbx2-/-</i>	22	22.25		17	12		27	20.25	
<i>Pbx1fl/+;Pbx2-/-</i>	22	22.25		11	12		23	20.25	

P values calculated using a Chi-square test.

Figure Legends

Fig. 1. Fast muscle differentiation genes are downregulated in *mzpbx2*^{-/-};*pbx4*^{-/-} zebrafish embryos relative to controls at 18 somite stage/18 hours post fertilization (hpf). (A-D) Whole mount RNA in situ hybridizations in 18 hpf embryos. Top panels of each set show control *pbx2*^{+/+};*pbx4*^{+/+} embryos. Bottom panels of each set show somite-stage matched *mzpbx2*^{-/-};*pbx4*^{-/-} embryos. Lateral views show anterior to the left and dorsal at the top. N=10-16 control and 3-5 mutant embryos for each condition. (A) Arrow in A points to reduced *myog* expression in *mzpbx2*^{-/-};*pbx4*^{-/-} embryo. (B-D) *atp2a1*, *myl1*, and *mylpfa* appear generally reduced in *mzpbx2*^{-/-};*pbx4*^{-/-} embryos. *krox20* is expressed in hindbrain rhombomeres 3 and 5 (r3, r5 in A) and is downregulated in *mzpbx2*^{-/-};*pbx4*^{-/-} embryos. *smyhcl* expression in the developing heart (arrowhead in A) is downregulated in *mzpbx2*^{-/-};*pbx4*^{-/-} embryos.

Fig. 2. Loss of Pbx1 has little effect on early mouse muscle differentiation. (A-B) qRT-PCR analysis of muscle gene expression in (A) E9.5 and (B) E11.5 *Pbx1*^{-/-} embryos relative to control *Pbx1*^{+/+} embryos. *Myh3*, *Mylpf*, and *Myf1* are fast muscle genes. *Myf4* and *Myf7* are slow muscle genes. *Pbx1* expression is included as a control gene. Expression levels are normalized to the gene *Odc1*. For each gene, the expression level in *Pbx1*^{+/+} embryos is set to 1 (dashed line in graphs). Box plots show the distribution of the normalized expression level ($2^{-\Delta\Delta C_t}$) of four control/mutant sibling pairs for each gene. The boxes extend from the 25th to 75th percentiles, the whiskers are at the minimum and maximum, the bar within the box represents the median, and the cross represents the mean. Statistics are described in Materials and Methods. * P<0.02,

** $P < 0.004$, *** $P < 0.0008$, **** $P < 0.0001$. (C-D) Whole mount *Myog* RNA in situ hybridization in (C) *Pbx1*^{+/+} and (D) *Pbx1*^{-/-} E11.5 embryos. N=3.

Fig. 3. *Pbx1* and *Pbx2* expression in mouse embryo somites. (A-B) E11.5 mouse embryo whole mount stained with anti-*Pbx1b* and anti-Myf5. A, low magnification, scale bar=20 μ m. B, high magnification of A, scale bar=20 μ m. (C-D) E11.5 mouse embryo sagittal section stained for anti-*Pbx2* and anti-Myod1. C, low magnification, scale bar=10 μ m. D, high magnification of C, scale bar=10 μ m. (E-F) E11.5 *Myf5*^{Cre/+};*Pbx1*^{fl/fl} embryo stained with anti-*Pbx1b* and anti-Myf5. E, low magnification, scale bar as in A. F, high magnification of E, scale bar as in B. Rostral is to the left. (G) Quantification of Myf5 and *Pbx1b* co-expressing cells (Myf5+*Pbx1*+) in E11.5 control embryos and *Myf5*^{Cre/+};*Pbx1*^{fl/fl};*Pbx2*^{-/-} mutant embryos.

Fig. 4. *Pbx1* and *Pbx2* are needed for mouse embryonic fast muscle gene expression. (A) Functional annotation terms associated with genes downregulated (Table S1) in E11.5 *Myf5*^{Cre/+};*Pbx1*^{fl/fl};*Pbx2*^{-/-} embryos relative to *Pbx1*^{fl/+};*Pbx2*^{-/-} control siblings. Numbers next to each bar indicate number of genes associated with each term. (B) Muscle fiber type-associated gene expression from RNA-seq in mutant *Myf5*^{Cre/+};*Pbx1*^{fl/fl};*Pbx2*^{-/-} E11.5 embryos relative to *Pbx1*^{fl/+};*Pbx2*^{-/-} control siblings. Genes showing significant changes are in bold. Color bar legend illustrates range of fold changes. (C) qRT-PCR analysis of muscle gene expression in E11.5 *Myf5*^{Cre/+};*Pbx1*^{fl/fl};*Pbx2*^{-/-} embryos relative to control siblings. Control siblings consisted of *Pbx1*^{fl/+};*Pbx2*^{-/-} (N=1) or *Myf5*^{Cre/+};*Pbx1*^{fl/+};*Pbx2*^{-/-} (N=4) embryos. *Tnnc2*, *Myh3*, *Atp2a1*, *Myl1* and *Mylpf* are fast muscle genes. *Myl4* and *Myh7* are slow muscle genes. Expression levels are normalized to the gene *Odc1*. For each gene, the expression level in control sibling embryos is

