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THE XENOPUS LEFTY (XLEFTY) PRODOMAIN NEGATIVELY REGULATES XLEFTY ACTIVITY AND IS NECESSARY FOR PROPER XLEFTY SECRETION

by

ADRIAN AMELIO VASQUEZ

THESIS

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

Master of Science

2013

BIOLOGICAL SCIENCES

Approved By:

_____

Advisor Date

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DEDICATION

This thesis is dedicated to my wife whose fortitude and constancy were determinant in my completion of this work. May God continue to perfect His work in us.

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ACKNOWLEDGMENTS

I would like to express my gratitude to Dr. William Branford in whose lab I completed this work. His unrelenting critique of my research and writing was part of my process to become more adroit in the sciences. I would also like to extend that gratitude to my committee members Dr. Markus Friedrich, Dr. Karen Beningo and Dr. Mark Russell who provided guidance and counsel throughout my academic training at Wayne State University. Lastly if it wasn't for my colleagues in the lab and the Department of Biological Sciences the journey would not have been the same. I also extend a sincere appreciation for Wayne State University and the City of Detroit where I lived and met many great friends from all over the nation and globe.

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LIST OF SCHEMES

BMP - Bone Morphogenetic Protein

BP - Blastopore

CCW - Counterclockwise

CMFM – Calcium-magnesium free medium

ct - Conotruncus

CW - Clockwise

DCM - Double cleavage mutant with both sites mutated

GAPDH – Glyceraldehyde-3-phosphate dehydrogenase

HA – Hemagglutinin epitope

LCM1 - Lefty cleavage mutant with site 1 mutated

LCM2 - Lefty cleavage mutant with site 2 mutated

LO - Left origin

Mat-Xlefty – Mature Xlefty

MEF - Mouse embryonic fibroblast

MO - Morpholinos

MYC - C-Myc epitope

ng - Nanogram

OE – Over-expression

PD – Prodomain

PDL – Xlefty without the PD region

PDmut-HA - Prodomain containing mutations (leucines 34, 35, 38, and 40 converted to alanines) and the HA epitope

PDmutXlefty - PDmut in the context of full-length Xlefty

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pg - Picogram

RO - Right origin

SMADS - Homologues of *Drosophila* Mothers against decapentaplegic and *C. elegans* SMA.

TGF-β – Transforming growth factor beta

Xlefty – *Xenopus* Lefty

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CHAPTER 1

THE LEFTY PRODOMAIN REGULATES LEFTY FUNCTION

Introduction

Embryogenesis is dependent on signaling ligands that instruct cells to migrate and differentiate into their respective fates. This process gives the embryo the capacity to give rise to a complete organism from a small pool of resources. How can an organism accomplish this process? An embryo is able to amplify the effects of a small number of signaling pathways. One such cell-signaling pathway that plays a prominent role in early vertebrate development is the Nodal-signaling pathway.

Nodal: Member of the TGFβ Superfamily

Nodal is a member of a large family of signaling molecules called the Transforming Growth Factor Beta (TGFβ) superfamily of secreted ligands (Schier, 2003). The TGFβ superfamily members function by sending extracellular signals to the cell nucleus via a cascade of intracellular components that then regulate the transcription of target genes. This pathway requires two receptors for signaling: a type I receptor and a type II receptor, both serine/threonine kinases. The type II receptors are constitutively active and phosphorylate and activate type I receptors when brought into close proximity by ligand interaction (Hill, 2001). Signals from the receptors are transduced to the nucleus via the phosphorylation of intracellular signaling proteins called smads (Hill, 2001). Once activated, the smads form complexes and accumulate in the nucleus where they are directly responsible for transcriptional activation [see figure 1] (Hill, 2001).

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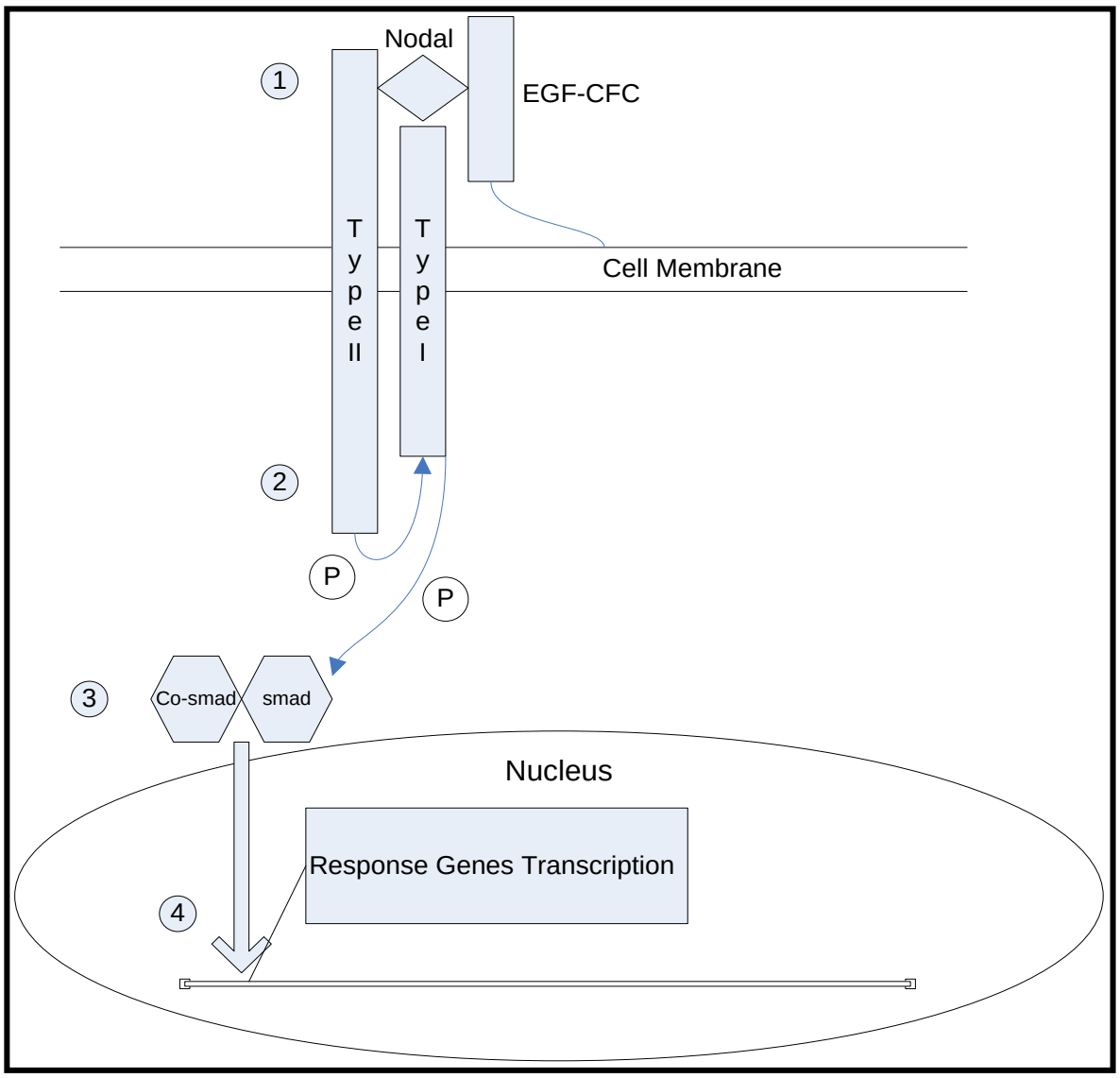

Figure 1. The Nodal-signaling pathway.
1. The secreted ligand Nodal interacts with its obligate co-receptor EGF-CFC and binds to the Type II constitutive serine – threonine transmembrane receptor. 2. Type II then phosphorylates the type I receptor thereby activating it. 3. This receptor in turn phosphorylates intracellular smad which complexes with a co-smad. 4. This smad complex is then translocated inside the nucleus to promote response genes' transcription.

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Lefty Negatively Regulates Nodal

Because Nodal signaling shares pathway components with other members of the TGFβ superfamily, there needs to be a mechanism that regulates Nodal signaling. Lefty, an atypical member of the TGFβ superfamily has been identified to function as the principal antagonist of the Nodal-signaling pathway. Studies have shown that Lefty is responsible for regulating the Nodal-signaling pathway by two possible mechanisms. The first is by interacting with Nodal itself and the second is by interacting with the EGF-CFC obligate Nodal co-receptor [see fig 2] (Chen and Shen, 2004).

The Effects of Lefty and Nodal Dysfunctional Signaling in *Xenopus laevis*

Previous work in *Xenopus* has shown that without Lefty function, Nodal is left unregulated and this leads to severe embryo perturbations known as exogastrulation (Branford and Yost, 2002; Cha et al., 2006). An exogastrulated embryo is observed as developing "inside-out". The tissue that normally lies within the embryo and under its skin extends outwards while the pigmented epidermis that normally covers the exterior of the embryo remains crumpled-up at the other extreme end of the developing embryo. These phenotypes are a result of excess endoderm and mesoderm formation as shown by phenotypic and *in situ* hybridization studies (Branford and Yost, 2002). A rescue of this phenotype was obtained by co-injecting Xlefty DNA with the anti-Xlefty morpholino (Branford and Yost, 2002). Based on these observations Lefty's function as the principal Nodal antagonist is very important since dysregulated Lefty signaling leads to an embryo that is incapable of undergoing proper development.

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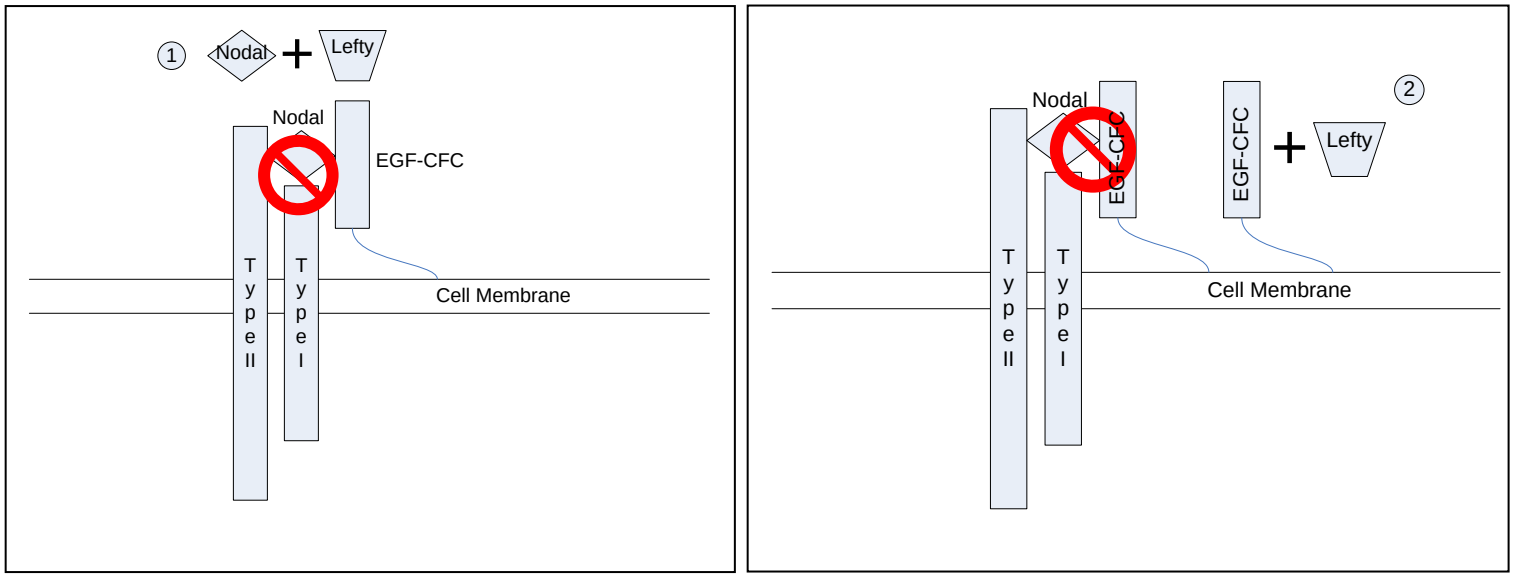

Figure 2. Two modes by which Lefty blocks the Nodal-signaling cascade.
1. Lefty antagonizes Nodal by directly interacting with it and thus preventing Nodal from signaling. 2. Lefty interacts with the EGF-CFC Nodal obligate co-receptor, which results in Nodal's inability to signal.

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TGFβ regulation by proteolysis

Post-translational modification is widespread for members of the TGFβ superfamily and presents a method of regulation (Freeman and Gurdon, 2002). Some members of this signaling family are secreted as inactive pro-proteins which are then processed by different species of proteases to release regulation so they can then function (Freeman and Gurdon, 2002). There is currently evidence for cleavage both prior and after secretion. For example, Nodal and Lefty are thought to be secreted as inactive pro-proteins and become active in the presence of proteases from the SPC superfamily (Ben-Haim et al., 2006; Constam and Robertson, 2000a; Sakuma et al., 2002; Westmoreland et al., 2007). Here we isolated the prodomain from the mature Xlefty so that we can elucidate the mechanisms that govern Lefty regulation by the prodomain.

The *Xenopus* Lefty (Xlefty) Prodomain

The Xlefty prodomain was isolated and tested in microinjection studies to elucidate the mechanisms that govern Lefty regulation by the prodomain. The Xlefty protein contains two consensus R-X-X-R cleavage sites that are acted upon by the calcium-dependent serine endoproteases of the subtilisin-like proprotein convertase family (SPCs) [see figure 3 and 4] (Westmoreland et al., 2007). These SPC sites are found in many intercellular signaling molecule pro-proteins, an example being Nodal. Cleavage at these sites releases the prodomain (PD) region from the mature Xlefty (Mat-Xlefty) protein (Fig. 4) (Sakuma et al., 2002; Westmoreland et al., 2007). Removal of the Lefty prodomain has been shown to be a prerequisite for normal functionality (Sakuma et al., 2002; Westmoreland et al., 2007). When an Xlefty cleavage mutant is made by mutating the two potential cleavage sites into non-cleavable sites, this mutated Xlefty derivative is unable to block Nodal in a frog animal cap assay (Sakuma et al., 2002;

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Westmoreland et al., 2007). For Lefty to function, cleavage of its prodomain is required yet there has been no study into what mechanism is used by the prodomain to regulate Lefty activity. The prodomains of other TGFβs have been shown to play roles in protein folding and stability, sequestration of inactive protein and regulation of signaling (Constam and Robertson, 1999, 2000a, b). The work presented here seeks to elucidate roles that the Lefty prodomain plays in regulating Lefty activity. Our studies into the Lefty prodomain considered recent evidence of TGFβ architecture where crystal structures revealed a novel fold for the prodomain that showed how the prodomain shields the growth factor from recognition by receptors and alters its conformation (Shi et al., 2011). This "strait-jacket" like conformation suggested that the domain at the C-terminus folds with or subsequent to the N-terminal prodomain (Shi et al., 2011). Additionally, since the Lefty protein is cleaved at the first cleavage site this would allow release of the straight-jacket and would be sufficient to enable access to growth factor domains (Shi et al., 2011). Our hypothesis has been that the Lefty prodomain is able to interact with the mature Lefty domain and thus folds the molecule into an inactive state.

