ISM1 regulates NODAL signaling and asymmetric organ morphogenesis during development

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Introduction

The TGF-β superfamily includes a large number of secreted signaling factors that are essential for embryonic development, tissue homeostasis, and human diseases such as cancer (Wakefield and Hill, 2013). These factors are secreted as precursors that undergo activation through cleavage by proprotein convertases (Constam, 2014). Based on the structural and sequence similarities, members of the TGF-β superfamily are categorized into several subfamilies that include TGB-β, ACTIVIN, NODAL, growth differentiation factors (GDFs), and bone morphogenetic proteins (BMPs). NODAL was first described to be critical for embryonic development in mice (Zhou et al., 1993; Conlon et al., 1994; Collignon et al., 1996). Evolutionarily conserved from hydras to human, NODAL plays key roles in embryonic patterning, namely, the formation of both anterior-posterior (AP) and left-right (LR) body axes (Rossant and Tam, 2009). NODAL is also important for the maintenance of pluripotency of embryonic stem cells and germ layer specification (Shen, 2007). Increasing evidence suggests that the cooperation between NODAL and GDF ligands is involved in the embryonic development. NODAL-GDF1 heterodimers rather than NODAL homodimers are required for mesendoderm and endoderm formation (Tanaka et al., 2007; Fueter et al., 2014; Montague and Schier, 2017), whereas GDF3 is an essential coligand for NODAL signaling in germ layer formation and LR patterning during early development (Levine et al., 2009; Peterson et al., 2013; Pelliccia et al., 2017). In addition to its roles in early embryogenesis, NODAL signaling has recently been reported to be involved in homeostasis of adult reproductive tissues (Park and Dufort, 2011; Park et al., 2012) and carcinogenesis (Kirsammer et al., 2014). NODAL binds to a coreceptor CRİPTO (also known as TDFG1) and a heterodimeric receptor complex composed of Activin receptor type IB (ACVRIB; also known as ALK4) and ACVRIA/IIB. This triggers the phosphorylation of SMAD2/3, which is then transported into the nucleus, where it interacts with nuclear factors such as FOXH1 (also known as FAST2) to regulate the transcription of targeted genes (Schier, 2009).

Isthmin 1 (ISM1) was first identified in Xenopus laevis gastrula embryos by an unbiased secretion cloning screen (Pera et al., 2002). Named after its prominent expression location in the isthmus region of the brain, ISM1 was described as a member of the FGF8 synexpression group. ISM1 has since been described as a target of WNT/β-Catenin and NODAL signaling in the zebrafish embryo (Weidinger et al., 2005; Bennett et al., 2007). In the chick embryo, ISM1 is among the top 20 genes expressed in the anterior primitive streak (Alev et al., 2010), a fundamental...
structure of the embryo whose formation marks the start of gastrulation and germ layer formation. Despite this information, the biological function of ISM1 remains largely unexplored. ISM1 has been reported as an angiogenesis inhibitor during tumor progression (Xiang et al., 2011) that has a dual function in endothelial cell survival and apoptosis through cell surface receptors vβ5 integrin (Zhang et al., 2011) and GRP78 (Chen et al., 2014). Based on its spatiotemporal distribution throughout mouse ontogeny (Osório et al., 2014), we speculate that ISM1 is likely to function as more than an angiogenesis inhibitor. Present in the embryo as early as embryonic day 6.75 in the anterior mesendoderm, ISM1 is dynamically expressed in several of its derivatives, such as paraxial mesoderm, lateral plate mesoderm (LPM), and endoderm. Since its expression persists in several adult tissues, ISM1 seems to be involved not only in embryogenesis but also in organ homeostasis. The sparse information available on ISM1 led us to search for a cue of its putative function based on its distinct structural features. The ISM protein family is characterized by the presence of thrombospondin type 1 repeat (TSR1) and an AMOP (adhesion-associated domain in MUC4 and other proteins) domain. While TSR1 is well characterized and present in many extracellular proteins (Tucker, 2004), AMOP is found only in MUC4, SUSD2, ISM1, and ISM2, with little-known functions (Ciccarelli et al., 2002). Several functions have been attributed to the TSR1 domain, among which is the ability to activate and regulate TGF-β signaling (Adams and Lawler, 2011). We therefore tested the potential involvement of ISM1 in TGF-β superfamily pathway signaling.

**Results**

**ISM1 is a secreted soluble N-glycosylated protein**

Similar to its counterpart in Xenopus, mouse ISM1 is a secreted protein that, when ectopically expressed in HEK293T cells, is detected as a single band of ~70 kD in both whole-cell lysates (WCLs) and conditioned medium (CM) samples (Fig. 1 A). Since the molecular weight of the detected ISM1 was larger than expected (52 kD), ISM1 is likely subject to posttranslational modifications. Glycosylation is commonly found in many secreted proteins (Moremen et al., 2012), and analysis of the ISM1 protein sequence predicted two putative N-glycosylation sites at positions N39 and N282 (Osório et al., 2014; see Fig. 6 A). Indeed, biochemical analyses showed that ISM1 is an N-glycosylated protein. PNGase F treatment reduced ISM1 molecular weight in both WCL and CM, whereas endoglycosidase H (Endo H) digestion removed almost all types of N-linked glycosylation while Endo H predominantly removes high-mannose N-glycans, this result suggests that intracellular ISM1 contains predominantly oligomannose N-glycans, whereas secreted ISM1 contains a more complex type of N-glycans. Glycosylation of ISM1 was further confirmed by mutating asparagine residues to glutamine at each single position (N39Q or N282Q) or in combination (N39Q/N282Q), which led to a shift of the intracellular ISM1 MW from 70 kD to ~65 kD and 60 kD, respectively (Fig. 1 B). Mutant ISM1 (N39Q/N282Q) was no longer sensitive to PNGase F treatment, indicating that both N39 and N282 residues are N-glycosylated. Treatment of cells expressing either ISM1 or its asparagine residue mutant forms with tunicamycin, an inhibitor of N-glycosylation, led to similar changes in ISM1 migration (Fig. 1 C), further supporting that ISM1 is N-glycosylated. N-glycosylation in ISM1 is essential for its secretion, as eliminating glycosylation, by either point mutations or tunicamycin treatment, dramatically reduced the amount of ISM1 in the CM (Fig. 1 C). These data provided the first experimental evidence that ISM1 is N-glycosylated at both N39 and N282 residues and that this modification is essential for its secretion.