set to 1 (dashed line). Box plots show the distribution of the normalized expression level ($2^{-\Delta\Delta C_t}$) of five control/mutant sibling pairs for each gene. The boxes extend from the 25th to 75th percentiles, the whiskers are at the minimum and maximum, the bar within the box represents the median, and the cross represents the mean. Statistics are described in Materials and Methods. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.003$.

Fig. 5. Fast muscle differentiation genes are downregulated in *Myf5^{Cre/+};Pbx1^{fl/fl};Pbx2^{-/-}* embryos relative to control siblings at E11.5. (A-I) Whole mount RNA in situ hybridizations in E11.5 embryos. Left column of each panel shows control embryos, and right column of each panel shows somite-stage matched *Myf5^{Cre/+};Pbx1^{fl/fl};Pbx2^{-/-}* embryos. The pictured control embryos for *Myog*, *Tnnc2*, *Myh3*, *Mylpf*, *Myl4*, *Myh7*, and *Myh6* are *Myf5^{Cre/+};Pbx1^{fl/+};Pbx2^{-/-}*. The pictured control embryo for *Atp2a1* is *Pbx1^{fl/fl};Pbx2^{-/-}*, and the control for *Myl1* is *Pbx1^{fl/+};Pbx2^{-/-}*. No differences in gene expression were noted among controls of different genotypes. Insets show 2X zoom views at the level of somite 17 (* in each panel). N=3-5 control and 3-5 mutant embryos for each condition. *Myh3*, *Myl4*, *Myh7*, and *Myh6* have strong heart expression (h in C) that appears unaffected in *Myf5^{Cre/+};Pbx1^{fl/fl};Pbx2^{-/-}* embryos.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Supplemental Figure 1

Supplemental Fig. 1. Generation of a zebrafish *pbx2* mutant allele with CRISPR/Cas9. (A) Top line: a portion of the wild-type (WT) genomic sequence of *pbx2* from the middle of Exon 3 showing the target sequence for the single-guide RNA (bold, underline) and the PAM sequence (green, bold). Bottom line: the sequence of the *pbx2^{scm10}* allele with a single “A” nucleotide (highlighted) inserted 3bp upstream of the PAM. (B) Alignment of the conceptual translation of the *pbx2^{scm10}* allele compared to wild-type Pbx2. Mis-sense amino acids are in lower case and the premature STOP codon is noted with an asterisk. The PBC-A and -B domains are conserved among the Pbx family. (C) Cartoon showing the domain structure of Pbx2. The scm10 mutation is predicted to produce a truncated product lacking the conserved PBC-B and Homeobox domains and thus is likely to be a loss of function allele.

Supplemental Table 1

Table S1 Genes downregulated in *Myf5^{Cre/+};Pbx1^{fl/fl};Pbx2^{-/-}* E11.5 embryos (FC>1.2, P<0.05)