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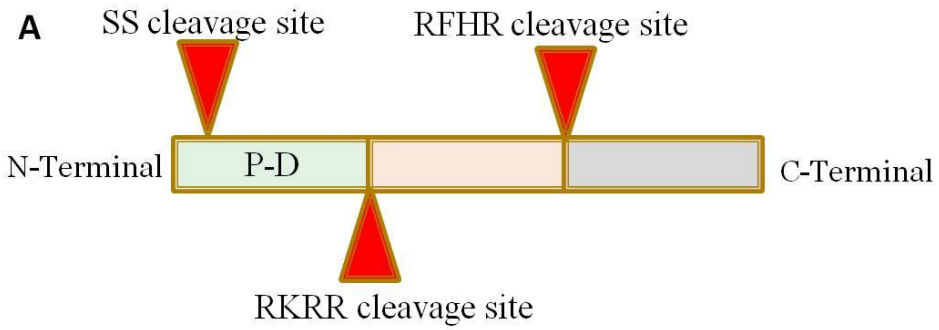

Figure 3. The Xlefty molecule.
Graphic depicting the approximate location of the predicted signal sequence (SS) site before the prodomain (PD), the consensus RKRR cleavage site and the second consensus RFHR cleavage site. PD stands for the prodomain which was studied in this work to determine its function in Lefty activity.

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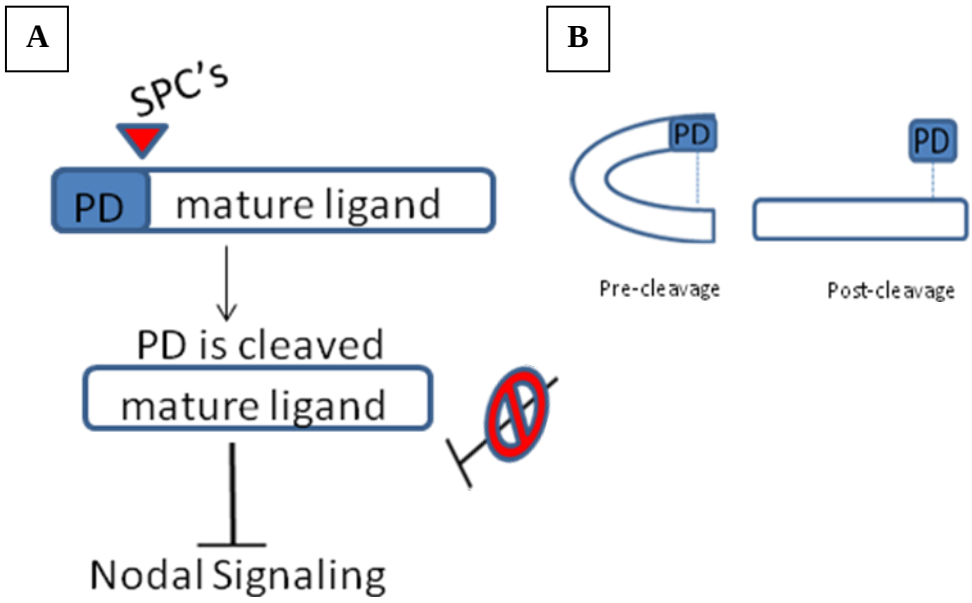

Figure 4. Xlefty proteolytic processing.
In "A" the schematic represents what is known about Xlefty processing and function. Briefly, the Xlefty prodomain is cleaved by subtilisin-like proprotein convertases (SPCs) which allow the mature ligand to function by blocking Nodal signaling either by interacting with Nodal itself or its obligate co-receptor Cripto. We hypothesize in "B" that the PD is most likely preventing Xlefty activity by interacting with the mature Xlefty ligand in either a pre-cleavage complex or a post-cleavage complex. There is also a possibility that it may interact with both a pre- and post-cleavage Xlefty.

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Insights into the Xlefty prodomain function

In this study, we utilized multiple Xlefty prodomain-related mutants to elucidate Lefty-prodomain regulatory mechanisms. Here we show that the Xlefty prodomain when over-expressed in *Xenopus* gastrula stage embryos results in exogastrulation. This phenotype has been shown by our lab to occur when Xlefty function is disrupted (Branford and Yost, 2002). This result led us to postulate that like other members of the TGFβ superfamily the Lefty prodomain has the capacity to interact molecularly with the mature portion of the growth factor and inhibits its function. This was then confirmed by co-immunoprecipitation (co-IP) and rescue studies. The prodomain is able to co-IP full length mature Xlefty (Mat-Xlefty) but a prodomain with mutated amino acids is unable to co-IP full length Mat-Xlefty. These results allowed us to propose a model in which the prodomain intramolecularly interacts with mature Lefty in order to regulate Lefty activity. Secretion studies implementing both an embryo and cell culture assay system were done on Xlefty and its mutated derivatives, including a prodomain-less Lefty (PDL), Lefty cleavage mutants (LCM), and a prodomain mutated Lefty (PDmut) and its derivatives. The collective results of our secretion studies led us to report on another function that the prodomain plays in Lefty regulation. It may regulate Lefty function by playing a role in proper Lefty secretion. The mutated Xlefty clones were not present in the secreted medium compared to wild-type Xlefty, which had secreted Mat-Xlefty in the secreted medium. This led us to hypothesize that the prodomain facilitates the secretion machinery to secrete the Lefty mature growth factor properly. Taken together these results show for the first time regulatory mechanisms of Lefty function by its prodomain.

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Results

PD microinjection causes left-right abnormalities at lower concentrations and exogastrulation at higher amounts.

Studies have shown that without the cleavage of the prodomain Lefty is unable to perform its function normally (Beck and Slack, 2001; Sakuma et al., 2002; Westmoreland et al., 2007). Our hypothesis was that the Lefty PD negatively regulates Lefty by molecularly interacting with it. Thus we predicted that over-expression of the PD would antagonize Lefty functionality. The PD corresponds to the 77 amino acid N-terminal sequence and the first 24 amino acids of the PD are predicted to be the signal sequence that is presumably cleaved from Xlefty in the endoplasmic reticulum. The remaining PD is cleaved from Xlefty by SPC proteases to release mature Xlefty. We tested the ability of PD to antagonize the normal functionality of Xlefty by microinjecting increasing amounts of PD into the cells of a 4 cell stage embryo. First, a concentration of 500 pg of PD mRNA was microinjected into each blastomere of a 4-cell stage embryo. This resulted in embryos having left-right defects, which are observed in an Xlefty mis-expression context (Fig.5) (Branford et al., 2000). An adapted scoring format previously designed by our lab was used to assess the presence of reversed hearts and the presence of reversed guts or guts having heterotaxia (Branford et al., 2000). At stage 45 and onwards the left-right asymmetry was assessed by observing the looping of the ventricle and the conotruncus (ct) in the three-chambered heart of the embryo (Branford et al., 2000). In normal embryos, the ct loops to the left whilst the ventricle is positioned to the right with the reverse orientation seen in reversed hearts. Coiling of the primitive gut commences at stage 43 and fully coils by stage 45 (P.D. Nieuwkoop, 1967). At stage 46, the orientation of the gut was assessed by its morphology and by the following scoring

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system. The system used to score left-right morphogenesis of the gut looks at two features defined as “coil origin” and “coil direction”. In normal embryos, the gut coil origin enters the abdominal cavity on the right side of the body and is referred to as the right origin (RO) (Branford et al., 2000). The coil direction refers to whether the coils spiral clockwise or counterclockwise when viewed ventrally (Branford et al., 2000). In normal embryos, the gut coils in a counterclockwise (CCW) manner. In this scoring system, a normal embryo has a normal heart and a gut with right origin (RO) and counterclockwise coiling (CCW) {RO-CCW}. A completely reversed embryo would have a reversed heart and a gut with left origin (LO) and clockwise coiling (CW). Other embryos were seen to express heterotaxic guts. Organs expressing heterotaxia have abnormal positioning within the organism that are not seen in normal *situs* without having total *situs inversus* (Branford et al., 2000). In embryos with heterotaxia the gut origin and gut coiling were not considered a full reversal and may be of RO-CCW or LO-CCW orientation (Branford et al., 2000).

Forty-six percent of the embryos injected with the PD had reversed hearts (Fig.6a). Thirty-one percent had gut heterotaxia, while 12% had full gut reversals (Fig.6b). Lefty is the principal antagonist of Nodal, therefore our results are consistent with the idea that the prodomain negatively regulates Lefty since randomization of left-right asymmetry was seen when Xlefty function was blocked (Branford et al., 2000). This led us to increase the concentration of PD being microinjected into the embryo to 1ng/cell at the 4-cell stage to see if we could elicit more phenotypes consistent with Xlefty over-expression. Surprisingly, we observed a spectrum of phenotypes at this concentration (Fig. 7). This spectrum included 100% dorsal closure, less than 50% dorsal closure, more than 50% dorsal closure and exogastrulation. The results showed that 60% (n=97) of PD-

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microinjected embryos resulted in exogastrulae, while 98% (n=98) of uninjected embryos were normal (Fig. 8). Our lab previously demonstrated that when the function of Xlefty is blocked using an anti-Xlefty morpholino, this resulted in exogastrulation (Branford and Yost, 2002). An exogastrula is an embryo that has developed inside out. This phenotype has also been observed when Nodal, Xlefty's primary target, is over-expressed (data not shown). The gradual loss of dorsal closure observed in the embryos and the presence of exogastrula led us to believe that proper mesendoderm differentiation and movement was inhibited (Branford and Yost, 2002). Taken together these observations were comparable to what we would expect in an anti-Xlefty context where Nodal activity occurs unregulated (Fig. 5).

The PD, when co-injected with Xlefty was able to rescue an Xlefty over-expression phenotype

Rescue experiments by co-injecting 1ng/cell of PD mRNA with 25pg/cell Xlefty mRNA at the 4-cell stage determined that the PD was able to rescue Xlefty over-expression (Fig. 9). Exogastrulation was absent in the co-injected embryos (0%, n=57), as well as a loss of ventral wrinkling (95%, n=57) [Fig. 10 and 11]. The result of this experiment demonstrates that the PD can rescue an Xlefty over-expression phenotype and led us to hypothesize that the PD regulates Mat-Xlefty function, possibly by interacting with it.

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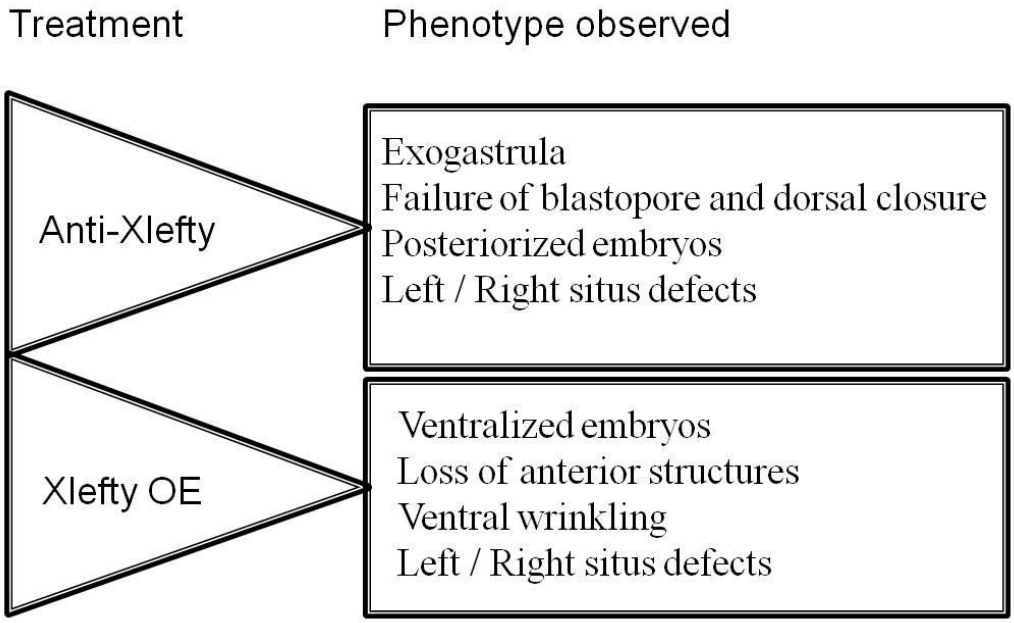

Figure 5: Schematic of phenotypes observed in an anti-Xlefty and an Xlefty context.
The published data, summarized in the schematic, provides a baseline with which subsequent phenotypes can be compared.

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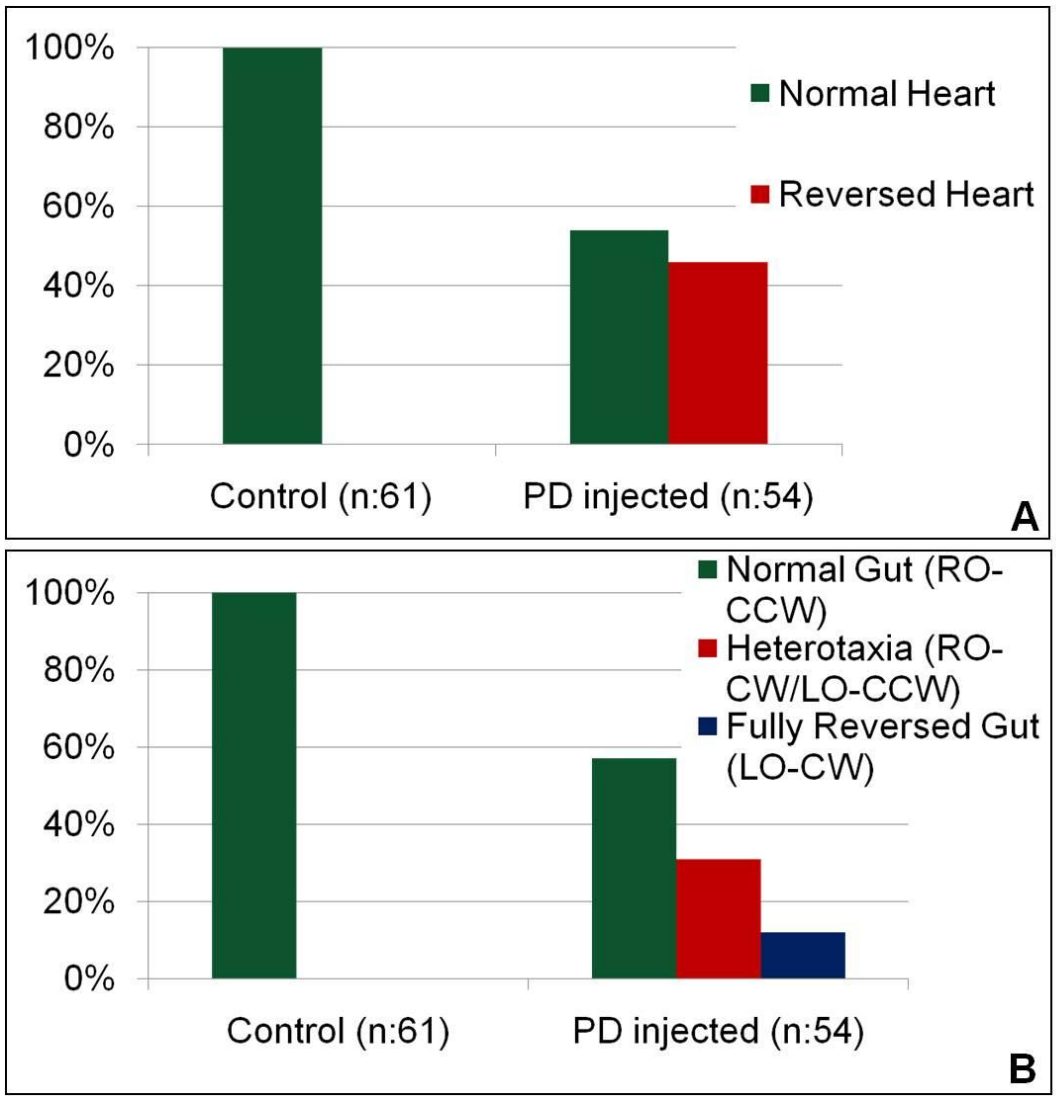

Figure 6. PD expression results in heart and gut reversals.
500 pg of PD mRNA was microinjected into each cell of a 4-cell stage embryo. Hearts and guts were scored at stage 46 for reversals. (A) 46% of the embryos injected had reversed hearts. (B) 31% had heterotaxia and 12% had full reversals in gut development. Controls were uninjected.

