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RESEARCH ARTICLE

Coco is a dual activity modulator of TGFβ signaling

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ABSTRACT

The TGFβ signaling pathway is a crucial regulator of developmental processes and disease. The activity of TGFβ ligands is modulated by various families of soluble inhibitors that interfere with the interactions between ligands and receptors. In an unbiased, genome-wide RNAi screen to identify genes involved in ligand-dependent signaling, we unexpectedly identified the BMP/Activin/Nodal inhibitor Coco as an enhancer of TGFβ1 signaling. Coco synergizes with TGFβ1 in both cell culture and *Xenopus* explants. Molecularly, Coco binds to TGFβ1 and enhances TGFβ1 binding to its receptor Alk5. Thus, Coco acts as both an inhibitor and an enhancer of signaling depending on the ligand it binds. This finding raises the need for a global reconsideration of the molecular mechanisms regulating TGFβ signaling.

KEY WORDS: Coco, TGFβ signaling modulators, Dual-activity pathway modulators

INTRODUCTION

The TGFβ pathway plays important roles in development and disease. Ligand binding by TGFβ superfamily members to type II receptors leads to recruitment and activation of a type I receptor, which in turn phosphorylates and activates receptor-regulated Smads (R-Smads). The activated R-Smads form complexes with Smad4, translocate to the nucleus and directly regulate the transcription of target genes (Schmierer and Hill, 2007; Massagué, 2012). The canonical TGFβ pathway consists of two branches. BMP/GDF ligands signal through the type I receptors Alk1/2/3/6 to activate R-Smads1/5/8, whereas TGFβ/Activin/Nodal ligands signal through the type I receptors Alk4/5/7 to activate R-Smad2/3. Notably, TGFβ1, TGFβ2 and TGFβ3 ligands signal through Alk5, whereas Nodal and Activin signal through Alk4 and Alk7, thus raising the possibility of differential regulation of these signals.

A large number of secreted inhibitors of the TGFβ signaling pathway have been identified. The correct timing and spatial localization of these inhibitors is crucial for proper embryonic development (Wu and Hill, 2009). Coco (also known as Dante, Cer2, Dand5, Cerl2, Grem3), which belongs to the DAN family of secreted proteins (Hsu et al., 1998; Pearce et al., 1999), is expressed maternally in the animal pole of the oocyte and fertilized egg, and its expression declines by mid-gastrulation (Bell et al., 2003). By contrast, other inhibitors, such as Noggin, Chordin and Follistatin, are expressed zygotically, in the embryonic organizer (node in mammals) and delineate the future neural territory (De Robertis and Kuroda, 2004). We originally discovered Coco in a gain-of-function screen for injected mRNAs with the ability to induce secondary head structures in *Xenopus* (Bell et al., 2003). Maternal Coco controls germ layer specification by inhibition of Activin, Nodal, and BMP signaling (Bates et al., 2013). Zygotic Coco expression, by contrast, is involved in proper specification of the right-left axis by inhibiting Nodal and Derriere (Hashimoto et al., 2004; Marques et al., 2004; Vonica and Brivanlou, 2007). Inhibition of BMP signaling by Coco also plays an important role in metastatic breast cancer (Gao et al., 2012). Thus, in all contexts examined to date, Coco acts as an inhibitor of TGFβ ligands.

We recently developed a system to measure TGFβ signaling dynamics by monitoring the localization of Smad4 (Warmflash et al., 2012; Sorre et al., 2014). We showed that Smad4 is a faithful reporter for TGFβ1 signaling dynamics and that TGFβ1 signaling responds to changes in ligand concentration before returning to baseline levels within 4 h (Warmflash et al., 2012). We took advantage of this system to investigate the molecular mechanism involved in the regulation of TGFβ1 signaling dynamics in a genome-wide RNAi screen. Among the factors that affect nuclear residency of Smad4, we rediscovered Coco. Unexpectedly, the results from this screen suggested that Coco is an enhancer of signaling through the TGFβ1 ligand. Here, we demonstrate that Coco is a dual-activity modulator of TGFβ signaling, enhancing activity through TGFβ1 while inhibiting Nodal and BMP ligands.

RESULTS

An siRNA screen identifies Coco as an enhancer of TGFβ1 signaling

To identify genes involved in ligand-dependent Smad4 nuclear translocation, we performed an unbiased, genome-wide RNAi screen using the C2C12 GFP-Smad4 reporter cell line (Warmflash et al., 2012) as a platform to monitor TGFβ1-induced changes in Smad4 localization (Fig. 1A). Application of 2 ng/ml TGFβ1 resulted in the rapid nuclear translocation of GFP-Smad4 that peaked within 1 h and its cytoplasmic relocalization within 6 h (Warmflash et al., 2012). Thus, we searched for genes that could either prevent GFP-Smad4 nuclear localization at 1 h or prevent its return to the cytoplasm by 6 h. To perform the screen, C2C12 GFP-Smad4 reporter cells were transfected with pools of three small interfering RNAs (siRNAs) targeting individual genes. Cells were stimulated with 2 ng/ml TGFβ1 48 h after transfection and fixed and analyzed for GFP-Smad4 localization at 1 and 6 h after TGFβ1 application. As controls for impairing and for enhancing GFP-Smad4 localization, we used the TGFβ1 type I receptor TGFβR1 (Alk5) and Exportin1 (Xpo1), a protein essential for nuclear export of Smad4 (Schmierer and Hill, 2007), respectively. Knockdown of TGFβR1 blocked translocation of GFP-Smad4 in the nucleus (Fig. 1B). Conversely, knockdown of Xpo1 led to the maintenance of GFP-Smad4 in the nucleus (Fig. 1B).

Screening of 17,582 genes, in two independent experiments, identified 321 genes (1.8%) that significantly affected the translocation of GFP-Smad4 when knocked down (supplementary material Fig. S1A and Table S1). Statistical analysis demonstrated a robust reproducibility between trials (r=0.93) (Fig. 1C). Of the

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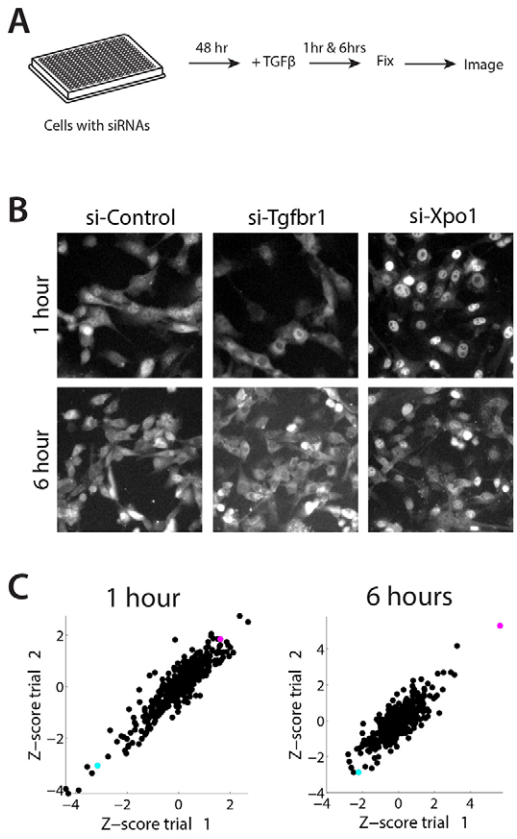

Fig. 1. A genome-wide siRNA screen identifies modulators of TGFβ1 signaling. See also supplementary material Fig. S1 and Table S1. (A) Schematic of the siRNA screen. C2C12 cells expressing GFP-Smad4 were incubated with pools of three siRNAs for each gene for 48 h, at which point cells were treated with 2 ng/ml TGFβ1 for 1 or 6 h. Cells were then fixed and imaged. (B) Validation of the siRNA screen. Images show the localization of GFP-Smad4 in C2C12 cells after 1 or 6 h of treatment with 2 ng/ml TGFβ1 incubated for 2 days with a non-targeting siRNA (si-Control), or siRNAs targeting the TGFβ receptor 1 (si-Tgfbr1) or Exportin1 (si-Xpo1). Knockdown of TGFβR1 leads to complete inhibition of GFP-Smad4 nuclear translocation at 1 h, while knockdown of Xpo1 prevents the cytoplasmic relocalization of GFP-Smad4 at 6 h. (C) Scatter plots showing the reproducibility of the screen results between the two replicates at 1 h and 6 h. The Z-score indicates a normalized measure of the nuclear/cytoplasmic ratio of GFP-Smad4 fluorescence. Data for Xpo1 is indicated in blue, data for TGFβR1 is indicated in purple.

genes impacting signaling, 166 (51.7%) blocked GFP-Smad4 translocation, and 155 (48.3%) enhanced the accumulation of GFP-Smad4 in the nucleus. Gene Ontology analysis and categorization of the candidates demonstrated that the genes identified in the screen belonged to a variety of classes (supplementary material Fig. S1B). These included transcriptional and translational regulators; channels, receptors, and other membrane proteins; secreted proteins; kinases and signaling proteins; and several proteins with unknown functions (supplementary material Table S1).

Modulation of Smad4 translocation can be due to an upstream effect on the pathway, for example modulation of R-Smads, or it can be due to an effect on Smad4 itself. To distinguish between these two possibilities, we examined the ability of R-Smad2 to translocate in response to TGFβ1 ligand in RFP-Smad2 reporter cells with knockdown of each of the 321 candidates. Nearly all genes for which knockdown prevented Smad4 translocation also prevented Smad2 translocation, whereas those enhancing Smad4 translocation were varied in their effect on Smad2 (supplementary material Fig. S1C and Table S1).

Among the genes that we discovered as modifiers of TGFβ1 signaling was a gene known as *Coco* (*Dand5/Dte/Dante*), which we originally identified as a BMP4 and Nodal inhibitor (Bell et al., 2003). Therefore, we expected Coco to behave in a similar manner in this context and also inhibit TGFβ1 signaling. However, knockdown of Coco strongly impaired, rather than enhanced, GFP-Smad4 translocation and thus TGFβ1 signaling. This finding led us to investigate Coco's mechanism of action further.

Coco knockdown impairs TGFβ1 signaling

To exclude nonspecific effects from the siRNAs, we first tested each individual siRNA from the pool of three in C2C12 cells. Transfection of two independent Coco siRNAs led to a significant reduction in *Coco* expression levels and also caused a reduction in Smad4 nuclear translocation (21-24% of the original expression level and 20-40% of the original Smad4 translocation; Fig. 2A,B). By contrast, the third siRNA as well as a non-targeting control had no significant effect on either *Coco* expression or Smad4 localization. siRNA-mediated Coco knockdown also significantly reduced the level of nuclear Smad2 as measured by immunofluorescence (Fig. 2C), further supporting the idea that Coco is required for TGFβ1 signaling and demonstrating that Coco exerts its function upstream of Smad4. Knockdown of Coco also impaired TGFβ1-mediated transcription, as measured by the CAGA-luciferase reporter (Fig. 2D). Taken together, our data suggest that Coco does not inhibit, but rather is necessary for, signaling by the TGFβ1 ligand. This is in contrast to the inhibitory activity of Coco on Nodal and BMP4 (Bell et al., 2003).

Coco overexpression enhances TGFβ1 signaling

We next examined whether overexpression of Coco would enhance TGFβ1 signaling. To address this, we used the ePiggyBac transposable element system (Lacoste et al., 2009) to generate a C2C12 cell line that expresses Coco under the control of the Tet-responsive-element (TRE) (Fig. 3A). This allows inducible Coco expression by addition of doxycycline (Dox). We then monitored the effects of Coco overexpression on GFP-Smad4 nuclear translocation in cells, in the presence or absence of TGFβ1 ligand. We found that Coco overexpression led to a significant increase in Smad4 nuclear localization after TGFβ1 treatment (Fig. 3B). These effects were dependent on the Dox dose, and thus dependent on the amount of Coco induction (Fig. 3B). Notably, Coco addition did not prolong Smad4 nuclear localization, but rather enhanced the magnitude of TGFβ1 signaling (Fig. 3B). Coco overexpression also enhanced activation of both the CAGA12-luciferase reporter construct as well as the direct transcriptional targets *Ctgf* and *Pai1* (also known as *Serpine1*) (Fig. 3C,D). Consistent with Coco exerting its effects extracellularly, in a non-cell-autonomous manner, conditioned media from Coco-expressing cells increased TGFβ1-induced reporter activity (Fig. 3E). Together, these results suggest that Coco overexpression enhances TGFβ1 signaling in C2C12 cells.

