Pinhead antagonizes Admp to promote notochord formation

Keiji Itoh, Olga Ossipova, and Sergei Y. Sokol*

Department of Cell, Developmental and Regenerative Biology,
Icahn School of Medicine at Mount Sinai, New York

*Correspondence: Sergei Y. Sokol, Ph. D.,

Phone: 1-212-241-1757; Fax: 1-212-860-9279

E-mail: sergei.sokol@mssm.edu

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Summary

Dorsoventral patterning of a vertebrate embryo critically depends on the activity of Smad1 that mediates signaling by several BMP proteins, anti-dorsalizing morphogenetic protein (Admp), and their antagonists. Pinhead (Pnhd), a cystine-knot-containing secreted protein, is expressed in the ventrolateral marginal zone during *Xenopus* gastrulation, however, its molecular targets and signaling mechanisms have not been fully elucidated. An unbiased mass spectrometry-based screen of the gastrula *secretome* identified Admp as a primary Pnhd-associated protein. We show that Pnhd binds Admp and inhibits its ventralizing activity by reducing Smad1 phosphorylation and suppressing its transcriptional targets. By contrast, Pnhd did not affect the signaling activity of BMP4. Importantly, the Admp gain-of-function phenotype and phospho-Smad1 levels have been enhanced after Pnhd depletion. Furthermore, Pnhd strongly synergized with Chordin and a truncated BMP4 receptor in the induction of notochord markers in ectoderm cells, and Pnhd-depleted embryos displayed notochord defects. Our findings suggest that Pnhd binds and inactivates Admp to promote notochord development. We propose that the interaction between Admp and Pnhd refines Smad1 activity gradients during vertebrate gastrulation.

Introduction

The specification of the dorsoventral axis and the three germ layers in vertebrate embryos is controlled by several signaling pathways triggered by Nodal, FGF and Wnt ligands (De Robertis and Kuroda, 2004; Harland and Gerhart, 1997; Kiecker et al., 2016). During gastrulation, the activity of the Spemann organizer results in the induction of the neural tissue in the ectoderm, whereas the mesoderm becomes subdivided into the notochord, paraxial (somites), intermediate (lateral plate, kidney) and ventral (blood) mesoderm (Kimelman, 2006; Niehrs, 2004). Both neuralization of the ectoderm and dorsalization of the mesendoderm primarily rely on a Smad1 activity gradient established by ventrolaterally expressed bone morphogenetic proteins (BMP4 and BMP7 ligands) and organizer-specific BMP antagonists Chordin, Noggin and Follistatin (De Robertis and Kuroda, 2004; Harland and Gerhart, 1997; Tuazon and Mullins, 2015).

Genetic and molecular studies implicated BMPs and their antagonists in the regulation of dorsoventral patterning during vertebrate gastrulation. BMPs specify ventral fates in a dose-dependent manner by stimulating the phosphorylation of Smad1/5/8 (referred to as Smad1, for simplicity). By contrast, Chordin secreted by the organizer, binds and inhibits BMPs to promote dorsal fates. Both Chordin and BMPs diffuse in the extracellular space and form opposing activity gradients in the embryo (Dale et al., 1992; De Robertis and Kuroda, 2004; Piccolo et al., 1996). Phospho-Smad1 is distributed along the dorsoventral axis in a graded fashion that corresponds to the putative BMP activity gradient (Faure et al., 2000; Schohl and Fagotto, 2002). In amphibians, anti-dorsalizing morphogenetic protein (Admp), a dorsally expressed BMP

ligand, also stimulates Smad1 phosphorylation to expand the zygotic BMP gradient (Dosch and Niehrs, 2000; Joubin and Stern, 1999; Moos et al., 1995; Reversade and De Robertis, 2005; Willot et al., 2002). Thus, the balance between the activators and inhibitors of Smad1 signaling is essential for dorsoventral patterning.

Pinhead is a conserved zygotic secreted protein implicated in *Xenopus* embryonic axis development (Kenwrick et al., 2004). Pnhd contains conserved cystine knot motifs and is induced in the ventrolateral marginal zone by Wnt and FGF signaling during gastrulation (Kjolby and Harland, 2017; Ossipova et al., 2020). Pnhd has been proposed to function synergistically with FGF, Nodal and BMP pathways during mesoderm development (Ossipova et al., 2020; Yan et al., 2019). Nevertheless, Pnhd-interacting proteins and signaling mechanisms remain to be characterized in further detail.

In this study, we initiated an unbiased mass spectrometry screen for *Xenopus* proteins that associate with Pnhd in the extracellular space. We identified the top candidate protein as Admp, an organizer-specific agonist of the BMP pathway. We find that Pnhd binds to Admp and inhibits its ventralizing activity mediated by phospho-Smad1 signaling. Moreover, Pnhd together with Chordin induces dorsal mesoderm markers. We also find that Pnhd is required for *Xenopus* notochord development consistent with the idea that Pnhd functions as Admp antagonist. We propose that the interaction of Pnhd and Admp in the marginal zone buffers Smad1 activity gradient during dorsoventral patterning.

Results

Screening for Pnhd-interacting proteins in the gastrula secretome.

Many cystine knot proteins, such as Cerberus or Gremlin (Hsu et al., 1998; Piccolo et al., 1999; Sasai et al., 1994), associate with other secreted proteins rather than cell surface receptors. Since Pnhd is readily secreted by cell lines and dissociated *Xenopus* gastrula cells (Ossipova et al., 2020; Yan et al., 2019), we decided to perform a screen for Pnhd-interacting partners in the extracellular space. This approach allows to enrich putative Pnhd-binding molecules by eliminating the abundant yolk proteins and common cytoplasmic contaminants, such as actin or tubulin.

In our gastrula secretome screen, the conditioned media from 200 dissociated control or Flag-Pnhd-expressing embryos were incubated with the media from 600 dissociated control embryos, and protein complexes have been pulled down using anti-Flag agarose beads (see Methods) (**Fig. 1A**). After protein separation in an SDS-polyacrylamide gel and Coomassie Blue staining, Flag-Pnhd was visible as prominent 36-38 kD protein bands in the sample from the Pnhd-expressing cells but not in the control sample (**Fig. 1B, C**). Mass spectrometry analyses of the gel slices identified several proteins that preferentially associated with Pnhd (**Fig. 1D**). Among these were Nodal inhibitors, Dand5/Coco/Cerl2 and Lefty/Antivin (Bell et al., 2003; Cheng et al., 2000; Marques et al., 2004; Meno et al., 1998; Montague et al., 2018), and Pnhd itself, consistent with the reported ability of Pnhd to form dimers (Yan et al., 2019). A top candidate (32 identified peptides) corresponded to Admp, a BMP family protein that is

expressed in the Spemann organizer and involved in dorsoventral patterning (Moos et al., 1995; Reversade and De Robertis, 2005). These results point to Admp as a candidate endogenous target of Pnhd in *Xenopus* gastrulae.

To validate the physical interaction between Pnhd and Admp, we combined the media conditioned by dissociated gastrula cells expressing either HA-Pnhd or Flag-Admp. When Flag-Admp was pulled down by anti-Flag antibodies, HA-Pnhd was readily detected in the precipitate (**Fig. 1E**). No signal was present in the control sample lacking Flag-Admp, indicating that the interaction is specific. This experiment shows that Admp binds to Pnhd.

To confirm this conclusion, we asked whether Admp associates with endogenous Pnhd using Pinhead-specific antibodies (**Suppl. Fig. 1**). The Pnhd protein was easily detected as 36-38 kDa double band in lysates of embryos overexpressing Pnhd but not in normal embryo lysates (**Suppl. Fig. 1A**). This suggests that Pnhd is not abundant but we could visualize endogenous Pnhd by immunoprecipitation from normal gastrula lysates (**Suppl. Fig. 1B**). We found that Pnhd was coprecipitated with Flag-Admp from conditioned media (**Fig. 1F**). Together, these studies indicate that endogenous Pnhd physically associates with Admp.

Pnhd inhibits Admp signaling but has no effect on BMP4

Admp has a strong ventralizing activity due to its ability to activate BMP receptors and increase Smad1 phosphorylation (Dosch and Niehrs, 2000; Joubin and Stern, 1999; Moos et al., 1995). To assess how Pnhd influences the function of Admp, the corresponding mRNAs were expressed in the marginal zone of four-cell embryos. Consistent with previous reports, *admp* RNA ventralized embryos in a manner similar to that of *bmp4* RNA (**Fig. 2A-C, H**). *Flag-pnhd* RNA did not cause ventralization on its own at the selected dose (**Fig. 2E, H**). Strikingly, when *pnhd* and *admp* RNAs were co-expressed, normal dorsal development has been restored (**Fig. 2D, H**). By contrast, BMP4-mediated ventralization was unaffected by Pnhd (**Fig. 2F-H**). These observations demonstrate an inhibitory effect of Pnhd on Admp.

We next analyzed Smad1 phosphorylation in the injected embryos. Consistent with the negative regulation of Admp, Pnhd inhibited Admp-dependent increase in phospho-Smad1 (**Fig. 2I**). By contrast, Pnhd had no effect on the stimulation of Smad1 by BMP4 (**Fig. 2J**). Levels of β -catenin were not altered by Pnhd, Admp and BMP4, suggesting no significant effects of these ligands on Wnt/ β -catenin signaling at the doses used (**Fig. 2I, J**). These results confirm that Pnhd is a selective antagonist of Admp. Supporting this conclusion, we found that higher doses of *pnhd* RNA reduced phospho-Smad1 levels in whole embryos (**Suppl. Fig. 2**).

To further support our observations, we analyzed known Smad1 target genes by RT-qPCR in whole embryos. Whereas Pnhd reversed Admp effects on both dorsal (*chrd1*,

dkk1) and ventral (*szl*, *bambi*, *ventx*) markers, it did not significantly modulate Bmp4 activity (**Fig. 3A-F**).

Admp activity is enhanced in Pnhd-depleted embryos

In vivo Admp activity is likely responsible for only a fraction of total phospho-Smad1 due to the presence of multiple BMP ligands in the early embryo. For this reason, our ability to detect endogenous Pnhd activity may be limited by the insufficient amount of Admp in the embryo. To sensitize the system, we injected embryos with exogenous *admp* RNA and examined Admp-dependent ventralization in *pnhd* morphants. In addition to previously characterized splicing-blocking and translation-blocking morpholino oligonucleotides (MOs) (Ossipova et al., 2020), we designed and validated a non-overlapping splicing-blocking MO, Pnhd-MO2^{sp} (**Suppl. Fig. 3**). Confirming the depletion, we observed a dramatic reduction of Pnhd protein level in embryos injected with Pnhd-MO2^{sp} but not in those injected with a control MO (**Suppl. Fig. 4**). Using Pnhd-MO2^{sp}, we found that the ventralization phenotype caused by *admp* RNA at a suboptimal dose has been significantly enhanced by the coinjection of Pnhd-MO2^{sp} (**Fig. 4A-F**). A similar effect has been observed for Pnhd-MO^{sp} (**Suppl. Fig. 5A-F**). These findings demonstrate the inhibitory role of Pnhd in the regulation of Admp activity.

Consistent with increased Admp function, we found an upregulation of phospho-Smad1 in Pnhd morphants injected with *admp* RNA. Pnhd-MO2^{sp}, Pnhd-MO^{sp} and Pnhd-MO^{atg}, but not the control MO, produced this effect (**Fig. 4G, H, Suppl. Fig. 5G, H**). We also

observed a small but significant increase in Smad1 phosphorylation in the samples with depleted Pnhd, even without Admp overexpression (**Fig. 4H**). Supporting these observations, the analysis of Smad1-sensitive dorsal (*chrd1*, *dkk1*) and ventral (*szl*, *bambi*) gene targets revealed an enhancement of Admp activity in the morphants (**Fig. 4I**).