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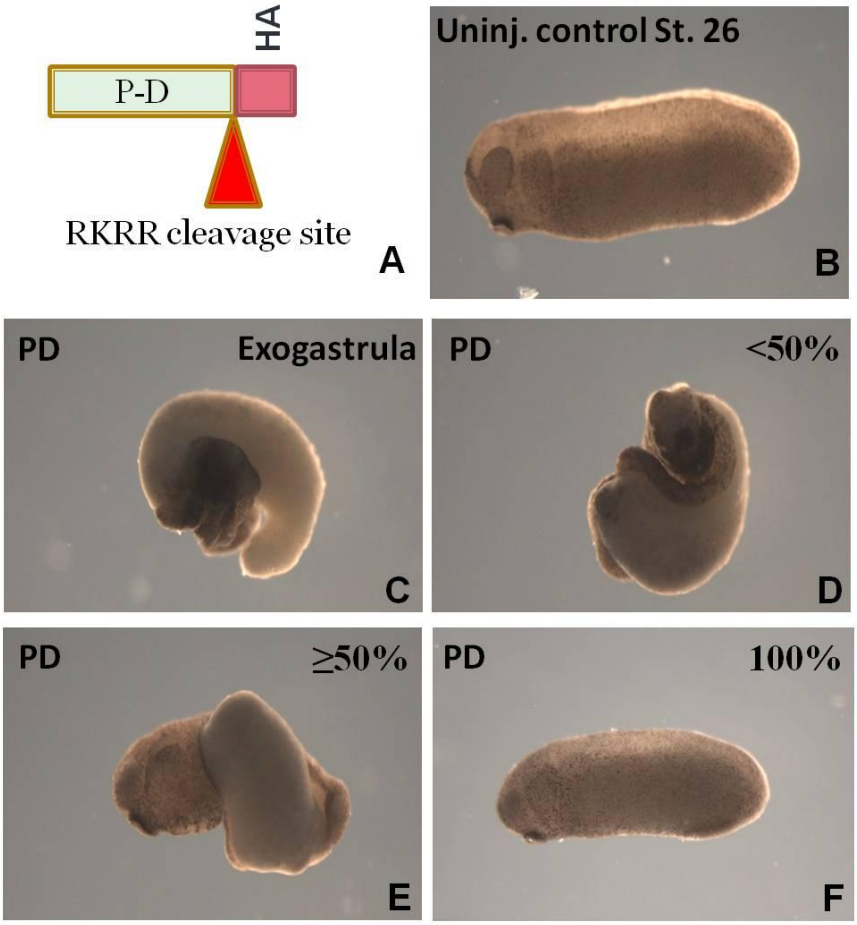

Figure 7. The PD causes exogastrulation.
(A) Schematic of the PD construct. The PD construct contains only the PD region. The molecule is tagged at the C-terminus with an HA tag. (B) Uninjected control embryo depicting normal phenotype. (C-F) PD microinjected embryos depicting exogastrulation (C), less than 50% dorsal closure (D), 50% or more dorsal closure (E) or full dorsal closure (F). (B, E and F) Anterior to left, lateral view. (C, D) Lateral views. 20X.

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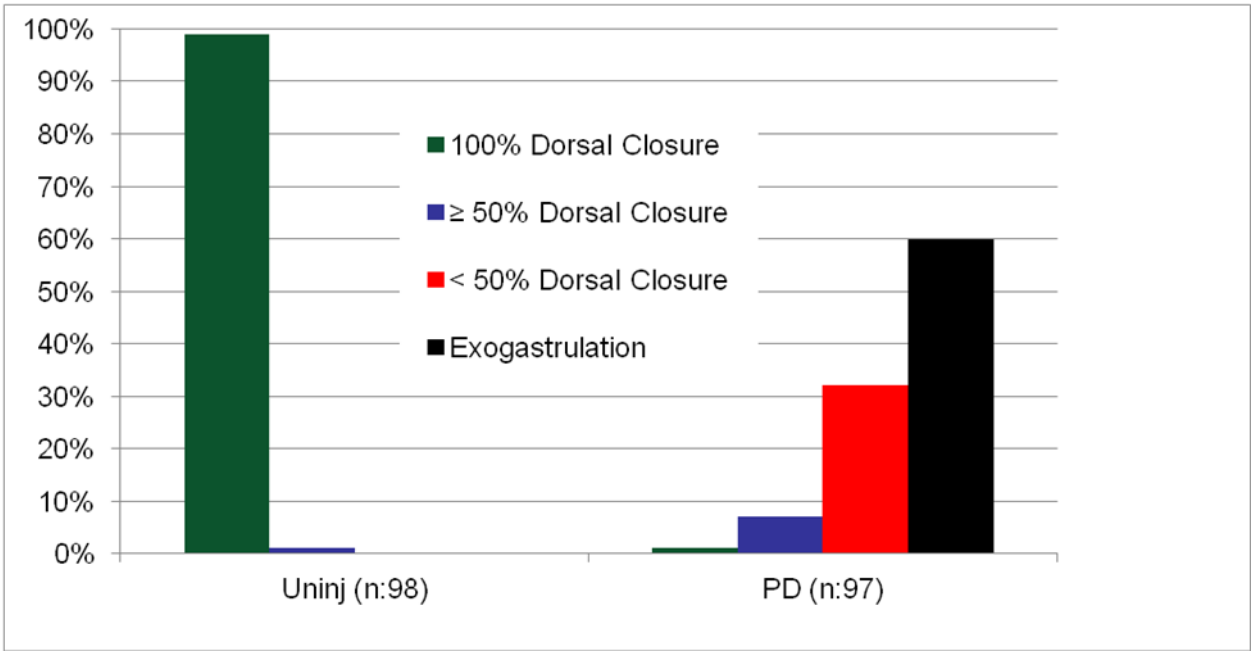

Figure 8. The PD causes a spectrum of dorsal closure loss.
Uninjected embryos did not exhibit any exogastrulation (n=98). PD microinjected embryos had 60% exogastrulation (n=97).

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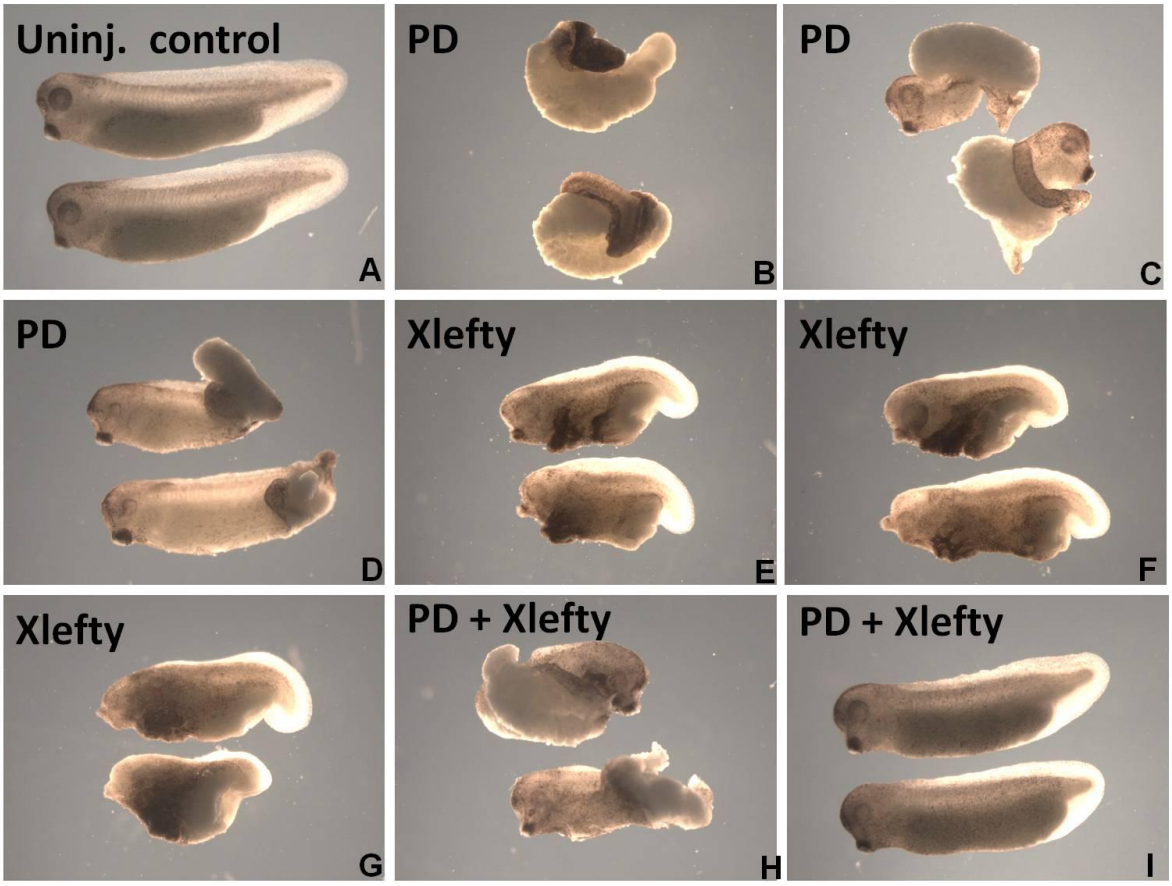

Figure 9. The PD is able to antagonize an Xlefty overexpression phenotype.
Spectrum of phenotypes seen when PD and Xlefty are overexpressed and co-expressed in the embryo. Xlefty PD mRNA was microinjected at 1ng/cell in each dorsal cell of a 4-cell stage embryo and Xlefty mRNA was injected at 25pg/cell in each dorsal cell of a 4-cell stage embryo. Xlefty PD mRNA (1ng/cell) was co-injected with Xlefty mRNA (25pg/cell) in each cell of a 4 cell stage embryo. (A) Uninjected controls. (B-D) Spectrum of PD-injected embryo phenotypes. (B) Exogastrulae. (C) <50% dorsal closure. (D) ≥50% dorsal closure. (E-G) Spectrum of Xlefty-injected embryos. (E) Bilateral eyes, ventral wrinkling. (F) Single eye, ventral wrinkling. (G) No eyes, ventral wrinkling. (H, I) Spectrum of PD+Xlefty-injected embryos. (H) Partial rescues which have ≥50% dorsal closure and rescue of eyes. (I) Fully rescued embryos. (A-I) Lateral view. (A, D-G, I) Anterior to left. (B) anterior to right. (C) Upper embryo anterior to left and lower to right. (H) Upper embryo anterior to right and lower to left. Embryos were scored and photographed at stage 36. 16X.

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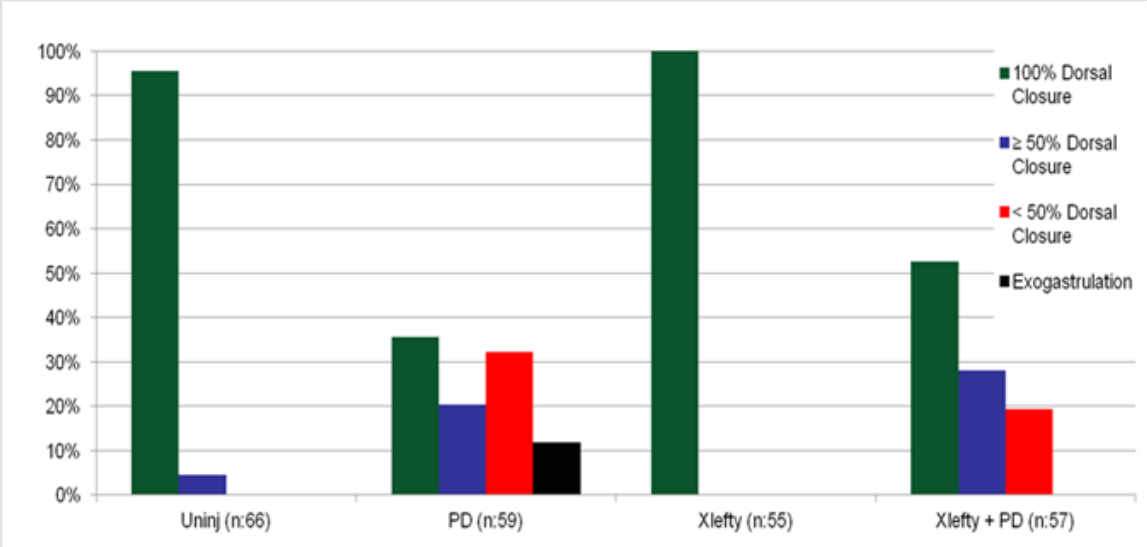

Figure 10. The PD co-injected with Xlefty rescues exogastrulation and dorsal closure.
Uninjected embryos did not exhibit any exogastrulation (n=66). PD-microinjected embryos had 12% exogastrulation and 52% failure of complete dorsal closure (n=59). Xlefty microinjected embryos had 0% exogastrulation or dorsal closure failure (n=55). PD+Xlefty microinjected embryos exhibited no exogastrulation and 52% of the embryos had 100% dorsal closure (n=57), thus demonstrating rescue.

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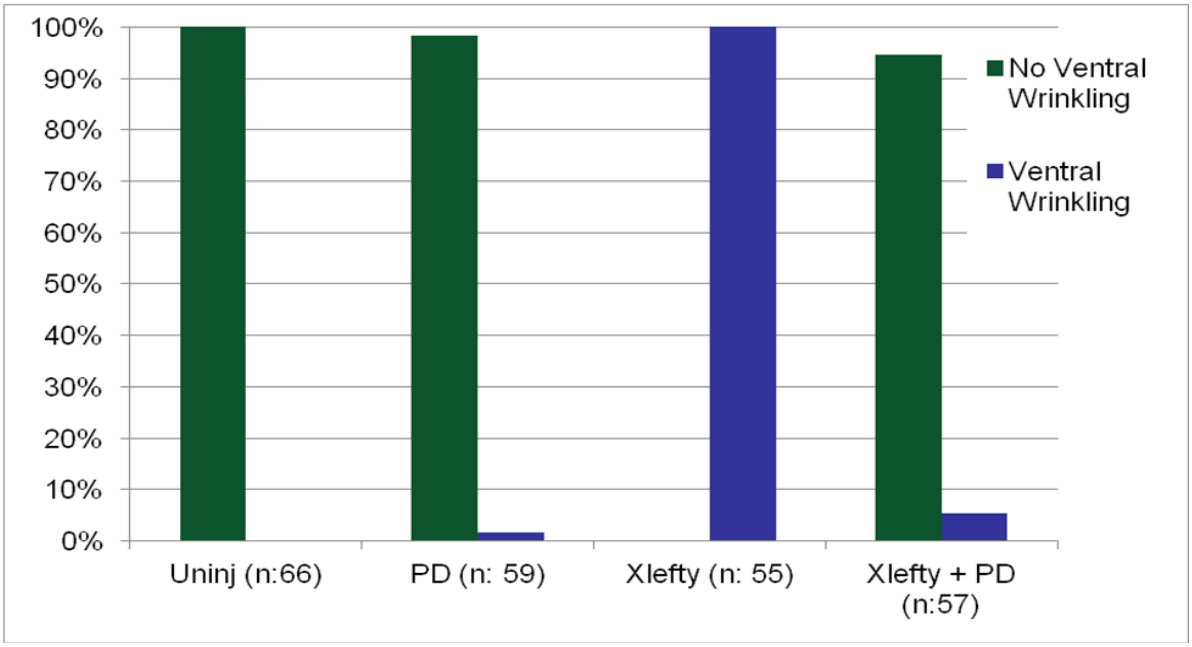

Figure 11. The PD co-injected with Xlefty rescues a ventral wrinkling phenotype. Uninjected embryos had no ventral wrinkling (n=66). 2% of the PD microinjected embryos had ventral wrinkling (n=59). In contrast, 100% of the Xlefty microinjected embryos had ventral wrinkling (n=55). Only 5% of the Xlefty+PD microinjected embryos had ventral wrinkling (n=57), thus demonstrating rescue.