Coco inhibits BMP signaling in C2C12

Coco was originally classified as an inhibitor of TGFβ signaling based on its function blocking Nodal and BMP signaling in *Xenopus* embryos (Bell et al., 2003). However, this is in contrast with the effect of Coco on the TGFβ1 ligand in mammalian cells. To rule out the possibility that the differential activity is due to species-specific differences between the amphibian and the mammalian system, we assessed the effects of mammalian Coco on BMP4 signaling in C2C12 cells. We monitored activation of the BMP4

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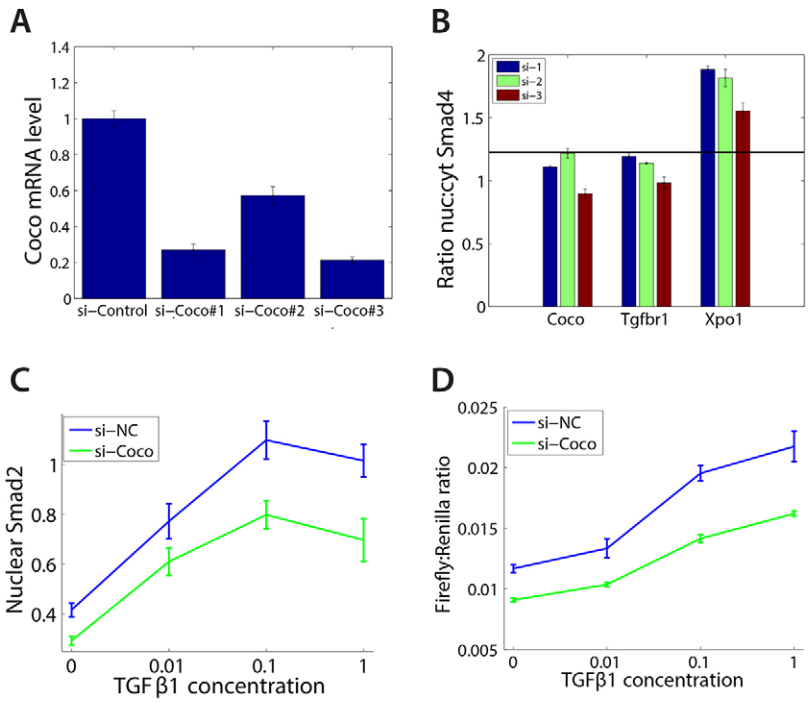

Fig. 2. Coco is required for TGFβ1 signaling. (A) Validation of the Coco siRNAs used in the screen. Transfection of C2C12 cells with two of the three siRNAs from the screen significantly reduces the levels of *Coco* mRNA by >75%. (B) Knockdown of Coco impairs Smad4 nuclear translocation. Transfection of C2C12 cells with the two siRNAs that reduce mRNA levels by >75% leads to a significant reduction in the Smad4 translocation in response to TGFβ1, similar to the effect caused by siRNAs targeting TGFβR1. Conversely, siRNAs targeting Xpo1 cause an accumulation of GFP-Smad4 in the nucleus. (C) Knockdown of Coco impairs Smad2 nuclear translocation, as measured by immunofluorescence. siRNA-mediated knockdown of Coco significantly decreases the nuclear accumulation of Smad2 after 1 h of treatment with various doses of TGFβ1. (D) Knockdown of Coco impairs TGFβ1-induced transcription. C2C12 cells expressing the CAGA12-firefly luciferase reporter were exposed to increasing doses of TGFβ1. siRNA-mediated knockdown of Coco decreases the transcriptional induction of the TGFβ1 reporter. Firefly luciferase signal was normalized to the co-expressed *Renilla* luciferase. Error bars represent s.d. from at least five images per condition or three independent replicates.

signaling cascade by measuring the levels of BMP4-induced phosphorylated Smad1 (P-Smad1). Treatment of C2C12 cells with increasing doses of BMP4 led to the accumulation of P-Smad1 as determined by immunostaining (Fig. 4A). However, expression of Coco dramatically reduced P-Smad1 levels, confirming the *Xenopus* finding that Coco is indeed an inhibitor of BMP4 signaling (Bell et al., 2003). Additionally, microinjection of synthetic mRNA encoding human COCO into early *Xenopus* embryos led to the emergence of two-headed tadpoles, similar to the phenotype observed after injection of the *Xenopus Coco* (Bell et al., 2003) (Fig. 4B). This excludes differences in Coco activity between the two species. Therefore, our data indicate that Coco activity is ligand specific, with Coco inhibiting some TGFβ ligands, such as Nodal and BMP4, while enhancing others, such as TGFβ1.

Interaction between Coco and TGFβ1 modulates cell fate decisions

To understand whether Coco enhancement of TGFβ1 signaling has biological relevance, we investigated how Coco levels affect TGFβ1 activity in two different contexts. We first determined the effects of Coco on the differentiation of C2C12 cells to myoblasts, a process that is inhibited by TGFβ1 (Kollias et al., 2006). We found that

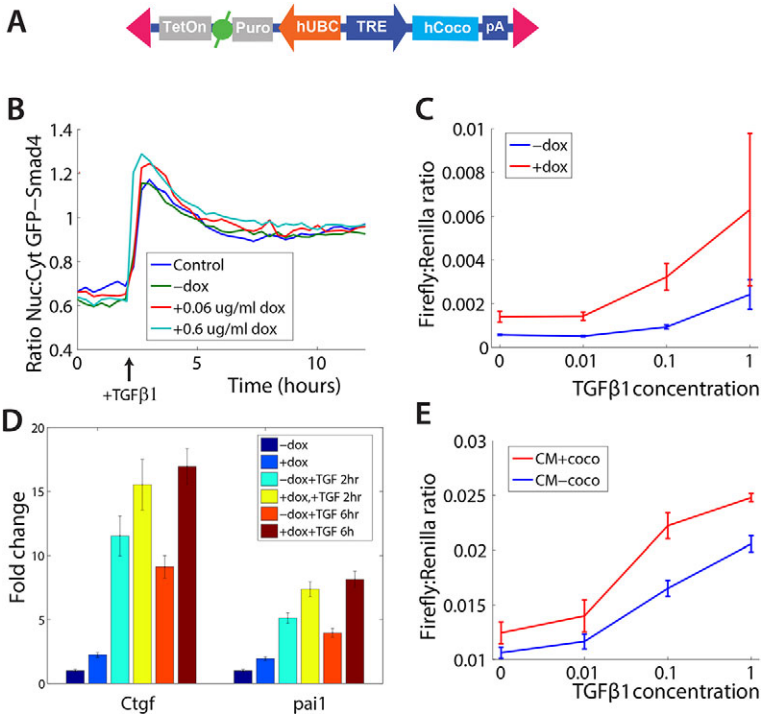

Fig. 3. Coco enhances TGFβ1 signaling. (A) Schematic of the cassette for the inducible expression of human *COCO* in ePiggyBac. (B) Coco enhances nuclear translocation of Smad4 in a dose-dependent manner. Live-cell imaging of C2C12 expressing GFP-Smad4 shows that expression of COCO increases nuclear accumulation of Smad4 in a dose-dependent manner. Cells were stimulated with TGFβ1 at the time indicated by the arrow. (C) COCO enhances TGFβ1-induced transcription. Expression of Coco leads to increased transcriptional output from the CAGA12-firefly luciferase reporter in response to TGFβ1 treatment. (D) Coco enhances TGFβ1 activity. COCO expression leads to higher levels of the endogenous TGFβ1 targets *Ctgf* and *Pai1*. (E) Coco functions extracellularly. Conditioned medium from COCO-expressing cells (CM+coco) leads to an increase in the transcription of the CAGA12-luciferase reporter compared with conditioned medium from cells not expressing Coco (CM–coco). Error bars represent s.d. from three independent replicates.

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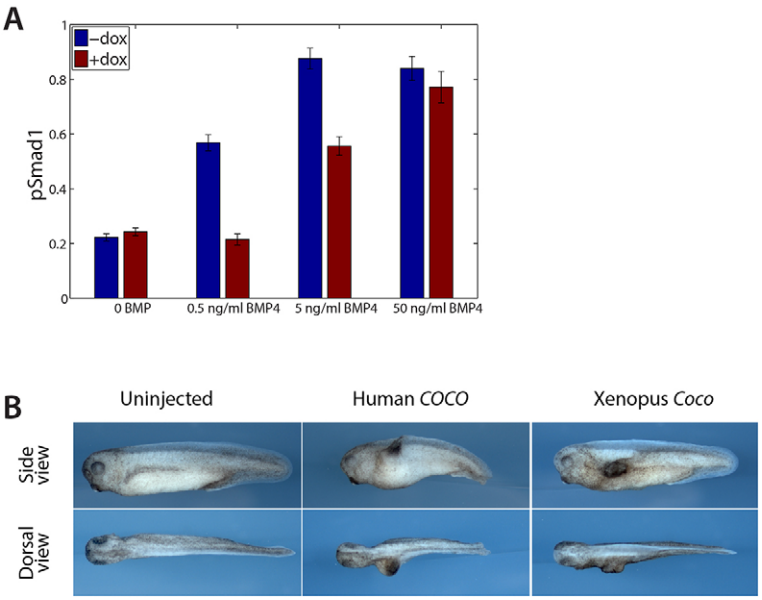

Fig. 4. Coco inhibits BMP signaling. (A) Coco impairs BMP4 signaling. Expression of Coco significantly reduces the levels of phospho-Smad1 (P-Smad1) in response to BMP4 in C2C12 cells. (B) Human COCO induces the formation of a secondary head in *Xenopus*. Injection of 2-cell-stage *Xenopus* embryos with mRNA encoding human or *Xenopus Coco* leads to the same phenotype of induction of a secondary head, consistent with Coco being an inhibitor of BMP signaling *in vivo*. Error bars represent s.d. from at least five images per condition.

C2C12 cells transfected with a Coco siRNA have a twofold higher rate of myoblast differentiation, as determined by myogenin immunostaining, compared with cells transfected with a control siRNA (Fig. 5A,B). Consistently, overexpression of Coco completely inhibited myoblast differentiation (Fig. 5A,B). This further supports the idea that Coco enhancement of TGFβ1 signaling has biological relevance.

Next, we investigated the effects of Coco overexpression in the context of maintenance of pluripotency in human embryonic stem cells (hESCs) using the RUES2 cell line (James et al., 2006). Maintenance of pluripotency requires signaling through Smad2/3, which can either be provided by Activin/Nodal ligands for hESCs cultured in conditioned medium (CM+FGF2) or TGFβ1 for hESCs cultured in the defined medium mTeSR1. Consistent with Coco inhibiting Activin/Nodal signaling, COCO overexpression in cells cultured in CM+FGF2 led to rapid loss of pluripotent morphology (Fig. 5C). Conversely, cells cultured in mTeSR1 did not change morphology, consistent with the idea that Coco does not inhibit TGFβ1 signaling (Fig. 5C). Furthermore, COCO expression in RUES2 cells grown in mTeSR strongly upregulated the TGFβ1-responsive genes *LEFTYA*, *LEFTYB* and *PAI1* (*LEFTY 2*, *LEFTY 1* and *SERPINE1*, respectively – Human Gene Nomenclature Database) (Fig. 5D), while downregulating the BMP4-responsive genes *ID1*, *ID2* and *ID3* (supplementary material Fig. S2A). Taken together, our data demonstrate that selective activity of Coco on different TGFβ ligands has a significant consequence on cell fate determination.