Taken together, these findings indicate that Pnhd antagonizes Admp to inhibit Smad1 signaling and promote dorsal development.

Synergistic effects of BMP inhibitors and Pnhd on ectoderm cells

Since both Pnhd and organizer-derived BMP antagonists, such as Chordin, reduce Smad1 signaling in the embryo, we asked whether embryonic cells would exhibit a stronger response to Pnhd in the presence of a BMP inhibitor. Consistent with this hypothesis, the combined inhibition of Admp and BMP ligands caused synergistic phenotypic changes (Reversade and De Robertis, 2005; Willot et al., 2002). To test this prediction, we examined ectodermal explants from the embryos injected with *pnhd* and *chordin* RNAs. By the end of gastrulation, *chordin*- or *pnhd*-expressing ectoderm explants had minimal changes in morphology, however, the explants from the embryos injected with both RNAs dramatically elongated (**Fig. 5A-E, Table 1**). This elongation was clearly detectable as early as the midgastrula stage (**Suppl. Fig. 6**). Importantly, the explant elongation was also stimulated by Pnhd in the presence of a truncated dominant-interfering BMP receptor (Graff et al., 1994; Suzuki et al., 1994) (**Fig. 5F, G**). We conclude that the observed synergy is not due to a unique activity of Chordin, but

can be mediated by another Smad1 signaling inhibitor, such as a truncated BMP receptor.

To examine which gene targets might be responsible for explant elongation, we assessed the synergy of Pnhd and Chordin in target gene activation. RNA sequencing identified 273 genes that were enriched in the animal pole cells expressing both *pnhd* and *chordin* RNAs as compared with the cells expressing *chordin* RNA alone (**Table 2**). The top upregulated genes were notochord-specific markers, including *admp* (Moos et al., 1995) and *shh* (Peyrot et al., 2011), suggesting that the complete inhibition of Smad1 by Chordin and Pnhd promotes dorsal mesoderm. Both *admp* and *shh* are expressed in the organizer at the onset of gastrulation and later in the notochord (Moos et al., 1995; Peyrot et al., 2011). The synergistic activation of *shh*, *admp*, and *emilin3/emi3*, a *Xenopus* orthologue of a zebrafish notochord-specific gene (Corallo et al., 2013), has been confirmed by RT-qPCR at stage 14 and stage 11, whereas the levels of *sox2* did not change (**Fig. 6A-C**; **Suppl. Fig. 6**). In the absence of BMP inhibitors, Pnhd activated the ventrolateral mesodermal genes, e.g. *cdx4* (**Fig. 6D**), as previously reported (Ossipova et al., 2020). Chordin reduced the induction of these markers, consistent with tissue dorsalization.

These studies suggest that Pnhd functions together with BMP antagonists to inhibit Smad1 activity and promote dorsal mesoderm formation.

A requirement for Pnhd in notochord development

To further test the hypothesis that Pnhd promotes dorsal cell fates by antagonizing Admp, we have studied effects of Pnhd depletion in whole embryos by wholemount in situ hybridization with the notochord markers *not* (von Dassow et al., 1993) and *chordin* (Sasai et al., 1994) and the somite marker *myod1* (Hopwood et al., 1989). Embryos were injected with Pnhd-MO^{sp} or Pnhd-MO2^{sp} into four blastomeres of four-cell embryos to target the broad expression of Pnhd in the ventrolateral marginal zone (Ossipova et al., 2020). Embryos injected with either MO revealed consistently narrower and/or weaker expression domains of *chordin* (Fig. 7A-D) and *not* (Fig. 7E-H), as compared to the uninjected embryos at stage 14. These findings suggest that Pnhd is required for notochord formation.

In agreement with these results, the gap between the two *myod1*-expressing domains that corresponds to the notochord was narrower in *pnhd* morphants as compared to the control embryos (Fig. 7I-L). Together, these experiments support a role of Pnhd in notochord formation. By contrast, the increased thickness of notochord has been reported for *admp* morphants (Inomata et al., 2013). Thus, we propose that Pnhd regulates notochord size by antagonizing Admp.

Discussion

Pnhd is expressed in multiple locations in *Xenopus* embryos and interacts with FGF and Nodal signaling during early stages of ventroposterior mesoderm specification (Ossipova et al., 2020). In this study, we have used mass spectrometry to analyze Pnhd-associated proteins pulled down from the medium conditioned by dissociated *Xenopus* gastrula cells (the gastrula secretome). We identified several candidate secreted proteins that appear to physically associate with Pnhd. The top hit in this screen was Admp, a member of the BMP family. Our biochemical and functional studies confirmed the specific binding of Admp by Pnhd and revealed that Pnhd inhibits Admp activity both phenotypically and molecularly by modulating phospho-Smad1 level. By inactivating Admp, Pnhd contributes to the specification of dorsal mesoderm including the notochord at later stages (**Fig. 8**). Thus, Pnhd might play multiple roles by interacting with distinct signaling pathways at different developmental stages.

Notably, *pnhd* and *admp* genes are positioned next to each other in the genome of lower vertebrates and birds, but have been eliminated from the mammalian genomes suggesting coevolution (Imai et al., 2012). While the physical binding between Pnhd and Admp has been already reported in *Ciona*, it remained unclear whether and how Pnhd influences Admp (Imai et al., 2012). Zebrafish *pnhd* mutant embryos do not have a strong morphological phenotype on their own (Yan et al., 2019). Nevertheless, in the zebrafish model, Pnhd was shown to activate BMP receptors and act redundantly with Admp (Yan et al., 2019). These observations indicate that the interaction between Pnhd

and *Admp* may be context-dependent. Our results suggest that *Pnhd* could directly sequester *Admp*, potentially interfering with the binding of BMP receptors and Smad1 phosphorylation. In support of this model, *Xenopus* *Pnhd* morphants are microcephalic (Kenwrick et al., 2004; Ossipova et al., 2020) and have a narrow notochord (this study), whereas *Admp* inhibition causes enlarged head structures and widened notochord (Inomata et al., 2013; Inui et al., 2012; Reversade and De Robertis, 2005). Alternatively, *Pnhd* might form heterodimers with *Admp* to prevent its signaling. This possibility is less likely, because *Pnhd* is structurally very different from BMPs, with multiple cystine knot domains. Unlike BMPs, *Pnhd* does not appear to undergo Furin-dependent proteolytic processing, because the proteins tagged at the N- and C-termini have the same mobility.

The *pnhd* and *admp* genes have been evolutionarily conserved and expressed in mutually exclusive domains in *Ciona*, zebrafish and *Xenopus* embryos, suggesting feedback regulation (Imai et al., 2012). Although *Pnhd* is produced in the ventrolateral marginal zone (Kenwrick et al., 2004; Ossipova et al., 2020), and *Admp* is in the dorsal organizer, we find that *admp* transcription is strongly upregulated by *Pnhd* in the presence of Chordin. Whereas this observation is at odds with the transcriptional repression predicted by enhancer analysis in *Ciona* (Imai et al., 2012), we hypothesize that Chordin dorsalizes the ventrolateral mesoderm induced by *Pnhd* to activate several notochordal markers, including *admp*. The induction of *admp* might be particularly strong because of the compensatory feedback needed to restore *Admp* levels after its

inactivation by Pnhd. Further analysis of the admp gene regulatory network is necessary to fully understand our observations.

The finding that Pnhd antagonizes Admp reveals an additional level of regulation of Smad1 signaling activity. This activity is primarily controlled by ventral BMP ligands and their dorsal antagonists Chordin and Noggin (De Robertis and Kuroda, 2004; Tuazon and Mullins, 2015). Other secreted molecules, such, as Admp, Tolloid-like proteases and Sizzled/Ogon (Lee et al., 2006; Muraoka et al., 2006) also contribute to Smad1 regulation. Dorsal stimulation of Smad1 signaling by Admp has been proposed to control embryo scaling (Ben-Zvi et al., 2008; Inomata et al., 2013; Reversade and De Robertis, 2005). Our study further extends these observations to indicate that the interaction of Pnhd and Admp is critical for the refining of Smad1 signaling activity during dorsoventral patterning.

Limitations of the Study

We have shown that *pnhd* is necessary to fine tune Smad1 signaling by antagonizing Admp. Although the mutants of Admp and Pnhd can be generated, it is not practical to pursue classical genetic analysis using *Xenopus laevis*, due to the long generation time. Moreover, the exact molecular mechanism underlying the observed interaction remains unclear. Additional studies are needed to determine whether Pnhd sequesters Admp to interfere with Admp binding to BMP receptors or promotes the interaction of Admp with other secreted inhibitors such as Chordin.

STAR Methods

Resource Availability

Lead Contact

Futher information and requests for reagents should be directed to and will be fulfilled by the Lead Contact, Sergei Sokol (Sergei.Sokol@mssm.edu).

Materials availability

All data generated in this study are included in this published article and its supplemental information.

Data and Code Availability

The RNA sequencing datasets generated during this study are available at the NIH GEO (submission number GSE168370). All other data produced in this study are included into this published article.

Experimental Model and Subject Details

Mature female and male *Xenopus laevis* frogs were obtained from NASCO (Fort Atkinson, WI). Animals were maintained in a recirculating tank system with regularly monitored temperature and water quality (pH, nitrate and nitrite levels) *Xenopus laevis* were housed at a temperature of 18-20C. Frogs were fed with food pellets (NASCO, cat#SA05960). All experimental protocols involving frogs were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory

Animals of the National Institutes of Health. The protocol 04-1295 was approved by the IACUC of the Icahn School of Medicine at Mount Sinai.

Method Details

All methods can be found in the accompanying Transparent Methods supplemental file.

Plasmids, in vitro RNA synthesis and morpholino oligonucleotides.

pCS2-Flag-Pnhd and pCS2-HA-Pnhd plasmids have been generated by PCR from the *X. laevis* DNA clone for *pnhd.L* (accession number NM_001127751) obtained from Dharmacon. Flag or HA tags were introduced after the predicted signal peptide sequence of Pnhd. pSP64T3-Flag-Admp is generated by PCR from *Xenopus laevis* *admp.S* cDNA obtained from Bill Smith (Kumano et al., 2006). pCS2-Chordin was from E. De Robertis (Sasai et al., 1995). Flag tag was introduced in Admp by PCR-based mutagenesis, four amino acids downstream of the conserved processing site RLGR.

Capped mRNAs were synthesized using Ambion mMessage mMachine kit (ThermoFisher). pCS2-Flag-Pnhd, pCS2-HA-Pnhd, pCS2-Pnhd-Flag, pCS2-Pnhd, pSP64T3-Flag-Admp, pSP64T3-Admp, pCS2+Chordin (obtained from Eddy De Robertis), pCS2-Flag-BMP4 (obtained from Andrei Zaraisky, (Bayramov et al., 2011)) and pSP64T-tBR (Graff et al., 1994) were used. Splicing blocking MOs, Pnhd MO^{sp}, 5'-CCTGTTTCATCACGCTACCATCTAAA-3' and Pnhd-MO2^{sp}, 5'-GGACTACCAGAGATATCTGTAATAA-3', translation blocking Pnhd MO^{atg}, 5'-ACAAGAAAAGATGTTCCATGTCTG-3'; and control MO (CoMO), 5'-

GCTTCAGCTAGTGACACATGCAT-3', were purchased from Gene Tools (Philomath, OR).

Xenopus embryo culture, microinjections, and production of secreted proteins.