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The PD is able to co-immunoprecipitate (co-IP) mat-Xlefty but PDmut cannot

To begin to test if the PD inhibits Mat-Xlefty by interacting with it, we asked if the PD could co-immunoprecipitate (co-IP) Xlefty. To do so, mRNAs encoding the PD and Xlefty were co-injected into a 4-cell stage embryo at a concentration of 750pg for the PD and 250pg for Xlefty. Because antibodies against the native Xlefty and its mutated derivatives are not available, all Xlefty constructs were C-terminally tagged with a Myc epitope tag or a hemagglutinin (HA) epitope via standard PCR cloning protocols. The results showed that the PD is able to co-IP mat-Xlefty, but not uncleaved Xlefty (Fig. 13). This was consistent with our hypothesis that the PD regulates Xlefty by molecularly interacting with it.

A comparative analysis of 33 human TGFβ family members identified a conserved hydrophobic motif Hyd-Hyd-X-X-Hyd-X-Hyd within the PD (Fig.12) (Walton et al., 2009). This conserved site suggests that this region serves a common role in governing the assembly and secretion of TGFβ ligands (Walton et al., 2009). When deleted in TGFβ1, the association between the pro- and mature domains, in addition to secretion, were inhibited (Walton et al., 2009; Young and Murphy-Ullrich, 2004). Our comparison of the Xlefty prodomain to the consensus residues revealed, that in *Xenopus*, the hydrophobic motif is present (Fig. 12). In the Xlefty PD, it corresponds to amino acids 34, 35, 38 and 40 (Fig.12). These were mutated by PCR mutagenesis from hydrophobic leucines to alanines to maintain hydrophobicity thereby reducing the effect on folding of the protein but disrupting the putative binding site (Fig. 12). To test if these conserved residues facilitate the interaction between the PD and Mat-Xlefty, a co-IP experiment was performed by co-injecting 1ng/cell of PDmut mRNA with 250pg/cell Xlefty mRNA into 4 cell embryos. The results demonstrated that the PDmut was unable

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to co-IP Mat-Xlefty (Fig. 13) and suggested that the interaction between the PD and Mat-Xlefty was dependent on the mutated motif.

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Inhibin α	22	LELARELV	LAKVRAL	F	L	-DA	L	G	P	PAV	46
Activin A	47	VPNSQPEM	VEAVKKH	I	L	-NM	L	H	L	KKR	71
Activin B	67	LGRVDGDF	LEAVKRH	I	L	-SR	L	Q	M	RGR	91
Activin C	36	LESQRELL	LDLAKRS	I	L	-DK	L	H	L	TQR	60
Activin E	36	PQAERALV	LELAKQQ	I	L	-DG	L	H	L	TSR	60
TGFβ1	38	MELVKRKR	IEAIRGQ	I	L	-SK	L	R	L	ASP	62
TGFβ2	29	MDQFMRKR	IEAIRGQ	I	L	-SK	L	K	L	TSP	53
TGFβ3	32	FGHIKKKR	VEAIRGQ	I	L	-SK	L	R	L	TSP	56
BMP2	34	AAASSGRP	SSQPSDE	V	L	-SE	F	E	L	RLL	58
BMP3	164	DLSAWTLK	FSRNQSQ	L	L	-GH	L	S	V	DMA	188
BMP4	46	RSGQSHEL	LRDFEAT	L	L	-QM	F	G	L	RRR	70
BMP5	41	RRLRNHER	REIQR-E	I	L	-SI	L	G	L	PHR	64
BMP6	69	RRLKTQEK	REMQK-E	I	L	-SV	L	G	L	PHR	92
BMP7	44	RRLRSQER	REMQR-E	I	L	-SI	L	G	L	PHR	67
BMP8a	34	RRLGARER	RDVQR-E	I	L	-AV	L	G	L	PGR	57
BMP8b	34	RRLGARER	RDVQR-E	I	L	-AV	L	G	L	PGR	57
BMP10	47	DGVDFNTL	LQSMKDE	F	L	-KT	L	N	L	SDI	71
BMP15	29	GQSSIALL	AEAPTLP	L	I	-EE	L	L	E	ESP	53
Nodal	42	AYMLSLYR	DPLPRAD	I	I	-RS	L	Q	A	EDV	66
GDF1	29	PV	PPGPAAA	L	L	-QA	L	G	L	RDE	48
GDF2	51	HTFNLKMF	LENVKVD	F	L	-RS	L	N	L	SGV	75
GDF3	67	SRDLCYVK	ELGVRGN	V	L	-RF	L	P	D	QGF	91
GDF5	220	QRYVFDIS	ALE-KDG	L	L	GAE	L	R	I	LRK	244
GDF6	146	QKYLFDVS	MLSDKEE	L	V	GAE	L	R	L	FRQ	171
GDF7	139	QSFLFDVS	SLNDADE	V	V	GAE	L	R	V	LRR	164
GDF8	45	RQNTKSSR	IEAIKIQ	I	L	-SK	L	R	L	ETA	61
GDF9	46	PWSLLOHI	DERDRAG	L	L	-PA	L	F	K	VLS	70
GDF10	158	SGRPLPLG	PPTRQHL	L	F	-RS	L	S	Q	NTA	182
GDF11	68	RQHSRELR	LESIKSQ	I	L	-SK	L	R	L	KEA	92
GDF15	48	TEDSRFRE	LRKRYED	L	L	-TR	L	R	A	NQS	72
MIS	68	SPLRVVGA	LSAYEQA	F	L	-GA	V	Q	R	ARW	92
Lefty1	22	LT	GEQLLGS	L	L	-RQ	L	Q	L	KEV	40
Lefty2	22	LT	EEQLLGS	L	L	-RQ	L	Q	L	SEV	40
Xlefty	22	IN	GFNIRDA	L	L	-KK	L	N	L	GEV	43

HYD-HYD-X-X-HYD-X-HYD

Figure 12. *Xenopus* lefty (Xlefty) shares a conserved hydrophobic motif with 33 other TGFβ family members.

A sequence alignment of the prodomains of 33 TGFβ family members was done using ClustalW by Walton et. al. They identified a conserved hydrophobic motif shown by the inverted parenthesis. We compared the Xlefty prodomain region (shown in green) that corresponds to the alignment and demonstrate that it was also conserved. The hydrophobic leucines highlighted were mutated to alanines in our PDmut. Adapted from Walton et. al. 2009.

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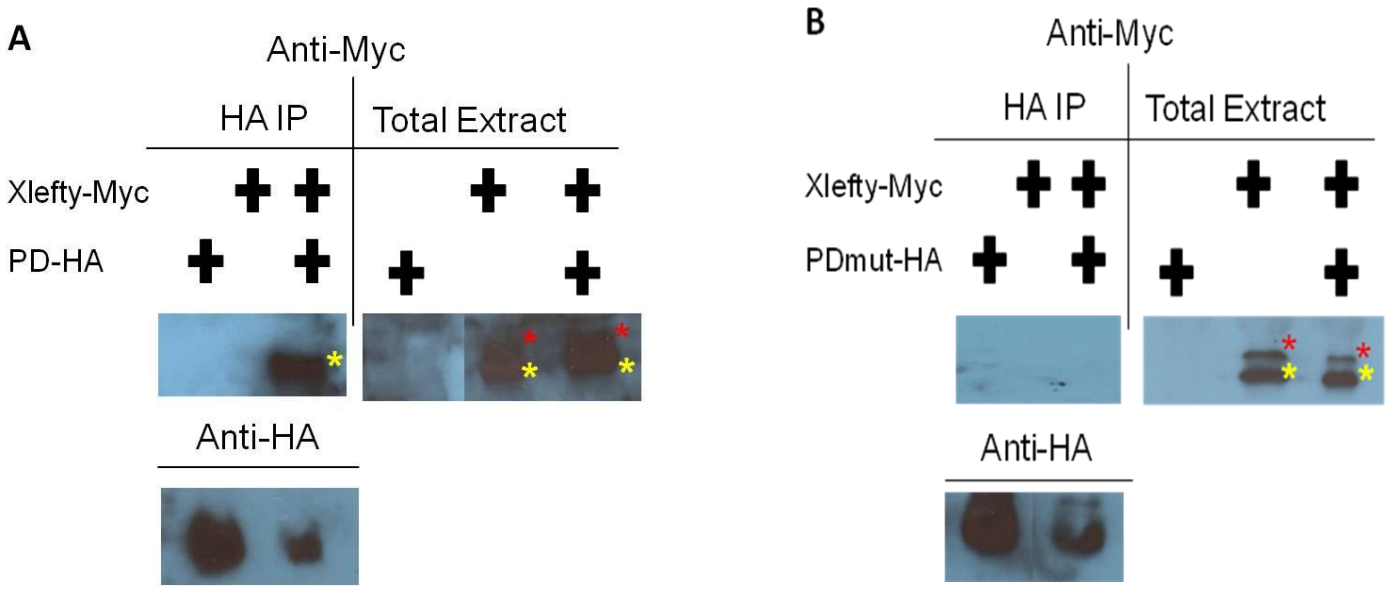

Figure 13. The PD, but not the PDmut, can co-IP Xlefty.
PD (A) is able to co-immunoprecipitate Xlefty but the PDmut (B) cannot. 2 cell *Xenopus* embryos were co-injected with RNAs encoding Xlefty-Myc and PD-HA or PDmut-HA. Embryonic extracts were immunoprecipitated with anti-HA antibodies and SDS-PAGE western blot analysis with anti-Myc antibodies was utilized to detect co-immunoprecipitation of Xlefty-Myc. Red asterisks in all blots indicate uncleaved Xlefty, or its mutated derivatives. The yellow asterisks in all blots indicate Mat-Xlefty or its mutated derivatives.

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PDmut does not cause exogastrulation yet weakly rescues Xlefty over-expression

Because PDmut was unable to co-IP Mat-Xlefty, we predicted that it would not inhibit Xlefty function. To test this, PDmut was microinjected at a concentration of 1ng/cell into 4 cell stage embryos. In contrast to PD injection (Figs.7-9), exogastrulation was not observed and 88% of the embryos exhibited normal development (n=78) (Fig.14). This inability of PDmut to inhibit Xlefty was consistent with our prediction.

We also tested if PDmut could rescue Xlefty over-expression. The PDmut was microinjected at a concentration of 1ng/cell into 4-cell stage and co-injected with Xlefty at a concentration of 1ng/cell for the PDmut and 25pg/cell for Xlefty at the 4-cell stage. For comparison, Xlefty was microinjected at a concentration of 25pg/cell at the 4-cell stage. The embryos were scored for normal blastopore (BP), delayed BP, no BP, and aberrant BP formation where the BP was either incomplete or not well defined.

Loss of BP formation is a definitive phenotype seen in an Xlefty over-expression context (Fig. 5). The Xlefty-microinjected embryos had 88% with a loss of BP and 12% with aberrant BP formation, a less severe phenotype (Fig. 15). Embryos co-injected with PDmut and Xlefty resulted in 63% loss of BP and 37% with aberrant BP formation (Fig. 15). Although there was not a complete rescue of the Xlefty phenotype, the co-injected embryos had fewer embryos exhibiting a complete loss of BP formation when compared to Xlefty alone. The increase in aberrant BP phenotypes in the co-injected embryos suggested that the PDmut had a weak ability to antagonize Xlefty. These results indicated that some interaction between Xlefty and PDmut might still occur.

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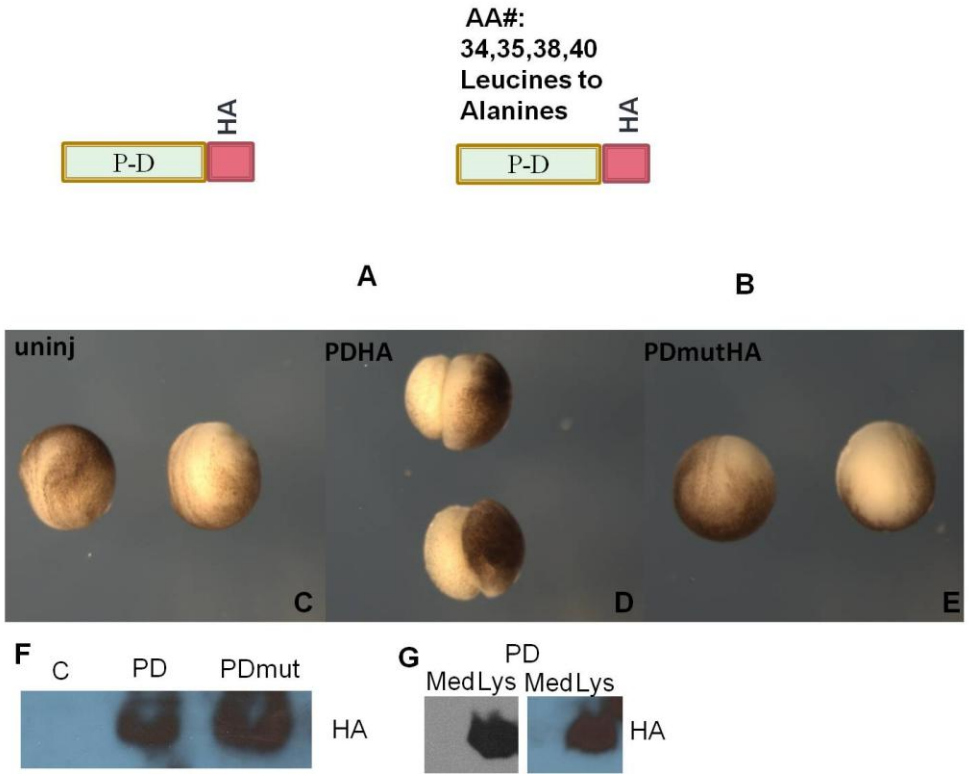

Figure 14. PD causes exogastrulation but the PDmut does not.
Schematics of PD-HA (A) and PDmut-HA (B) are compared where the PDmut-HA has mutations that change amino acid numbers 34, 35, 38, and 40 from leucines to alanines. (C) Uninjected controls. (D) PD-HA was microinjected at 1ng/cell into the dorsal cell of a 4-cell stage embryo and resulted in exogastrulation. (E) PDmut-HA was microinjected at 1ng/cell into the dorsal cell of a 4-cell stage embryo and resulted in normal gastrulation. (F) Western analysis of the C (control uninjected embryos), PD-HA microinjected embryos, and the PDmut-HA microinjected embryos showing the presence of the protein in the embryo lysates but not in the control. (G) Western analysis of secretion studies done on the PD. The first blot is the result of the PD secretion properties in an embryo assay showing no secretory product in the medium (Med) but presence of the PD in the lysate (Lys). The second blot is the result of the PD secretion properties in a cell culture assay showing no secretory product in the Med but presence of the PD in the Lys. (C, E) Dorsal view, anterior to top. (D) Lateral view, anterior to left. Embryos are at stage 14. 16X.