Using a variety of assays, we previously demonstrated that Coco is a potent inhibitor of the BMP and Smad1 branch of signaling of the TGFβ pathway in *Xenopus* (Bell et al., 2003). To address the role of Coco on TGFβ1 in the same system, we investigated whether Coco expression is able to synergize with TGFβ1 in inducing the dorsal-specific gene Goosecoid (*gsc*). Increasing concentrations of synthetic *coco* mRNA were injected together with RNA encoding TGFβ1 and TGFβRII, which are necessary for TGFβ1 signaling at this stage, and *gsc* expression was measured by qRT-PCR. We found that *gsc* expression is enhanced by Coco in a dose-dependent manner (supplementary material Fig. S2B). Consistent with its inhibitory role in BMP signaling, Coco expression significantly decreased the levels of the BMP-responsive gene *vent2.2* (also known as *ventx2.2*) (supplementary material Fig. S2C). In addition, we determined the effects of Coco on cement gland formation. The cement gland is an organ located at the extreme anterior of the frog embryo that can be induced by both inhibitory and stimulatory signals (Bradley et al., 1996). We found that Coco induces expression of the cement gland marker *Xag* (also known as *ag1*; supplementary material Fig. S2D). This induction is increased by co-expression of TGFβ1, suggesting that Coco and TGFβ1 have a synergistic role in *Xag* induction. Similarly, Coco and TGFβ1 synergize in inducing ectopic cement glands in the developing embryo (supplementary material Fig. S2E,F). These results confirm that Coco functions as a dual activity modulator of TGFβ ligands in *Xenopus* embryonic explants.

Coco binds to TGFβ1 and enhances TGFβ1-ALK5 receptor interactions

The synergistic effect of Coco on TGFβ1 prompted us to investigate the mechanism by which Coco functions. We had previously demonstrated that Coco is able to bind BMP4 and Nodal (Bell et al., 2003; Vonica and Brivanlou, 2007), and therefore asked first if Coco can also bind TGFβ1. Extracts of *Xenopus* embryos injected with HA-tagged *TGFβ1* and Flag-tagged *coco* mRNA were immunoprecipitated. Precipitation with anti-Flag antibody resulted in co-precipitation of HA-tagged TGFβ1, indicating that Coco does indeed bind to TGFβ1 (Fig. 6A). This finding made us wonder whether Coco enhances TGFβ1 signaling by affecting ligand-receptor binding. To test this possibility, we microinjected *Xenopus* embryos with mRNAs encoding TGFβ1, Coco and ALK5 and performed immunoprecipitation experiments. We found that Coco enhances the binding of TGFβ1 to ALK5 (Fig. 6B). Taken together, the mechanistic evidence presented above strongly suggests that Coco-mediated enhancement of TGFβ1 signaling is due to an increase in the level of Smad2 phosphorylation and nuclear translocation of Smad2-Smad4 complexes (Fig. 6C).

DISCUSSION

We took advantage of our previously described fluorescent assay for TGFβ signaling dynamics to perform an RNAi-based screen for factors that modulate Smad4 nuclear translocation in response to the TGFβ1 ligand in C2C12 cells. Among the 321 genes that we identified, we discovered Coco as an enhancer of TGFβ1 signaling

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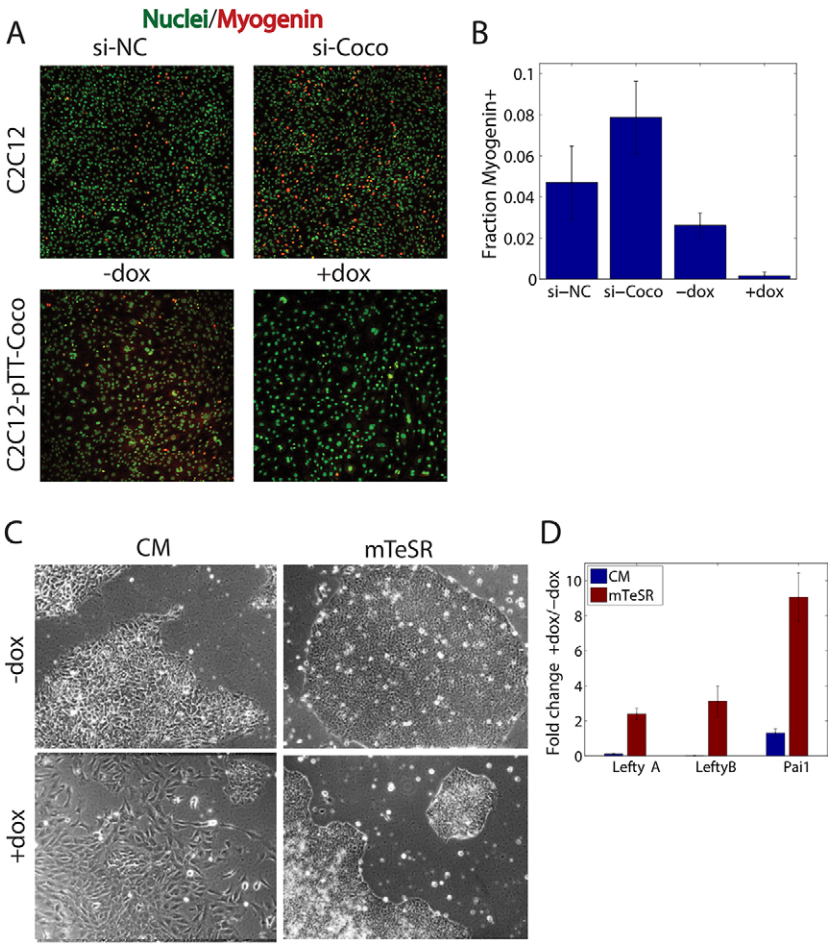

Fig. 5. Coco modulates TGFβ1-dependent cell fate decisions. See also supplementary material Fig. S2. (A) Coco levels affect myoblast differentiation. C2C12 transfected with an siRNA against Coco (si-Coco) differentiate more readily into myotube precursors compared with cells treated with a non-targeting siRNA (si-NC) as determined by myogenin immunostaining. Overexpression of Coco (+dox) completely abolishes myoblast differentiation. These results support a role for Coco as an enhancer of TGFβ1. Green, nuclei; red, myogenin. (B) Quantification of results shown in A. (C) Coco expression affects maintenance of pluripotency in hESCs. Expression of Coco (+dox) leads to the rapid differentiation of stem cells grown in conditioned media (CM) but not in mTeSR, further suggesting that Coco inhibits Activin/Nodal while enhancing TGFβ1 activity. (D) Coco induces the expression of TGFβ1-responsive genes in hESCs. Expression of the TGFβ1-responsive genes *LeftyA*, *LeftyB* and *Pai1* is increased in Coco-expressing hESCs grown in mTeSR. Data are shown as a ratio of the mRNA levels in Coco-expressing (+dox) versus not expressing (–dox) cells. Error bars represent s.d. from at least five images per condition or three independent replicates.

that increases the magnitude of Smad4 translocation into the nucleus. Coco is required for signaling by the TGFβ1 ligand and synergizes with TGFβ1 in modulating cell-fate decisions. At the molecular level, Coco binds to TGFβ1 ligand and enhances the interaction of TGFβ1 with its receptor Alk5, thus leading to increased signaling.

We had originally discovered Coco based on a gain-of-function screen for factors that induce secondary dorsal and head structures in *Xenopus* embryos (Bell et al., 2003). Coco was shown to be an inhibitor of Activin, Nodal and BMP4, providing a molecular explanation for the embryonic phenotype. However, here we demonstrate that Coco has the opposite activity with regard to the TGFβ1 ligand. We therefore propose that Coco functions as both an inhibitor and an enhancer, depending on the TGFβ ligand it is exposed to. To our knowledge, Coco is the first secreted protein that has been shown to exert opposing activities on signaling by different TGFβ ligands.

We had previously proposed two embryonic roles for Coco. Maternal Coco functions by blocking incoming mesendoderm-inducing signals in the early blastula to delineate the boundaries of the marginal zone. During this time window, TGFβ1 ligand is not expressed and therefore Coco acts solely as an inhibitor. Zygotic Coco is involved in the specification of the right-left axis at the neurula stage (Vonica and Brivanlou, 2007). Coco restricts Nodal signaling to the left side of the embryo by inhibiting Xnr1 (Nodal1 – Xenbase) and derrière (Gdf3 – Xenbase) signaling on the right side. Given that TGFβ1 is also expressed in this region and that Coco enhances TGFβ1 signaling, the interaction of TGFβ1 and Coco might play an important role in the specification of the right-left axis. Consistent with these results is the fact that ectopic expression of TGFβ1 randomizes the right-left axis of the embryo. Therefore, during early embryogenesis, the activating and inhibitory functions of Coco are functionally and temporally separated. The function performed by Coco depends on which TGFβ ligands are concurrently expressed (supplementary material Fig. S3A,B).

Alternatively, it is possible to speculate that TGFβ1 could compete with BMPs and Nodal for binding to Coco in the embryo. In the absence of TGFβ1-specific receptors, TGFβ1 might saturate Coco and prevent the inhibitory effects of Coco on BMPs and Nodal.

We find that knockdown of Coco leads to increased myogenin expression in a myoblast differentiation paradigm in C2C12 cells. Conversely, Coco overexpression completely abolishes myogenin expression. As the process of myoblast differentiation is negatively regulated by TGFβ1 signaling, our results support the idea that Coco enhances the activity of TGFβ1. Indeed, these results cannot be explained by only considering the inhibitory effects of Coco on BMP signaling, as BMP signaling has been shown to inhibit myoblast formation in favor of osteoblast formation (Katagiri et al., 1994).

Coco expression has also been shown to be sufficient for dormant breast cancer cells to undergo reactivation and metastasis in the lung of adult mice (Gao et al., 2012). This effect was ascribed to the function of Coco in blocking lung-derived BMP ligands. Our data, however, raise the possibility that the role of Coco in metastasis might also include an effect mediated via enhancement of TGFβ1 signaling, in addition to BMP inhibition.

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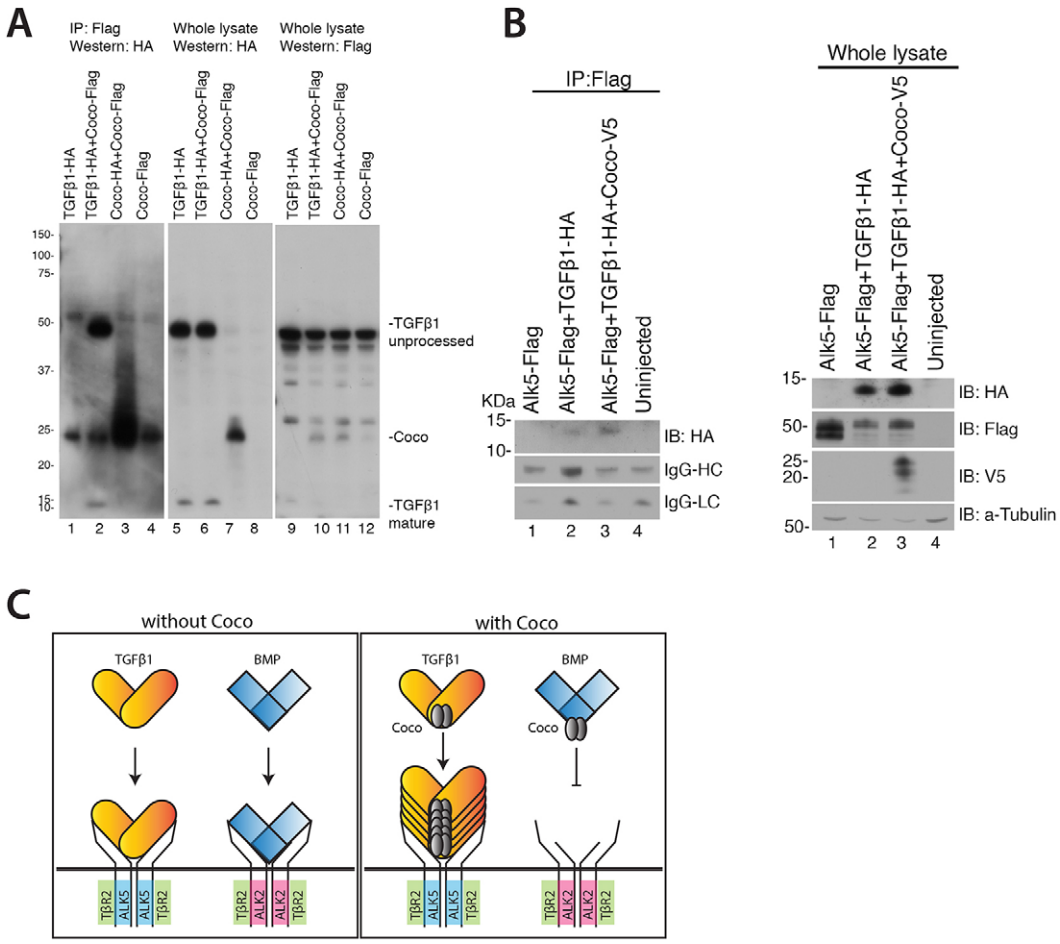