Ensembl Gene ID	Gene Symbol	log2FoldChange	PValue
ENSMUSG00000059634	Gm11942	1.997625366	0.00281949
ENSMUSG00000016327	Atp1b4	1.251227808	1.02E-06
ENSMUSG00000073489	Ifi204	1.124550446	0.00015198
ENSMUSG00000073643	Wdfy1	1.123038354	4.92E-06
ENSMUSG00000074634	Gm7120	1.119924629	0.00116979
ENSMUSG00000021758	Ddx4	1.102087178	0.00053919
ENSMUSG00000006542	Prkag3	0.973063584	0.00075995
ENSMUSG00000019933	2310015B20Rik	0.926526652	0.00014017
ENSMUSG00000017300	Tnnc2	0.903462414	7.97E-19
ENSMUSG00000024578	Il17b	0.900941787	9.94E-09
ENSMUSG00000085494	3110045C21Rik	0.888868424	0.00015497
ENSMUSG00000025488	Cox8b	0.881086172	0.00501959
ENSMUSG00000025610	ORF63	0.87635424	0.00059409
ENSMUSG00000087692	Gm11940	0.875068601	0.00995758
ENSMUSG00000033044	Dhrs7c	0.83950002	0.00038984
ENSMUSG00000020429	Igfbp1	0.83784793	1.94E-06
ENSMUSG00000031097	Tnni2	0.819467555	1.04E-09
ENSMUSG00000042828	Trim72	0.817775515	2.70E-09
ENSMUSG00000074635	3110070M22Rik	0.766527767	0.03872035
ENSMUSG00000062345	Serpinb2	0.760078317	0.01888044
ENSMUSG00000057280	Musk	0.756434002	1.17E-06
ENSMUSG00000086866	4930512H18Rik	0.753218651	0.00156951
ENSMUSG00000030730	Atp2a1	0.748005332	8.00E-21
ENSMUSG00000085614	1700123M08Rik	0.719703582	0.02952474
ENSMUSG00000020722	Cacng1	0.701568796	0.00014105
ENSMUSG00000070645	Ren1	0.695455825	0.00644048
ENSMUSG00000042895	Abra	0.693791851	0.02726867
ENSMUSG00000001508	Sgca	0.687273872	0.00187296
ENSMUSG00000062077	Trim54	0.68587758	0.00055611
ENSMUSG00000033313	Fbxl8	0.685161621	0.00346938
ENSMUSG00000042045	Sln	0.672438007	3.16E-07
ENSMUSG00000026459	Myog	0.671548455	3.52E-31
ENSMUSG00000042451	Mybph	0.663330345	3.13E-21
ENSMUSG00000079363	Gbp4	0.658339619	0.00629997
ENSMUSG00000025701	Alox5	0.657609781	0.01013828
ENSMUSG00000090045	Tmem229a	0.655849126	0.02848229
ENSMUSG00000026574	Dpt	0.653574052	0.00189961
ENSMUSG00000040533	Matn1	0.640553476	0.00617057
ENSMUSG00000028584	Lrrc38	0.639594229	0.04050359
ENSMUSG00000022479	Vdr	0.637646441	0.00893736

ENSMUSG00000026725	Tnn	0.634312573	1.33E-06
ENSMUSG000000031461	Myom2	0.628934127	8.06E-09
ENSMUSG000000034555	Tex16	0.628500694	0.01715763
ENSMUSG000000048442	2210020M01Rik	0.625970394	0.00461295
ENSMUSG000000042677	Zc3h12a	0.623725016	0.01930386
ENSMUSG000000025081	Tdrd1	0.618142031	9.79E-05
ENSMUSG000000070072	Mir433	0.616452103	0.04237151
ENSMUSG000000044938	Klhl31	0.616401756	1.16E-05
ENSMUSG000000004038	Gstm3	0.61330437	0.0094936
ENSMUSG000000002104	Rapsn	0.609688932	4.59E-07
ENSMUSG000000071540	3425401B19Rik	0.604462204	0.00091145
ENSMUSG000000055489	Ano5	0.597258767	0.00092375
ENSMUSG000000038086	Hspb2	0.589741021	2.88E-05
ENSMUSG000000039824	Myl6b	0.586596978	1.88E-08
ENSMUSG000000020908	Myh3	0.582859624	3.85E-12
ENSMUSG000000002007	Srpk3	0.582406793	4.87E-05
ENSMUSG000000021211	Akr1c12	0.580411755	0.02404366
ENSMUSG000000032860	P2ry2	0.572441945	0.01732682
ENSMUSG000000021702	Thbs4	0.566751503	8.09E-15
ENSMUSG000000005681	Apoa2	0.565319962	6.79E-10
ENSMUSG000000021680	Crhbp	0.565076794	0.01781884
ENSMUSG000000031074	Fgf3	0.561368745	0.03998839
ENSMUSG000000058914	C1qtnf3	0.560414393	6.26E-08
ENSMUSG000000001260	Gabrg1	0.560085266	0.01823623
ENSMUSG000000055994	Nod2	0.558336216	0.03278935
ENSMUSG000000020583	Matn3	0.555032035	0.00975114
ENSMUSG000000000435	Myf5	0.554993541	0.00097489
ENSMUSG000000026100	Mstn	0.554436286	3.49E-05
ENSMUSG000000044086	Lmod3	0.553440981	3.07E-05
ENSMUSG000000031932	Gpr83	0.550355575	0.01823693
ENSMUSG000000053063	Clec12a	0.550353594	0.02898404
ENSMUSG000000024471	Myot	0.54995695	3.09E-05
ENSMUSG000000009214	Tmem8c	0.544022249	9.25E-08
ENSMUSG000000049565	Aknad1	0.54361441	0.02271281
ENSMUSG000000058715	Fcer1g	0.542167393	0.00021779
ENSMUSG000000026971	Itgb6	0.53923265	0.00141368
ENSMUSG000000030672	Mylpf	0.538601704	7.15E-28
ENSMUSG000000028414	Fktn	0.533896702	2.46E-08
ENSMUSG000000074882	Cyp2c68	0.529203976	0.00604621
ENSMUSG000000079173	Zan	0.526269092	0.03147969
ENSMUSG000000038354	Ankrd35	0.521552035	0.02320364
ENSMUSG000000022215	Fitm1	0.52050739	0.00029212
ENSMUSG000000045589	6430704M03Rik	0.511679318	2.21E-05
ENSMUSG000000039376	Synpo2l	0.511592176	2.50E-12
ENSMUSG000000026950	Neb	0.509892859	8.69E-08
ENSMUSG000000045404	Kcnk13	0.508874051	0.00519331