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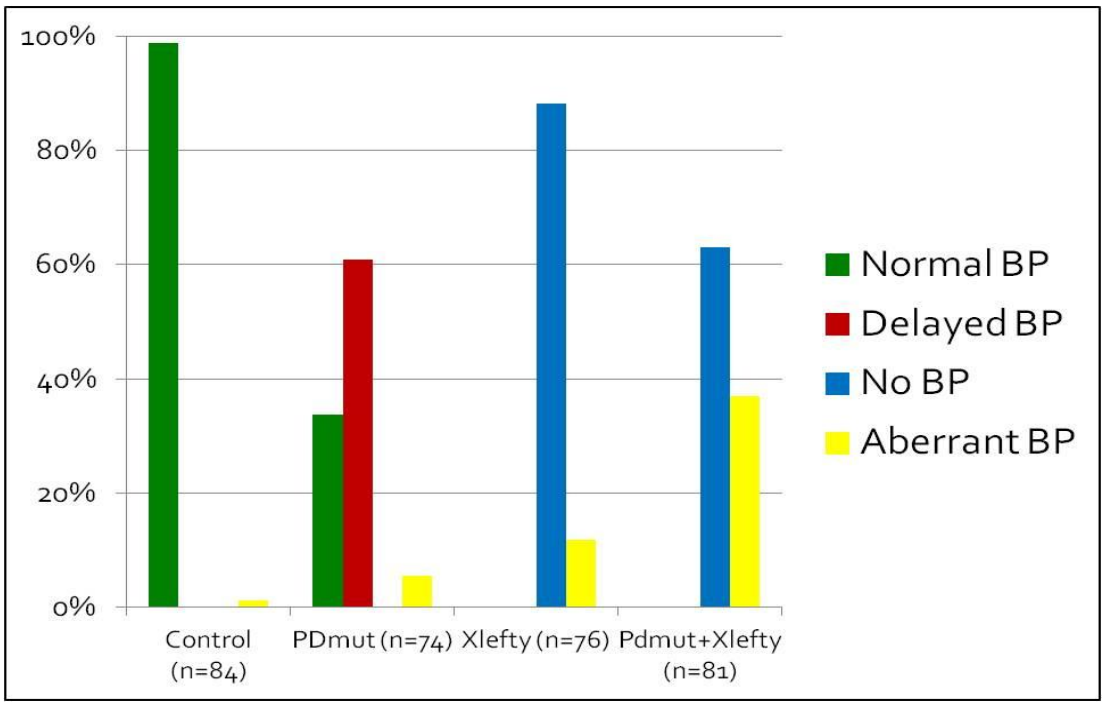

Figure 15. The PDmut weakly rescues Xlefty over-expression.
The graph represents the percentage of embryos that had normal BP formation compared to delayed BP, no BP and aberrant BP formation. Xlefty-microinjected embryos had 88% with no BP and 12% with aberrant BP formation. Embryos co-injected with PDmut and Xlefty had 63% with no BP and 37% with aberrant BP formation. The decrease in the loss of BP formation and the increase in the aberrant BP formation in the co-injected embryos suggest that the PDmut potentially still may interact with Xlefty.

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PDmutXlefty over-expression is similar to Xlefty over-expression embryos

Our hypothesis states that the PD negatively regulates Xlefty and it might do so by molecularly interacting with it as seen by our rescue and co-IP studies. To further test this hypothesis, we created a full length Xlefty with the PDmut (PDmutXlefty) by PCR mutagenesis. We predicted that PDmutXlefty would have increased activity compared to wild-type Xlefty because inhibition by the PD should be attenuated in PDmutXlefty.

PDmutXlefty was tested by microinjecting 500pg/cell of PDmutXlefty into 2 cell stage embryos. The resulting phenotypes were compared with embryos microinjected with 500pg/cell of Xlefty (Fig. 16). Xlefty and PDmutXlefty microinjected embryos were scored for a lack of blastopore formation at stage 11 and both were observed to have similar numbers of embryos with lack of BP formation (Xlefty (%; n=75) and PDmutXlefty (%, n=79)). Uninjected control embryos were normal (n=80). Embryos were allowed to develop to stage 37/38 and no phenotypic disparity was apparent between the PDmutXlefty and Xlefty microinjected embryos (Fig. 16 E-G). Thus, our results do not demonstrate any difference in activity between Xlefty and PDmutXlefty. More quantitative assays of Xlefty activity might be necessary to detect any differences.

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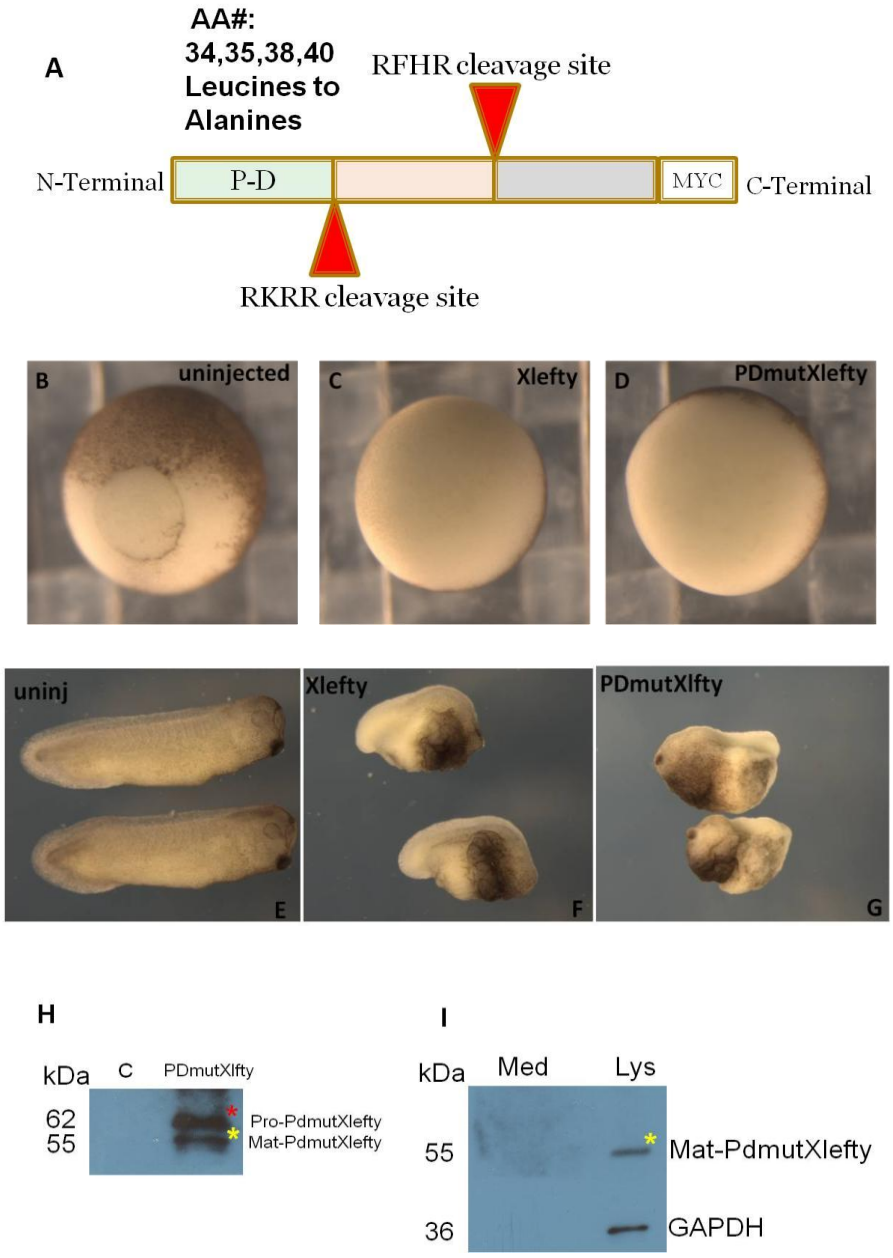

Figure 16. PDmutXlefty over-expression resembles Xlefty over-expression but its secretory properties do not.

(A) Schematic of the PDmutXlefty-Myc showing the mutation where amino acids 34, 35, 38 and 40 were changed from leucines to alanines (B) Uninjected embryos at St. 11.5 showing normal blastopore formation (C) Xlefty microinjected embryos showed no blastopore formation. (D) PDmutXlefty-Myc microinjected embryos showed no blastopore formation. (E) Uninjected embryos at St. 31 with normal development (F) Xlefty microinjected embryos showed typical Xlefty OE phenotype with ventral wrinkling, loss of anterior structures and stunting. (G) PDmutXlefty-Myc over-expression resembles Xlefty over-expression. (H) Western analysis of PDmutXlefty-Myc microinjected embryos revealed proper cleavage and presence of the two forms of Xlefty. (I) PDmutXlefty secretory properties tested using the cell culture assay showed the presence of Mat-PDmutXlefty in the cell lysate but not in the medium. (B-D) Vegetal views. (E, F) Lateral views, anterior to right. (G) Lateral view, anterior to left. (B-D) 20X. (E-G) 16X.

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Nodal-dependent mesodermal markers *Goosecoid* and *Xbrachyury* have an expanded expression pattern in PD over-expressing embryos compared to PDmut injected embryos

Our hypothesis posits that the PD negatively regulates Xlefty, thus we would predict an upregulation in Nodal signaling and subsequently in Nodal-dependent gene expression in PD injected embryos. To test this, we examined the expression of Goosecoid and Brachyury in PD injected embryos. Expansion in the Nodal-dependent expression of these two genes has previously been seen when an anti-Xlefty morpholino was used to block Xlefty (Branford and Yost, 2002).

Goosecoid (Gsc) is a transcription factor that was isolated from the dorsal blastopore lip of the early *Xenopus* gastrula and plays a key role in patterning mesoderm in the early gastrula (Blumberg et al., 1991; Niehrs et al., 1994). At stage 10.25, Gsc is expressed at the dorsal blastopore lip and is confined to the immediate lip region, but in embryos microinjected with an anti-Xlefty morpholino this region expanded away from the lip (Branford and Yost, 2002). Similarly, when the PD was over-expressed, the expression pattern of Gsc was clearly expanded away from the boundaries observed in the uninjected embryos (n=16/20) (Fig. 17).

Brachyury (Bra) is a transcription factor that is necessary for the formation of posterior mesoderm and axial development. *Xenopus* brachyury (Xbra) is expressed transiently throughout the presumptive mesodermal tissue (Schulte-Merker and Smith, 1995). At stage 10.5, Xbra is expressed at the boundary of the blastopore as a distinct tight ring that does not expand away from the blastopore, but in anti-Xlefty morpholino microinjected embryos, thickening of the ring and expansion away from the blastopore is

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observed (Branford and Yost, 2002). In PD injected embryos, the Xbra ring of expression thickened and expanded away from the blastopore (n=10/10) (Fig. 17 D-F).

The changes in the expression of Gsc and Xbra in PD injected embryos are indicative of expanded Nodal signaling and further support our hypothesis that the PD negatively regulates Xlefty activity. In contrast, the majority of the PDmut injected embryos did not exhibit changes in the expression patterns of Gsc (14/20) and Xbra (20/22) (Fig. 18). Taken together with the PD co-IP (Fig.13), this suggests that the residues mutated in PDmut are necessary for the PD-dependent regulation of Xlefty function.

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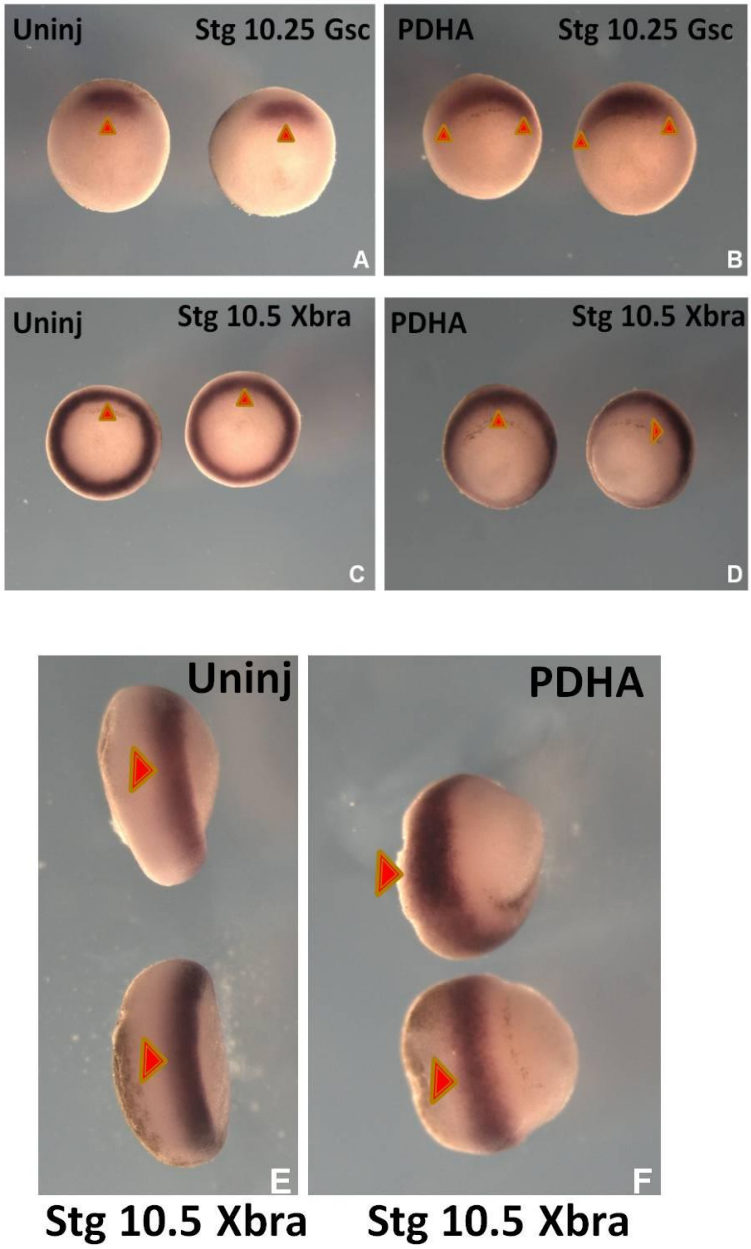

Figure 17. PD over-expression alters Nodal-dependent gene expression.
Induction of ectopic *gsc* and *Xbra* in the embryo by microinjection of PD. (A) Uninjected embryos with red arrowheads indicating limits of wild-type *gsc* expression at the blastopore lip. (B) PD-HA injected embryos with red arrow heads showed expansion of *gsc* expression beyond the boundaries of the blastopore lip. (C) Uninjected embryos with red arrowheads indicating *Xbra* expression on the periphery of the blastopore. (D) PD-HA injected embryos with red arrowheads showed *Xbra* expression has moved away from the periphery of the blastopore. (E) A lateral view of the uninjected embryos with red arrowheads indicated the boundary of the expression pattern of *Xbra.* (F) Lateral view of PD-HA injected embryos showed the expression pattern of *Xbra* and the expansion away from the periphery of the blastopore. (A-D) Vegetal view. (E, F) Lateral view. RNAs were injected into the animal pole cells of a 4-cell embryo and then embryos were fixed during gastrulation (St. 10.25 and St. 10.5) for in situ hybridization. (A-D) 16X. (E-F) 20X.