Fig. 6. Coco enhances the interactions of TGFβ1 with its receptor Alk5. See also supplementary material Fig. S3. (A) Coco binds to TGFβ1. Immunoprecipitation of proteins extracted from animal caps injected with HA-tagged *TGFβ1* and Flag-tagged *coco* mRNA shows that Coco forms complexes with TGFβ1. (B) Coco expression leads to enhanced binding of TGFβ1 to Alk5. Flag-tagged Alk5, HA-tagged TGFβ1 and V5-tagged Coco were co-expressed in *Xenopus* embryos. Alk5 immunoprecipitation leads to pull-down of TGFβ1, with more TGFβ1 being pulled down in the presence of Coco. (C) Model for the mechanism of action of Coco as an enhancer of TGFβ1 signaling and inhibition of BMP signaling.

Our data show that Coco binds TGFβ1, and that the presence of Coco enhances the interactions between TGFβ1 and its receptor Alk5. This strongly supports a model in which Coco functions as a direct enhancer of TGFβ1. However, we cannot exclude the possibility that, in addition to directly enhancing TGFβ1 signal transduction, Coco might also inhibit an inhibitor of TGFβ1, thus making more TGFβ1 available for interactions with the Alk5 receptor.

It is tempting to speculate that the dual function of Coco might also be shared by other 'classic' inhibitors of TGFβ ligands, such as cerberus, noggin, chordin and follistatin. If this were the case, it would require reconsideration of our current understanding of the extracellular regulation of the TGFβ signaling pathway.

Coco was only one of the 321 hits that we identified in the loss-of-function screen for modulators of Smad4 nuclear translocation. Dissection of the function of the remaining hits might lead to further understanding of the molecular mechanisms controlling TGFβ1 signaling and will most likely highlight new players and regulators of the pathway.

MATERIALS AND METHODS

Screening

The siRNA screen was performed with the GFP-Smad4 C2C12 line described previously (Warmflash et al., 2012) using a library of Ambion silencer siRNAs. Each well of a 384-well plate contained three siRNAs targeted to the same gene for a total of 1.5 pmol of siRNA in 5 µl volume. On each plate, we manually added controls to two wells – siRNA against TGFβR1 and siRNA against Xpo1 in the same volume and concentration as the remainder of the siRNAs. To each well, we added 5 µl Lipofectamine2000 solution (Invitrogen; 1:100 in Optimem), incubated for 20 min, added 40 µl of cell suspension (30,000 cells/ml in growth medium) and incubated the plates for 48 h. TGFβ1 solution was prepared at a concentration of 20 ng/ml in growth medium, and 5 µl of this solution was dispensed into each well (final concentration of 2 ng/ml). After either 1 or 6 h the plates were washed once with PBS, fixed for 20 min with 4% paraformaldehyde and then washed twice with PBS. Plates were sealed and imaged with an ArrayScan instrument. Images were analyzed using custom software written in MATLAB (Mathworks) with an algorithm that we have described previously (Warmflash et al., 2012). Gene Ontology analysis was performed using DAVID Functional Annotation Tool (NCBI). Annotation terms were derived from the Swiss-Prot and Protein Information Resource database (SR-PIR-keywords). The RNAi dataset has been submitted to the GEO database under the accession number GSE70924.

Constructs

The ePiggyBac (Lacoste et al., 2009) construct for conditional expression of hCoco was cloned into the *Bam*HI-*Not*I sites of the ePB-TT vector described previously (Arduini and Brivanlou, 2012). *Xenopus tgfb1* (formerly *tgfb5*) cDNA was obtained from GE Healthcare Dharmacon (Clone Id:8548338) and subcloned into pCS105. This *tgfb1* was HA-tagged at three amino acids downstream of the cleavage site (RKKR) by standard PCR methods. Human

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TGFBR2 was obtained from Addgene (#15012). *Xenopus coco* and *coco-Flag* were previously described (Bell et al., 2003). *Xenopus coco* was HA-tagged and V5-tagged at the C-terminus by standard PCR methods. Human Coco was obtained from GE (8069306) and subcloned into pCS105.

Cell culture, transfections, and selection of stable lines

C2C12 cells were maintained in DMEM medium containing 10% fetal bovine serum. The GFP-Smad4 and RFP-Smad2 reporter cell lines were described previously. The C2C12-hCoco-expressing line was created by transfecting cells with the ePB-P-TT-hCoco plasmid together with the ePB-transposase plasmid followed by selection with 4 µg/ml puromycin for 3 days. The RUES2 line of human embryonic stem cells was maintained on Matrigel (BD biosciences; 1:40 in DMEM-F12)-coated dishes either in mouse embryonic fibroblast conditioned medium (CM) supplemented with 20 ng/ml bFGF or in mTeSR1 as indicated. The hCoco-expressing RUES2 line was created by nucleofecting RUES2 cells with ePB-P-TT-hCoco and the ePB-transposase plasmids using an Amaxa nucleofector and nucleofection solution L followed by 3 days of selection with 4 µg/ml puromycin.

Xenopus explant dissection, cell culture and RNA preparation

Xenopus laevis embryos were obtained by *in vitro* fertilization and staged according to Nieuwkoop and Faber (1967). Microinjection, explant dissection and cell culture were performed as described (Hemmati-Brivanlou and Melton, 1994; Wilson and Hemmati-Brivanlou, 1995). RNA for injections was prepared using the mMessage mMachine *in vitro* SP6 Transcription Kit (Life Technologies).

Immunofluorescence

Cells were plated in glass-bottomed dishes (MatTek) at least one day before the experiment. After stimulation, cells were rinsed once with PBS, fixed with 4% paraformaldehyde for 20 min, rinsed twice with PBS, blocked for 30 min with 3% donkey serum, 0.1% Triton X-100 in PBS and then incubated overnight at 4°C with primary antibodies diluted in blocking buffer. Antibodies used were against Smad2/3 (BD Transduction Labs 610842; 1:100), pSmad1 (Cell Signaling; 1:100) and Myogenin (clone F5D; abcam 1835; 1:100). Cells were then washed three times with PBS+0.1% Tween20 for 30 min each wash, incubated for 30 min with secondary antibodies (Alexa 488 or Alexa 647; 1:500) and Sytox (1:50,000) diluted in blocking buffer, and washed twice more with PBS+0.1% Tween20 for 30 min each wash. When performing immunofluorescence against pSmad1, the protocol was modified to add a 30 min incubation with 1% SDS in PBS at 37°C before blocking.

Live cell imaging

C2C12 cells were plated in glass-bottomed dishes (MatTek) at least one day before imaging. Immediately before imaging, we switched the cell medium to custom culture medium (DMEM without Phenol Red or riboflavin) containing 10% fetal bovine serum. We collected images every 15 min using an Olympus Vivaview microscope with a 20×, 0.80NA lens.

Gene expression analysis

C2C12 cells were grown in 60-mm dishes and RUES2 cells were grown in 35-mm dishes. Total RNA was isolated with the TRIzol reagent (Life Technologies), treated with DNase (Ambion), and cDNA was synthesized with the Transcriptor First Strand cDNA Synthesis Kit (Roche). Real-time qRT-PCR analysis was performed with the LightCycler 480 SYBR Green I kit (Roche). A complete list of primers is provided in the supplementary materials and methods.

For cell culture, gene expression was normalized to the expression of mouse or human ATP50 as appropriate. For *Xenopus* experiments, *ornithine decarboxylase 1* (*odc1*) was used to normalize gene expression.

Luciferase assays

C2C12 cells were transfected with 3.6 µg of CAGA12-luc plasmid and 0.4 µg of pRL-Tk for normalization. Cells were treated as described in the text and lysed and analyzed for luciferase activity using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

Co-immunoprecipitation assay

Immunoprecipitation assays were performed as described (Hama et al., 2002) with minor modifications. Two nanograms of Alk5-Flag and/or 2 ng TGFβ1-HA and/or 250 pg of Coco-V5 were injected into the animal hemisphere of two-cell-stage embryos. Embryos were lysed when sibling embryos had reached stage 23. Lysates were incubated overnight at 4°C with anti-Flag polyclonal antibody (1:1000) followed by incubation with Dynabeads Protein G (Life Technologies) for 1 h at 4°C. After four washes with lysis buffer, proteins were boiled and eluted in NuPAGE LDS Sample Buffer (Life Technologies) with 0.1 M DTT. Antibodies used were anti-HA (Abcam, ab18181), anti-Alk2 (LifeSpan Biosciences, LS-B2010), anti-Alk5 (R&D Systems, AF587), anti-Flag (Sigma-Aldrich, F3165, F7425) and anti-V5 (Abcam, ab9116).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

A.D., A.W., T.H. and A.H.B. planned experiments; A.D., A.W., T.H. and B.S. performed and analyzed experiments; A.D., A.W. and A.H.B. wrote the manuscript.

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Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.122358/-/DC1