ENSMUSG00000031972	Acta1	0.50797014	4.86E-16
ENSMUSG00000064179	Tnnt1	0.504897667	8.17E-20
ENSMUSG00000062515	Fabp4	0.502155826	0.01847878
ENSMUSG00000073490	Al607873	0.498061198	0.0179164
ENSMUSG00000041828	Abca8a	0.497036606	0.003378
ENSMUSG00000045259	Klhdc9	0.496090908	0.00044735
ENSMUSG00000028758	Kif17	0.495159619	0.04926557
ENSMUSG00000059775	Rps26-ps1	0.490654785	0.00238278
ENSMUSG00000007682	Dio2	0.488216039	0.01209769
ENSMUSG00000028396	2310002L09Rik	0.484231204	0.01232068
ENSMUSG00000031962	Cdh15	0.477951759	7.25E-08
ENSMUSG00000035606	Ky	0.475709302	0.00504494
ENSMUSG00000042429	Adora1	0.474981096	5.98E-07
ENSMUSG00000055775	Myh8	0.473679617	2.71E-11
ENSMUSG00000084897	Gm14226	0.469395994	0.02938826
ENSMUSG00000036855	Gjd4	0.469300148	0.01647953
ENSMUSG00000074001	Kbtbd5	0.467147461	0.00015936
ENSMUSG00000078521	2610002D18Rik	0.462381336	0.04991346
ENSMUSG00000027861	Casq2	0.462242954	1.43E-08
ENSMUSG00000060180	Myh13	0.459267373	0.00596266
ENSMUSG00000036214	Znrd1as	0.452895392	0.01072842
ENSMUSG00000020787	P2rx1	0.452229276	0.01509239
ENSMUSG00000086327	1700003F17Rik	0.451494344	0.01304189
ENSMUSG00000025450	Gm9752	0.448148532	0.02543087
ENSMUSG00000020067	Mypn	0.447078889	0.00166056
ENSMUSG00000047631	Apof	0.444096313	0.02441692
ENSMUSG00000075307	Kbtbd10	0.443384263	5.80E-07
ENSMUSG00000041556	Fbxo2	0.442022852	0.03041434
ENSMUSG00000037086	1110059M19Rik	0.439579622	0.00588576
ENSMUSG00000085236	2610206C17Rik	0.435065022	0.02246406
ENSMUSG00000023993	Trem1	0.433037879	0.01750325
ENSMUSG00000062822	4833420G17Rik	0.432153462	0.00041592
ENSMUSG00000038403	Hfe2	0.430554005	0.00018901
ENSMUSG00000061816	Myl1	0.429046152	2.41E-15
ENSMUSG00000040287	Stac3	0.42305648	0.00195412
ENSMUSG00000019936	Epyc	0.422547599	0.00393017
ENSMUSG00000018920	Cxcl16	0.421573156	0.0159116
ENSMUSG00000043687	1190005I06Rik	0.421154892	0.02896011
ENSMUSG00000006403	Adamts4	0.417012512	0.00010599
ENSMUSG00000043789	Vwce	0.416472145	0.01681858
ENSMUSG00000026390	Marco	0.416332664	0.01417638
ENSMUSG00000000290	Itgb2	0.415374413	0.01923706
ENSMUSG00000052374	Actn2	0.415039379	7.07E-05
ENSMUSG00000031312	Itgb1bp2	0.410967671	0.0004314
ENSMUSG00000057606	Colq	0.407707638	0.03184891
ENSMUSG00000042485	Mustn1	0.40547905	0.00715716