In vitro fertilization and culture of *Xenopus laevis* embryos were carried out as previously described (Dollar et al., 2005). Staging was according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). For microinjections, 2 to 4-cell embryos were transferred into 3 % Ficoll in 0.5x Marc's Modified Ringer's (MMR) solution (50 mM NaCl, 1 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 2.5 mM HEPES pH 7.4) (Peng, 1991) and 10 nl of mRNA or MO solution were injected into two blastomeres of 2 cell embryos or four blastomeres of 4 cell embryos either animally or sub-equatorially. Injected embryos were transferred into 0.1 MMR at blastula stages. Amounts of injected mRNA per embryo have been optimized in preliminary dose-response experiments and are indicated in figure legends.

Secreted proteins were produced after dissociating whole embryos in Ca/Mg-free medium essentially as described (Eroshkin et al., 2016). At early gastrula stage, vitelline membrane was removed and 60 embryos were transferred into 600 µl of Ca/Mg-free medium (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 7.5 mM Tris-HCl, pH 7.6) per well of 4 well dish. After shaking at 80-100 rpm for 3 hrs (with 2 mM EDTA for the last hour), the medium (500 µl) was collected from each well and cleared by centrifugation at 100 g for 1 min. For immunoprecipitation, 4.5 mM CaCl₂, 1.6 mM MgCl₂ and 1 mM phenylmethylsulfonylfluoride (PMSF, Sigma) were added. The remaining cell pellets

were lysed in 500 μ l of the lysis buffer (50 mM Tris-HCl pH 7.6, 50 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10 mM NaF, 1 mM Na_3VO_4 , 1 mM PMSF) and the lysates were collected after centrifugation for 4 min at 16000 g for immunoblotting.

For whole embryo lysates, 5 embryos were lysed in 85 μ l of the lysis buffer and the lysates were collected after the same centrifugation. For animal cap experiments, both blastomeres of the 2-cell embryo were injected in the animal pole region. Ectoderm explants were prepared at stages 9+ to 10, and cultured in 0.6xMMR in the presence of gentamicin (10 μ g/mL) until the indicated time. Embryo phenotypes at stage 28 were quantified according to the dorso-anterior index (DAI)(Kao and Elinson, 1988). DAI 5, normal embryos; DAI 4, small head with eyes and cement glands; DAI 3, small head with cement glands; DAI 2, rudimentary head without eyes nor cement glands; DAI 1, posterior body axis lacking head; DAI 0, completely ventralized.

Immunoprecipitation, western blot analysis and blue staining

For immunoprecipitation of Flag-Pnhd protein in mass spectrometry experiments, 1.5 ml of conditioned medium from 200 Flag-Pnhd-expressing or control embryos were combined with 5 ml of conditioned medium from 600 control embryos and incubated with 25 μ l of anti-FLAG agarose beads (Sigma) at 4°C overnight. For coprecipitation of Flag-Admp and HA-Pnhd, the HA-Pnhd- and Flag-Admp-containing or control conditioned media (500 μ l each) were combined and incubated with 2 μ l of anti-Flag agarose beads at 4°C overnight. For endogenous Pnhd, the conditioned medium (500 μ l) from embryos injected with PnhdMO2^{sp} or control MO (40 ng x 4) was precipitated

with 20 μ l of the supernatant containing anti-Pnhd 5F9 antibodies (a gift of D. Alfandari), followed by incubation of 4 μ l of Protein A sepharose beads. The beads were washed with phosphate buffered saline (PBS) and boiled in the sample buffer. For immunoblotting, 3 or 10 μ l of immunoprecipitated proteins, 10 μ l of conditioned medium or dissociated cell lysates or 10 μ l of embryo lysates were separated by SDS-PAGE. Immunoblotting was carried out essentially as described (Itoh et al., 2005). For Coomassie blue staining, 15 μ l of immunoprecipitated material (25 μ l total) was loaded and separated on a gel followed by SDS-PAGE and the gel was stained with Simply Blue (Pierce) according to the manufacturer's protocol. Gel slices were excised after Simple Blue staining and subjected to medium gradient LC-MS/MS analysis carried out by the Keck Proteomics Laboratory at Yale University.

The following primary antibodies were used: mouse anti-FLAG (M2, Sigma), mouse anti-HA (12CA5), anti-Myc (9E10), rabbit anti-Erk1 (K23, Santa Cruz), anti-pSmad1/5 S463/465 (41D10, Cell Signaling Technology), anti-Smad1 (Invitrogen), rabbit anti- β -catenin (Invitrogen) and anti-Pnhd antibodies (5F9, 8G12, 11D5; gifts of D. Alfandari). The detection was carried out by enhanced chemiluminescence as described (Itoh et al., 2005), using the ChemiDoc MP imager (BioRad). Normalized ratios of band intensities of pSmad1 to Smad1 were calculated using NIH imageJ software.

Ectoderm explants, RNA sequencing, RT-PCR and qRT-PCR

Embryos were injected with various MOs and RNAs as indicated in figure legends. Ectoderm explants were prepared and cultured until the required stage. RNA was

extracted from 15-30 animal pole explants or 3-4 whole embryos using the RNeasy kit (Qiagen). For RT-PCR, cDNA was made from 1-2 µg of total RNA using the first strand cDNA synthesis kit (Invitrogen) or iScript (Bio-Rad) according to the manufacturer's instructions.

For RNA sequencing, ectoderm explants expressing *chordin* RNA (150 pg), *pnhd* RNA (0.5 ng) or both, and control uninjected explants were cultured until stage 13/14. cDNA library preparation and paired-end 150 bp sequencing were performed by Novogene (Sacramento, CA) using Illumina HiSeq2000 analyzers. The raw reads (FASTQ files) were filtered to remove reads containing adapters or reads of low quality. The sequences were mapped to the *Xenopus* genome version XL-9.1_v1.8.3.2 available at <http://www.xenbase.org/other/static/ftpDatafiles.jsp> using the software hisat2 (Kim et al., 2015). The differentially expressed genes (DEGs) were detected using DESeq (Anders and Huber, 2010) with the two-fold change cutoff. The p-value estimation was based on the negative binomial distribution, using the Benjamini-Hochberg estimation model with the adjusted $p < 0.05$. *Pnhd* and *chordin*-induced DEGs have been assessed using two independent samples in the same RNA sequencing experiment. The entire dataset has been submitted to the NIH GEO (submission number GSE168370).

For RT-qPCR, the reactions were amplified using a CFX96 light cycler (Bio-Rad) with Universal SYBR Green Supermix (Bio-Rad). Primer sequences used for RT-PCR and RT-qPCR are shown in **Table 3**. The reaction mixture consisted of 1X Power SYBR Green PCR Master Mix, 0.3 µM primers, and 1 µl of cDNA in a total volume of 10 µl.

The $\Delta\Delta CT$ method was used to quantify the results. All samples were normalized to control uninjected embryos or explants. Transcripts for *elongation factor 1a1* (*ef1a1*) were used for normalization. Data are representative of two to three independent experiments and shown as means $\pm$ standard deviation. Statistical significance was assessed by two-tailed Student's *t*-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$).

Wholemout in situ hybridization

Wholemout in situ hybridization (WISH) was carried out as described (Harland, 1991) and pigments were bleached as described (Mayor et al., 1995). Digoxigenin-rUTP-labeled RNA probes were prepared by in vitro transcription of linearized plasmids of pBS-*chrd.1* (Sasai et al., 1994), pBSKS-*not* (von Dassow et al., 1993), and pBSSK-*myod1* (Hopwood et al., 1989) with T3 or T7 RNA polymerases and the RNA labeling mix containing digoxigenin-rUTP (Roche). All data are representative of two to three independent experiments. Measurement of width of expression domains of *chrd.1* and *not* or expression gap of *myod1* as well as embryo width was performed with NIH ImageJ software.

Quantification and statistical analysis

Each figure legend contains quantification details, including frequencies of embryonic phenotypes or numbers of explants examined (n), the mean values, and the s.d. In addition, information about the statistical tests used for measuring significance and

interpretation of p values is provided. Statistical analyses and graphic results for RT-qPCR were generated using the CFX Maestro software (Biorad).

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Figure legends

Fig. 1. Screening gastrula secretome for Pnhd-associated proteins. A, Experimental scheme. Embryos were injected four times animally with Flag-Pnhd RNA (500 pg) and dissociated at stage 10. After 3 hrs, conditioned media (CM) from dissociated Flag-Pnhd-expressing and control embryos were combined and immunoprecipitated with anti-Flag beads. B. Coomassie blue staining of Flag-Pnhd-containing and control protein pulldowns. Two bands of 36-38 kD correspond to Flag-Pnhd (arrow). C. Immunoblot analysis of immunoprecipitates, CM and cell lysates with anti-Flag antibody. Heavy and light antibody chains are visible in addition to the specific Pnhd bands (arrowhead). Anti-Erk1 antibody serves as loading control for the lysates. D. Numbers of identified peptides for top candidate secreted Pnhd-interacting proteins that were identified by mass spectrometry. E. CM were combined from embryos expressing HA-Pnhd and Flag-Admp as described in (A), and precipitated with anti-Flag antibody. Supernatant (S) or cell pellet (P) fractions from dissociated cell lysates are also shown. Anti-HA antibody recognizes HA-Pnhd as 37-39 kD bands, while anti-Flag antibody detects the unprocessed form of Flag-Admp (45 kD) and mature Flag-Admp (17 kD, arrows). Anti-Erk1 antibody validates the separation of cytoplasmic and secreted proteins. F, Admp binds endogenous Pnhd. CM from dissociated normal embryos (stage 10) or sibling embryos expressing Flag-Pnhd were immunoprecipitated with anti-Flag or anti-Pnhd (5F9) antibodies. After gel separation of protein precipitates, immunoblotting was carried out with the indicated antibodies.

Fig. 2. Pnhd inhibits Admp ventralizing activity. A, Experimental scheme. Marginal zone of four-cell embryos was injected with *admp* or *flag-bmp4* RNA (50 pg), and *flag-pnhd* RNA (150 pg) as indicated. B-G, Typical embryo phenotypes at stage 20 are shown. Arrows (B, D) points to neural tubes, asterisk in C marks a ventralized embryo. Bar in G is 400 μ m. H, Quantification of the experiments shown in B-G. Ventralization activity in stage 20 embryos has been scored as indicated at the bottom of the panel. Numbers of scored embryos are shown above each bar. Data are representative of three independent experiments. I, J, Pnhd inhibits Smad1 phosphorylation in response to Admp but not BMP4. Embryos were injected as shown in A and harvested at stage 11 for immunoblotting with anti-phospho-Smad1 (pSmad1) and anti-Smad1 antibodies. Normalized ratios of pSmad1 to Smad1 levels are shown. Independent biological replicas are included for each group. Erk1 and β -catenin (β Cat) levels control loading.

Fig. 3. Pnhd regulates Admp-dependent dorsoventral markers. Embryos were injected as described in Fig. 2A legend. RNA was extracted from injected embryos at stage 11.5. RT-qPCR analysis of ventral (*vent1*, *vent2*, *szl* and *bambi*, A-D) or dorsal (*chrd1* and *dkk1*, E, F) markers is shown. These data are representative of four experiments. Means $\pm$ s. d. are shown. Significance was determined by the Student's t-test, $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p > 0.05$, non-significant (N.S.).

Fig. 4. Admp activity is enhanced by Pnhd depletion. A, Experimental scheme. Marginal zone of four-cell embryos was injected with *admp* RNA (50 pg) or PnhdMO2^{sp} (40 ng) as indicated. B-F, Pnhd MO2^{sp} enhanced ventralization caused by Admp.