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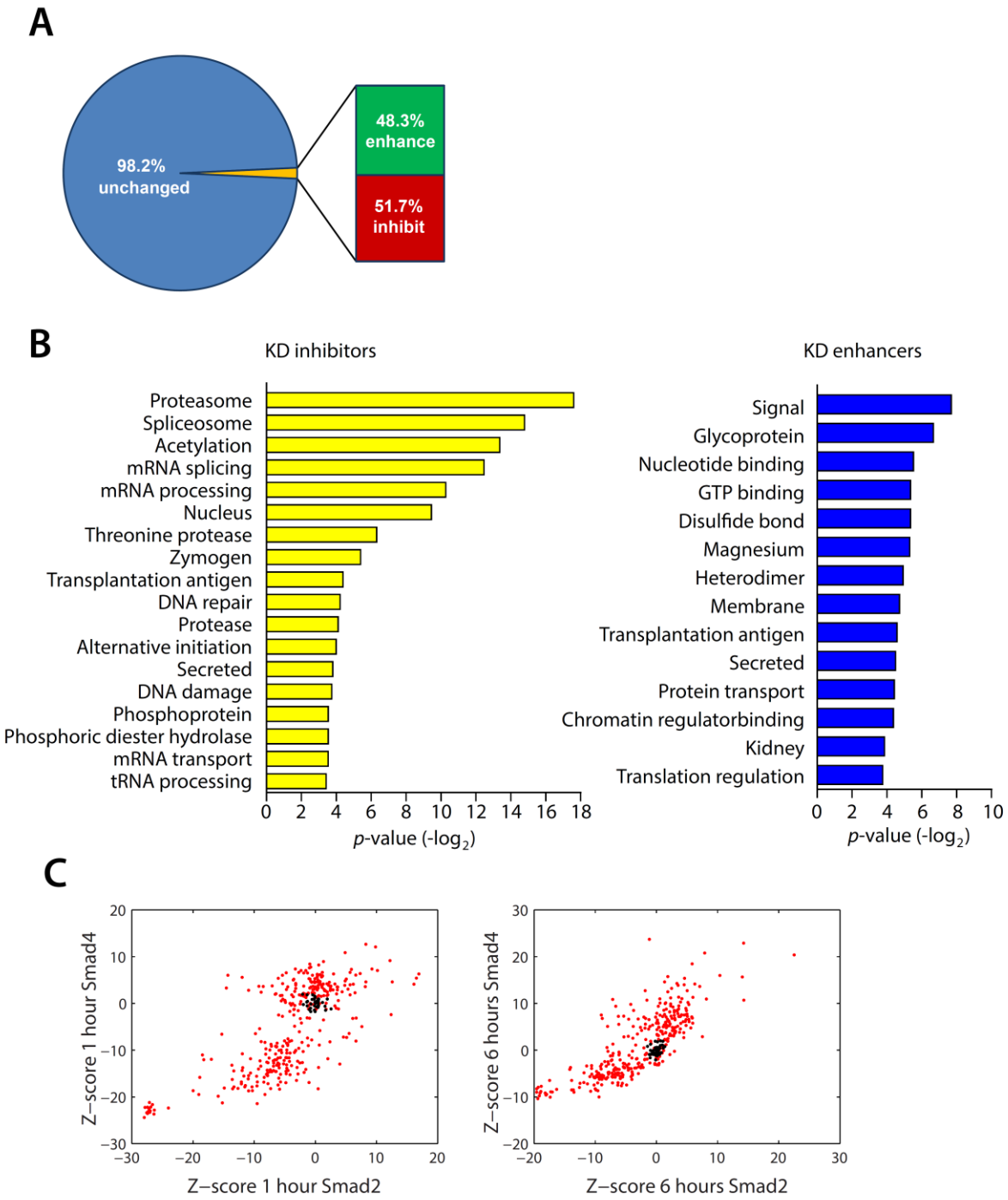

Fig.S1. A genome-wide siRNA screen identifies modulators of TGFβ1 signaling.

A. A siRNA screen identifies 321 genes (yellow, 1.8%) to affect TGFβ1-induced Smad4 nuclear translocation when knocked down. Knockdown of 48.3% of these genes (green) leads to an enhancement of Smad4 translocation, while knockdown of 51.7% of them (red) leads to an inhibition of

Smad4 translocation. The great majority of genes in the screen (98.2%, blue) does not change Smad4 behavior.

B. Gene ontology analysis of genes that when knocked down inhibit (yellow) or enhance (blue) Smad4 nuclear translocation.

C. Correlation of Smad4 and Smad2 nuclear translocation in response to 1 hour and 6 hours TGFβ1 treatment when the genes identified in the siRNA screen were knocked down.

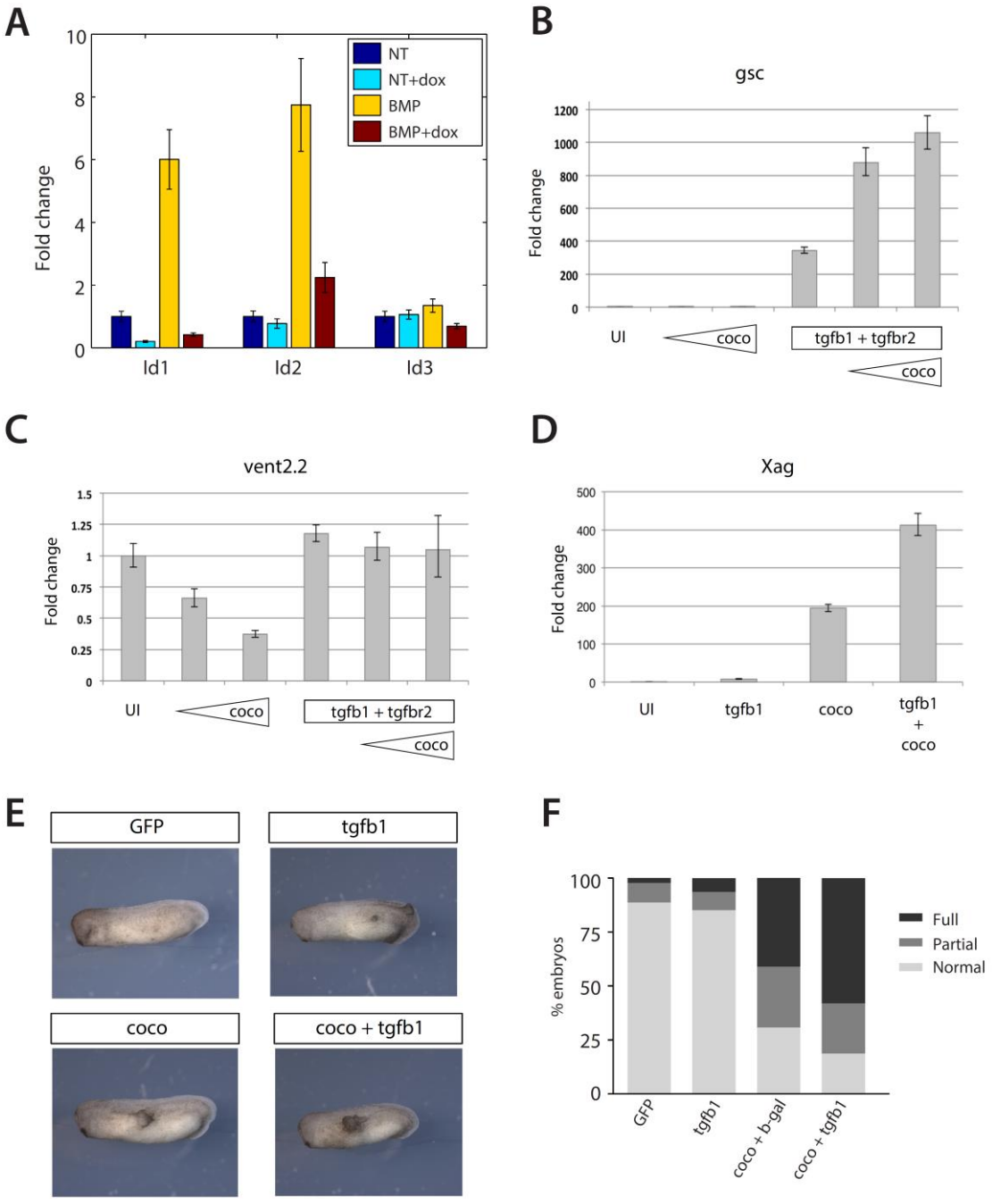

Fig.S2. Coco modulates TGFβ signaling

A. qRT-PCR quantification of the changes in expression levels of the BMP-responsive genes *ID1*, *ID2*, and *ID3* in RUES2 treated with vehicle (NT) or BMP4 (BMP) in the absence or presence (+dox) of doxocycline. Doxocycline treatment leads to the production of Coco.

B. Coco enhances expression of the Smad2-responsive gene *gsc* in animal cap explants injected with *tgfb1* and *tgfbr2* in a dose-dependent manner.

C. Expression of Coco in *Xenopus* animal caps leads to a dose-dependent decrease in the expression levels of the BMP-induced gene *vent2.2.* When animal caps are injected with *tgfb1* and *tgfbr2*, *vent2.2* levels are not affected.

D. Coco and TGFβ1 synergize to induce the cement gland marker *Xag*

E. Coco and TGFβ1 synergize to induce cement gland in *Xenopus* embryos. Embryos were injected with constructs encoding the indicated genes and the emergence of ectopic cement glands was observed.

F. Quantification for experiment in E. Embryos were scored based on the presence of ectopic cement glands and given a score of “normal” if no ectopic cement glands were observed, “partial” if ectopic extrusions were observed in the embryo, and “full” if ectopic, fully formed cement glands were observed. N>40 per condition

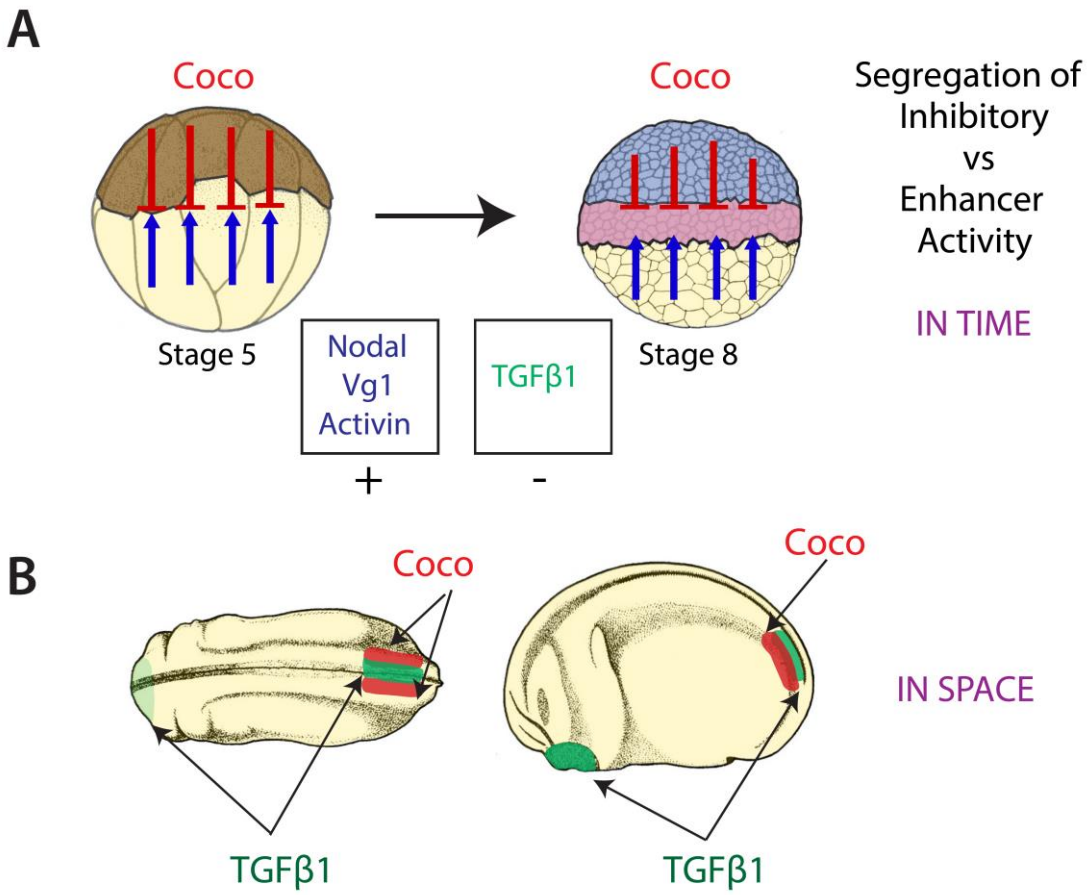

Fig.S3. Model of Coco activity during early embryo development

A-B. Model for the segregation of inhibitory and enhancer activities of Coco (A) in time and (B) in space.

Table S1

List of genes that affect Smad4 nuclear translocation at 1 hour or 6 hours after application of TGFβ1 as identified in the siRNA screen.