ENSMUSG00000047011	Mtap4	0.405248997	0.0176333
ENSMUSG000000061723	Tnnt3	0.405037407	0.00025145
ENSMUSG000000031618	Nr3c2	0.401968702	0.01138455
ENSMUSG000000021200	Asb2	0.401899298	0.00062097
ENSMUSG000000026418	Tnni1	0.399544026	2.95E-13
ENSMUSG000000031765	Mt1	0.396005941	6.60E-12
ENSMUSG000000028417	Tal2	0.393486658	0.02103196
ENSMUSG000000051747	Ttn	0.393208884	0.00015848
ENSMUSG000000024887	Asah2	0.391290823	0.00251144
ENSMUSG000000027188	Pamr1	0.390065372	3.59E-05
ENSMUSG000000025229	Pitx3	0.38984228	0.00477099
ENSMUSG000000060791	Gmfg	0.389490721	0.02270391
ENSMUSG000000020216	Jsrp1	0.38913719	0.03574297
ENSMUSG000000028470	Hint2	0.388875622	0.00196966
ENSMUSG000000079110	Capn3	0.388867779	0.03968014
ENSMUSG000000040694	Apobec2	0.38794292	0.00061565
ENSMUSG000000028116	Myoz2	0.387903641	0.00293157
ENSMUSG000000086566	2810429I04Rik	0.387281461	0.01446724
ENSMUSG000000029372	Ppbp	0.386538655	0.00688941
ENSMUSG000000001334	Fndc5	0.385176976	7.31E-08
ENSMUSG000000086741	Gm15816	0.384866487	0.00192303
ENSMUSG000000038763	Alpk3	0.383775593	0.00091421
ENSMUSG000000031762	Mt2	0.380790014	1.05E-08
ENSMUSG000000010609	Psen2	0.37909022	0.00164963
ENSMUSG000000062329	Cytl1	0.375253939	0.00945664
ENSMUSG000000029925	Tbxas1	0.37494231	0.04030196
ENSMUSG000000021388	Aspn	0.374378291	0.00264657
ENSMUSG000000041189	Chrnbl	0.373828981	0.00568736
ENSMUSG000000030785	Cox6a2	0.369946973	0.00490543
ENSMUSG000000026678	Rgs5	0.368894323	0.0001231
ENSMUSG000000064361	mt-Tr	0.367063972	0.01987099
ENSMUSG000000030607	Acan	0.365270209	0.00602465
ENSMUSG000000036278	Macrod1	0.362187382	0.00858772
ENSMUSG000000030996	Art1	0.357918724	0.04952297
ENSMUSG000000079471	Gm7325	0.356200875	0.00371319
ENSMUSG000000053963	6330403A02Rik	0.355211687	0.00294897
ENSMUSG000000032925	Itgbl1	0.348910538	0.03793757
ENSMUSG000000028464	Tpm2	0.345856658	7.27E-09
ENSMUSG000000027487	Cdk5rap1	0.345253026	0.0072262
ENSMUSG000000027107	Chrna1	0.344822171	0.00128325
ENSMUSG000000037318	Traf3ip3	0.342951693	0.00952413
ENSMUSG000000079428	Tceal7	0.34187202	0.00031241
ENSMUSG000000052305	Hbb-b1	0.340499141	2.47E-06
ENSMUSG000000023067	Cdkn1a	0.338367609	2.87E-06
ENSMUSG000000024330	Col11a2	0.337093452	0.00588635
ENSMUSG000000036249	Rbm43	0.333843039	0.00682483