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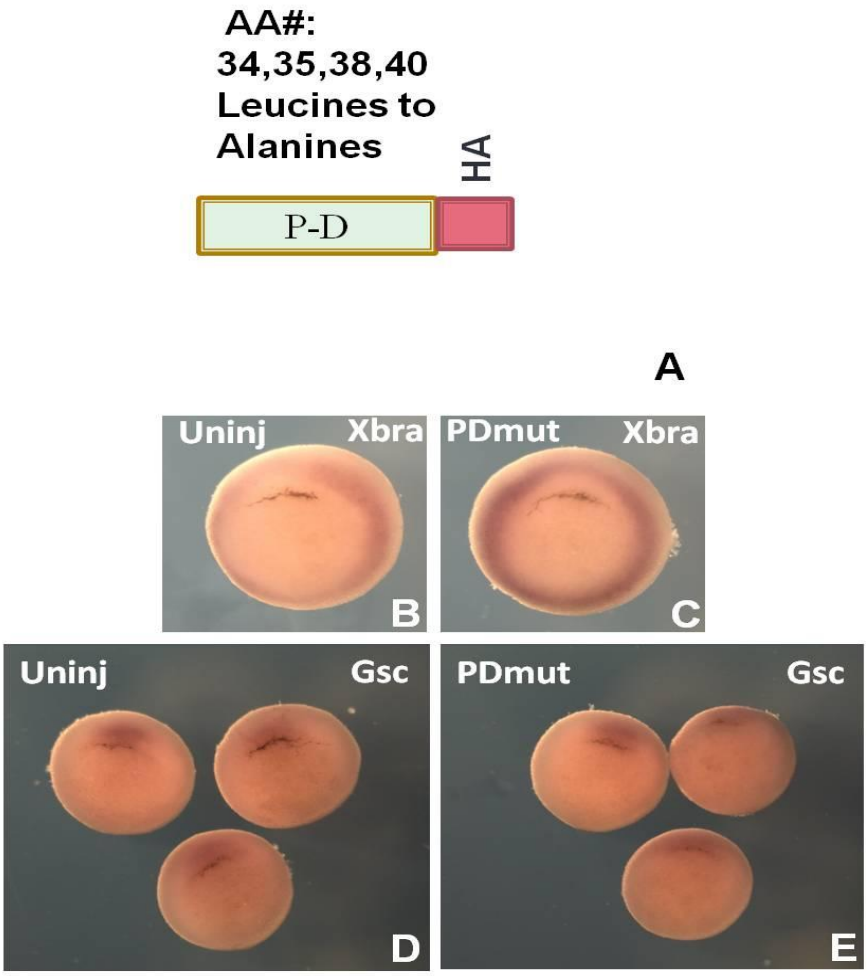

Figure 18. PDmut microinjection does not affect Nodal-dependent gene expression. (A) Schematic of the PDmut-HA showing the mutation where amino acids 34, 35, 38 and 40 were changed from leucines to alanines. (B) Uninjected embryos were assessed for *Xbra* expression where the ring of staining could be seen around the periphery of the blastopore. (C) When compared to PDmut microinjected embryos there was not a visible shift in the expression pattern unlike PD-HA embryos seen in Fig.17. (D) Uninjected embryos were assessed for *Gsc* expression and normal *Gsc* expression was seen above the dorsal blastopore lip. (E) PDmut microinjected embryos revealed no difference in *Gsc* expression compared to uninjected embryos. Embryos were photographed at St. 10.25 for *Gsc* and St. 10.5 for *Xbra*. All embryos are vegetal views. (B, C) 20X. (D, E) 16X.

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Cleavage of the Xlefty prodomain is a necessary prerequisite for proper function

Removal of the prodomain by SPCs has been shown to be a necessary prerequisite for normal functionality of the members of the TGFβ superfamily (Sengle et al., 2011). Lefty is an atypical member of the TGFβ superfamily and accordingly cleavage of its prodomain is necessary for it to function and properly antagonize Nodal signaling (Westmoreland et al., 2007). Xlefty has two canonical SPC cleavage sites located at amino acids 74-77 and amino acids 132-135 (Branford et al., 2000; Hamada et al., 2002; Westmoreland et al., 2007).As done previously, we mutated canonical cleavage site 1 from RXXR to GVDG and site 2 from RFHR to GVDG in addition to creating a double cleavage mutant that contains the two mutated cleavage sites. (Hamada et al., 2002; Westmoreland et al., 2007). Our hypothesis was that the Xlefty PD is able to interact with the mature region of Xlefty and negative regulation by the PD would be exacerbated by these mutations since the PD would not be able to be cleaved.

Phenotypic analysis of embryos microinjected with the Xlefty cleavage mutants LCM1 (RKRR→GVDG), LCM2 (RFHR→GVDG) and an Xlefty double cleavage mutant L-DCM was performed by microinjecting 500pg of LCM into the left dorsal cell of a 4-cell embryo. Embryos were comparatively assessed with Xlefty microinjected embryos for loss of blastopore closure at stage 11, a distinctive phenotype seen in an Xlefty over-expression context (Fig. 5) (Branford and Yost, 2002). Consistent with what was seen in previous studies, the LCM2 and L-DCM had an Xlefty over-expression phenotype and no phenotype respectively. Briefly, 83% of the LCM2 microinjected embryos (n=96) microinjected embryos exhibited Xlefty over-expression suggesting that site 2 is not active in the embryo. The L-DCM did not affect the embryo and only had 21% (n=97) loss of blastopore closure. We postulate that since the L-DCM has both sites

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mutated it is rendered inert. On the other hand, the LCM1 had some very interesting phenotypes. LCM1 had a 19% (n=92) loss of blastopore closure in comparison to Xlefty which had a 90% (n=95) loss of blastopore closure. Uninjected embryos had no prominent aberrations (n=98). Phenotypes were also assessed at stage 28 and we noted that 60% of the LCM1 injected embryos had a ventral bending phenotype that stunted the embryo (Fig. 19). Furthermore, LCM1 over-expression resulted in ventral wrinkling, a phenotype associated with Xlefty over-expression (Fig. 19) (Branford et al., 2000; Branford and Yost, 2002). These results were intriguing because previous studies reported a loss of Xlefty activity in the absence of PD cleavage (Westmoreland et al., 2007).

Lefty cleavage mutants (LCM) gives insight into the PD's regulatory role

Our results show that LCM1 has some weak activity while L-DCM is not active. The L-DCM has both the first and second cleavage site mutated unlike LCM1 which only has the first site mutated (Fig.20A). Taking this difference into account, we postulated that in LCM1 the second cleavage site may mediate Xlefty processing which results in a partially active protein. To assess this possibility, we examined LCM1 protein by western blot analysis (Fig. 20). For these studies mRNA was microinjected into a 2 cell stage embryo at a concentration of 500 pg, embryos were harvested at stage 11 and whole embryo extract was analyzed. As seen in Fig. 20B LCM1 was unable to be normally cleaved. However, a band was present immediately below the un-cleaved LCM1 indicated by a yellow arrow in Fig. 20B. It is possible that site 2 could mediate some other type of post-translational modification that enable LCM1 to have some activity. Further studies are necessary to validate this observation. Unlike previous studies, we

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report here that the LCM1 has some activity in the embryo and western analysis suggests that a processed protein is present that may mediate this activity.

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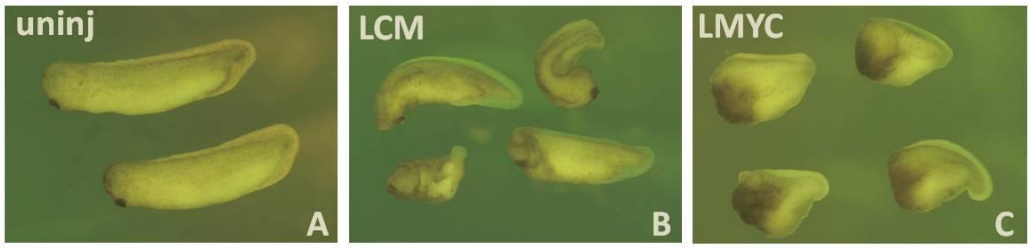

D Treatment	N	Lefty phenotype(%)	Ventral bending phenotype(%)
Uninjected	100	0	0
LMYC (500pg)	100	98	2
LCM1 (500pg)	100	2	60

Figure 19. L-CM1 (RKRR→GVDG) exhibits function in *X. laevis* embryos.
Embryos were microinjected with 500 pg mRNA in the left dorsal cell. (A) Normal uninjected embryos. (B) LCM1 injected embryos. (C) Xlefty injected embryos. (A-C) Stage 28 embryos. (A, C) Left lateral view, anterior to left. (B) Left lateral view (upper left and lower right embryos); right lateral view (upper right embryo); dorsal view (lower left embryo). Anterior to left (two lower and left upper embryos); anterior to right (upper right embryo). (A, C) 20X. (B) 16X.

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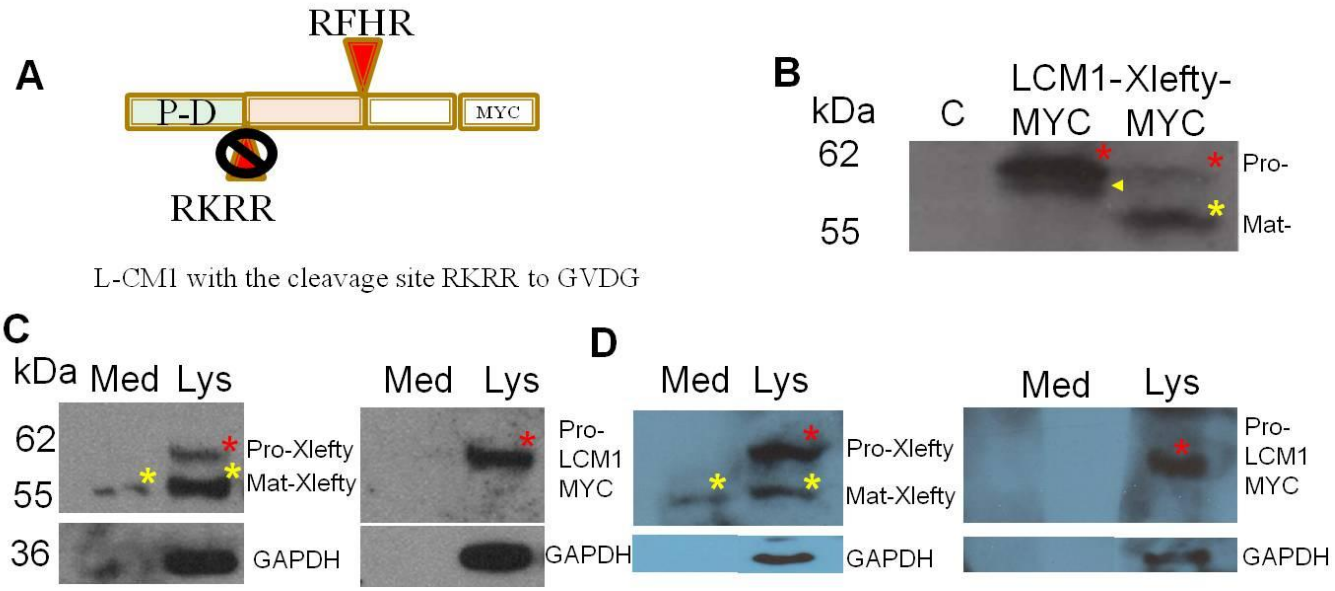

Figure 20. The LCM1 is not cleaved and is not secreted.
(A) Schematic of an Xlefty cleavage mutant 1 (LCM1). The active first cleavage site was mutated as indicated (LCM1 = RKRR to GVDG). At the N-terminus, a Myc tag was added since there is no available antibody that consistently detects Xlefty. (B) Western blot analysis on whole embryo extract reveals the inability of the LCM1 to be cleaved. Red asterisks in all blots indicate the Pro-Xlefty protein (or its mutated derivatives) which still have the prodomain present. The yellow asterisks in all blots indicate Mat-Xlefty (or its mutated derivatives) which have had the prodomain cleaved. The yellow arrow in the LCM1-MYC is postulated to be a processed protein resulting from cleavage. (C) Results of the embryo secretion assay done on Xlefty and LCM1. The first blot is of Xlefty protein secretion demonstrating normal secretion of Mat-Xlefty seen in the medium. The second blot demonstrates LCM1 unable to be cleaved and no secretory products in medium. (D) Results of the cell secretion assay done on Xlefty and LCM1. The first blot is of Xlefty normal secretion with the Mat-Xlefty seen in the medium. The second blot is of the LCM1 unable to be cleaved and no secretory product in the medium. GAPDH detection was used to demonstrate proof of principle of the secretion assay. GAPDH should not be detected in the medium.

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An Xlefty with the PD removed (PDL) has weak activity

To further expand our analysis of the function of the PD, an Xlefty without the PD (PDL) was created by PCR mutagenesis. The PDL is an Xlefty with the PD domain removed but retaining the first 25 amino acids predicted to be the signal sequence cleavage site (SS) (as computationally determined using SignalP 3.0 Server). Our hypothesis is that since the PD is negatively regulating Mat-Xlefty once PD inhibition is removed (PDL) there would be no negative regulation and the PDL would behave in a strong Xlefty over-expression fashion. The PDL was over-expressed in the embryo by microinjecting 1ng/cell of a 4-cell embryo. Initial observations revealed that at St. 10.5 PDL injected embryos were delayed in blastopore closure compared to uninjected controls (Fig. 21B-C). At stage 28, PDL microinjected embryos were observed to have a defect in eye formation. Defects in blastopore closure and eye formation are both consistent with Xlefty over-expression phenotypes (Branford et al., 2000; Branford and Yost, 2002) and suggest that the PDL has weak Xlefty activity. This is contrary to our hypothesis and indicates that the PD is necessary for more than just regulating Xlefty activity negatively.

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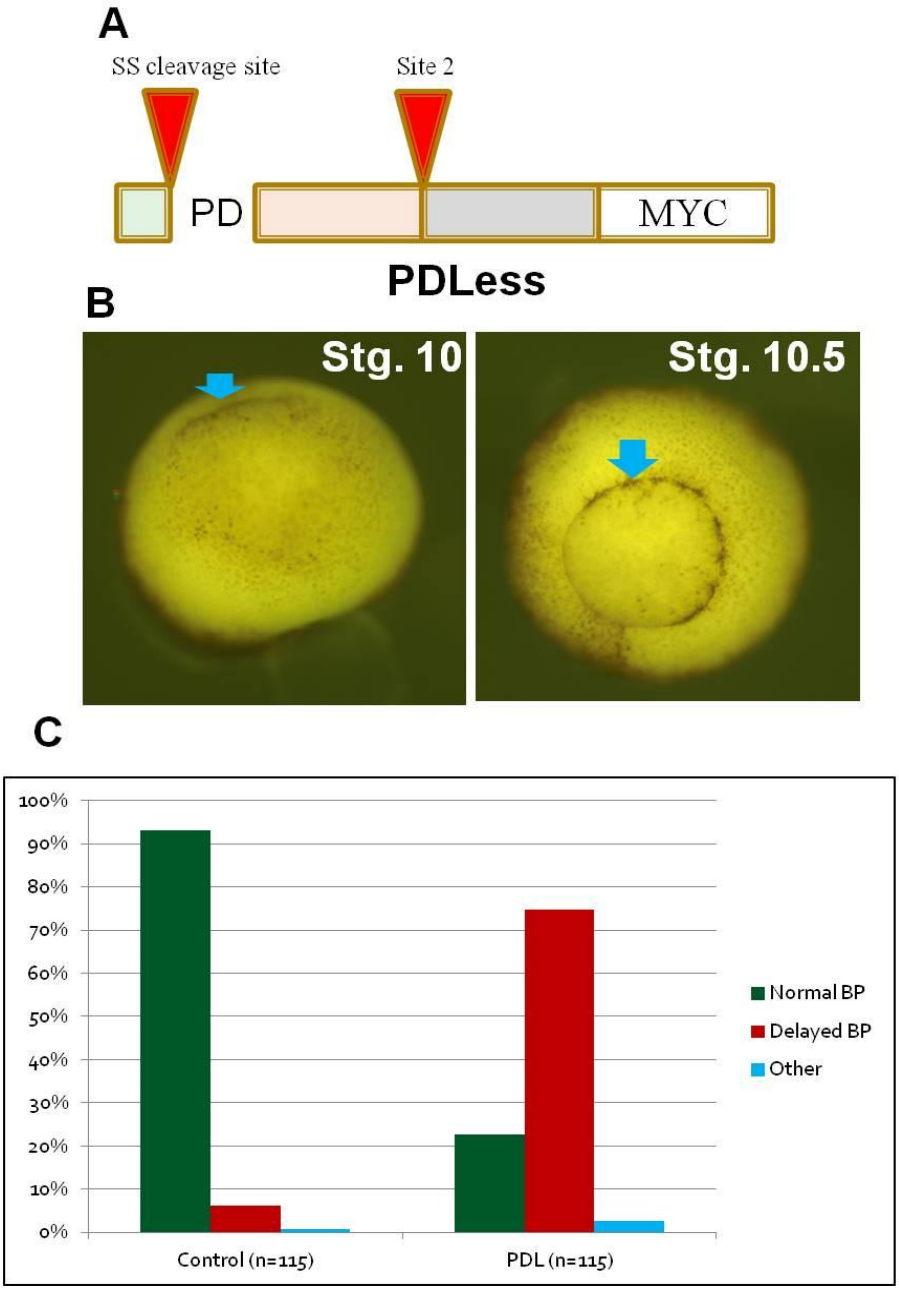

Figure 21. The PDL when microinjected into 4-cell stage embryos results in delay of blastopore closure.
(A) A schematic of the PDL protein. (B) Uninjected embryos at Stg. 10 and 10.5 respectively. The blue arrows point to the blastopore lip forming at St. 10 and already starting to close at stage 10.5. Both are vegetal views. 20X. (C) Graph depicting that embryos were delayed at St. 10 and were developing on time at 10.5.