Gene name	siRNA	Smad4 1h - trial1	Smad4 1h - trial2	Smad4 6h - trial1	Smad4 6h - trial2	Smad2 - 1h	Smad2 - 6h
0610030I09Rik	75400	1.664	2.1385	2.0195	2.0844	1.026612774	0.23208093
1110001A23Rik	68472	-4.0538	-5.5601	-3.0741	-4.0956	-3.80939617	-2.8845832
1110004B15Rik	74165	2.1254	2.1372	2.4198	2.0521	-1.055870247	-1.784663
1110012M11Rik	73711	-4.4023	-3.9954	-3.6414	-3.287	-4.116401895	-2.7311913
1110018G07Rik	68497	-3.6142	-3.7287	-2.9521	-2.2546	-2.828468812	-1.2052422
1300007B12Rik	57439	1.9214	1.2244	2.2817	2.4787	0.933861984	0.28780813
1700016D06Rik	76413	1.7408	1.2759	2.6434	1.9359	-0.915484618	-0.5748452
1810009M01Rik	65963	-4.3446	-3.9764	-2.4946	-2.869	-3.104351661	-1.5113031
2010003K11Rik	69861	1.9857	1.5716	2.999	2.5732	0.95847377	0.39288047
2210415F13Rik	70163	-3.4281	-3.7383	-1.7487	-2.0166	-2.019776433	-0.9077318
2300003C06Rik	71912	1.682	1.5323	2.5602	1.9648	0.115555732	-0.137986
2310056K19Rik	75689	0.74815	0.87339	2.1244	2.0244	1.645071321	-0.203623
2400006A19Rik	66413	2.1427	3.1321	3.0424	3.5059	2.547159488	3.91705751
2400009B11Rik	66985	1.8991	1.6324	2.4511	2.5595	0.699123252	0.39851497
2510048O06Rik	66537	1.2071	1.2909	2.9229	3.8901	3.711456536	3.82603435
2810032G03Rik	72669	2.0565	1.9268	2.4338	2.1488	0.820389767	-0.0178512
2810406C15Rik	68298	-4.1397	-3.0314	-2.1263	-1.4581	-2.042188435	-1.8786331
2900027G03Rik	72865	-3.5174	-3.7285	-2.2043	-2.7047	-3.194434194	-1.6214416
2900073H19Rik	68205	2.3656	2.4836	2.0778	2.3612	1.021697206	0.0962536
2900090M10Rik	329977	-3.5385	-2.8673	-1.7268	-2.7171	-1.16726489	-0.5284162
3632451O06Rik	67419	-3.2644	-3.3845	-2.1919	-2.8973	-1.717428275	-1.2089862
4921511C20Rik	245598	-3.9738	-3.899	-2.4402	-2.9279	-3.354434568	-1.4034444
4930412M03Rik	320201	1.1335	0.88291	2.1742	2.452	0.582242728	0.62189949
4930429A08Rik	74648	1.0692	1.1471	2.1345	2.7026	0.768640516	1.01031561
4930449I24Rik	67410	-3.6372	-3.6273	-2.8825	-2.2769	-4.508825796	-2.6030435
4930529M08Rik	78774	1.3171	1.5153	2.6845	1.4015	0.172064393	0.05820836
4930534B04Rik	75216	1.5968	1.6715	2.7829	2.2456	0.045780469	-0.0570765
4933421H10Rik	68178	-3.9326	-3.7393	-1.2865	-1.571	-2.965833132	-2.9514312
5530600A18Rik	71452	1.6941	1.5973	3.0773	2.5962	-2.291490554	-1.4131133
5530600A18Rik	77582	0.7408	0.40804	2.2073	2.5653	0.834274209	1.44564266
5530600A18Rik	432450	-3.5852	-4.1159	-4.0744	-2.5189	-1.4274573	-0.9923853
5730589L02Rik	74552	-3.9977	-2.7313	-2.0469	-2.4623	-2.534804372	-3.1585053
9930117H01Rik	320782	2.8625	2.4885	3.326	2.8708	-1.175880712	-0.0761918
A2lp	233871	2.5482	2.0897	2.3547	2.0764	0.475417391	-0.0493538
A630007B06Rik	213993	-3.1695	-3.3922	-2.006	-2.6316	-1.218077196	-0.7353482
A930006D11	278304	1.2226	1.2096	2.3801	2.1688	0.631534195	0.06562835
A930008A22Rik	235283	1.8486	1.8931	2.1482	2.2232	0.696319206	0.41963818
A930013B10Rik	414094	1.3309	2.0182	2.8371	2.4594	2.008232656	0.99257187
AA407526	231279	-3.8253	-3.489	-1.0107	-1.7075	0.231091944	0.3233265
AB041803	232685	2.0183	1.5037	2.1422	2.4683	-0.467421106	0.25875832
Accn4	241118	-3.8334	-2.7027	-1.7686	-1.2842	-3.156657648	-2.5098703
Acp5	11433	0.77669	1.0269	2.1484	2.0113	-0.602375169	1.32192457
Actg	11465	0.82704	1.1729	2.1797	2.1111	1.691864267	1.25875884
Actr3	74117	-4.2777	-2.4767	-3.4422	-3.0043	0.16846598	0.45437191
Adam21	56622	-3.5149	-3.134	-2.3048	-3.1901	-1.201965811	0.27272101
Agtrap	11610	-4.4631	-3.8006	-2.4087	-2.4567	-3.979893047	-2.329628
Ahsg	11625	-3.4473	-3.4417	-2.0295	-1.2521	-1.139916952	-1.2690443
AI118078	244886	-4.0926	-4.0332	-3.0788	-3.2749	-2.681490618	-1.6489345
AI507495	105866	1.2629	1.8684	2.688	2.2151	1.293662538	0.22189311
AI847670	330050	1.3456	1.2829	2.0639	2.1159	0.545640758	0.0492191
AK122209	382038	-4.8392	-3.8581	-2.3746	-1.9541	-3.300152849	-1.532173
Apba2	11784	-3.1533	-3.2089	-2.0242	-2.2422	-1.183206538	-0.7833774
Apg7l	74244	1.7753	1.6365	2.0908	2.2373	0.571624015	0.18238364
Arch	67106	-3.2581	-3.1196	-2.8231	-3.4799	-1.088215226	-0.9041176
Arhgap10	78514	-3.3146	-3.0201	-2.9258	-2.2983	-1.54052215	-0.8223803
Asrij	68095	1.7169	2.9291	2.0695	2.1259	0.904035894	-0.0654788
Atp4a	11944	1.2568	1.1761	2.3833	2.458	-0.417179114	-1.4519741
Atp6v0a1	11975	0.96987	0.60082	2.1658	2.1476	1.510898046	1.54548208
AW742319	57874	2.8576	1.3132	3.4732	1.8784	-0.821708619	-2.326366
B230354K17Rik	320063	-2.4488	-3.5461	-1.6169	-2.1246	-0.169010834	-0.9123717
BC005752	233189	1.9745	2.0317	2.3262	2.6102	1.808133741	3.01831739
BC011210	70461	1.8045	1.8767	2.5234	2.4521	0.999903045	0.596328
BC016235	216019	-4.1057	-4.1918	-2.5586	-2.062	-3.734508444	-2.0288371
BC024561	232983	0.68459	1.4334	2.1088	2.1843	0.685279547	0.15374776
BC034054	217125	-4.4652	-3.8043	-2.0132	-1.3508	-1.672556745	-1.4689641
BC048082	332110	-4.3555	-4.6338	-3.6749	-3.5804	-2.073895205	-1.6075469
BC050092	235048	-3.0264	-3.9104	-2.2492	-1.2765	-0.949364015	-1.0961849
BC057627	330474	-3.3855	-3.1081	-1.7417	-2.0908	-0.730539773	-0.869699

BC066223	407786	-3.3786	-4.6417	-2.8388	-1.9606	-3.070642	-1.1440967
Brd4	57261	2.6571	2.5183	3.9225	4.6362	4.851067113	4.00914331
Brsk2	75770	-3.6334	-3.2506	-0.38198	-0.36405	-2.028989728	-2.5610504
Btbd12	52864	1.3642	1.3577	2.4559	2.2269	0.117558622	-1.0616056
Btf3	218490	2.305	2.0732	2.8354	2.4081	-0.464678165	1.14701406
C130099A20Rik	235534	1.6293	2.0626	2.9123	2.8995	0.023157994	1.3712141
C1qb	12260	0.61111	0.91771	2.5444	2.4915	1.472055555	-0.785237
C230004F18Rik	331424	2.0132	1.6837	2.5131	1.7966	0.265534866	0.71999049
C330027G06Rik	652994	1.2535	1.2937	3.5831	1.1235	0.514083356	-0.1093563
C630007C17Rik	241514	1.7346	1.2606	2.9438	1.5182	1.587965187	0.13176579
C730027E14Rik	216871	-3.9352	-4.3338	-2.9888	-3.2754	-2.451613139	-0.9252655
Cacnb3	12297	1.9748	2.1755	2.6743	1.9364	1.397636302	1.88470751
Cd19	12478	-3.9174	-3.7164	-2.5497	-3.5353	-1.683046458	-1.2367077
Cd207	246278	-3.5213	-3.1191	-1.8436	-2.5576	-2.120036364	-1.282383
Cdk3	69681	-4.0409	-4.0076	-3.4624	-4.8825	-2.46413969	-0.9456844
Cdyl2	75796	1.2018	0.99463	2.3859	2.4014	0.958663875	0.05156681
Chd3	216848	1.6735	1.5247	2.9968	3.2713	1.015851482	2.47171511
Chd9	109151	-3.5637	-2.9728	-2.8161	-3.4354	-0.740241909	0.02513655
Clcn4-2	12727	-4.89	-4.5971	-3.7897	-3.8565	-4.020819901	-3.4653138
Cldn15	60363	-9.9629	-0.1886	0.67826	0.044912	0.095289199	0.02041024
Clecsf9	56619	-3.8497	-4.03	-2.7959	-1.8695	-4.622467749	-4.3935115
Clns1a	12729	-0.27718	0.39345	3.3154	-0.53586	0.750213926	1.07192444
Col1a1	12842	-3.232	-3.6625	-2.4746	-2.6161	-1.013490448	-1.4829885
Col4a3	12828	-3.5292	-4.2191	-2.3418	-2.1092	-1.183715747	-0.5757225
Copa	12847	-3.9262	-4.4704	-2.2548	-1.9629	-2.656668359	-3.9997574
Copb1	70349	-5.6244	-5.0193	-2.0148	-2.138	-2.701485573	-3.3268793
Copb2	50797	-4.8246	-4.6046	-2.9873	-1.9178	-3.182790273	-4.0499984
Copg	54161	-3.5074	-2.7638	-3.2044	-2.6018	-1.644618124	-2.5476499
Cox8a	12868	1.4568	0.71121	2.2226	2.0778	1.869333918	1.67201124
Cpsf4	54188	-4.6113	-3.6211	-2.2341	-2.7888	-2.606623262	-1.961013
Crygd	12967	1.6796	1.7767	2.8338	1.0357	0.663451438	0.26052528
Ctnnb1	12387	0.67612	-0.66977	2.7657	1.804	-3.150798345	-3.1026607
Cul3	26554	2.0184	2.4861	2.6237	2.5967	0.57958126	-1.0701562
D430014M15	269902	-3.3369	-3.3644	-1.4829	-2.6491	-2.375326785	-1.0107098
D530030D03Rik	98417	-4.8782	-5.2332	-3.7892	-4.0114	-2.815562052	-1.1752595
D5Ertd577e	320549	1.4254	1.3399	3.2879	1.6305	-2.579309271	-2.5692365
D8Ertd325e	66855	0.91618	1.1274	2.3715	2.0733	1.892098972	1.30391517
Dctd	320685	0.90999	1.6147	2.6063	2.9114	0.677912985	0.48130261
Dcx	13193	1.3468	2.0784	2.4746	2.8902	0.471180769	0.22497602
Dfna5h	54722	-4.895	-4.6578	-3.6425	-3.1717	-3.342899279	-1.7621744
Dnahc8	13417	-4.5377	-5.1413	-1.7543	-2.9147	-1.198211241	-1.0744439
Dner	227325	0.74793	0.68891	2.1805	2.6246	0.735087013	-0.969631
Dpp4	13482	1.2974	1.8177	2.3425	2.7868	0.437973528	0.70156715
Dte	23863	-4.0276	-3.1736	-1.5109	-1.7942	-1.774357883	-1.6854352
Dufd1	71804	-3.7126	-3.483	-1.8073	-2.7996	-1.99562633	-1.3534012
Dullard	67181	1.2656	1.4943	3.3954	3.6075	3.24639901	0.9706455
E030041M21Rik	75089	2.4128	2.2345	2.11	2.3123	0.006876873	-1.2280335
E330040A16Rik	330660	-4.1696	-3.7484	-2.1179	-2.2564	-1.752237828	-1.4002936
E430028B21Rik	211948	-4.4924	-4.3927	-2.2186	-3.3399	-3.278270425	-2.9363935
Ecg2	408198	0.0027139	-6.0515	0.14739	1.9023	-2.029111938	-1.8620571
Eif2s1	13665	-3.7325	-3.7761	-2.6397	-2.4464	-2.306984098	-0.5885917
Emd	13726	-3.8433	-3.6866	-2.7001	-2.8948	0.011873914	-0.0938861
Enpp5	83965	2.4636	1.6954	3.0787	2.7643	0.340958958	0.28375524
Epx	13861	-3.3697	-3.6998	-2.9606	-2.8217	-1.675191055	-2.0222264
Ern2	26918	0.92642	1.2601	2.7593	1.8509	0.135781528	0.18635621
Esx1	13984	-3.6266	-2.5375	-2.3902	-1.9753	-2.495887197	-0.8967532
Exosc3	66362	-3.4206	-3.1542	-2.4516	-2.2672	-4.862339311	-3.2393283
Faim2	72393	-1.4342	-3.731	-0.50773	-0.83627	-0.035475768	-0.1325986
Fgf21	56636	1.2565	0.74757	2.0283	2.0031	0.294532642	-0.6094492
Fgf3	14174	2.2144	2.0974	2.5115	2.6835	0.652425358	0.28170409
Frmd3	242506	-4.0374	-3.996	-2.0302	-2.8907	0.194585026	0.1123663
G0s2	14373	1.1221	0.60656	2.34	2.0588	-0.14963372	-0.6095913
Gbp2	14469	1.985	2.0677	2.6688	2.5181	1.090128156	0.40532951
Gcet2	14525	-4.2405	-3.936	-1.6875	-2.5176	-0.385200766	-0.3628409
Ghsr	208188	2.3494	2.742	3.2586	4.1687	1.109776849	0.45906733
Gm14	195046	-3.6907	-4.2075	-2.3478	-2.53	1.331785346	1.01627756
Gm185	235497	1.3417	1.1401	3.9929	1.2115	-0.149063406	0.31445463
Gm867	333670	-3.9656	-4.1497	-1.3853	-2.5028	-0.775248356	-1.920169
Gmppa	69080	0.91705	1.0295	3.3294	0.45913	0.340354696	-0.5728311
Gnat1	14685	-3.6161	-2.8099	-1.8677	0.041448	-0.477951556	-0.0742333
Grinl1a	28015	-3.4865	-3.2196	-2.3063	-0.91011	-2.187252001	-0.300225
Gsn	227753	-3.2066	-3.5012	-2.6866	-1.9239	-1.838810211	-1.3296337
H2-Q6	110557	2.3804	2.222	3.1616	3.634	1.329633088	1.66606783
H2-T24	15042	1.2397	1.5808	2.394	2.081	1.217505184	-0.0185308
H2-Tw3	547339	-3.4448	-3.5982	-2.5219	-2.345	-2.255832319	-1.7213613
H2afy2	67552	-4.8256	-4.6595	-2.8043	-3.1132	-3.092639845	-1.2354844
Hapln1	12950	-0.13856	0.71139	2.283	2.4507	1.652505778	0.70434733
Hdac7a	56233	-3.0534	-3.2186	-1.589	-3.0998	-0.27648117	-0.437356
Hddc2	69692	-3.6826	-2.2849	-0.11309	-0.74505	-0.101102672	-0.403759
Hnrpab	15384	-3.199	-3.027	-2.3514	-2.7298	-1.242308772	-0.6244375
Hp	15439	-4.0972	-4.1657	-1.9048	-2.9111	-2.940712137	-2.3234869