ENSMUSG00000087107	Al62270	0.333264522	0.03271734
ENSMUSG00000086596	Susd5	0.330333555	0.02086009
ENSMUSG00000044951	Mylk4	0.330267863	0.00592378
ENSMUSG00000027199	Gatm	0.32912307	0.00033889
ENSMUSG00000035296	Sgcg	0.327349369	0.03261574
ENSMUSG00000068122	Agtr2	0.320310535	1.00E-05
ENSMUSG00000025076	Casp7	0.31925688	0.00219929
ENSMUSG00000055027	Smyd1	0.316585047	7.11E-06
ENSMUSG00000004730	Emr1	0.315186362	0.00499263
ENSMUSG00000068614	Actc1	0.313852677	9.43E-07
ENSMUSG00000009246	Trpm5	0.313253164	0.0009709
ENSMUSG00000026407	Cacna1s	0.311114329	0.01526524
ENSMUSG00000073940	Hbb-b2	0.309187296	4.58E-05
ENSMUSG00000067235	H2-Q10	0.309078837	0.02397856
ENSMUSG00000057457	Phex	0.308873813	0.01362792
ENSMUSG00000047910	Pcdhb16	0.307913156	0.03913632
ENSMUSG00000024427	Spry4	0.307038996	0.00012221
ENSMUSG00000043518	Rai2	0.304910395	0.00774812
ENSMUSG00000021798	Ldb3	0.304832892	0.00714162
ENSMUSG00000074818	Pdzd7	0.304509786	0.01792369
ENSMUSG00000060913	Trim55	0.30356309	0.01127983
ENSMUSG00000023885	Thbs2	0.301062714	1.91E-07
ENSMUSG00000090066	1110002E22Rik	0.299868154	0.01615601
ENSMUSG00000048612	Myof	0.297460533	1.38E-06
ENSMUSG00000011148	Adssl1	0.295776045	0.02495573
ENSMUSG00000029084	Cd38	0.294192572	0.01011198
ENSMUSG00000054484	Tmem62	0.293070552	0.04439612
ENSMUSG00000032503	Arpp21	0.290096675	0.00241346
ENSMUSG00000025068	Gsto1	0.286751633	0.00090928
ENSMUSG00000032735	Ablim3	0.285535424	2.73E-06
ENSMUSG00000089902	Gm13625	0.284828783	0.04901378
ENSMUSG00000038239	Hrc	0.284401846	0.02072195
ENSMUSG00000069919	Hba-a1	0.284350875	0.00116311
ENSMUSG00000020475	Pgam2	0.283618949	0.00943925
ENSMUSG00000069917	Hba-a2	0.282161757	0.00129652
ENSMUSG00000025017	Pik3ap1	0.282020849	0.02903027
ENSMUSG00000002992	Apoc2	0.279395317	0.01275751
ENSMUSG00000002944	Cd36	0.279227521	0.02499893
ENSMUSG00000060600	Eno3	0.27803025	0.00058382
ENSMUSG00000020150	Gamt	0.277184632	0.00685728
ENSMUSG00000038132	Rbm24	0.276930268	0.00133199
ENSMUSG00000001493	Meox1	0.275090349	0.00313707
ENSMUSG00000053684	BC048403	0.274289185	0.03833233
ENSMUSG00000022025	Lect1	0.27417929	0.01631824
ENSMUSG00000052160	Pld4	0.271590944	0.03456755
ENSMUSG00000054150	4831426I19Rik	0.271509199	0.04166042

ENSMUSG00000020061	Mybpc1	0.271137725	0.00033988
ENSMUSG000000064349	mt-Tc	0.269482522	0.01920889
ENSMUSG000000049538	Adamts16	0.267382379	0.02498982
ENSMUSG000000043903	Gm22	0.264526387	0.02548835
ENSMUSG000000028736	Pax7	0.263736512	0.01444815

Supplemental Table 2

Table S2 Genes upregulated in *Myf5^{Cre/+};Pbx1^{fl/fl};Pbx2^{-/-}* E11.5 embryos (FC<-1.2, P<0.05)

Ensembl Gene ID	Gene Symbol	log2FoldChange	PValue
ENSMUSG00000032080	Apoa4	-6.306315557	3.17E-14
ENSMUSG00000074373	Gm10680	-5.515828015	1.72E-11
ENSMUSG00000078503	Gm13225	-5.47922862	7.48E-11
ENSMUSG00000040808	S100g	-3.325509795	1.54E-05
ENSMUSG00000025172	Ankrd2	-2.928952639	5.43E-13
ENSMUSG00000062518	Zfp534	-2.538250615	0.00029127
ENSMUSG00000031410	Nxf7	-2.326189513	2.23E-06
ENSMUSG00000064193	Gm13699	-1.862530812	2.50E-05
ENSMUSG00000085715	Tsix	-1.64619603	0.003453398
ENSMUSG00000026726	Cubn	-1.588274999	0.000301274
ENSMUSG00000086503	Xist	-1.42707307	0.00966999
ENSMUSG00000042357	Gjb5	-1.389972315	0.000216672
ENSMUSG00000031098	Syt8	-1.22251762	0.004651279
ENSMUSG00000025400	Tac2	-1.208829904	0.009045378
ENSMUSG00000026535	Ifi202b	-1.190125984	0.00012606
ENSMUSG00000021278	Amn	-1.118509657	0.004797454
ENSMUSG00000034427	Myo15b	-1.117488525	0.002139268
ENSMUSG00000063011	Msln	-1.024260596	0.010304563
ENSMUSG00000048528	Nkx1-2	-1.018518435	0.000299412
ENSMUSG00000048087	Gm4737	-0.98277365	0.012815925
ENSMUSG00000048538	Gm9826	-0.965443669	0.005130603
ENSMUSG00000049908	Gja8	-0.887394669	0.007521134
ENSMUSG00000074000	Gm10606	-0.823043723	0.007204508
ENSMUSG00000029195	Klb	-0.803722238	0.001863233
ENSMUSG00000086219	2410137F16Rik	-0.797311711	0.003548871
ENSMUSG00000073125	Xlr3b	-0.780298726	0.000136367
ENSMUSG00000087130	D230004N17Rik	-0.778579788	0.02928641
ENSMUSG00000070900	Gm10306	-0.76539987	0.002570925
ENSMUSG00000057836	Xlr3a	-0.759084446	0.000858649
ENSMUSG00000075589	Gm11536	-0.741431847	0.019912768
ENSMUSG00000086983	Gm16972	-0.720476111	0.002334959
ENSMUSG00000091344	1700037F24Rik	-0.716684418	0.017844048
ENSMUSG00000036279	Zbtb20	-0.709206915	0.005800701
ENSMUSG00000090300	Gm17119	-0.699302536	0.025911127
ENSMUSG00000087126	1700109K24Rik	-0.695473973	0.015247948
ENSMUSG00000020609	Apob	-0.689822562	0.006034631
ENSMUSG00000087445	Gm14286	-0.682320993	0.006918723
ENSMUSG00000058147	Xlr3c	-0.663193337	0.007801348
ENSMUSG00000025905	Oprk1	-0.662227666	0.034090499