**ISM1 is an extracellular antagonist of the NODAL signaling pathway**

The TSR1 domain is well known for its involvement in the regulation of TGF-β signaling (Adams and Lawler, 2011). We therefore asked if ISM1 regulates the TGF-β superfamily
signaling pathway. To test our hypothesis, we examined whether ISM1 has any effect on SMAD activation mediated by the major ligands of the TGF-β superfamily, including TGF-β, ACTIVIN, and NODAL, using the CM collected from HEK293T cells ectopically expressing ISM1. While TGF-β, ACTIVIN, and NODAL signals are largely transduced through the phosphorylation of SMAD2/3, BMPs activate SMAD1/5/8. We found no significant changes in phosphorylated SMAD2 (pSMAD2) levels in the presence or absence of ISM1 CM, in response to the stimulation of either TGF-β1 or ACTIVIN-A (Fig. 2 A). However, CM containing ISM1 led to a significant reduction in SMAD2 phosphorylation elicited by NODAL (Fig. 2 B). A similar observation was found in the presence of GDF1, a NODAL-related TGF-β ligand that uses the same core components of the signaling pathway (Shen, 2007; Schier, 2009) and forms NODAL-GDF1 heterodimers (Tanaka et al., 2007; Fuerer et al., 2014; Montague and Schier, 2017; Fig. 2 B). On the other hand, in response to BMP4, no obvious alteration in pSMAD1/5/8 levels was observed between ISM1 CM treatment and mock CM treatment (Fig. 2 C). These results indicate that ISM1 modulates SMAD-mediated TGF-β superfamily signaling in a ligand-dependent manner. This was further confirmed by purified recombinant mouse ISM1 protein (Fig. 2, D and E). The antagonizing effect on NODAL signaling by ISM1 was further examined on the transcription of SMAD2 downstream targeted genes. One of the best understood SMAD2 targets is Mix.2 gene (Chen et al., 1996, 1997; Liu et al., 1997). In the nucleus, pSMAD2 associates with FOXH1 to form a transcriptional complex on the ACTIVIN-NODAL-TGF-β responsive element of the Mix.2 promoter. Its activity can be measured by the A3-luc reporter gene assay, which has been extensively used as “readout” of the NODAL signaling pathway (Liu et al., 1997; Iratni et al., 2002; Yan et al., 2002; Gray et al., 2003; Chen and Shen, 2004; Cheng et al., 2004). Consistent with the results on SMAD2 phosphorylation, both recombinant ISM1 (rISM1) and ISM1 CM inhibited SMAD2/FOXH1 transcriptional activity induced by NODAL (Fig. 3, A and B). In addition, the NODAL signaling antagonism by ISM1 was dose dependent in the A3-luc reporter gene assay, with stronger inhibitory effects at higher ISM1 concentrations (Fig. 3, A and B). However, ISM1 CM did not significantly impact the SMAD2/FOXH1 transcriptional activity induced by either TGF-β1 or ACTIVIN-A (Fig. 3, C and D). These results suggest that ISM1, acting as a secreted protein, specifically antagonizes NODAL signaling, leading to reduced activation of its major intracellular effector SMAD2 and decreased SMAD2/FOXH1 transcriptional activity. In addition to Mix.2, several other genes have been reported, including NODAL gene itself, to be NODAL targets (Shen, 2007; Schier, 2009). In chick embryos, it has been shown that NODAL-soaked beads induce ectopic expression of NODAL in ∼50% of embryos when implanted in the right side of the embryo (Schlueter and Brand, 2009). To test the inhibitory effect of ISM1 on NODAL signaling in vivo, we used the same assay to examine how ISM1 affects the ability of NODAL to regulate its own expression (Fig. 3 E). As shown in Fig. 3 (F and G), 58% of embryos (7 of 12) implanted with rNODAL + PBS beads showed ectopic NODAL expression in the right LPM, whereas ectopic NODAL expression was detectable in only 14% of embryos (2 of 14) implanted with rNODAL + rISM1 beads. Taken together, these results are in agreement with our previous observations and identify ISM1 as a new extracellular antagonist of the NODAL signaling pathway.