Representative embryos are shown. F. Quantification of the data shown in B-E. Phenotypic scoring was done when uninjected embryos reached stage 28 using dorsoanterior index (DAI). G, H, Immunoblotting of lysates of stage 11 embryos with anti-pSmad1, anti-Smad1 and anti-Erk1 antibodies. G, control MO; H, PnhdMO2^{sp}. Normalized ratio of band intensities of pSmad1 relative to total Smad1 is indicated. Erk1 serves as a loading control. Independent biological replicas are included for each group (G, H). Data represent two to three independent experiments. I, The effect of Admp on marginal zone markers is enhanced in Pnhd morphants. RNA was extracted from injected embryos at stage 11.5. RT-qPCR analysis of ventral (*szl* and *bambi*) or dorsal (*chrd1* and *dkk1*) markers is shown. These data are representative of three experiments. Means +/- s. d. are shown. Significance was determined by the Student's t-test, p<0.01 (**), p<0.001 (***).

Fig. 5. Pnhd cooperates with BMP inhibitors to trigger explant elongation. A, Two-cell stage embryos were injected with *pnhd* (0.5 ng), *chordin* (0.15 ng) or truncated BMP receptor (*tBR*, 0.45 ng) RNA as indicated. Ectoderm explants were dissected at stages 9.5-10 and cultured until stage 14 for morphological examination. B-G, Representative group morphology is shown. Arrows in D and F point to elongating explants. These experiments were repeated 3-7 times.

Fig. 6. Pnhd synergizes with BMP inhibitors to induce notochord markers. A, Experimental scheme. Two-cell embryos were injected into animal pole region with *pnhd* (0.5 ng) RNA, *chordin* (0.15 ng) or truncated BMP receptor (*tBR*, 0.45 ng) RNA as

indicated in B, C. Ectoderm explants were dissected at stages 9.5-10 and cultured until stage 14 to examine notochord markers *admp*, *shh* and *emi3* by RT-qPCR. B, *Pnhd* synergizes with *Chordin* to induce *admp.S*, *shh.L* and *emi3*, but not *sox2*, at stage 14. C, *Pnhd* synergizes with *tBR* to induce *admp.S* and *shh.L*, but not *sox2*. D, *Chordin* inhibits the induction of *cdx4.L* by *pnhd*. These data are representative of three experiments. Means +/- s. d. are shown. Significance was determined by the Student's t-test, $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

Fig. 7. Pnhd is required for notochord specification. A-L, *Pnhd*MO^{sp} or *Pnhd*MO2^{sp} (40 ng each) were injected four times into marginal zone as shown in Fig. 2A. When control embryos reached stage 14, injected or uninjected embryos were fixed for wholemount *in situ* hybridization with the notochord-specific probes *chrd.1* (A-D) and *not* (E-H) or the somitic marker *myod1* (I-L). Dorsal view is shown, anterior is up. Arrows in A, E, I demarcate the width of *chrd.1* or *not* expression domains or the gap in *myod1* expression. D, H, L, Quantification of notochord width (marked by *chrd.1*, *not* or the gap in *myod1*) relative to embryo width. Means +/- s. d. are shown. Number of scored embryos per group are shown above each bar. The data are representative of three independent experiments.

Fig. 8. A model of Pnhd effects on dorsal mesoderm, Admp and Smad1 activity. Major activators of Smad1 phosphorylation in the *Xenopus* gastrula are ventrolaterally-expressed Bmp4/7 and dorsal *Admp*. *Pnhd* transcripts are distributed in the ventrolateral marginal zone, complementary to the dorsal expression of *admp*. Dorsal

organizer signals, such as Chordin, reduce Smad1 phosphorylation triggered by Bmp4/7. A similar antagonistic effect of Pnhd on Admp is proposed to further modulate Smad1 activity gradients.

Supplementary Figure legends

Suppl. Fig. 1. Both exogenous and endogenous Pnhd are recognized by specific antibodies, related to Figure 1. A, Embryos were injected with *flag-pnhd* RNA (75 pg) and the lysates of the injected and control uninjected embryos at stage 11.5-12 were immunoblotted with 5F9 antibodies. There is no visible signal in control embryos, but a strong 35-37 kDa band is detected in pnhd-expressing embryos. B, Lysates were prepared from normal embryos at stage 12, immunoprecipitated with the Pnhd-specific antibodies 5F9, 8G12, 11D5 or anti-Myc as indicated and immunoblotted with 5F9.

Suppl. Fig. 2. Inhibition of endogenous phospho-Smad1 levels by Pnhd, related to Figure 2. Two-cell embryos were injected into each blastomere with *flag-pnhd* RNA (1 ng) or *bmp4* RNA (50 pg). The injected embryos were collected when control siblings reached stage 11 for immunoblotting with anti-pSmad1, anti-Smad1 and anti-Flag antibodies. Pnhd reduced Smad1 phosphorylation whereas Bmp4 upregulated it.

Suppl. Fig. 3. Characterization of the effects of PnhdMO2^{sp} on *pnhd* RNA splicing, related to Figure 4. A, Experimental scheme. Four-cell embryos were injected four

times into marginal zone with 30 ng of PnhdMO2^{sp}. RNAs were collected at stage 11 or 14 for RT-PCR. B, Pnhd-L gene structure with indicated exon (ex) and intron (int) boundaries. PnhdMO2^{sp} targets the junction of int2 and ex3. C, RT-PCR results show the addition of the intron 2 in the morphants. *ef1a* is a loading control.

Suppl. Fig. 4. Validation of Pnhd knockdown by immunoprecipitation, related to Figure 4.

Four-cell embryos were injected in the marginal zone with PnhdMO2^{sp} or control MO (40 ng each) as indicated. Sixty injected embryos were dissociated at stage 10 and cultured for 3 hrs. Conditioned media were immunoprecipitated with anti-Pnhd (5F9) or anti-HA antibodies and immunoblotted with 5F9. Pnhd-specific band has been eliminated in embryos injected with PnhdMO2^{sp}.

Suppl. Fig. 5. Enhancement of Admp ventralizing activity by PnhdMO^{sp}, related to Figure 4. A, Experimental scheme. Marginal zone of four-cell embryos was injected with *admp* RNA (50 pg) or Pnhd MO^{sp} (60 ng) as indicated. B-E, PnhdMO^{sp} enhanced ventralization caused by Admp. Representative embryos are shown. F. Quantification of the data shown in B-E. Phenotypes were scored at stage 28 using dorsoanterior index (DAI). G, H, Immunoblotting of lysates of stage 11 embryos with anti-pSmad1, anti-Smad1 and anti-Erk1 antibodies. Normalized ratio of band intensities of pSmad1 relative to total Smad1 is indicated. Both PnhdMO^{sp} (60 ng) and PnhdMO^{atg} (10 ng) enhanced pSmad1 levels by Admp. Erk1 serves as a loading control. Data represent two to three independent experiments.

Suppl. Fig. 6. Pnhd promotes elongation of ectodermal explants in the presence of Chordin at midgastrula stage, related to Figure 5. A-D. Two cell embryos were injected with 0.5 ng of *pnhd* and/or 0.15 ng of *chordin* RNA as indicated. Ectodermal explants were dissected at stage 9.5-10 as shown in Fig. 5A. The explants were cultured until stage 11. E. Embryos were injected as described in A-D. Ectoderm explants were dissected at stages 9.5-10 and cultured until stage 11 to examine *admp* and *shh* by RT-qPCR. Data are representative of three experiments. Means $\pm$ standard errors are shown. Significance was determined by the Student's t-test, $p < 0.05$ (*), $p < 0.01$ (**).

Tables 1-3

Table 1. Pnhd cooperates with Chordin to promote animal cap elongation.

Table 2. Genes differentially expressed in the ectoderm of embryos injected with *chordin* and *pnhd* RNAs as compared to only *chordin* RNA.

Table 3. Primers used for RT-qPCR and RT-PCR.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

A

Chrd or *tBR*
+/- *pnhd* RNA

Explants
dissected
at st. 9.5-10

Elongation
at st. 11 or 14

B

Uninjected
0/35

st 14

C

Chordin
0/43

st 14

D

Chordin+Pnhd
53/54

st 14

E

Pnhd
0/45

st 14

F

tBR+Pnhd
22/23

st 14

G

tBR
0/15

st 14

Figure 6

Figure 7

Figure 8

Suppl. Fig.1

A

B

Suppl. Fig.2

Suppl. Figure 3

A

B

C

Suppl. Fig.4

Suppl. Figure 5

Suppl. Figure 6

Table 1. Pnhd cooperates with Chordin to promote animal cap elongation

RNA injected	Dose	Elongation	Elongation, %	Total N
Uninjected st 14	NA	0/35	0	35
Chordin	0.15 ng	0/43	0	43
Chordin +Pnhd	0.15 ng 0.5 ng	53/54	98	54
Pnhd	0.5 ng	0/45	0	45
tBR +Pnhd	0.43 ng 0.5 ng	22/23	96	23
tBR	0.43 ng	0/15	0	15
Uninjected st 11	NA	0/21	0	21
Chordin	0.15 ng	0/28	0	28
Chordin +Pnhd	0.15 ng 0.5 ng	67/67	100	67
Pnhd	0.5 ng	0/75	0	75
Uninjected st 14	NA	0/39	0	39
Chordin	0.15 ng	0/52	0	52
Chordin +Pnhd	0.15 ng 0.5 ng	106/107	99	107
Pnhd	0.5 ng	9/54	17	54

RNA injected	Dose	Elongation	Elongation, %	Total N
Uninjected st 15	NA	0/13	0	13
Chordin	0.15 ng	0/15	0	15
Chordin +Pnhd	0.15 ng 0.5 ng	18/18	100	18
Pnhd	0.5 ng	1/13	7.7	13
tBR +Pnhd	0.43 ng 0.5 ng	22/22	100	22
tBR	0.43 ng	0/16	0	16

Table 2. Genes induced by Pnhd in the presence of Chordin.