ENSMUSG00000064797	snoZ40	-0.654614859	0.036797891
ENSMUSG00000003354	Ccdc65	-0.645778509	0.019246502
ENSMUSG00000088524	Snord2	-0.630430525	0.041410335
ENSMUSG00000026175	Vil1	-0.612509025	0.024694391
ENSMUSG00000081382	Rpl18-ps1	-0.600675167	0.014347111
ENSMUSG00000026656	Fcgr2b	-0.594487947	0.029671309
ENSMUSG00000013611	Snx31	-0.590706189	0.041840498
ENSMUSG00000078736	Gm2023	-0.586318407	0.015163515
ENSMUSG00000086903	Gm16258	-0.577412052	0.014299005
ENSMUSG00000052450	2810055G20Rik	-0.56102587	0.006218148
ENSMUSG00000043773	1700048O20Rik	-0.552712293	0.035969575
ENSMUSG00000085144	A930029G22Rik	-0.550742938	0.027274843
ENSMUSG00000073374	C030034I22Rik	-0.545615984	0.02575865
ENSMUSG00000058773	Hist1h1b	-0.54060025	0.043517707
ENSMUSG00000071550	Wdr52	-0.538737931	0.038204208
ENSMUSG00000071005	Ccl19	-0.537158827	0.031752102
ENSMUSG00000034472	Rasd2	-0.526510352	0.035130209
ENSMUSG00000006143	2310043J07Rik	-0.524123792	0.028261176
ENSMUSG00000087490	A330076H08Rik	-0.520190635	0.015905084
ENSMUSG00000073877	Gm13306	-0.517113532	0.001602514
ENSMUSG00000043488	Gm9783	-0.51683802	0.032243702
ENSMUSG00000037660	Gdf7	-0.513695846	0.017432151
ENSMUSG00000075296	Aldh3b2	-0.51265723	0.027387874
ENSMUSG00000082585	Gm15387	-0.512017147	0.026753475
ENSMUSG00000040148	Hmx3	-0.510956447	0.048865905
ENSMUSG00000011008	Mcoln2	-0.508964718	0.033444389
ENSMUSG00000066202	Ccl27b	-0.508429922	0.001916313
ENSMUSG00000086103	Gm11832	-0.505445927	0.048687777
ENSMUSG00000050328	Hoxc12	-0.504155076	0.004718282
ENSMUSG00000078734	Gm2506	-0.499562449	0.00232082
ENSMUSG00000061991	Hist1h2af	-0.499256211	0.022297201
ENSMUSG00000091455	Otogl	-0.498867697	0.036221036
ENSMUSG00000078747	Gm13308	-0.492689343	0.005162536
ENSMUSG00000034570	Inpp5j	-0.482996943	0.037139714
ENSMUSG00000093629	RP24-89J3.3.1	-0.482861525	0.032686405
ENSMUSG00000086843	E030013I19Rik	-0.482844259	0.002581848
ENSMUSG00000027070	Lrp2	-0.481726552	0.005171399
ENSMUSG00000064968	Snord47	-0.47430961	0.019388459
ENSMUSG00000048015	Neurod4	-0.453916921	0.00827315
ENSMUSG00000033200	Tpsg1	-0.452988883	0.037538228
ENSMUSG00000025176	Hoga1	-0.448387514	0.013597914
ENSMUSG00000072572	Slc39a2	-0.441203678	0.029232621
ENSMUSG00000090454	Gm17341	-0.439560692	0.04550548
ENSMUSG00000060470	Gpr97	-0.437686664	0.00936483
ENSMUSG00000039110	Mycbpap	-0.436628091	0.038426709