Magnitude of inhibition of NODAL signaling by ISM1, LEFTY1, and CER1

At present, the LEFTY and DAN/CER families of proteins are known extracellular antagonists of NODAL signaling (Shen, 2007; Schier, 2009). LEFTY1 and 2 are divergent noncanonical TGF-β ligands that, often through a negative-feedback mechanism (Meno et al., 1998, 1999; Branford and Yost, 2002; Feldman et al., 2002), block NODAL signaling by binding to NODAL itself and its coreceptor CRIPTO (Chen and Shen, 2004; Cheng et al., 2004). Members of the DAN/CER family are cysteine-rich extracellular proteins that inhibit NODAL signaling through their direct interaction with NODAL ligand (Piccolo et al., 1999; Perea-Gomez et al., 2002; Marques et al., 2004). To compare the inhibitory effect of ISM1 on NODAL signaling with known extracellular antagonists, ectopic FLAG-tagged LEFTY1, FLAG-tagged ISM1, and FLAG-tagged CER1 were expressed in HEK293T. CM was collected from these cells individually for the evaluation. NODAL signaling was examined in HEK293T-CRIPTO cells by Western blotting of pSMAD2 upon NODAL stimulation in the presence of LEFTY1, ISM1, or CER1. As shown in Fig. 4 (A and B), NODAL treatment readily activated SMAD2, represented by a significant elevated phosphorylation of SMAD2. LEFTY1, ISM1, and CER1 all caused a significant decrease in pSMAD2 levels in a dose-dependent manner. Similar dose-dependent inhibitory effects for LEFTY1, ISM1, and CER1 on SMAD2/FOXH1 transcriptional activity were observed in the A3-luc luciferase reporter activity assay (Fig. 4 C). In both sets of experiments, LEFTY1 exhibited the strongest inhibition to NODAL signaling and almost completely abolished pSMAD2 (∼90% of reduction) at higher concentrations, whereas the inhibition to NODAL signaling by CER1 was much weaker (∼40% of reduction at similar concentrations; Fig. 4, A-C). These observations are in line with the previous report showing CER1 as a potent antagonist of NODAL signaling (Meno et al., 1999). Compared with CER1, similar or lower concentrations of ISM1 (determined by Western blotting using anti-FLAG antibodies) could achieve comparable (A3-luc luciferase reporter activity assay) or slightly stronger (pSMAD2 level) inhibition to NODAL signaling (Fig. 4, A–C). In the presence of undiluted ISM1 CM, pSMAD2 levels and SMAD2/FOXH1 transcriptional activity are reduced to ∼40 and ∼60%, respectively (Fig. 4, A–C). Taken together, these comparative analyses of NODAL signaling inhibition, at the level of both SMAD2 activation and its subsequent transcriptional activity, revealed that ISM1 is an antagonist of NODAL signaling with a similar inhibitory activity to CERI.

ISM1 interacts with NODAL ligand and type I receptor ACVR1B

To understand the molecular mechanism underlying the inhibitory effect of ISM1 on NODAL signaling, we examined the potential interaction between ISM1 and the distinct components of the NODAL signaling pathway through a series of ligand-binding assays. As shown in Fig. 5 A, ISM1 was found to interact with
both the proprotein and mature forms of NODAL ligand in the CM. GST pull-down assays and surface plasmon resonance (SPR)–based interaction analysis further confirmed the direct interaction between ISM1 and NODAL ligand (Fig. S1). We then tested whether ISM1 interacts with the receptor complex by an in situ proximity ligation assay (PLA). As shown in Fig. 5 B, significantly higher numbers and percentages of PLA+ signals were found in ACVR1B-transfected cells treated with ISM1 CM, compared with those treated with mock CM, suggesting that ISM1 could form a complex with ACVR1B. Since ISM1 acts as a secreted protein, we hypothesize that its interaction with the receptor complex is likely to occur through their extracellular domains. To test this hypothesis, we generated soluble forms of the N-terminal ectodomain (ECD) of ACVR1B and ACVR2B (ACVR1B_ECD and ACVR2B_ECD; Fig. 5 C) that allowed us to discriminate which particular element of the receptor complex is involved in the interaction. We found that ISM1 interacted with ACVR1B_ECD in a cell-independent manner (Fig. 5 D). GST pull-down assays and SPR-based interaction analysis further confirmed the direct interaction between ISM1 and ACVR1B ligand (Fig. S1). In contrast, no interaction between ISM1 and ACVR2B_ECD (Fig. 5 E) or between ISM1 and the coreceptor CRIPTO was observed (Fig. 5 F). As ACVR2A has been shown to interact with NODAL (Kelber et al., 2008; Wang et al., 2016), we then tested if ISM1 could also interact with ACVR2A. As shown in Fig. S2, coimmunoprecipitation experiments indicated interaction between ISM1 and ACVR2A. These data suggested that ISM1 could bind intimately to both ligand and receptor complex components of NODAL signaling.

**Inhibition of NODAL signaling by ISM1 requires the AMOP domain**

To understand which domain of ISM1 mediates the interaction with components of the NODAL signaling pathway, we generated domain-deleted ISM1 constructs lacking either TSR1 (ΔTSR1) or AMOP (ΔAMOP) domains (Fig. 6 A). The boundaries for TSR1 and AMOP domains have been previously described (Osório et al., 2014): TSR1 domain spans amino acids 215–259, and AMOP domain spans amino acids 286–449. While ISM1 expression is not affected by the absence of either domain, several bands were detected in WCL samples of ISM1 lacking AMOP (Fig. 6 B). Treatment with PNGase F or Endo H eliminated the higher-molecular weight bands, suggesting that these correspond to different N-glycosylated
forms of ISM1. In addition, deletion of TSR1 or AMOP domain does not affect N-glycosylation and secretion of ISM1 (Fig. 6 B). Ligand-binding experiments showed that AMOP domain is required for ISM1 interaction with the components of the NODAL signaling pathway, as the interactions between ISM1 and NODAL or ACVR1B were lost when AMOP domain was deleted. However, deletion of TSR1 domain in ISM1 did not significantly affect the interactions (Fig. 6 C and D). In line with this, the inhibition of NODAL-induced phosphorylation of SMAD2 by ISM1 was attenuated only when AMOP domain was deleted (Fig. 6 E). These results indicated that the inhibition of NODAL signaling by ISM1 is dependent on its AMOP domain, which mediates the physical interaction of ISM1 with NODAL and ACVR1B.