Hspa14	50497	-3.13	-3.0315	-3.6821	-3.4545	-2.732452293	-1.1991443
Hspa8	15481	-1.2952	-1.5636	2.821	1.6732	0.31975548	-0.1899136
Hspg2	15530	2.2702	2.3927	2.4529	2.0516	0.192113664	-0.9309
Hyal3	109685	-4.7214	-0.75392	-1.6954	-0.65599	0.031244238	-0.0589361
Ifna12	242519	-4.2366	-3.5138	-2.5412	-2.3353	-2.3663647	-1.5627612
Ik	24010	1.8216	1.4787	2.0755	2.0042	-1.093965898	-0.6517265
Il27	246779	-3.2404	-3.1111	-2.6308	-2.7879	0.275739422	-0.9561935
Itgad	381924	-5.3181	-5.3209	-2.5634	-2.8129	-3.223914022	-0.5071941
Ixl	67224	-4.1633	-4.5526	-2.8399	-3.4751	0.88465199	-0.4151207
Kcnc4	99738	2.5668	2.0096	2.4368	2.7192	1.734094698	0.83521976
Kcnk15	241769	2.0778	1.6369	2.2626	3.5385	1.208244363	0.5944313
Kcnmb2	72413	-4.3828	-4.8846	-2.9586	-2.7484	-3.285888197	-2.0025922
Kif5a	16572	0.81579	0.39417	2.6241	1.9576	-3.870637083	-2.5956976
Klkb1	16621	1.1884	1.4207	2.0409	2.1238	1.065204055	1.20127086
Krtcap3	69815	-3.4315	-4.1288	-1.6607	-2.3414	1.407800121	0.8112237
L1cam	16728	-3.3247	-3.7529	-0.95586	-1.6737	-2.587633147	-1.1365099
Lgi1	56839	0.96119	1.0701	2.912	2.6937	1.213309298	0.55557671
Lmo1	109594	1.8783	1.9154	2.9317	1.0813	1.28272472	-0.1994898
LOC434448	434448	-0.27118	-0.22462	5.208	-0.987	0.24204334	-1.05607
LOC547347	547347	-3.6262	-3.7794	-2.674	-2.0881	-2.444552102	-1.525167
Lrrc15	74488	-4.2493	-4.1573	-2.6247	-2.6077	-2.972072645	-1.6662643
Ltbp2	16997	1.2823	1.9614	2.2252	2.1679	0.747613564	0.448515
Magea7	17143	-3.4996	-4.2711	-2.9371	-2.7901	-1.318364284	-1.0600735
Mdm2	17246	2.3058	2.1634	3.1038	2.3548	-0.543008145	0.14908324
Mical3	194401	1.2342	1.1919	2.6614	2.1708	1.095899196	0.58357623
Mknk2	17347	-3.4287	-3.4168	-1.9674	-1.373	-2.026606628	-2.9247105
Mlycd	56690	1.7261	1.539	2.0727	2.0791	-0.213373155	-0.6670361
Mmp14	17387	0.52535	0.54504	2.3062	3.0581	1.405783652	0.14317689
Mmp1b	83996	0.82865	0.70032	2.8045	2.0046	0.89028724	0.6451171
Mrpl4	66163	-3.5136	-4.7314	-1.565	-2.3892	-2.913615409	-2.3462103
Mrpplf4	107849	-4.1236	-3.495	-2.3042	-2.1975	-1.521416614	-1.637579
Ms4a4d	66607	2.6711	2.5076	3.1145	2.5597	1.737292533	0.40553339
Mtnr1a	17773	-0.094032	-0.81067	2.0933	2.3291	0.453596071	-0.1472038
Mum1	68114	1.6838	2.1351	3.1247	1.7194	-0.858955588	-1.9468773
Mup5	17844	1.562	1.6215	2.5979	2.5569	1.176204909	0.22616223
Myst1	67773	-3.6709	-3.3411	-2.7412	-2.9203	-3.072909679	-1.6215404
Naca	17938	2.4349	3.0195	4.8456	5.1971	-0.782003867	0.53592392
Nanos3	244551	-4.0489	-4.9706	-3.1256	-3.4742	-2.286147251	-1.2283301
Ndrg2	29811	1.9739	1.6072	2.5721	2.8598	1.874887695	0.40423597
Nfkbie	18037	-3.2785	-3.6645	-2.2882	-1.737	-4.298053852	-2.4504424
Nkx1-2	20231	-4.1358	-3.8101	-3.0269	-2.501	-2.74188964	-1.6975568
Nphp4	260305	0.99167	1.4379	2.2739	2.0498	0.904293893	0.9761132
Nqo2	18105	1.6125	2.1551	2.0257	2.0069	0.644237271	-0.2840135
Nucb2	53322	-0.89456	-4.1663	-1.0163	-1.6029	-1.228790961	-0.7336925
Nudt3	56409	-2.9129	-3.6178	-1.9315	-2.3655	-2.635254407	-1.3044885
Nutf2	68051	0.32109	0.932	2.0847	2.2382	-0.331638729	-1.0851445
Nxf	225872	-3.3048	-3.0557	-2.0802	-2.0284	-1.545872243	-1.2412733
Olfr101	258831	-3.6138	-2.6229	-1.9659	-1.8407	-1.227820068	-0.8564653
Olfr1181	258060	-3.7346	-4.3202	-2.8755	-3.5646	-2.62356975	-0.4605241
Olfr1269	258339	1.8107	1.6195	2.4376	2.8046	-0.027769733	-0.4648551
Olfr133	258828	-3.2808	-4.0204	-2.3656	-2.3903	-2.066175591	-0.2652256
Olfr1361	258534	-4.5246	-4.1346	-2.2027	-1.9157	-4.728777088	-4.1469093
Olfr525	258958	1.6822	2.063	2.8517	2.692	1.749907346	0.01944027
Olfr575	259118	3.0013	3.0973	2.3529	2.7408	1.282317353	1.4975641
Olfr808	258930	0.60977	0.77736	2.5374	0.73421	0.50625511	-0.665683
Olfr809	258321	0.96139	0.94398	2.4242	2.1522	0.68486539	-0.9223
Olfr860	258521	-0.064173	0.47345	6.1901	0.094614	-1.968067918	-0.4393268
Ormdl2	66844	2.4619	2.5897	2.2191	2.963	2.17914369	1.81991692
Ott	18422	2.278	2.1297	2.5855	2.6809	1.357666761	0.71204535
Pcdhga2	93710	1.8682	2.256	2.542	3.1085	1.78295164	0.6758968
Pcdhgb4	93701	1.1578	0.7995	3.5124	3.1837	3.28086909	2.93688278
Pcsk4	18551	1.5064	1.3853	2.1186	2.4094	1.550059641	1.66721698
Pcyt1a	13026	-3.1331	-3.4355	-2.7416	-3.266	-2.292760183	-0.9333466
Pde6b	18587	1.1558	1.377	2.032	2.4262	0.887944877	-0.0454306
Pdlim3	53318	-3.267	-3.2916	-1.0262	-0.95565	-0.064120493	-1.534842
Pes1	64934	-4.8624	-4.7872	-1.7871	-2.5716	-5.198125551	-4.1857454
Pex12	103737	-3.0572	-3.747	-1.6931	-1.8074	-2.873455763	-2.4678154
Pglyrp4	384997	1.1996	1.3462	2.1884	2.0871	0.789049628	0.22264067
Phf5a	68479	2.3154	1.5096	2.4515	2.7979	0.006415189	1.26730326
Phyh	16922	-3.4283	-3.2491	-3.6669	-3.5704	-1.914139251	-2.011359
Pik3cb	74769	-2.5987	-4.1801	-1.7141	-2.5355	-1.710638817	-1.2909398
Pip	18716	-3.9578	-3.2747	-2.2761	-2.5049	-3.181473118	-1.3962345
Pkp2	67451	1.7906	1.862	2.2867	2.2661	0.636531236	-0.3774338
Plac8	231507	-3.6444	-4.0372	-2.054	-2.3369	-2.354870148	-1.4960245
Plaur	18793	1.9081	2.1122	3.6335	3.4412	1.395640201	1.41457258
Plg	18815	0.4848	-4.57	-0.33269	-0.43048	-0.111300439	0.18660951
Pofut1	140484	1.9411	2.3082	2.7453	3.3244	1.118983354	1.45355691
Ppargc1b	170826	-4.8757	-5.4442	-5.0552	-4.4096	-1.297126859	-0.3241964
Ppp3r2	19059	-3.5103	-3.7085	-2.6057	-2.9751	0.191258192	-1.7208794
Prcc	94315	1.4276	1.8078	2.1868	3.1174	0.555532999	-0.5742398
Prkwnk4	69847	0.69653	0.224	2.0477	2.4485	-1.274219226	-1.1118899