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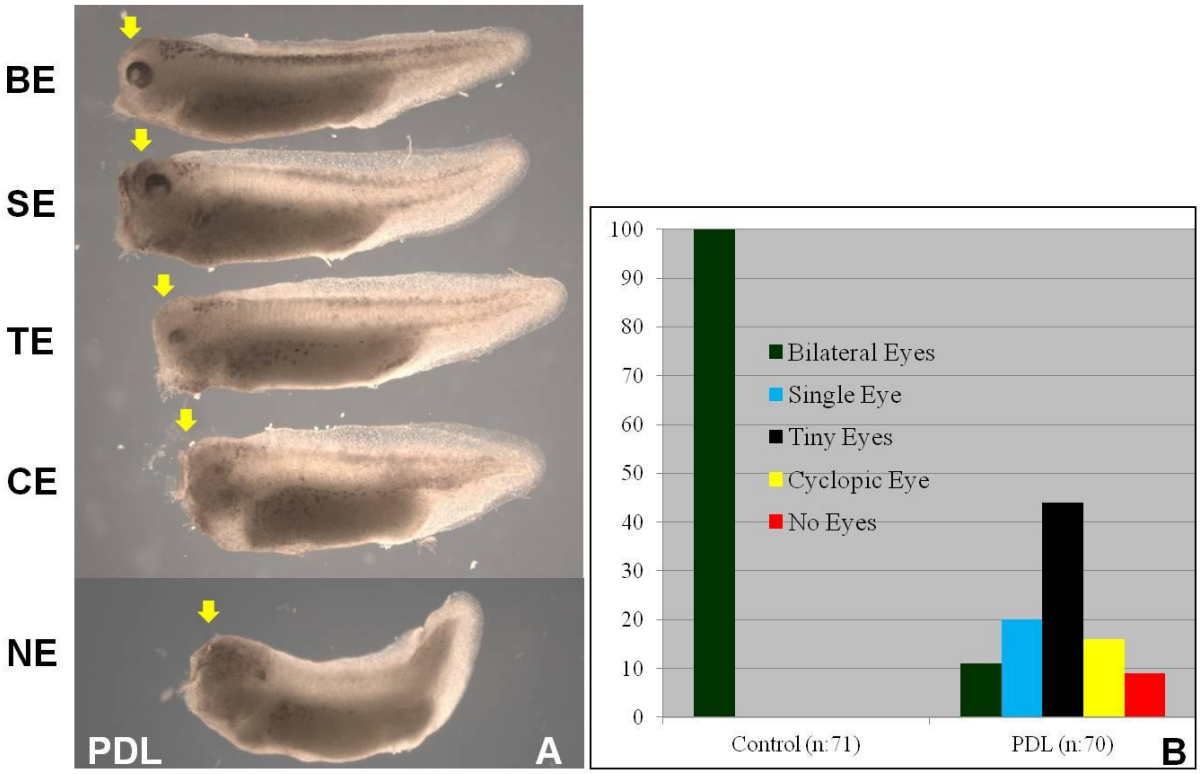

Figure 22. The PDL when microinjected into 4 cell stage embryos results in eye defects. (A) Spectrum of eye defects seen in embryos at St. 28. Top embryo is the uninjected control and has bilateral eyes (BE), followed by a single eye (SE), tiny eye (TE), cyclopic eye (CE) and no eye (NE). Yellow arrowheads indicate dorsally anterior region where the eyes or lack of can be seen. All are lateral views with anterior to the left. 16X. (B) Graph depicting number of embryos with eye defects.

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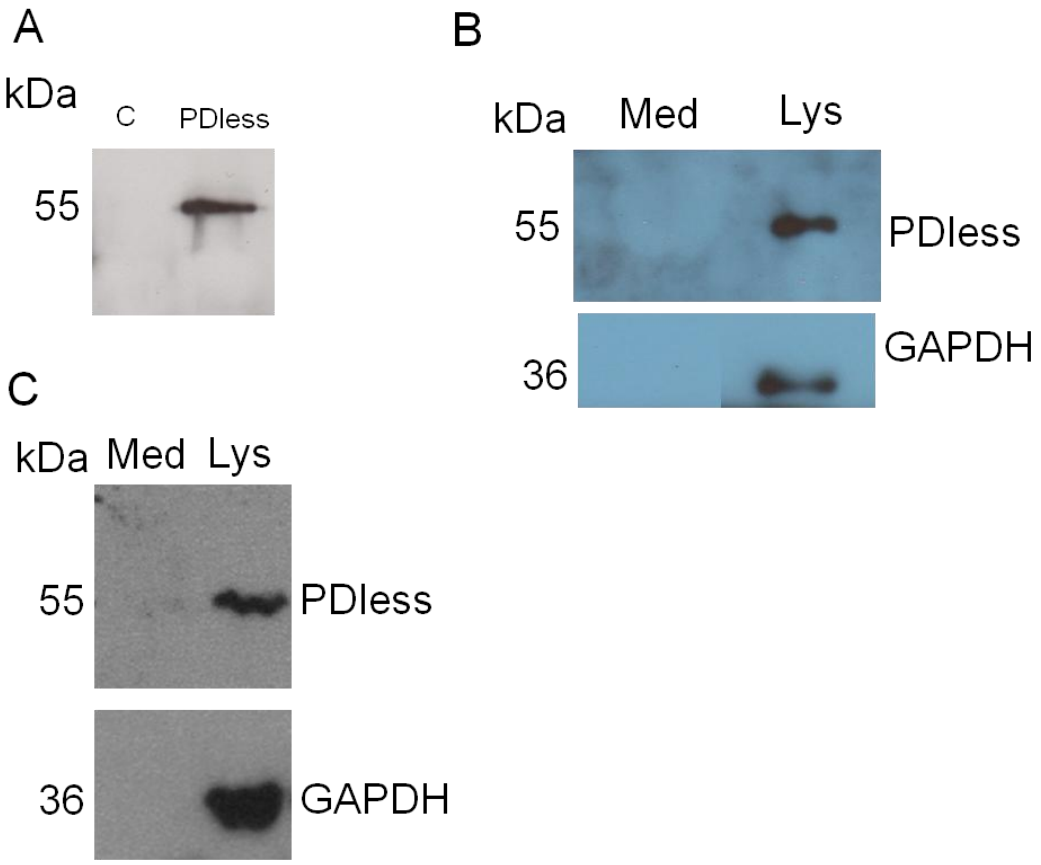

Figure 23. The secretory properties of the PDL construct.
(A) The PDL mRNA is translated when microinjected in the embryo. (B, C) The PDL is translated but not secreted in both the cell culture system (B) and the embryo secretion assay (C). GAPDH detection was used to demonstrate proof of principle of both assays. GAPDH is expected not to be found in the medium but present in the lysate. GAPDH also doubles as a loading control.

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The secretory properties of Xlefty-mutated derivatives indicate that the PD may mediate proper Xlefty secretion

Given that TGFβ members signal extracellularly, including the secreted Nodal antagonist Xlefty, the secretory properties of the constructs that had a weak or no phenotype were investigated (Hill, 2001; Serra, 2008). The lack of phenotypes led us to postulate that mutations affecting the PD may impact secretion therefore constructs were assessed using an embryo and cell culture secretion assay.

Briefly, the embryo secretion assay was done by microinjecting mRNA into the left dorsal cell of 4-cell stage embryos. The embryos were left to develop until the 8-cell stage at which time the animal cap was punctured and embryos were incubated for 3-4 hours. After this incubation period the conditioned medium and embryo lysates were collected for protein analysis. Briefly, the cell culture secretion assay used mouse embryonic fibroblast (MEF) cells that were transformed with plasmid DNA by the Nucleofection method. 4ug of plasmid DNA was transformed into MEF cells and the conditioned medium and cell lysates were collected at 24 and 48 hrs.

Lefty cleavage mutants (LCM) gives insights into the PD's role in secretion

The secretory properties of the LCM1 were assessed by the embryo secretion assay. Embryos were microinjected with 600pg of Xlefty or LCM1 mRNA into the left dorsal cell. The results indicate that Xlefty-Myc is cleaved and present in the medium. In contrast, the LCM1 is not able to be cleaved and is not secreted. LCM1 was also assessed by the cell culture secretion assay where 4ug of Xlefty-Myc or LCM1 plasmid were transformed into MEF cells. The LCM1 was not observed in the cell medium but was clearly translated and present in the cell lysates (Fig. 20D). The results showed that the LCM1 was unable to be cleaved but its secretory properties were affected. The LCM1

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has some activity in the embryo but its activity was probably limited because failure to cleave its PD seemed to affect secretion negatively. These results suggest that the PD plays a prominent role not only in regulating Xlefty function but also in mediating secretion.

An Xlefty with the PD removed (PDL) is not secreted

The PDL was studied by over-expression and it was noted that besides delay of blastopore formation in early embryos and loss of eye at St.28 we did not observe strong Xlefty over-expression phenotypes. This was unexpected because our premise was that loss of the PD would remove inhibition and thus promote Xlefty over-expression. The lack of a strong Xlefty over-expression phenotype led us to look at the secretory properties of the PDL using both secretion assays. For the embryo secretion assay, 750pg of PDL mRNA was microinjected into the dorsal cell of 4 cell stage embryos. For the cell culture secretion assay, 4ug of PDL plasmid was transformed in MEF cells. Both secretion assays suggested that the PD has a role in the secretion of Mature-Xlefty since we did not see any processed form of the PDL in the secretion medium.

The PD and the PDmutXlefty were not found to be secreted

Given the lack of secretion seen for our mutated constructs, we also tested if the PD was secreted (Fig. 14G). In both assays no secreted product was detected. The PDmut was tested in a cell culture secretion assay and secretion was not observed (results not shown). The absence of the PD or PDmut in the secreted medium was intriguing to us since we expected that the PD would be secreted because it caused severe phenotypes such as exogastrulation. It is possible that in the context of the secreted medium the PD and PDmut are easily degraded and would be undetectable by our assays.

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The PDmutXlefty was also tested in the cell culture secretion assay and the results showed the absence of PDmutXlefty processed forms in the secreted medium. This was interesting since the PDmutXlefty had a strong Xlefty over-expression phenotype and this suggests that it was most likely secreted. The mutations in the PDmut could potentially affect the stability of PDmutXlefty's processed forms in the secreted medium and further studies are necessary to test this.

DISCUSSION

The TGFβ superfamily is comprised of active signaling ligands that pattern the early embryo. Members of this superfamily include Nodal, BMP, Activin and Lefty. Lefty is considered an atypical member since it does not signal per se but its primary function is to antagonize the signaling capacity of Nodal. The mode of Lefty action is known, but so far there has not been any study that demonstrates the regulation of Lefty itself. In this study, we showed for the first time that *Xenopus* lefty (Xlefty) is regulated by its prodomain and that the prodomain also may play a role in proper Lefty secretion.

A putative model of PD function in Xlefty regulation and secretion

Figure 24 is a schematic that shows our proposed model of PD regulation of Xlefty and its role in secretion. First, we show that a putative molecular interaction (indicated by dotted lines) occurs between the PD and Xlefty in either a pre-cleavage or a post-cleavage complex. Both forms result in negative regulation of Xlefty function. Xlefty's primary function is to antagonize Nodal signaling and our model shows that this is blocked. Secondly, our work on the secretory properties of Xlefty and its mutated derivatives suggest that the PD is a necessary core component that facilitates proper secretion (red arrow).

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PD Over-expression Causes Anti-Xlefty Phenotypes and Can Rescue Xlefty Over-expression Phenotypes

Over-expression of a PD alone construct (PD) at a concentration of 500pg per cell of a 2 cell embryo resulted in left right abnormalities in the embryos (Figure 6). Left-right abnormalities are indicative of dysregulated Nodal signaling so we increased the concentration of the PD to 1ng/cell in 4-cell stage embryos.

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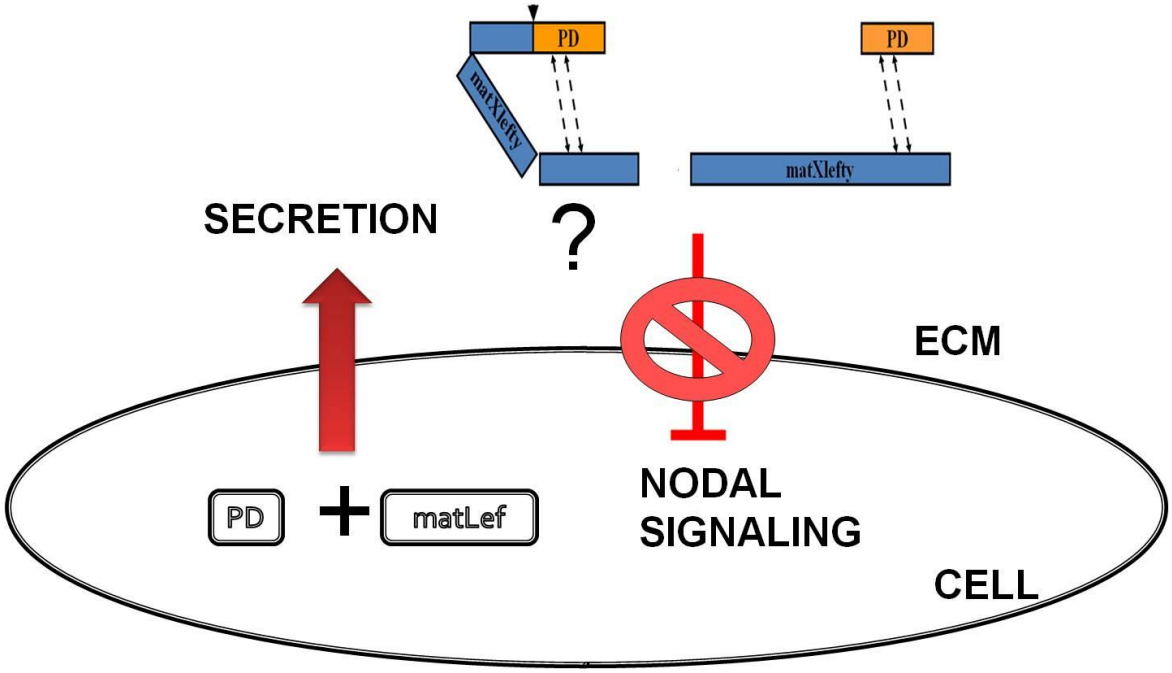

Figure 24. Model of PD role in regulation and secretion of Xlefty.
This graphic illustrates our model that the PD interacts with the mature Xlefty peptide inhibiting normal functionality. This may be occurring in a pre-cleavage complex as indicated in the first complex with the cleavage site intact (black arrow) and the interaction present (dotted lines). The second complex is more likely with the PD interacting with the mature Xlefty in a post-cleavage complex. Both complexes have the capacity to block proper Xlefty function (red circle). Based on our work the PD is a core component in proper Xlefty secretion (red arrow).