ISM1 compromises the formation of NODAL–ACVR1B complex
The interactions of ISM1 with NODAL, ACVR1B, and ACVR2A raise the possibility that ISM1 may disturb NODAL signaling through interfering with the interaction between NODAL ligand and receptor complex. To test if ISM1 could potentially prevent NODAL and/or ACVR1B interaction with the coreceptor CRIPTO, we performed competitive binding assays. As shown in Fig. 7 A, the interaction between NODAL and a soluble form of CRIPTO (sCRIPTO) was not disturbed by increasing concentrations of ISM1. Similarly, the interaction between sCRIPTO and ACVR1B also remained largely unchanged with increasing concentrations of ISM1 (Fig. 7 B). However, NODAL–ACVR1B interaction was significantly compromised by the presence of ISM1. In a cell-independent assay, the physical
interaction between NODAL and ACVR1B was greatly reduced by ISM1 CM in a dose-dependent manner (Fig. 7 C). In line with this result, NODAL–ACVR1B complex formation represented by PLA+ signals was significantly disturbed in the presence of ISM1 CM compared with mock CM (Fig. 7 D). On the other hand, the interaction between NODAL and ACVR2A was not disturbed in the presence of increasing concentrations of ISM1 (Fig. S3). These data provided strong evidence that ISM1 impacts the interaction between NODAL ligand and its receptor complex by specifically compromising NODAL–ACVR1B interaction.

ISM1 causes defective LR patterning in the chick embryo

NODAL signaling has a conserved role in the generation of LR asymmetry (Nakamura and Hamada, 2012; Grimes and Burdine, 2017). LR asymmetry is initiated in the node region during gastrulation and subsequently transmitted to the LPM. The asymmetric expression of NODAL in the left LPM, which depends on NODAL signaling, initiates a left-sided regulatory genetic cascade that involves downstream targets such as CER1 and PITX2 and that ultimately controls the shape and position of the internal organs. Our previous study (Osório et al., 2014) showed that ISM1 mRNA is asymmetrically distributed in the anterior mesendoderm of E7.5 mouse embryos and in the foregut endoderm of Hamburger and Hamilton stage 10 (HH10; Hamburger and Hamilton, 1951) chicks, suggesting that ISM1 may be potentially involved in LR asymmetric organ morphogenesis. We therefore explored the biological relevance of ISM1 as an antagonist of NODAL signaling during LR asymmetry. To address this question, we used the chick embryo assay in which embryos at HH4–5 were cultured ex ovo in the presence or absence of ISM1 CM. To test if ISM1 disturbs the left-sided gene expression downstream of NODAL signaling, we examined NODAL expression by whole-mount in situ hybridization, given that the NODAL gene itself is a downstream target of NODAL signaling. As shown in Fig. 8 (A and B), 87% of mock-treated embryos (21 of 24) exhibited strong expression of NODAL asymmetrically in the left LPM. In contrast, asymmetric expression of NODAL gene was observed in only 60% of ISM1-treated embryos (18 of 30). By quantitative real-time PCR (qPCR) analyses, NODAL relative transcription was significantly reduced by ∼70% in the left side of LPM in ISM1 CM–treated embryos (Fig. 8 C). In addition, the expression of other NODAL downstream targets was also disturbed by ISM1. As shown in Fig. 8 (D and E), 89% of mock-treated embryos (23 of 26) exhibited asymmetric CER1 expression on the left side of LPM
compared with only 67% (22 of 33) in ISM1-treated embryos. Moreover, the relative transcriptional levels of CER1 and PITX2 were significantly reduced in the left side of LPM in ISM1 CM–treated embryos (Fig. 8, F and G).

LEFTY1 is another NODAL target gene that has important functions in LR asymmetry, as it prevents NODAL signals from crossing to the right side of the embryo. In contrast to CER1 and PITX2, we observed no obvious differences in LEFTY1 expression along the midline between embryos treated with mock and ISM1 CM (Fig. 8, H and I), suggesting that LEFTY1 is regulated by signals other than NODAL. This is in agreement with midline LEFTY1 being regulated by at least two parallel pathways involving NODAL and BMP (Yamamoto et al., 2003; Smith et al., 2011). To test if disrupted NODAL signaling in left LPM leads to defective organ asymmetry, we examined the direction of the heart looping, the first overt morphological asymmetry in the embryo. In mock-treated embryos, ∼88% embryos (36 of 41) manifested the correct heart position to the right side, whereas in ISM1-treated embryos, 56% (33 of 59) exhibited normal heart position (Fig. 8 J). Taken together, these data show that treatment of embryos with ISM1 results in defective NODAL signaling in the left LPM and impaired asymmetric heart morphogenesis.
ISM1 antagonizes NODAL signaling