ENSMUSG000000031450	Grk1	-0.431999342	0.049762882
ENSMUSG000000085730	Gm13307	-0.430442756	0.035219898
ENSMUSG000000073888	Ccl27a	-0.429593487	8.06E-05
ENSMUSG000000073442	Gm16386	-0.429037138	0.03385672
ENSMUSG000000092827	Mir3091	-0.427362633	0.021540545
ENSMUSG000000071033	Gm10308	-0.418041797	0.036558743
ENSMUSG000000089417	snoU90	-0.413098433	0.001259728
ENSMUSG000000081669	Npm3-ps1	-0.412365229	0.00186434
ENSMUSG000000051977	Prdm9	-0.410887138	0.045070606
ENSMUSG000000034145	Tmem63c	-0.406131619	0.032119561
ENSMUSG000000028392	Bspry	-0.402511396	0.018211081
ENSMUSG000000036078	Sigmar1	-0.402257195	0.000247912
ENSMUSG000000038422	Hdhd3	-0.401825763	0.004742039
ENSMUSG000000090386	2810055G20Rik	-0.401158534	0.026295633
ENSMUSG000000066201	Il11ra2	-0.399345083	0.000443053
ENSMUSG000000073876	Gm13305	-0.398860782	0.000567427
ENSMUSG000000078735	Gm2002	-0.397553566	0.000735166
ENSMUSG000000084869	Gm13063	-0.391921682	0.010075281
ENSMUSG000000034258	Mfsd7c	-0.385244072	0.020670293
ENSMUSG000000092901	Mir5130	-0.374405672	0.022756003
ENSMUSG000000093485	RP23-268N22.1.1	-0.372013329	0.03427877
ENSMUSG000000020159	Gabrp	-0.367993171	0.012219199
ENSMUSG000000037649	H2-DMA	-0.36483157	0.035183311
ENSMUSG000000091890	A830073O21Rik	-0.36175489	0.011968231
ENSMUSG000000083327	Vcp-rs	-0.361193389	0.008325311
ENSMUSG000000020911	Krt19	-0.360532345	0.025384835
ENSMUSG000000043639	Rbm20	-0.358514829	0.004938199
ENSMUSG000000028447	Dctn3	-0.358475068	3.54E-05
ENSMUSG000000036073	Galt	-0.358319934	0.000709789
ENSMUSG000000078727	Gm2542	-0.35560714	0.023357824
ENSMUSG000000039457	Ppl	-0.347810887	0.005002175
ENSMUSG000000036114	2810432D09Rik	-0.346478055	2.11E-05
ENSMUSG000000086180	Rmst	-0.342215289	0.012717115
ENSMUSG000000078746	Gm13298	-0.336072322	0.031630909
ENSMUSG000000028393	Alad	-0.335187017	0.002193137
ENSMUSG000000035451	Foxa1	-0.334889071	0.013207033
ENSMUSG000000040536	Necab1	-0.327347544	0.031856674
ENSMUSG000000086796	4932702P03Rik	-0.321666574	0.014966049
ENSMUSG000000051397	Tacstd2	-0.319453391	0.029637862
ENSMUSG000000026107	Obfc2a	-0.31300041	0.024603458
ENSMUSG000000073889	Il11ra1	-0.307002006	0.001566128
ENSMUSG000000042448	Hoxd1	-0.306611254	0.04328941
ENSMUSG000000045591	Olig3	-0.305054297	0.023278691
ENSMUSG000000037369	Kdm6a	-0.304827298	0.011985765
ENSMUSG000000084941	Gm11944	-0.304758751	0.047579588

ENSMUSG00000062785	Kcnc3	-0.302012229	0.022765758
ENSMUSG00000019845	Tube1	-0.301628925	0.008287381
ENSMUSG00000022790	Igsf11	-0.300830029	0.030328379
ENSMUSG00000042564	4933432B09Rik	-0.300756901	0.048734933
ENSMUSG00000042985	Upk3b	-0.298811587	0.036736019
ENSMUSG00000047965	Rpl9-ps7	-0.295016302	0.009639905
ENSMUSG00000070282	3000002C10Rik	-0.291815924	0.020409602
ENSMUSG00000019913	Sim1	-0.287834164	0.011229479
ENSMUSG00000041930	BC057022	-0.284038412	0.011750179
ENSMUSG00000086370	B230206F22Rik	-0.28135244	0.036810276
ENSMUSG00000092315	Gm20440	-0.276850894	0.022301176
ENSMUSG00000046341	Gm11223	-0.274585873	0.022273274
ENSMUSG00000000690	Hoxb6	-0.26718011	0.009502245
ENSMUSG00000038253	Hoxa5	-0.265256538	0.000531584
ENSMUSG00000085133	B930095G15Rik	-0.263275388	0.045735525