chor-Pinh_vs_chor-wNames								
gene symbol	log2 Fold Change	padj	Human symbol	gene product	lfcSE	stat	pvalue	Human PID
admp.L	9.63	4.4E-05	BMP2	bone morphogenetic protein 2 preproprotein	1.9	-4.9	7.8E-07	NP_001191
emilin3.S	9.58	5.0E-05	EMILIN3	EMILIN-3 precursor	2.0	-4.9	9.1E-07	NP_443078
Xelaev18002200m.g	9.32	1.3E-07	ATP2A1	sarcoplasmic/endoplasmic reticulum calcium ATPase 1 isoform a	1.5	-6.1	1.3E-09	NP_004311
cdx1.S	9.26	9.2E-04	CDX2	homeobox protein CDX-2	2.2	-4.2	2.4E-05	NP_001256
Xelaev18003389m.g	9.19	2.3E-07	ATP2A1	sarcoplasmic/endoplasmic reticulum calcium ATPase 1 isoform a	1.5	-6.0	2.4E-09	NP_004311
shh.L	9.16	3.5E-04	SHH	sonic hedgehog protein preproprotein	2.1	-4.5	8.2E-06	NP_000184
MGC75582.L	9.09	3.5E-07	ACTA1	actin, alpha skeletal muscle	1.5	-5.9	3.8E-09	NP_001091
MGC75582.S	9.05	5.0E-07	ACTA1	actin, alpha skeletal muscle	1.6	-5.8	5.5E-09	NP_001091
admp.S	8.91	3.1E-03	BMP2	bone morphogenetic protein 2 preproprotein	2.3	-3.9	9.8E-05	NP_001191
t2.S	8.88	7.1E-04	T	brachyury protein isoform 1	2.1	-4.3	1.8E-05	NP_003172
Xelaev18034590m.g	8.87	3.5E-03	MMP21	matrix metalloproteinase-21 preproprotein	2.3	-3.9	1.1E-04	NP_671724
shh.S	8.84	2.4E-03	SHH	sonic hedgehog protein preproprotein	2.2	-4.0	7.5E-05	NP_000184
LOC100496628-like.	8.30	1.8E-05	GPR34	PREDICTED: probable G-protein coupled receptor 34 isoform X1	1.6	-5.1	2.8E-07	XP_005272654
kng1.L	8.28	2.1E-05	KNG1	kininogen-1 isoform 1 precursor	1.6	-5.1	3.4E-07	NP_001095886
des.1.L	8.24	1.9E-05	NA	NA	1.6	-5.1	3.0E-07	NA
act3.L	8.16	2.2E-05	ACTA1	actin, alpha skeletal muscle	1.6	-5.1	3.5E-07	NP_001091
foxb1.S	8.06	1.4E-11	FOXB1	forkhead box protein B1	1.1	-7.5	7.7E-14	NP_036314
foxb1.L	8.05	6.2E-06	FOXB1	forkhead box protein B1	1.5	-5.4	8.8E-08	NP_036314
foxa4.S	8.04	5.7E-05	FOXA1	hepatocyte nuclear factor 3-alpha	1.6	-4.9	1.0E-06	NP_004487
lrat.S	8.00	6.3E-06	LRAT	PREDICTED: lecithin retinol acyltransferase isoform X1	1.5	-5.3	8.9E-08	XP_006714475
wnt1.L	7.99	6.6E-06	WNT1	proto-oncogene Wnt-1 precursor	1.5	-5.3	9.5E-08	NP_005421
hoxb7.L	7.93	6.9E-05	HOXB7	homeobox protein Hox-B7	1.6	-4.8	1.3E-06	NP_004493
cdx4.S	7.92	3.7E-05	CDX4	homeobox protein CDX-4	1.6	-5.0	6.4E-07	NP_005184
Xelaev18047312m.g	7.91	6.9E-05	MYL1	myosin light chain 1/3, skeletal muscle isoform isoform 1f	1.6	-4.8	1.3E-06	NP_524144
angptl7.S	7.88	1.4E-04	ANGPTL7	angiopoietin-related protein 7 precursor	1.7	-4.7	3.0E-06	NP_066969
dmrt2.L	7.83	9.6E-05	DMRT2	PREDICTED: doublesex- and mab-3-related transcription factor 2	1.6	-4.8	2.0E-06	XP_005251409
neurog1.L	7.79	1.9E-04	NEUROG1	neurogenin-1	1.7	-4.6	4.0E-06	NP_006152
nkx2-8.L	7.77	3.1E-05	NKX2-8	homeobox protein Nkx-2.8	1.5	-5.0	5.1E-07	NP_055175
LOC101732630.S	7.71	1.5E-04	THBS4	thrombospondin-4 precursor	1.7	-4.7	3.1E-06	NP_003239
foxa4.L	7.69	5.1E-05	FOXA1	hepatocyte nuclear factor 3-alpha	1.6	-4.9	9.3E-07	NP_004487
pnhd.L	7.68	1.5E-202	#N/A	#N/A	0.3	-30.7	7.0E-207	NA
LOC100492804.L	7.57	1.7E-06	RNF220	PREDICTED: E3 ubiquitin-protein ligase RNF220 isoform X3	1.4	-5.6	2.1E-08	XP_005271051
col8a1.L	7.53	6.7E-05	COL8A1	collagen alpha-1(VIII) chain precursor	1.6	-4.8	1.3E-06	NP_065084
Xelaev18044867m.g	7.51	4.3E-04	MYL1	myosin light chain 1/3, skeletal muscle isoform isoform 1f	1.7	-4.4	1.0E-05	NP_524144
cdx2.S	7.49	1.3E-04	CDX2	homeobox protein CDX-2	1.6	-4.7	2.6E-06	NP_001256
plod2-like.S	7.49	2.7E-05	PLOD2	procollagen-lysine,2-oxoglutarate 5-dioxygenase 2 isoform 2 prec	1.5	-5.0	4.4E-07	NP_000926

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col8a1.S	7.45	4.7E-04	COL8A1	collagen alpha-1(VIII) chain precursor	1.7	-4.4	1.1E-05	NP_065084
LOC100497280.S	7.39	1.5E-03	PSKH1	serine/threonine-protein kinase H1	1.8	-4.1	4.2E-05	NP_006733
des.1.S	7.37	6.7E-04	NA	NA	1.7	-4.3	1.7E-05	NA
actc1.L	7.33	1.3E-08	ACTC1	actin, alpha cardiac muscle 1 proprotein	1.1	-6.4	1.1E-10	NP_005150
klhl31-like.L	7.29	8.5E-04	KLHL31	PREDICTED: kelch-like protein 31 isoform X1	1.7	-4.2	2.2E-05	XP_005249164
kctd12.L	7.28	1.2E-03	KCTD12	BTB/POZ domain-containing protein KCTD12	1.8	-4.2	3.2E-05	NP_612453
twist1.S	7.27	1.6E-03	TWIST2	PREDICTED: twist-related protein 2 isoform X1	1.8	-4.1	4.6E-05	XP_006712305
Xelaev18044831m.g	7.27	7.5E-04	GBX2	homeobox protein GBX-2	1.7	-4.3	1.9E-05	NP_001476
itga2.L	7.23	2.3E-04	ITGA2	integrin alpha-2 precursor	1.6	-4.6	4.9E-06	NP_002194
Xelaev18002814m.g	7.17	1.1E-03	#N/A	#N/A	1.7	-4.2	3.1E-05	NA
hoxa7.L	7.14	1.9E-03	HOXA7	homeobox protein Hox-A7	1.8	-4.0	5.6E-05	NP_008827
hoxc8.L	7.14	2.1E-03	HOXC8	homeobox protein Hox-C8	1.8	-4.0	6.3E-05	NP_073149
hoxa10.L.g	7.14	2.3E-03	NA	NA	1.8	-4.0	7.2E-05	NA
hoxc8.S	7.14	2.3E-03	HOXC8	homeobox protein Hox-C8	1.8	-4.0	7.2E-05	NP_073149
hoxa1.L	7.11	1.8E-03	HOXA1	homeobox protein Hox-A1 isoform a	1.8	-4.0	5.4E-05	NP_005513
klhl41.L	7.07	1.8E-03	KLHL41	kelch-like protein 41	1.8	-4.0	5.4E-05	NP_006054
rfx4.L	7.06	4.9E-20	RFX4	transcription factor RFX4 isoform c	0.7	-9.8	8.9E-23	NP_998759
vgll3.L	7.06	3.5E-16	VGLL3	transcription cofactor vestigial-like protein 3	0.8	-8.8	1.0E-18	NP_057290
LOC100486832.L	7.06	2.3E-03	FGL2	fibroleukin precursor	1.8	-4.0	6.9E-05	NP_006673
LOC100496061-like.	7.02	5.9E-04	RAB38	ras-related protein Rab-38	1.6	-4.3	1.5E-05	NP_071732
stmnd1.L	7.02	2.8E-04	STMND1	stathmin domain-containing protein 1	1.6	-4.5	6.1E-06	NP_001177695
hes5.2.S	7.00	2.7E-03	NA	NA	1.8	-3.9	8.5E-05	NA
hoxb8.L	6.98	3.9E-03	HOXB8	homeobox protein Hox-B8	1.8	-3.8	1.3E-04	NP_076921
LOC100485775.L	6.96	5.0E-03	TTC6	PREDICTED: tetratricopeptide repeat protein 6	1.9	-3.8	1.8E-04	XP_006726617
hoxa3.S	6.95	2.5E-03	HOXA3	PREDICTED: homeobox protein Hox-A3 isoform X3	1.8	-4.0	7.7E-05	XP_005249789
col9a2.L	6.93	2.1E-07	COL9A2	PREDICTED: collagen alpha-2(IX) chain isoform X2	1.2	-6.0	2.1E-09	XP_006710428
LOC101733581.S	6.91	5.3E-03	TGFB1	transforming growth factor-beta-induced protein ig-h3 precursor	1.8	-3.7	1.9E-04	NP_000349
hoxc6.S.g	6.91	6.8E-03	NA	NA	1.9	-3.7	2.5E-04	NA
pou5f3.2.S	6.88	8.7E-09	NA	NA	1.1	-6.5	7.1E-11	NA
hoxb7.S	6.85	5.6E-03	HOXB7	homeobox protein Hox-B7	1.8	-3.7	2.0E-04	NP_004493
hoxd1.S	6.84	6.3E-03	HOXD1	homeobox protein Hox-D1	1.9	-3.7	2.3E-04	NP_078777
ebf2.L	6.84	5.9E-04	EBF2	transcription factor COE2	1.6	-4.3	1.5E-05	NP_073150
Xetrov90024189m.S	6.80	7.7E-03	TTN	titin isoform novex-3	1.9	-3.6	3.0E-04	NP_596870
neurod4.L	6.75	5.9E-03	NEUROD4	neurogenic differentiation factor 4	1.8	-3.7	2.1E-04	NP_067014
st6gal1.S	6.71	6.5E-03	ST6GAL1	PREDICTED: beta-galactoside alpha-2,6-sialyltransferase 1 isofo	1.8	-3.7	2.4E-04	XP_005247777
hoxb9.S.g	6.66	1.2E-03	NA	NA	1.6	-4.2	3.3E-05	NA
atp6v0d2.L	6.61	2.0E-03	ATP6V0D1	V-type proton ATPase subunit d 1	1.6	-4.0	6.0E-05	NP_004682
fam181a-like.L	6.59	8.8E-03	FAM181A	protein FAM181A isoform 2	1.8	-3.6	3.5E-04	NP_001194003
foxa2.S	6.52	1.2E-05	FOXA2	PREDICTED: hepatocyte nuclear factor 3-beta isoform X1	1.3	-5.2	1.8E-07	XP_006723625
vgll3.S	6.41	2.5E-06	VGLL3	transcription cofactor vestigial-like protein 3	1.2	-5.5	3.2E-08	NP_057290
cdx2.L	6.38	1.7E-07	CDX2	homeobox protein CDX-2	1.1	-6.0	1.7E-09	NP_001256
LOC100492804.S	6.35	3.1E-30	RNF220	PREDICTED: E3 ubiquitin-protein ligase RNF220 isoform X6	0.5	-12.0	2.4E-33	XP_005271054