Prpf8	192159	-3.0517	-3.7249	-1.6648	-1.3206	-0.794605102	-0.8785029
Psmb2	26445	1.3751	1.9084	2.2341	2.2269	0.995001056	2.03891493
Psmb5	19173	1.5246	1.7391	2.966	2.2966	2.687952485	1.57211004
Psmb6	19175	1.1802	1.9424	2.5382	2.5474	1.850662908	0.73295231
Psmb9	16912	-3.7046	-2.6763	-1.841	-2.3989	-1.706918194	-2.1881044
Psmd14	59029	1.8594	2.1207	2.9153	3.6471	0.553489372	2.97414957
Psmd2	21762	-0.21305	-0.16682	4.1679	4.2935	0.129216122	-0.5870162
Ptprn2	19276	-4.9234	-4.3594	-3.8057	-2.7681	-2.322599852	-0.7814313
Qtrt1	60507	1.2126	1.5865	3.2161	3.0147	0.58709719	0.41701245
Rab18	19330	-3.3166	-4.5921	-3.3774	-3.6364	-3.008396247	-2.5684642
Rad21	19357	1.5912	0.82627	2.2296	2.4865	1.904476154	0.33052408
Ran	19384	0.027267	0.52175	2.8128	1.6313	5.515089715	1.453248
Ranbp1	19385	-0.3905	-0.18495	3.8501	-0.26907	0.14014715	0.62366028
Rara	19401	-3.9301	-3.5015	-2.1078	-2.1464	-2.983153041	-1.3273169
Rasgrp4	233046	-3.6339	-3.16	-1.0599	-1.808	-2.765421902	-1.2867324
Rasl12	70784	-4.3513	-0.24929	1.0889	0.4314	0.66706343	-0.9785215
Rbed1	232089	-4.4339	-3.8407	-3.2492	-2.3689	-3.128107975	-1.1930156
Rbm8	60365	2.5786	1.4697	2.9667	2.9797	1.396767251	2.01569115
Resp18	19711	-3.6429	-2.9531	-2.18	-1.7573	-1.789776743	-1.2833468
Rheb	19744	-5.4918	1.0072	0.37925	0.41899	0.037687434	-0.6881654
Rnf150	330812	-3.9984	-4.415	-2.9003	-2.6654	-3.587822198	-0.9133231
Rnf2	19821	-6.2025	-4.2946	-2.3225	-2.3544	-2.47854692	-3.7208743
Rpn1	103963	-4.2997	-3.2	-1.7694	-1.8072	-1.628737581	-0.4210765
Rps16	20055	0.20822	-0.44299	8.5599	1.8748	-0.131125657	0.93368144
Rragc	54170	-4.9669	-4.4249	-3.6881	-3.4307	-0.111741754	-1.7459814
Rrm1	20133	-0.020454	2.4533	5.4226	1.914	0.037205383	2.3964216
Rsl1	380855	1.3692	1.5308	2.5307	2.1977	0.446351719	1.63601098
S100a8	20201	-6.1628	-0.022413	-0.45882	-0.30108	-0.279427795	-0.4828953
Sbp	20234	-3.2483	-3.2025	-2.3424	-3.1865	-1.8577528	-1.1461973
Scgb1a1	22287	-4.8181	-3.3217	-3.3412	-2.4128	-2.91823903	-1.6021657
Scgb3a1	68662	0.79269	0.90988	2.1678	2.036	1.144722191	0.10477332
Scrib	105782	-4.6754	-3.8166	-2.2237	-2.226	-2.104970556	-1.1917367
Sdk1	330222	-3.3371	-3.1001	-1.7914	-1.9665	0.232938676	-1.907961
Sec61g	20335	-3.6828	-3.7025	-2.0262	-1.2713	-2.621675491	-2.2555208
Seh1l	72124	1.3115	1.3647	2.4353	2.7349	0.345657263	0.33137049
Sertad1	55942	1.1021	0.54988	2.3327	2.2538	1.003107669	-0.2788424
Set7	73251	-3.2404	-3.1676	-2.5434	-3.0679	-2.538042943	-0.8084237
Sf3a1	67465	0.60254	1.0565	2.1207	2.1222	-1.496872722	-0.7903649
Sf3a3	75062	0.78954	1.0691	2.6001	3.4574	0.350973409	0.47681725
Sf3b1	81898	-0.82982	-0.98753	2.8362	2.7068	1.090148525	0.55619453
Sf3b2	319322	0.9341	0.44153	2.0357	2.6308	0.286704397	0.12217725
Sf3b3	101943	1.2735	1.94	2.562	2.5407	1.012769068	0.49712493
Sh2bp1	22083	1.6843	1.4877	2.3161	2.1227	-0.476790558	-1.3367077
Siat8b	20450	-3.5041	-2.6184	-2.4303	-3.3061	0.308104769	-0.3414397
Slamf9	98365	-3.1964	-3.2938	-1.949	-1.6205	-1.434701652	-0.9456535
Slc12a1	20495	-4.1215	-4.1832	-2.2062	-1.8378	-2.815473789	-2.2563363
Slc24a5	317750	1.9599	1.6455	2.7213	2.3713	0.423430508	-0.0073483
Slc2a10	170441	1.9828	1.9329	2.099	2.154	0.985794551	0.2546004
Slc31a2	20530	-3.3796	-3.7355	-2.5824	-2.1179	-1.653899314	-0.4898952
Slc35f2	72022	-3.099	-3.409	-1.654	-2.509	-2.140751001	-1.3220036
Slc7a5	20539	0.075711	0.93623	2.1528	2.4784	2.033720283	0.94824959
Smc5l1	226026	1.5422	1.6135	2.3104	2.657	-0.482480125	-0.6436949
Snrp116	20624	-3.4579	-3.8712	-2.8169	-1.6778	-1.463081588	-0.1371828
Snrpa1	68981	1.0604	0.74998	2.7363	3.3875	-0.020871644	0.37398759
Snrpd2	107686	-1.5675	-0.69583	2.4108	3.4429	2.008898023	0.18112329
Snrpe	20643	-0.64843	-1.2852	2.6303	3.5164	0.140873622	0.87771947
Spata4	69281	1.4491	1.3204	2.0013	2.0512	0.652289569	-0.2866145
Sprr2d	20758	1.1371	0.33248	2.9382	2.7454	1.087059321	0.20485368
Sprrl5	68720	-5.9185	-2.4117	-3.7818	-4.2915	-0.654782997	-0.6350578
Ssrp1	20833	2.6391	1.4896	2.998	3.7887	0.883789729	-0.2768962
Sstk	83984	-3.9491	-3.5272	-5.3094	-3.255	-1.826765712	-0.5410691
Supt16h	114741	1.1934	0.39815	3.0022	2.5059	0.275427107	-0.7473092
Surb7	108098	0.36339	0.12385	3.9012	0.7386	1.68406318	-0.1050192
Tada3l	101206	1.3702	1.1058	3.1103	2.5284	0.685849861	1.24691526
Tcp10a	21460	-3.8989	-3.3437	-2.3719	-2.0098	-3.658534406	-2.8754519
Tdrd6	210510	-3.9543	-4.2451	-3.9744	-2.9522	-2.482654542	-0.1964069
TGFβr1	21812	-6.5442	-5.8763	-3.4675	-4.0597	-4.380124824	-3.3026299
TGFβr2	21813	-3.8618	-3.0052	-2.0338	-1.7408	-0.064684018	0.26173003
Timd2	171284	1.4287	1.2057	2.1042	2.0733	1.719612784	0.88096302
Tle4	21888	1.5555	1.2399	2.0667	2.0703	0.503478222	-0.0316533
Treh	58866	-4.8226	-4.9743	-4.4767	-3.272	-3.475952293	-2.1986382
Trem3	58218	-4.3154	-1.6425	-3.8048	-3.1871	-1.981728308	-0.8686116
Trip6	22051	0.96094	1.0947	2.8715	1.9783	-0.275924434	0.26009899
Trpv1	193034	1.1402	1.4814	2.0435	3.0483	1.19461113	0.11601143
Ube2m	22192	1.3332	1.4471	3.5574	1.8902	-0.708847454	-0.7231834
V1rc15	171188	-3.6239	-3.5336	-2.7207	-1.7368	-2.64964127	-1.857201
V1rh3	171246	0.71908	0.60122	2.0887	2.3914	0.612815658	-0.2252899
V1ri7	171258	-3.8336	-3.3767	-2.6146	-2.9549	-2.014630023	-0.9067124
V2r4	22310	1.0285	1.142	2.5855	2.629	-0.128090769	-0.877156
Vcl	22330	-1.5929	-0.73445	6.1486	-1.6715	0.704072767	-0.6198286
Vps28	66914	1.332	1.5623	3.4496	3.3182	2.588833184	0.4176488

Wbp5	22381	-3.0482	-3.531	-2.2663	-3.2408	0.554786158	0.30471164
Whsc2	24116	-4.1696	-2.231	-3.2038	-2.768	-1.914451566	-1.1204034
Xlr4	27083	-3.0013	-3.1439	-1.6345	-1.7496	-1.961359933	-0.8061811
Xpo1	103573	-0.28535	0.2653	3.3469	1.906	0.983255293	2.03654251
Ythdf3	229096	1.126	1.2413	2.5651	1.3649	1.359391283	0.34027325
Zcchc9	69085	1.9333	1.4463	3.3276	2.5059	1.629822197	2.26301612
Zf	233490	-0.036777	0.037508	2.576	1.6824	0.5058749	-0.3713915
Zfp265	53861	0.4178	0.94988	2.6213	1.8523	0.642444854	0.03937726
Zfp451	98403	1.3621	1.0896	2.4968	2.3594	0.112161003	-1.1453138
Zpbp	53604	-3.0864	-4.1214	-2.0254	-1.5383	-1.721617371	-2.0732582

SUPPLEMENTAL MATERIALS AND METHODS

Primers. List of primers used for quantification of gene expression.

	FORWARD	**REVERSE**
Mouse genes		
Atp5o	TCTCGACAGGTTCGGAGCTT	AGAGTACAGGGCGGTTGCATA
Coco	ATGACCCTGTTCTGGGGACA	TTATGGTTCCCACGGAATGC
Ctgf	GGGCCTCTTCTGCGATTTC	ATCCAGGCAAGTGCATTGGTA
Pai1	GTAGCACAGGCACTGCAAAA	GCCGAACCACAAAGAGAAAG
Human genes		
ATP5O	ACTCGGGTTTGACCTACAGC	GGTACTGAAGCATCGCACCT
ID1	ATCGCATCTTGTGTCGCTGA	GTGGAATCCCACCCCCTAAA
ID2	GCAAAGCACTGTGTGGCTGA	CCAACTGCAGAAAGGGCATT
ID3	CACCTTCCCATCCAGACAGC	TCCAGGAAGGGATTTGGTGA
LEFTY1	ACCTCAGGGACTATGGAGCTCAGG	AGAAATGGCCAATTGAAGGCCAGG
LEFTY2	TGCTACAGGTGTCGGTGCAGAGG	AGAAACGGCCACTTGAAGGCCAGG
PAI1	GAGAAACCCAGCAGCAGATT	TGGTGCTGATCTCATCCTTG
***Xenopus* genes**		
gsc	TTCACCGATGAACAACTGGA	TTCCACTTTTGGGCATTTTC
vent2.2	TGACACTTGGGCACTGTTCTG	CCTCTGTTGAATGGCTTGCT