**Discussion**

ISM1 is the founding member of a new family of proteins whose function remains largely elusive. Based on its spatiotemporal distribution throughout mouse ontogeny, we suggested that ISM1 plays roles other than as an angiogenesis inhibitor (Osório et al., 2014). In the present work, for the first time, we reveal
that mouse ISM1 is a secretary protein with posttranslational modifications. We provide evidence that ISM1 is an N-glycosylated protein in which complex glycans are linked to the asparagine residues 39 and 282. We found that either point mutations at these residues or tunicamycin treatment abolished the secretion of ISM1. This observation indicates that caution should be taken when interpreting previous findings in functional studies on ISM1 produced in Escherichia coli lacking N-glycosylation. Our results also suggest that ISM1 is likely to carry other types of posttranslational modifications, as its molecular weight is still larger than expected even when N-glycosylation is abolished. Particularly, O-fucosylation as well as C-mannosylation have been identified within the TSR1 domain of several proteins (Hofsteenge et al., 2001; Luo et al., 2006) and were also predicted to occur in ISM1 (Du et al., 2010), though not yet experimentally validated.

Given limited information available, we searched for cues of potential putative functions of ISM1 based on its structural features. The presence of the TSR1 domain in ISM1 suggested the potential involvement of ISM1 in TGF-β superfamily signaling. To test this hypothesis, we analyzed if ISM1 affects the response of cells to TGF-β, ACTIVIN, and NODAL as well as BMP. We found that ISM1 modulates SMAD-mediated TGF-β superfamily signaling in a ligand-dependent manner. While ISM1 causes no alteration in signaling induced by TGF-β1, ACTIVIN, or BMP4, it specifically inhibits pSMAD2 induced by NODAL and its related member GDF1. This observation is consistent with previous reports showing that NODAL-GDF1 heterodimers rather than NODAL homodimers are required for NODAL signaling (Tanaka et al., 2007; Fuerer et al., 2014; Montague and Schier, 2017). These data identified ISM1 as a new extracellular antagonist of the NODAL signaling pathway. In ligand-binding assays, although ISM1 did not interact with the extracellular domain of ACVR2B, one of the NODAL receptors, or the transmembrane coreceptor CRIPTO, it bound to NODAL ligand and two other NODAL receptors, ACVR1B and ACVR2A (Figs. 5, S1, and S2). Although ISM1 interacted with ACVR2A, it did not interfere with the association between NODAL ligand and ACVR2A (Fig. S3).
However, through a series of competitive-binding assays, we revealed that ISM1 specifically compromised the association between NODAL and ACVR1B (Fig. 7). Hence, the way by which ISM1 suppresses NODAL signaling is distinct from other secretory antagonists, LEFTY and CER (Fig. 9). Similar to GDF1, GDF3 is also a coligand essential for NODAL signaling (Levine et al., 2009; Peterson et al., 2013; Pelliccia et al., 2017). It is unclear whether ISM1 interacts with GDF1/GDF3 and jeopardizes NODAL signaling through interfering with NODAL/GDF heterodimer formation. Unexpectedly, the inhibitory effect of ISM1 on NODAL signaling requires the presence of the AMOP domain but not the TSR1 domain. The TSR1 domain is involved in the activation and regulation of TGF-β signaling, among other functions, and has been shown to have antiangiogenic activity (Adams and Lawler, 2011). However, none of these functions can be attributed to the TSR1 domain in ISM1 (Xiang et al., 2011; present work). On the other hand, the interaction between ISM1 and the components of NODAL signaling is lost upon AMOP domain deletion. Consistently, the inhibition of NODAL-induced SMAD2 activation is abrogated in the absence of AMOP, an uncommon and rarely studied motif implicated in the regulation of tumor angiogenesis in ISM1 and MUC4 (Zhang et al., 2011; Tang et al., 2016). ISM1 function in endothelial cells has been described to be dependent on a KGD motif located in the C-terminal portion of AMOP, important for integrin interaction. Our findings provide evidence of a new function of AMOP domain in the regulation of NODAL signaling.

NODAL signaling is subject to tight spatiotemporal regulation, fundamental for homeostasis and tumorigenesis in not only embryogenesis but also the adult reproductive tissues (Shen, 2007; Park and Dufort, 2011; Park et al., 2012; Kirsammer et al., 2014). Secreted antagonists (DAN/CER and LEFTY, identified previously, and ISM1, described in this study) exhibited different magnitudes of inhibition on NODAL signaling. Although the method we employed in the study to compare the inhibitory strength of three NODAL antagonists has limitations and is not quantitative, it showed that LEFTY1 was the most potent inhibitor of NODAL signaling, whereas ISM1 exhibited a considerably weaker activity than LEFTY1 but similar or slightly higher inhibitory potential compared with CER1 (Fig. 4).
difference in their inhibitory activities is likely attributable to the distinct underlying mechanisms through which NODAL signaling is regulated. CER1 uses a single mechanism, whereas LEFTY1 and ISM1 use distinct dual mechanisms (Fig. 9; Piccolo et al., 1999; Perea-Gomez et al., 2002; Chen and Shen, 2004; Cheng et al., 2004; Marques et al., 2004).