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LOC101734664.S	6.32	6.4E-06	#N/A	#N/A	1.2	-5.3	9.1E-08	NA
actc1.S	6.30	1.4E-14	ACTC1	actin, alpha cardiac muscle 1 proprotein	0.8	-8.4	4.8E-17	NP_005150
lhx1.S	6.27	6.1E-03	LHX1	LIM/homeobox protein Lhx1	1.7	-3.7	2.2E-04	NP_005559
flnc.S	6.26	1.8E-05	FLNC	filamin-C isoform b	1.2	-5.1	2.8E-07	NP_001120959
Xelaev18013563m.g	6.23	9.2E-03	HOXC6	homeobox protein Hox-C6 isoform 1	1.8	-3.6	3.7E-04	NP_004494
foxj1.S	6.23	3.4E-08	FOXJ1	forkhead box protein J1	1.0	-6.3	3.1E-10	NP_001445
Xelaev18044833m.g	6.21	2.1E-08	GBX2	homeobox protein GBX-2	1.0	-6.4	1.8E-10	NP_001476
hes5.1.S	6.15	1.5E-05	NA	NA	1.2	-5.2	2.4E-07	NA
Xelaev18000756m.g	6.06	1.3E-14	ELAVL3	PREDICTED: ELAV-like protein 3 isoform X3	0.7	-8.4	4.5E-17	XP_006722746
not.L	6.04	2.8E-08	EMX1	homeobox protein EMX1	1.0	-6.3	2.5E-10	NP_004088
col5a1-like.S	6.00	8.3E-03	COL5A1	collagen alpha-1(V) chain isoform 1 preproprotein	1.7	-3.6	3.2E-04	NP_000084
foxa1.L	5.86	6.3E-05	FOXA1	hepatocyte nuclear factor 3-alpha	1.2	-4.9	1.2E-06	NP_004487
olig3.S	5.86	8.7E-05	OLIG3	oligodendrocyte transcription factor 3	1.2	-4.8	1.7E-06	NP_786923
Xelaev18039828m.g	5.76	1.6E-06	MYH2	myosin-2	1.0	-5.6	1.9E-08	NP_060004
foxd4l1.1.L	5.72	2.8E-04	NA	NA	1.3	-4.5	6.2E-06	NA
foxa2.L	5.68	1.7E-03	FOXA2	PREDICTED: hepatocyte nuclear factor 3-beta isoform X1	1.4	-4.1	4.8E-05	XP_006723625
hoxb8.S	5.56	9.0E-04	HOXB8	homeobox protein Hox-B8	1.3	-4.2	2.4E-05	NP_076921
wnt1.S	5.51	5.0E-06	WNT1	proto-oncogene Wnt-1 precursor	1.0	-5.4	6.8E-08	NP_005421
Xelaev18003936m.g	5.43	8.9E-04	#N/A	#N/A	1.3	-4.2	2.3E-05	NA
col9a1.L	5.35	1.3E-06	COL9A1	collagen alpha-1(IX) chain isoform 2 precursor	0.9	-5.7	1.5E-08	NP_511040
col5a3-like.S	5.28	9.5E-05	COL11A1	collagen alpha-1(XI) chain isoform A preproprotein	1.1	-4.8	1.9E-06	NP_001845
iqub-like.L	5.26	2.3E-03	IQUB	PREDICTED: IQ and ubiquitin-like domain-containing protein isoform	1.3	-4.0	7.2E-05	XP_005250218
cdx4.L	5.24	8.0E-06	CDX4	homeobox protein CDX-4	1.0	-5.3	1.2E-07	NP_005184
Xelaev18004302m.g	5.22	3.7E-05	NKX6-1	PREDICTED: homeobox protein Nkx-6.1 isoform X1	1.0	-5.0	6.3E-07	XP_006714293
iqca1.L	5.20	4.8E-05	IQCA1	IQ and AAA domain-containing protein 1 isoform 2	1.1	-4.9	8.6E-07	NP_001257513
nkx6-2.S	5.16	2.2E-11	NKX6-2	homeobox protein Nkx-6.2	0.7	-7.4	1.3E-13	NP_796374
Xetrov90026956m.S	5.14	5.1E-03	THBS3	thrombospondin-3 isoform 1 precursor	1.4	-3.7	1.8E-04	NP_009043
plod2-like.L	5.11	1.3E-07	PLOD2	procollagen-lysine,2-oxoglutarate 5-dioxygenase 2 isoform 1 precursor	0.8	-6.1	1.3E-09	NP_891988
Xelaev18030725m.g	5.11	6.5E-12	NRP1	neuropilin-1 isoform a precursor	0.7	-7.6	3.6E-14	NP_003864
irx1.L	5.10	1.1E-22	IRX1	iroquois-class homeodomain protein IRX-1	0.5	-10.4	1.8E-25	NP_077313
en2.S	5.04	1.2E-07	EN2	homeobox protein engrailed-2	0.8	-6.1	1.2E-09	NP_001418
mylpf.S	5.01	3.2E-04	MYLPF	myosin regulatory light chain 2, skeletal muscle isoform	1.1	-4.5	7.2E-06	NP_037424
foxj1.L	4.97	1.5E-10	FOXJ1	forkhead box protein J1	0.7	-7.1	1.0E-12	NP_001445
irx3.S	4.95	5.2E-26	IRX1	iroquois-class homeodomain protein IRX-1	0.4	-11.2	7.1E-29	NP_077313
Xelaev18015140m.g	4.93	1.6E-03	TEKT2	PREDICTED: tektin-2 isoform X1	1.2	-4.1	4.6E-05	XP_005270810
fam154b.L	4.91	9.7E-03	FAM154B	PREDICTED: protein FAM154B isoform X1	1.4	-3.5	4.0E-04	XP_005254374
Xelaev18042772m.g	4.88	3.6E-153	CRABP2	PREDICTED: cellular retinoic acid-binding protein 2 isoform X1	0.2	-26.7	3.2E-157	XP_006711232
col5a3-like.L	4.88	2.2E-13	COL11A1	collagen alpha-1(XI) chain isoform E preproprotein	0.6	-8.0	9.6E-16	NP_001177638
dmbx1.L	4.85	1.9E-16	DMBX1	Otx3 / diencephalon/mesencephalon homeobox protein 1 isoform	0.5	-8.9	5.0E-19	XP_006710418
wnt4.S.g	4.84	5.1E-04	NA	NA	1.1	-4.4	1.3E-05	NA
Xelaev18027783m.g	4.80	3.4E-17	PAX3	paired box protein Pax-3 isoform PAX3i	0.5	-9.1	8.4E-20	NP_001120838
lrrc36.L	4.79	6.7E-13	LRRC36	leucine-rich repeat-containing protein 36 isoform 2	0.6	-7.9	3.2E-15	NP_001155047

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Xelaev18017637m.g	4.78	8.5E-03	CAV1	caveolin-1 isoform alpha	1.3	-3.6	3.3E-04	NP_001744
Xelaev18016667m.g	4.77	3.7E-09	UPK2	uroplakin-2 precursor	0.7	-6.7	2.9E-11	NP_006751
barhl2.S	4.74	1.5E-08	BARHL2	barH-like 2 homeobox protein	0.7	-6.4	1.2E-10	NP_064447
igf3.L	4.69	6.3E-05	NA	NA	1.0	-4.9	1.2E-06	NA
nkx6-2.L	4.65	2.8E-14	NKX6-2	homeobox protein Nkx-6.2	0.6	-8.3	1.0E-16	NP_796374
cbx6.S	4.58	5.1E-03	CBX6	chromobox protein homolog 6	1.2	-3.7	1.8E-04	NP_055107
Xetrov90025669m.S	4.57	4.7E-06	DNAH9	dynein heavy chain 9, axonemal isoform 2	0.8	-5.4	6.3E-08	NP_001363
Xelaev18037602m.g	4.56	1.0E-06	HES5	transcription factor HES-5	0.8	-5.7	1.2E-08	NP_001010926
pou3f2.L	4.52	4.8E-05	POU3F2	POU domain, class 3, transcription factor 2	0.9	-4.9	8.6E-07	NP_005595
fgf3.S	4.51	6.9E-06	FGF3	fibroblast growth factor 3 precursor	0.8	-5.3	1.0E-07	NP_005238
ltbp1.S	4.51	3.5E-03	LTBP1	latent-transforming growth factor beta-binding protein 1 isoform L1	1.2	-3.9	1.1E-04	NP_996826
not.S	4.51	3.7E-28	EMX1	homeobox protein EMX1	0.4	-11.6	4.2E-31	NP_004088
stmnd1.S	4.50	4.3E-03	STMND1	stathmin domain-containing protein 1	1.2	-3.8	1.5E-04	NP_001177695
Xelaev18009356m.g	4.46	5.1E-06	NEUROG2	neurogenin-2	0.8	-5.4	7.0E-08	NP_076924
Xelaev18042726m.g	4.44	5.5E-04	ONECUT2	one cut domain family member 2	1.0	-4.3	1.4E-05	NP_004843
sp5.L	4.43	1.2E-06	SP5	transcription factor Sp5	0.8	-5.7	1.5E-08	NP_001003845
hes5.2.L	4.43	3.9E-03	NA	NA	1.2	-3.8	1.3E-04	NA
pou5f3.2.L	4.43	9.1E-08	NA	NA	0.7	-6.1	9.0E-10	NA
Xelaev18035683m.g	4.42	5.2E-07	WNT4	protein Wnt-4 precursor	0.8	-5.8	5.8E-09	NP_110388
LOC101734338.L	4.42	4.9E-03	C11orf63	PREDICTED: uncharacterized protein C11orf63 isoform X1	1.2	-3.8	1.7E-04	XP_005271736
fam181b.S	4.35	2.8E-04	FAM181B	protein FAM181B	1.0	-4.5	6.2E-06	NP_787081
Xelaev18013523m.g	4.35	4.5E-09	COL2A1	collagen alpha-1(II) chain isoform 1 precursor	0.7	-6.6	3.6E-11	NP_001835
pcdh18.L	4.32	2.3E-05	PCDH18	PREDICTED: protocadherin-18 isoform X1	0.9	-5.1	3.8E-07	XP_005263127
pou5f3.1.S.g	4.28	8.0E-09	NA	NA	0.7	-6.5	6.5E-11	NA
foxd4l1.1.S	4.27	5.9E-05	NA	NA	0.9	-4.9	1.1E-06	NA
hepacam2.L	4.27	7.1E-03	HEPACAM2	HEPACAM family member 2 isoform 1 precursor	1.2	-3.6	2.7E-04	NP_001034461
LOC100487796-like.	4.25	3.3E-04	SAPCD2	suppressor APC domain-containing protein 2	0.9	-4.5	7.5E-06	NP_848543
ntn1-like.1.L	4.25	6.3E-03	NA	NA	1.2	-3.7	2.3E-04	NA
slc7a8.S	4.18	3.8E-08	SLC7A8	large neutral amino acids transporter small subunit 2 isoform a	0.7	-6.3	3.5E-10	NP_036376
Xelaev18031753m.g	4.16	6.1E-06	SALL3	sal-like protein 3	0.8	-5.4	8.6E-08	NP_741996
fzd10.S	4.11	1.4E-12	FZD10	frizzled-10 precursor	0.5	-7.8	7.1E-15	NP_009128
foxd1.S	4.06	5.6E-11	FOXD2	forkhead box protein D2	0.6	-7.3	3.6E-13	NP_004465
LOC100496628-like.	4.01	1.9E-09	GPR34	PREDICTED: probable G-protein coupled receptor 34 isoform X1	0.6	-6.8	1.4E-11	XP_005272654
ccdc78.1	4.00	4.7E-03	CCDC78	PREDICTED: coiled-coil domain-containing protein 78 isoform X3	1.1	-3.8	1.6E-04	XP_005255159
sd3.L	3.92	1.5E-03	SDC3	syndecan-3 precursor	1.0	-4.1	4.3E-05	NP_055469
Xelaev18004445m.g	3.91	6.5E-28	ELAVL3	PREDICTED: ELAV-like protein 3 isoform X1	0.3	-11.5	7.7E-31	XP_005259869
dmbx1.S	3.91	6.2E-08	DMBX1	Otx3 / diencephalon/mesencephalon homeobox protein 1 isoform	0.6	-6.2	5.9E-10	XP_006710418
fzd10.L	3.89	6.4E-23	FZD10	frizzled-10 precursor	0.4	-10.5	9.6E-26	NP_009128
sfrp1.S	3.88	2.4E-12	SFRP1	secreted frizzled-related protein 1 precursor	0.5	-7.7	1.2E-14	NP_003003
sp5l.S	3.83	2.3E-11	SP5	transcription factor Sp5	0.5	-7.4	1.4E-13	NP_001003845
hes9-1.S.g	3.81	3.3E-05	NA	NA	0.8	-5.0	5.7E-07	NA
col5a1-like.L	3.80	4.7E-05	COL5A1	collagen alpha-1(V) chain isoform 1 preproprotein	0.8	-4.9	8.4E-07	NP_000084

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Xelaev18037604m.g	3.76	1.5E-03	HES5	transcription factor HES-5	0.9	-4.1	4.2E-05	NP_001010926
hes7_x1.S.g	3.74	5.0E-03	NA	NA	1.0	-3.8	1.7E-04	NA
barhl2.L	3.74	6.4E-08	BARHL2	barH-like 2 homeobox protein	0.6	-6.2	6.1E-10	NP_064447
LOC100494180.L	3.73	3.2E-04	KIAA2012	PREDICTED: uncharacterized protein KIAA2012 isoform X2	0.8	-4.5	7.3E-06	XP_006712235
chrd.2.L.g	3.72	4.6E-13	NA	NA	0.5	-7.9	2.1E-15	NA
plekhg4.S	3.65	2.5E-10	PLEKHG4B	pleckstrin homology domain-containing family G member 4B	0.5	-7.1	1.7E-12	NP_443141
shisa2.S	3.64	4.3E-03	SHISA2	protein shisa-2 homolog precursor	1.0	-3.8	1.5E-04	NP_001007539
prickle2.S	3.62	1.4E-03	PRICKLE2	PREDICTED: prickle-like protein 2 isoform X3	0.9	-4.1	3.9E-05	XP_006713069
Xelaev18005657m.g	3.60	1.9E-03	NEUROG2	neurogenin-2	0.9	-4.0	5.8E-05	NP_076924
tubb2b.S	3.59	1.5E-08	TUBB2B	tubulin beta-2B chain	0.6	-6.4	1.3E-10	NP_821080
efemp2.L	3.56	2.5E-03	EFEMP2	EGF-containing fibulin-like extracellular matrix protein 2 precursor	0.9	-3.9	8.0E-05	NP_058634
Xelaev18011776m.g	3.56	2.3E-20	ARX	homeobox protein ARX	0.4	-9.9	4.0E-23	NP_620689
ror2.L	3.52	7.2E-31	ROR2	tyrosine-protein kinase transmembrane receptor ROR2 precursor	0.3	-12.2	4.6E-34	NP_004551
wnt2b.L	3.49	9.0E-03	WNT2B	protein Wnt-2b isoform WNT-2B1	1.0	-3.6	3.6E-04	NP_004176
tdgf1.3.L	3.49	9.4E-04	NA	NA	0.8	-4.2	2.5E-05	NA
Xelaev18015990m.g	3.47	5.3E-04	COL2A1	PREDICTED: collagen alpha-1(II) chain isoform X1	0.8	-4.4	1.3E-05	XP_006719305
lhx5.S	3.45	1.2E-05	LHX5	LIM/homeobox protein Lhx5	0.7	-5.2	1.8E-07	NP_071758
chrd.1.L.g	3.41	1.2E-11	NA	NA	0.5	-7.5	6.6E-14	NA
pou3f3.S	3.39	8.8E-12	POU3F3	POU domain, class 3, transcription factor 3	0.4	-7.5	5.0E-14	NP_006227
lhx5.L	3.37	2.9E-11	LHX5	LIM/homeobox protein Lhx5	0.5	-7.4	1.8E-13	NP_071758
wnt11b.L	3.35	6.2E-08	WNT11	PREDICTED: protein Wnt-11 isoform X1	0.5	-6.2	5.9E-10	XP_005274288
pou3f2.S	3.35	1.1E-04	POU3F2	POU domain, class 3, transcription factor 2	0.7	-4.7	2.3E-06	NP_005595
crtap.S	3.32	3.6E-03	CRTAP	cartilage-associated protein precursor	0.9	-3.9	1.2E-04	NP_006362
cyyr1.L	3.31	9.8E-16	CYYR1	cysteine and tyrosine-rich protein 1 precursor	0.4	-8.7	3.1E-18	NP_443186
mn1.S	3.29	1.8E-14	MN1	probable tumor suppressor protein MN1	0.4	-8.4	6.5E-17	NP_002421
wdr16.L	3.28	1.8E-06	CFAP52	WD repeat-containing protein 16 isoform b	0.6	-5.6	2.2E-08	NP_659491
pou5f3.1.L.g	3.25	2.3E-39	NA	NA	0.2	-13.7	9.6E-43	NA
foxd1.L	3.24	1.1E-13	FOXD2	forkhead box protein D2	0.4	-8.1	4.3E-16	NP_004465
sp5l.L	3.23	2.0E-28	SP5	transcription factor Sp5	0.3	-11.7	2.1E-31	NP_001003845
egr2.L	3.20	4.1E-05	EGR2	E3 SUMO-protein ligase EGR2 isoform a	0.6	-5.0	7.2E-07	NP_001129650
ngfr.L	3.17	2.9E-09	NGFR	tumor necrosis factor receptor superfamily member 16 precursor	0.5	-6.7	2.2E-11	NP_002498
Xelaev18042375m.g	3.09	6.6E-10	NOVA1	RNA-binding protein Nova-1 isoform 2	0.4	-6.9	4.8E-12	NP_006480
adamts10-like.L	3.05	8.7E-03	ADAMTS10	PREDICTED: A disintegrin and metalloproteinase with thrombospi	0.9	-3.6	3.4E-04	XP_006722979
Xelaev18040362m.g	3.03	1.2E-08	ONECUT2	one cut domain family member 2	0.5	-6.5	9.9E-11	NP_004843
xarp-like.S	3.01	1.4E-20	XARP	PREDICTED: axin-1 isoform X3	0.3	-10.0	2.4E-23	XP_005255666
pfkfb3.L	3.00	6.4E-29	PFKFB3	PREDICTED: 6-phosphofructo-2-kinase/fructose-2,6-bisphosphat	0.3	-11.8	5.3E-32	XP_005252520
hes5.1.L	2.97	3.5E-03	NA	NA	0.8	-3.9	1.2E-04	NA
tnfrsf19.L	2.97	3.2E-07	TNFRSF19	tumor necrosis factor receptor superfamily member 19 isoform 2 p	0.5	-5.9	3.5E-09	NP_001191387
ptch2.L	2.96	4.4E-12	PTCH1	protein patched homolog 1 isoform L	0.4	-7.6	2.4E-14	NP_000255
adamts4.S	2.93	3.4E-05	ADAMTSL4	ADAMTS-like protein 4 isoform 1 precursor	0.6	-5.0	5.8E-07	NP_061905
Xelaev18043762m.g	2.85	1.8E-35	SLC16A3	monocarboxylate transporter 4	0.2	-13.0	8.8E-39	NP_001193881
rspo2.L	2.84	2.2E-13	RSPO2	PREDICTED: R-spondin-2 isoform X1	0.4	-8.0	9.6E-16	XP_005250963

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tril.L	2.81	1.5E-06	TRIL	TLR4 interactor with leucine rich repeats precursor	0.5	-5.6	1.8E-08	NP_055632
cttnbp2-like.S	2.80	5.6E-04	CTTNBP2	cortactin-binding protein 2	0.6	-4.3	1.4E-05	NP_219499
olig2.S	2.77	3.0E-11	OLIG2	PREDICTED: oligodendrocyte transcription factor 2 isoform X1	0.4	-7.4	1.8E-13	XP_005260965
xarp-like.L	2.76	4.2E-17	AXIN2	axin-2	0.3	-9.1	1.1E-19	NP_004646
cyyr1.S	2.75	1.8E-03	CYYR1	cysteine and tyrosine-rich protein 1 precursor	0.7	-4.0	5.4E-05	NP_443186
irx2.L	2.73	7.9E-19	IRX2	iroquois-class homeodomain protein IRX-2	0.3	-9.5	1.7E-21	NP_001127694
mn1.L	2.72	3.1E-16	MN1	probable tumor suppressor protein MN1	0.3	-8.9	8.5E-19	NP_002421
LOC100145154.L	2.71	5.9E-03	GPR21	PREDICTED: probable G-protein coupled receptor 21 isoform X1	0.7	-3.7	2.1E-04	XP_005251990
Xelaev18033587m.g	2.71	6.3E-03	APCDD1	protein APCDD1 precursor	0.7	-3.7	2.3E-04	NP_694545
Xelaev18006859m.g	2.70	6.4E-06	CYP2J2	cytochrome P450 2J2	0.5	-5.3	9.1E-08	NP_000766
insm1.L	2.68	6.7E-09	INSM1	insulinoma-associated protein 1	0.4	-6.6	5.3E-11	NP_002187
hes3.L	2.64	1.1E-20	NA	NA	0.3	-10.0	1.8E-23	NA
Xelaev18031847m.g	2.62	9.4E-05	APCDD1	protein APCDD1 precursor	0.5	-4.8	1.9E-06	NP_694545
Xelaev18034617m.g	2.61	1.2E-08	VENTX	homeobox protein VENTX	0.4	-6.5	1.0E-10	NP_055283
tspan18.L	2.61	6.0E-03	TSPAN18	PREDICTED: tetraspanin-18 isoform X3	0.7	-3.7	2.2E-04	XP_006718436
ppap2b.S	2.60	2.0E-06	PPAP2B	lipid phosphate phosphohydrolase 3	0.5	-5.6	2.5E-08	NP_003704
prickle1.L	2.59	3.7E-05	PRICKLE1	prickle-like protein 1	0.5	-5.0	6.3E-07	NP_001138355
p4ha1-like.1.L	2.58	3.6E-03	NA	NA	0.7	-3.8	1.2E-04	NA
kcp.L	2.56	3.5E-16	KCP	kielin/chordin-like protein isoform 1 precursor	0.3	-8.8	9.8E-19	NP_001129386
zc4h2.L	2.56	7.7E-29	ZC4H2	zinc finger C4H2 domain-containing protein isoform 1	0.2	-11.8	7.0E-32	NP_061154
LOC100492840-like.	2.54	1.0E-06	MATN2	PREDICTED: matrilin-2 isoform X2	0.4	-5.7	1.2E-08	XP_005250977
ccdc39.L	2.53	5.4E-03	CCDC39	coiled-coil domain-containing protein 39	0.7	-3.7	1.9E-04	NP_852091
zic3.L	2.51	6.5E-21	ZIC3	zinc finger protein ZIC 3	0.2	-10.0	1.0E-23	NP_003404
lmx1b.1.S	2.50	1.4E-03	NA	NA	0.6	-4.1	4.1E-05	NA
LOC100487093-like.	2.48	5.4E-04	EFCAB6	EF-hand calcium-binding domain-containing protein 6 isoform a	0.6	-4.4	1.3E-05	NP_073622
ankrd65.L	2.45	5.8E-05	ANKRD65	PREDICTED: ankyrin-3 isoform X13	0.5	-4.9	1.1E-06	XP_006717850
pou3f1.S	2.45	6.9E-08	POU3F4	POU domain, class 3, transcription factor 4	0.4	-6.2	6.6E-10	NP_000298
Xelaev18010085m.g	2.43	5.4E-13	CYP2J2	cytochrome P450 2J2	0.3	-7.9	2.5E-15	NP_000766
Xetrov90013456m.L	2.43	7.5E-04	SYNE1	PREDICTED: nesprin-1 isoform X10	0.6	-4.3	1.9E-05	XP_006715479
rin2.S	2.40	1.7E-03	RIN2	PREDICTED: ras and Rab interactor 2 isoform X6	0.6	-4.1	5.0E-05	XP_006723636
grpel2.S	2.39	1.6E-05	GRPEL2	grpE protein homolog 2, mitochondrial precursor	0.5	-5.2	2.4E-07	NP_689620
irx1.S	2.38	1.9E-08	IRX1	iroquois-class homeodomain protein IRX-1	0.4	-6.4	1.6E-10	NP_077313
bean1-like.L	2.38	1.2E-03	BEAN1	protein BEAN1 isoform 1	0.6	-4.1	3.3E-05	NP_001171491
Xelaev18033531m.g	2.38	2.3E-03	SALL3	sal-like protein 3	0.6	-4.0	6.9E-05	NP_741996
itga5.S	2.36	7.7E-04	ITGA5	integrin alpha-5 precursor	0.6	-4.3	2.0E-05	NP_002196
rxf4.S	2.36	5.2E-04	RFX4	transcription factor RFX4 isoform d	0.5	-4.4	1.3E-05	NP_001193620
stox1.L	2.34	6.5E-03	STOX1	storkhead-box protein 1 isoform a	0.6	-3.7	2.4E-04	NP_001123633
Xelaev18014509m.g	2.30	6.7E-12	ARX	homeobox protein ARX	0.3	-7.6	3.7E-14	NP_620689
ptch2.S	2.29	4.8E-08	PTCH1	protein patched homolog 1 isoform L	0.4	-6.2	4.5E-10	NP_000255
insm1.S	2.28	2.6E-07	INSM1	insulinoma-associated protein 1	0.4	-5.9	2.8E-09	NP_002187
Xelaev18037875m.g	2.28	1.2E-07	GPRC5C	G-protein coupled receptor family C group 5 member C isoform b	0.4	-6.1	1.2E-09	NP_061123
ccdc37.L	2.26	4.2E-03	CCDC37	PREDICTED: coiled-coil domain-containing protein 37 isoform X9	0.6	-3.8	1.4E-04	XP_006713686