The differences in inhibition potentials and mechanisms used also seem to be in agreement with genetic studies in mice. Deletion of LEFTY genes in mice gives rise to phenotypes related to enhanced NODAL signaling, such as excess of mesoderm formation and defects in LR patterning (Meno et al., 1998, 1999). On the other hand, Cer1 is not essential for mouse development, as Cer1 knockout mice show no defects in anterior patterning and are fertile (Simpson et al., 1999; Belo et al., 2000; Stanley et al., 2000). The mild phenotypes observed in Cer1 knockout mice may be attributable to the relatively mild inhibition of NODAL signaling, in addition to the proposed genetic compensation. Although a recent study reported that knockdown of ISM1 in Xenopus resulted in craniofacial dysmorphologies, the potential link between the phenotype and nald signaling was not investigated (Lansdon et al., 2018). In addition, the loss of ISM1 may not significantly disturb NODAL signaling given that LEFTY genes have much stronger inhibitory effects on NODAL signaling. Whether loss- or gain-of-function in Ism1 gene in mice will lead to phenotypes related to disturbed NODAL signaling remains to be determined. However, our analyses in ex ovo culture of chick embryos do support the biological relevance of ISM1 as an antagonist of NODAL signaling during embryonic development, as reduced signaling in left LPM and abnormal asymmetric heart morphogenesis were observed in the presence of ectopic ISM1 (Fig. 8). Although ISM1 may have other unidentified functions that contribute to these phenotypes, such as signaling pathways determining the right-side identity, our results do not support this possibility because of two lines of evidence. ISM1 has no effect on BMP signaling (Fig. 2 C), and ectopic ISM1 does not cause obvious changes in the expression of right-side marker SNAI1 (Fig. 8, K and L).

ISM1 involvement in LR asymmetric organ morphogenesis was initially proposed because of its asymmetric expression in the anterior mesendoderm of E7.5 mouse embryos and the foregut endoderm of HH10 chicks (Osório et al., 2014). Data from the current study provide the first evidence of the involvement of ISM1 in the negative regulation of NODAL signaling during early embryogenesis. As ISM1 is expressed in several adult tissues, it is expected that ISM1 has additional roles in development yet to be explored. Taken together, our results reveal a novel regulatory paradigm for NODAL signaling, providing evidence of the complexity and plasticity in the fine-tuning of NODAL signaling by the combinatorial effects of secretory antagonists. Our study sheds light on the molecular mechanism underlying NODAL signaling-mediated developmental processes as well as tumorigenesis.

**Materials and methods**

**Cell culture and CM preparation**

HEK293T and HeLa cells were maintained in DMEM (12100-046; Gibco) containing 10% FBS under 37°C and humidified 5% CO₂.
conditions. P19C6 cells were maintained in DMEM/F12 medium with 10% FBS. HEK293T-RIPTO cells are HEK293T cells stably expressing CRIPTO. FLAG-RIPTO expression plasmid was transfected into HEK293T cells using Lipofectamine 2000 (1668-500; Invitrogen) according to the manufacturer’s protocol. After selection of expressing clones, HEK293T-RIPTO cells were maintained in DMEM containing 10% FBS and 750 μg/ml of Geneticin (10131-027; Gibco).

For CM preparation, the plasmids encoding the proteins of interest were transiently transfected into HEK293T cells. After transfection, cells were washed with FBS and the medium was changed to serum-free DMEM. CM samples were collected after 16 h and centrifuged at 4,000 rpm at 4°C before further usage/analysis.

**Growth factor stimulations**

HEK293T-RIPTO or HEK293T cells were grown to 80–90% and serum starved for 12 h before stimulation with 40 ng/ml TGF-β1 (100-B; R&D Systems), 100 ng/ml NODAL (1315-ND/CF; R&D Systems), 100 ng/ml GDF1 (6937-GD/CF; R&D Systems), 40 ng/ml ACTIVIN-A (338-AC; R&D Systems), or 50 ng/ml BMP4 (314-BP; R&D Systems) in the presence of CM as indicated. Mouse rISM1 (577502; BioLegend) was used at 100 or 200 ng/ml. CM were preincubated at 37°C for 30 min with gentle shaking before being added to the cells. For P19C6 cells, cells were grown to 60–80% and serum starved for 18 h followed by stimulation for 1 h with 100 ng/ml NODAL in the presence of prewarmed CM as indicated.

**Plasmid vectors**

Mouse ISM1 cdNA plasmids were prepared as described previously (Osório et al., 2014). Epitope-tagged and chimeric constructs were generated by PCR-based strategy, and domain deletions and amino acid substitution by site-directed mutagenesis. Positive clones were confirmed by restriction enzyme and sequencing analyses. In FLAG-NODAL, the FLAG epitope was introduced 4 amino acids downstream of the proteolytic cleavage site of the NODAL proprotein. ACVR1B-HA was generated by fusion of the HA epitope to the C terminus of the mouse ACVR1B cDNA sequence. ACVR1B-HA contains the cDNA encoding amino acids 1–126 (ECD) of ACVR1B fused to the HA epitope. Chimeric ACVR1B(ECD)-Fc receptor was generated by cloning the cdNA encoding amino acids 1–126 of ACVR1B fused in-frame with the human IgG Fc fragment derived from pRKS-mF2SCRD-IgG (Semenov et al., 2001; 16689 Plasmid; Addgene; from Dr. Xi He, Harvard Medical School, Boston, MA). ACVR2B(ECD)-MYC was obtained by fusion of the cDNA corresponding to amino acids 1–134 (ECD) of mouse ACVR2B to the MYC epitope. MYC-ACVR2A and CER1-FLAG plasmids were purchased from Sino Biological (MGS0613-NM and MGS1613-CF, respectively). HIS-sRIPTO was obtained by amplification of CRIPTO cDNA corresponding to amino acids 1–150 (lacking the GPl motif) and tagged with HIS epitope. A list of the primers used is available in Table S1. Plasmid pcDNA3-FLAG-LEFTY1 was a kind gift from Dr. Michael Shen (Robert Wood Johnson Medical School, New Brunswick, NJ; Chen and Shen, 2004). pFLAG-CMV1-ISM1 mammalian-expressing plasmid was subcloned using HindIII/EcoRI from pcDNA3-ISM1 plasmid into pFLAG-CMV1 vector (E7273; Sigma-Aldrich).

**N-glycosylation modification assay**

WCL and CM were obtained from HEK293T cells transiently transfected with mock, ISM1, N39Q, N282Q, N39Q/N282Q, ΔTSRI, or ΔAMOP. According to the manufacturer’s instructions, the samples were treated with PNGase F (P0704S; New England Biolabs) or Endo H (P0702S; New England Biolabs) and analyzed by Western blotting. For tunicamycin treatment, transfected cells were treated with 1 ng/ml tunicamycin (654380; Calbiochem) for 8 h before harvesting.

**Protein extraction and Western blotting**

Cells were lysed in radioimmunoprecipitation assay buffer containing a protease inhibitor cocktail (1183614501; Roche) and sodium orthovanadate. The protein concentration was determined using DC Protein Assay (500-0111; Bio-Rad), and 15–30 μg of total protein lysates were used. Samples were resolved by SDS-PAGE under reducing conditions and blotted onto a polyvinylidene fluoride membrane (03010040001; Roche). Membranes were probed with specific primary antibodies followed by peroxidase-conjugated secondary antibodies. The bands were detected using SuperSignal West Pico Chemiluminescent Substrate (1859674; Thermo Fisher Scientific) according to the manufacturer’s protocol. Densitometry analyses were performed using the ImageJ program (http://imagej.nih.gov/ij/), and the quantification results were normalized to the loading control. The following primary antibodies were used: mouse anti-β-actin (1:5,000, A5316; Sigma-Aldrich), mouse anti-FLAG (1:2,000, F1804; Sigma-Aldrich), mouse anti-HA (1:1,000, MMS-101P; Covance), rabbit anti-HIS (1:1,000, sc-803; Santa Cruz Biotechnology), goat anti-IgG (Fc; 1:2,000, 109–035–008; Jackson ImmunoResearch), rabbit anti-ISM1 (1:5,000, Genescript; Osório et al., 2014), mouse anti-MYC (1:1,000, sc-40; Santa Cruz Biotechnology), mouse anti-SMAD2/3 (1:2,000, 610845; BD Transduction Laboratories), rabbit anti-pSMAD2 (1:1,000, 3101; Cell Signaling), and mouse anti-GST (1:3,000, sc-138; Santa Cruz Biotechnology). The secondary antibodies used were HRP-conjugated rabbit anti-goat IgG (1:3,000, 81-1620; Invitrogen), HRP-conjugated rabbit anti-mouse IgG (1:3,000, 64-6420; Invitrogen), and HRP-conjugated goat anti-rabbit IgG (1:3,000, 65-6120; Invitrogen).

**Dual luciferase reporter assay**

HEK293T-RIPTO cells were cotransfected with plasmids encoding A3-lux firefly reporter, FLAG-FOXH1, and RS-SV40 renilla reporter and serum starved for 12 h before stimulation with 100 ng/ml NODAL, 100 ng/ml GDF1, 40 ng/ml ACTIVIN-A, and 40 ng/ml TGF-β1 for 18–24 h. The CM were preincubated at 37°C for 30 min with gentle shaking before being added to the cells. Dual luciferase reporter assay was performed following the manufacturer’s instructions (E1960; Promega). A3-lux firefly reporter, FLAG-FOXH1, and RS-SV40 renilla reporter plasmids were a kind gift from Dr. Michael Shen (Chen and Shen, 2004). A3-lux contains three copies of the ACTIVIN-NODAL-TGF-β responsive element of the well-known SMAD2 target Mix.2 promoter.
CM were gently placed on top of the embryo. For the beads previously described (Chapman et al., 2001). Briefly, the embryos were washed three times in Pannett starved for 12 h. CM were preincubated at 37°C for 30 min with gentle shaking before being added to the cells as indicated. The cells were fixed in 4% PFA for 10 min and then processed for PLA using Duolink II Red Starter Kit (92101; OLink) according to the manufacturer’s instructions. Cells were counterstained with DAPI (D1306; Molecular Probes), mounted with SlowFade Gold antifade reagent (S36936; Molecular Probes), and photographed using an Olympus BX51 microscope equipped with a Spot RT3 charge-coupled device camera. Images and figures were processed with Adobe Photoshop CS5. For each experimental group, the number of PLA signals was counted in a total of 200 cells.

Chick embryos and ex ovo culture
Fertilized chick eggs were obtained from Jinan Poultry Co. (Tin Hang Technology) and incubated in a humidified atmosphere. Embryos were staged according to the table in Hamburger and Hamilton (1951). For the experiments using CM, whole embryos at HH4–5 were cultured ex ovo using the easy culture method as previously described (Chapman et al., 2001). Briefly, the embryos were cultured ventral-side up on top of a 20-µl drop of CM placed on the semisolid agar-albumin substrate. Another 20 µl of CM were gently placed on top of the embryo. For the beads experiment, embryos at HH4 or HH6 were cultured using the new culture method as reported (Alev et al., 2013). 2 µl of PBS-washed heparin-acrylic beads (HS5262; Sigma-Aldrich) were soaked in 2.5 µl of rNODAL mixed with either 2.5 µl PBS or 2.5 µl rSM1 (577502; BioLegend) for ~3 h on ice. Before implantation, the beads were washed three times in Pannett–Compton solution. For both experiments, the embryos were incubated until they reached stages HH7–9 or HH10–12 and processed for whole-mount in situ hybridization and/or qPCR analyses. Embryos exhibiting gross morphological defects were equally excluded from control and experimental groups and not considered in further analysis.