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foxh1.L	2.26	2.3E-03	FOXH1	forkhead box protein H1	0.6	-4.0	7.1E-05	NP_003914
LOC100127584-like.	2.25	2.1E-04	AKAP12	PREDICTED: A-kinase anchor protein 12 isoform X3	0.5	-4.6	4.5E-06	XP_005267292
rcn3.L	2.25	6.6E-16	CALU	calumenin isoform b precursor	0.3	-8.8	2.0E-18	NP_001124146
Xelaev18038401m.g	2.23	1.9E-18	#N/A	#N/A	0.2	-9.4	4.1E-21	NA
entpd2.S	2.23	6.2E-06	ENTPD2	ectonucleoside triphosphate diphosphohydrolase 2 isoform 1	0.4	-5.4	8.7E-08	NP_982293
rhbdl3.L	2.23	2.1E-03	RHBDL3	rhomboid-related protein 3	0.6	-4.0	6.5E-05	NP_612201
pfkfb3.S	2.20	7.8E-12	PFKFB3	PREDICTED: 6-phosphofructo-2-kinase/fructose-2,6-bisphosphat	0.3	-7.6	4.3E-14	XP_005252520
cldn5.S	2.18	9.2E-05	CLDN5	PREDICTED: claudin-5 isoform X1	0.5	-4.8	1.8E-06	XP_005261329
pou3f1.L	2.17	5.4E-03	POU3F4	POU domain, class 3, transcription factor 4	0.6	-3.7	1.9E-04	NP_000298
Xetrov90019615m.L	2.17	7.9E-41	HSPG2	PREDICTED: basement membrane-specific heparan sulfate prote	0.2	-14.0	2.5E-44	XP_006710659
ackr3.L	2.17	1.9E-04	ACKR3	PREDICTED: atypical chemokine receptor 3 isoform X2	0.5	-4.6	4.2E-06	XP_005246155
barhl1.L	2.15	1.0E-19	BARHL1	barH-like 1 homeobox protein	0.2	-9.7	2.1E-22	NP_064448
Xetrov90025030m.S	2.15	2.1E-03	METRNL	PREDICTED: meteorin-like protein isoform X1	0.5	-4.0	6.4E-05	XP_006725354
MGC89906.L	2.14	4.2E-04	DLEU7	PREDICTED: leukemia-associated protein 7 isoform X1	0.5	-4.4	1.0E-05	XP_005266343
tspan18.S	2.13	2.9E-03	TSPAN18	PREDICTED: tetraspanin-18 isoform X3	0.5	-3.9	9.3E-05	XP_006718436
gpr37l1.S	2.13	3.7E-03	GPR37L1	prosaposin receptor GPR37L1 precursor	0.6	-3.8	1.2E-04	NP_004758
zic3.S	2.13	1.2E-16	ZIC3	zinc finger protein ZIC 3	0.2	-9.0	3.3E-19	NP_003404
fkbp10.L	2.13	7.3E-03	FKBP10	peptidyl-prolyl cis-trans isomerase FKBP10 precursor	0.6	-3.6	2.8E-04	NP_068758
LOC100490274.S	2.11	2.8E-05	PPP1R18	phostensin	0.4	-5.0	4.6E-07	NP_597728
pnp.L	2.10	1.1E-28	PNP	purine nucleoside phosphorylase	0.2	-11.7	1.0E-31	NP_000261
Xelaev18016063m.g	2.10	2.9E-06	MUC19	PREDICTED: mucin-19-like	0.4	-5.5	3.8E-08	XP_005275972
Xelaev18040968m.g	2.09	6.4E-07	ATP6V1A	V-type proton ATPase catalytic subunit A	0.4	-5.8	7.2E-09	NP_001681
pappa.S	2.08	1.1E-03	PAPPA	pappalysin-1 preproprotein	0.5	-4.2	3.0E-05	NP_002572
Xelaev18030359m.g	2.08	4.8E-03	ZNF41	PREDICTED: zinc finger protein 41 isoform X5	0.6	-3.8	1.7E-04	XP_005272725
Xelaev18034615m.g	2.07	7.5E-06	VENTX	homeobox protein VENTX	0.4	-5.3	1.1E-07	NP_055283
Xelaev18013898m.g	2.06	3.2E-04	FZD4	frizzled-4 precursor	0.5	-4.5	7.2E-06	NP_036325
ebf2.S	2.06	4.1E-04	EBF2	transcription factor COE2	0.5	-4.4	9.6E-06	NP_073150
lrat.L	2.06	2.0E-03	LRAT	PREDICTED: lecithin retinol acyltransferase isoform X1	0.5	-4.0	5.9E-05	XP_006714475
Xetrov90015445m.L	2.04	7.2E-03	#N/A	#N/A	0.6	-3.6	2.7E-04	NA
cyp26a1.L	2.04	2.0E-06	CYP26A1	cytochrome P450 26A1 isoform 1	0.4	-5.6	2.6E-08	NP_000774
sall1.L	2.02	1.0E-09	SALL1	PREDICTED: sal-like protein 1 isoform X1	0.3	-6.8	7.6E-12	XP_006721304
cdk5r1.L	2.02	1.0E-03	CDK5R1	cyclin-dependent kinase 5 activator 1	0.5	-4.2	2.7E-05	NP_003876
dkkx.L	-2.03	2.4E-19	DKK3	PREDICTED: dickkopf-related protein 3 isoform X2	0.2	9.6	5.0E-22	XP_006718241
Xelaev18033759m.g	-2.07	7.2E-31	BHLHE22	class E basic helix-loop-helix protein 22	0.2	12.2	4.3E-34	NP_689627
etv2.L	-2.08	2.1E-11	ETS1	protein C-ets-1 isoform 3	0.3	7.4	1.2E-13	NP_001155894
rorb.L	-2.12	3.5E-06	RORB	nuclear receptor ROR-beta	0.4	5.5	4.7E-08	NP_008845
Xelaev18036457m.g	-2.15	5.4E-08	FLI1	Friend leukemia integration 1 transcription factor isoform 1	0.3	6.2	5.0E-10	NP_002008
samd11.L	-2.16	6.9E-05	SAMD11	sterile alpha motif domain-containing protein 11	0.4	4.8	1.3E-06	NP_689699
myb.L	-2.21	1.1E-08	MYB	transcriptional activator Myb isoform 8	0.3	6.5	9.4E-11	NP_001155132
Xetrov90006664m.S	-2.22	9.3E-14	NBEA	neurobeachin isoform 1	0.3	8.1	3.6E-16	NP_056493
she-like.L	-2.25	1.0E-03	SHE	SH2 domain-containing adapter protein E	0.5	4.2	2.7E-05	NP_001010846
nos1.L	-2.33	5.7E-11	NOS1	PREDICTED: nitric oxide synthase, brain isoform X3	0.3	7.3	3.7E-13	XP_006719491

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Xelaev18028200m.g	-2.38	6.5E-03	CRISP2	PREDICTED: cysteine-rich secretory protein 2 isoform X10	0.6	3.7	2.4E-04	XP_005249415
mab21l1.S	-2.55	1.8E-17	MAB21L1	protein mab-21-like 1	0.3	9.2	4.1E-20	NP_005575
Xetrov90016643m.S	-2.60	1.9E-11	SOGA2	PREDICTED: protein SOGA2 isoform X3	0.3	7.4	1.1E-13	XP_006722381
Xelaev18023452m.g	-2.97	9.1E-09	LHX9	LIM/homeobox protein Lhx9 isoform 1	0.5	6.5	7.5E-11	NP_064589
tubb3.L	-3.02	6.2E-08	TUBB3	tubulin beta-3 chain isoform 1	0.5	6.2	5.8E-10	NP_006077
mab21l1.L	-3.37	1.2E-13	MAB21L1	protein mab-21-like 1	0.4	8.1	4.9E-16	NP_005575
vsx1.L	-3.54	9.5E-67	VSX1	visual system homeobox 1 isoform a	0.2	17.8	1.3E-70	NP_055403
vsx1.S	-3.69	2.4E-28	VSX1	visual system homeobox 1 isoform a	0.3	11.6	2.6E-31	NP_055403

Table 3

Primers used for RT-qPCR

Gene name	Forward primer	Reverse primer
ef1a.1.S	5'-acc ctc ctc ttg gtc gtt tt -3',	5'-ttt ggt ttt cgc tgc ttt ct -3'
admp.S	5'-atg tat act gtc tga aag gg -3'	5'-ggg ggc tat aaa tga tac tt -3'
emilin3	5'- ggg cac cag tgg gga act gc -3'	5'- tc cca cgc agc agg tct gtc -3'
cdx4.L	5'- tga ttt atc acc taa cca g -3'	5'- gtc cca gat gga tga gga ga -3'
shh.L	5'- aac aca cct ggg cac acc tc -3'	5'- tcc aaa agc caa gtc cct at -3'
sox2.L	5'-tca cct ctt ctt ccc att cg -3'	5- cga cat gtg cag tct gct tt -3'
ventx1.2.S	5'-ttc cct tca gca tgg ttc aac-3'	5'- gca tct cct tgg cat att tgg-3'
ventx2.1.L	5'-tga gac ttg ggc act gtc tg-3'	5'-cct ctg ttg aat ggc ttg ct-3'
szl.L/S	5'- gtc ttc ctg ctc ctc tgc-3'	5'- aac agg gag cac agg aag-3'
chrd1.S	5'-cat gct ctt tcg aag gtc aa-3'	5'- gat cac aaa tca cgg tac gc-3'
bambi.L	5'- gca caa tgg tcc agt agg gg-3'	5'-ttc gtc ctc cta aag atc ag-3'
dkk1.S	5'-aca gag cct gaa gga cct tc-3'	5'-gtt cag gga aga cca gag ca-3'

Primers used for RT-PCR

Gene name	Forward primer	Reverse primer
ef1a	5'- cag att ggt gct gga tat gc -3'	5- act gcc ttg atg act cct ag -3'
pnhd.L	5'- tga tga aca ggc aga atg ac-3'	5- gat tca aag gga gaa tct gg -3'