Whole-mount in situ hybridization
RNA probes for in situ hybridization were prepared by in vitro transcription as previously described (Osório et al., 2014). Briefly, plasmids were linearized, purified by phenol-chloroform, and used as template for the transcription reaction containing digoxigenin (DIG) RNA Labeling Mix (II27073910; Roche). RQ1 DNase I (M6101; Promega) was used to remove the excess of template DNA. DIG-labeled RNA was purified by ProbeQuant G-50 Micro Columns (28-9034-08; GE Healthcare). Whole-mount in situ hybridization in young embryos was performed as described (Streit and Stern, 2001). In short, the fixed embryos were treated with methanol and proteinase K and postfixed. The embryos were then prehybridized for 3 h before incubation with DIG-labeled probes overnight at 68°C. After extensive washing, the embryos were blocked in 5% goat serum and 1 mg/ml BSA and then incubated with sheep anti-DIG-AP antibody (11093274910; Roche) overnight at 4°C. AP activity was detected using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate substrate. Once color developed, the embryos were washed and fixed. Whole-mount embryos were photographed on a Leica M210F stereomicroscope coupled with a Leica DFC310FX camera. Images and figures were processed with Adobe Photoshop CS5. For histological analyses, the fixed embryos were incubated in 15% sucrose overnight at 4°C, embedded in gelatin, frozen, and serially sectioned on a Leica CM3050S cryostat. Sections were photographed using an Olympus BX51 microscope equipped with a Spot RT3 CCD camera. Images and figures were processed with Adobe Photoshop CS5.

RNA extraction and qPCR analyses
Embryos cultured until HH7–9 were dissected along the midline, and the extracellular embryonic tissues were removed. 10–12 embryos were used per group. The left half of the embryos were mixed together and processed for total RNA extraction using Trizol (15596-026; Invitrogen) according to the manufacturer’s instructions. RQ1 DNase I was used to eliminate genomic DNA contamination from the RNA samples before synthesis of first-strand cDNA. Briefly, 2 µg of total RNA was reverse transcribed using Oligo(dT)15 primer (C1101; Promega) and Moloney murine leukemia virus reverse transcription (M170; Promega). Real-time PCR was performed on a StepOne PCR machine (Applied Biosystems) using Power SYBR Green PCR Master Mix (4368577; Applied Biosystems). All absolute data were first normalized to 18S RNA and then normalized to the control sample treated with mock CM. The relative transcriptional levels of the genes were determined by the ΔΔCt method. Two biological experiments were included, and for each, two independent PCR reactions with three replicates each were performed. The sequences of the primers used are listed in Table S2.

Statistical analysis
For all experiments, no statistical method was used to predefine the size sample, and investigators were not blinded to allocation during experiments and outcome assessment. Statistical analysis was performed using Prism 6 software (GraphPad). The data are represented as mean ± SEM, and unpaired, two-tailed Student’s t test was used to compare two groups of individual samples. The data analyzed meet normal distribution, and an F test showed that variances were not significantly different between groups. Statistically significant P values are indicated.
ISM1 antagonizes NODAL signaling


Ośório et al. ISM1 antagonizes NODAL signaling

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Figure S1. ISM1 directly interacts with NODAL and ACVR1B. (A) Purified GST-tagged ACVR1B and NODAL were used in a GST pull-down assay to pull down ISM1. GST was used as negative control. (B and C) Analyses of interaction affinity between ISM1 and ACVR1B or Nodal by SPR using Biacore X100. Purified ISM1 protein was covalently immobilized to a Biacore CM5 sensor chip. ACVR1B or NODAL proteins with increasing concentrations (6.25–100 nM) were applied for binding assays. The corresponding plots of steady-state binding data from the end of the association phases against the analyte’s concentrations were used to calculate the steady-state affinities ($K_D$). RU, shown in the y-axis, represents resonance units. Purified NODAL, ACVR1B, and ISM proteins were purchased from Origene (NODAL-TP761839; ACVR1B-TP761428; and Ism1-TP723759).
Figure S2. **ISM1 interacts with type II receptor ACVR2A.** ISM1 and Myc-tagged ACVR2A were transiently expressed in HEK293T-Cripto cells. WCLs were immunoprecipitated (IP) with anti-Myc antibodies or IgG using agarose beads. Western blotting was performed to detect the presence or absence of ISM1 in the precipitant.

Figure S3. **ISM1 does not significantly affect the interaction between NODAL and ACVR2A.** (A and B) Two repeats of competitive binding assays: FLAG-NODAL and MYC-ACRV2A were ectopically expressed in HEK293T-CRIPTO cells transfected with increased dosage of ISM1-expressing plasmid. 2–3 d after transfection, cell lysates were immunoprecipitated (IP) with anti-MYC antibodies. The potential influence of ISM1 on the interaction between ACVR2A and NODAL was examined by Western blotting using antibodies against FLAG.
### Table S1. Primer sequences used for generation of the different constructs for mammalian expression

<table>
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<th>Plasmid name</th>
<th>Primer</th>
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### Table S2. Primer sequences used in qPCR

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