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PD over-expression at this concentration resulted in exogastrulation, a phenotype we reported as a result of blocking Xlefty function with an anti-Xlefty morpholino (Fig. 7) (Branford and Yost, 2002). This result suggested that the PD was inhibiting mature Xlefty function since targeting Xlefty with anti-Xlefty morpholino results in a similar phenotype (Branford and Yost, 2002). The phenotypes of PD-injected embryos also included loss of dorsal closure. They had varying degrees of dorsal closure and this suggests that there is a defect in convergent extension as a result of Nodal-expanded signaling which leads to unregulated mesodermal tissue expansion impeding dorsal closure (Fig. 8).

To examine whether the PD inhibits Xlefty, we tested if the PD could rescue Xlefty over-expression. Co-injections with the PD and Xlefty were performed to test this (Fig. 9). Co-injecting both the PD and Xlefty demonstrated that the PD was able to rescue ventral wrinkling, an Xlefty OE phenotype (Fig. 9, 11). The PD was also able to rescue the open dorsal phenotype observed in PD alone injections (Fig. 7-10). This suggested that interaction between the co-injected PD and Xlefty resulted in most of the embryos having a "rescued" phenotype as seen in Fig. 9I.

The PD co-IPs Xlefty: Evidence of a Molecular Interaction

The prodomains of other TGFβ family members have been shown to play an inhibitory regulatory role in TGFβ activity. Other studies suggest that the prodomain is able to sequester the mature ligand by binding to members of the extra-cellular membrane (Serra, 2008). The Xlefty PD, a protein of 77 amino acids, was co-injected with Xlefty and a co-IP experiment was carried out to test if the PD is able to interact with Xlefty molecularly (Fig. 13A). Seeing that the PD was able to co-IP the Mat-Xlefty

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protein, this indicated that a molecular interaction occurs between the PD and Mat-Xlefty.

A comparative analysis of the prodomain of 33 TGFβ family members was previously done and a conserved hydrophobic motif that constitutes four consensus amino acids was identified (Walton et al., 2009). We compared the prodomain region of *Xenopus* lefty (Xlefty) to the conserved motif and saw that this site was conserved in Xlefty as well (Fig. 12). We predicted that this site played a role in the PD-mature Xlefty interaction seen in the co-IP experiments. Based on what is known about other TGFβ ligands known to be secreted non-covalently bound to the PD, we predicted that mutation of this site would disrupt the interaction between the PD and Mat-Xlefty. PDmut, a PD with mutations at the aforementioned site, was made and co-injected with Xlefty and a co-IP experiment was carried out to test if the interaction was disrupted. The results of the co-IP experiments seen in Fig. 13 show that the PD has the capacity to co-IP Mat-Xlefty while the PDmut cannot. These results are consistent with our hypothesis that the PD regulates Xlefty function by a molecular interaction, which we are just starting to understand.

The PDmut is Unable to Rescue an Xlefty OE

The PDmut was also over-expressed in the embryo to test if there was any activity as was seen in the PD over-expression experiments. In a comparative analysis seen in Fig. 14 the PDmut was not seen to cause exogastrulation. The PDmut was also co-injected with Xlefty to determine if it could rescue an Xlefty over-expression phenotype. The results demonstrated that the PDmut was not able to rescue the loss of blastopore formation fully, but was able partially to restore total loss of blastopore to aberrant blastopore formation (Fig. 15). These results led us to consider that it is possible for there

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to be other relevant molecular interaction sites within the PD molecule that our mutations did not affect. We propose that further investigation of other potentially important residues should be done. Western blot analysis of whole embryo extract of PD and PDmut microinjected embryos did not reveal any difference in protein concentration (Fig. 14F).

***In situ* hybridization of PD microinjected embryos suggests loss of antagonism of Nodal, further evidence of negative regulation of Xlefty by the PD**

In the PD microinjected embryos, we studied the ectopic expression of Nodal-dependent markers Goosecoid (Gsc) and Xbrachyury (Xbra) to ascertain if the PD was affecting Xlefty function. At stage 10.25, we observed significant expansion of Gsc away from the dorsal blastopore lip region as can be seen in Fig. 17B. At stage 10.5 Xbra was seen to expand farther away from the blastopore than the endogenous ring of Xbra in the control embryos (Fig. 17C-F). The expansion of endogenous Gsc and Xbra expression in the PD-expressing embryos and the subsequent exogastrulation of these embryos are consistent with the phenotypes previously seen with the injection of anti-Xlefty morpholinos (Branford and Yost, 2002).

The results of the PDmut *in situ* hybridization experiment in Fig. 18, demonstrate that the mutations in the PDmut prevented it from negatively regulating Xlefty, which did not alter the expression pattern of Nodal-dependent markers Gsc and Xbra. The observations of the PDmut *in situ* hybridization experiments led us to consider looking at the PDmut in the context of full length Xlefty. A PDmutXlefty construct was made that has all the characteristics of the Xlefty molecule but contains the mutations in the PDmut (Fig. 16). We expected to see the PDmutXlefty with a stronger Xlefty over-expression phenotype than un-mutated Xlefty since PD negative regulation would have been

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removed. Our results showed that there was no phenotypic difference from the Xlefty over-expression phenotypes (Fig.16). Lefty, when compared to Nodal, has been shown to have higher mobility, thus we postulated that Xlefty and PDmutXlefty had comparable levels of activity in the embryo (Muller et al., 2012). It is possible that our over-expression assay needs to be modified to tell if there was a difference in activity levels.

The PD is more than meets the eye

TGFβ ligands have diverse roles such as cell cycle regulation, cell morphology and adhesion, ECM deposition and tissue differentiation (Serra, 2008). Our studies so far suggest that the versatility of the TGFβ superfamily members can be attributed to the regulatory functionality of the prodomain. Further studies, done on the secretory properties of Xlefty and its mutated derivatives, revealed that the PD is also necessary for proper secretion.

Xlefty Cleavage Mutant 1 (LCM1) holds clues to the PD's role in secretion

Our initial investigation into the Lefty prodomain started with repeating a previous study on Lefty cleavage mutants (LCM). We mutated cleavage site 1 (LCM1), site 2 (LCM2) and made a double cleavage site mutant (DCM) and tested their functional and secretory properties as done previously (Sakuma et al., 2002; Westmoreland et al., 2007). Phenotypic assessment of the cleavage mutants revealed that the DCM did not have activity and the LCM2 had phenotypes similar to Xlefty over-expression. However, LCM1 revealed interesting phenotypes unlike previously reported (Fig. 19). This led us to study the secretory properties of LCM1 and we demonstrated that prevention of cleavage affected the secretion properties of the LCM1 using both an embryo and cell culture assay (Fig.20). The LCM was not cleaved as seen in Fig.20B. When tested in both our assays and compared to un-mutated Xlefty, LCM1 was not secreted (Fig. 20C-D).

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Inversely, the un-mutated Xlefty had both the unprocessed Pro-Xlefty and the processed Mat-Xlefty indicating cleavage (Fig. 20 C-D). Additionally, the processed Mat-Xlefty is seen in both the embryo and cell culture secreted medium thus demonstrating secretion of Mat-Xlefty but not uncleaved Xlefty. This is consistent with the processing of TGFβ ligands intracellularly and the requisite for proper secretion in some members (Harrison et al., 2011). It is probable that the LCM1 is unable to be secreted from the cell since the PD first has to be cleaved. Our model suggests that a molecular interaction is present in an extracellular context. Could this same molecular interaction be used to mediate a specific conformation that is required for Xlefty secretion?

Given that TGFβ secretion is dependent on non-covalent molecular interactions between the prodomain and the mature domain it is possible that this interaction could occur intracellularly (Walton et al., 2009). It is possible that the PD is a core component for a portion of the Xlefty secretory pathway but since the PD is still attached in LCM1 the inhibitory non-covalent hydrophobic motif may be impeding proper secretion. The ventral bending phenotype seen in the LCM1 microinjected embryos could mean that the second cleavage site was mediating a processed form that requires further validation. We propose that a PDmut combined with a LCM1 mutant would reveal interesting results that could elucidate the dual mechanism behind the PD's negative regulation of Xlefty and its equally important role for proper Xlefty secretion.

The PD and the PDmutXlefty are not secreted

We also studied the secretory properties of the PD itself and it was not found to be secreted in our secretion assays (Fig. 14). These results were unexpected since it is believed that cleavage of the PD occurs once Xlefty is secreted out of the cell and the spectrum of PD phenotypes seen would suggest secretion. Perhaps the PD was not

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detectable in the media of our secretion assays due to sensitivity issues of the assay or when secreted, the PD is in a conformation that masks it from detection. The PD could also be unstable in the context of secreted media and thus would degrade rapidly upon secretion making it undetectable.

The secretory properties of the PDmutXlefty were also studied and surprisingly a processed form was not detected in the medium (Fig.16). Our results show that the PDmutXlefty when microinjected, has similar phenotypes to Xlefty so we strongly believe that it is secreted. However, our cell secretion assay did not detect a secreted form in the medium. This finding suggests that the secretion assay is not sensitive enough since we detect translated PDmutXlefty from whole embryo extract. As previously mentioned, mutations in the PD may disrupt non-covalent binding sites that prevent proper secretion resulting in undetectable levels of processed PDmutXlefty in the secreted medium.

A PD Less Lefty (PDL) mutant opens a Pandora's box

The results from my LCM1 study led to the construction of an Xlefty protein that lacks the PD domain named PD Less (PDL). This clone contains all the components of unaltered Xlefty, but is missing the domain after the signal sequence and immediately after the first cleavage site (Fig. 21). Our preliminary investigations revealed that PDL microinjected embryos had a delay in blastopore closure (Fig. 21). At stage 28 we saw that the embryos had defects in eye formation (Fig. 22). Our hypothesis suggested that the PDL would have a strong Xlefty over-expression phenotype since PD regulation is no longer there but we did not see this. At most, the PDL had a weak Xlefty activity leading us to look at the secretory properties of the PDL. The results showed that the PDL is not found to be secreted when tested with both the embryo and cell secretion assay (Fig. 23).

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These results along with the LCM1 results indicate that the PD is a necessary core component of Lefty secretory processing and function since mutations within the PD or the PD deletion constructs seem to abrogate proper secretion of Xlefty. This is paradoxical since many of the mutants looked at had phenotypic effects and we expected them to be secreted based on canonical behavior of other TGFβ family members. It appears that not only does the PD regulate Lefty function by interacting with it but it also plays a role in Lefty secretion. The results of the secretory studies suggest a previously unknown role of the Xlefty PD and warrant further investigation.

The PD is a bio-molecule that presents a potential therapeutic application for the treatment of diseases caused by Nodal and Lefty mis-expression

Further studies are necessary since potential therapies to address malignancies can be devised based on Lefty's antagonism of Nodal. Studies have shown that exposure of tumor cells to Lefty leads to a downregulation of Nodal signaling resulting in reduced clonogenicity and tumorigenesis (Postovit et al., 2008). This along with the fact that Nodal can auto-induce its own transcription and also directly regulates that of Lefty led us to consider the regulatory mechanisms that govern Lefty functionality (Schier, 2009). Since its isolation, abnormal Lefty activity has been implicated in several diseased states in vertebrates, such as *situs inversus* in mice (Meno et al., 1996). Lefty was also isolated in the endometrial tissue and was named endometrial bleeding associated factor (ebaf) where it possibly regulates normal menstruation (Kothapalli et al., 1997). Further, investigations showed that dysregulation of ebaf was associated with abnormal endometrial bleeding (Tabibzadeh et al., 2000).

The role of prodomains in the TGFβ family are different from species to species, however their sequences have conserved residues between species (Derynck R., 2008).

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For some of the members of the superfamily, the prodomain is non-covalently associated with its growth factor dimer in an inactive complex. For others, the prodomain/growth factor complex is active even though the prodomain is non-covalently associated with its growth factor dimer (Sengle et al., 2011). Here we have reported a mechanism by which the Xlefty prodomain may regulate the function of Xlefty and this represents a new study of prodomain functionality in the TGFβ signaling peptides. We believe that studies like these on the Lefty prodomain could potentially lead to the development of small bio-molecule candidates with the capacity to prevent disease manifestations that arise from dysregulated cell-signaling pathways.

Materials and Methods

Frogs and embryo culture.

The frog colony was maintained in an Aquatic Habitats XR5 system (Apopka, FL) chilled by a Marine Biotech System (Apopka, FL) and kept according to Institutional Animal Care and Use Committee (IACUC) and Wayne State Universities Division of Laboratory Animal Resources (DLAR) facility standards. Embryos were obtained by in vitro fertilization, cultured in 1/3 Marc's modified Ringer's solution (MMR), and staged according to Nieuwkoop and Faber (P.D. Nieuwkoop, 1967).

Microinjection.

Embryos were microinjected at 2 through 4 cell stage in 2.5% Ficoll in 1/3 MMR. Synthetic mRNAs were injected in the embryo with the location determined by experiment. All embryos used in these studies were staged using the Nieuwkoop and Faber Table of *Xenopus laevis* development (P.D. Nieuwkoop, 1967). For microinjection, a PLI-90 microinjection unit (Harvard Apparatus) fitted with an adjustable Narishige needle mount (Japan) was used. Total RNA concentrations for the different constructs

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ranged from 25pg to 4ng. After microinjection embryos were left to develop in 1/3 MMR with 50ug/ml Gentamycin. For the embryo secretion assay 1/3 MMR was replaced with calcium free conditioned medium (CFCM). Uninjected controls as well as embryos microinjected with inert mRNA (GFP mRNA) at the same concentrations served as controls. Uninjected controls allowed us to show that there is no background phenotype in our wild type embryos. The use of GFP mRNA tests if our results are due to a high concentration of mRNA resulting in embryo toxicity.

Plasmid constructs and synthetic RNA construction.

All constructs were generated by cloning into a pCS2+ vector. These were subsequently transformed into XL1 blue super-competent cells (Stratagene). Epitope tagged and mutant constructs were made by PCR mutagenesis. For pCS2+Lefty Cleavage Mutant 1st site and 2nd site and combined, the 1st and/or 2nd cleavage site were mutated from RXXR to GVDG using a Quick Change Site - Directed Mutagenesis Kit (Stratagene). Clones including pCS2+PD, pCS2+PDless, pCS2+PDmut and pCS2+PDmutXlefty were made using PCR mutagenesis. All constructs were sequenced to confirm the mutations. The 5' primer sequence used for the LCM first cleavage site amplification was 5' CTA TGC TGC ACA GTC ACA GAG AGG GGG TGG ATG GAT CAC TGC CCA GCT TGG CTG GC 3'; second cleavage site 5' GAA CGT CCC AGA GAG GGG AGT CGA CGG ACC AGT CAG CAA TGC 3'; for the PDless forward 5' GAA GCC ATT GAT GGC AGC CAG GCA CAG GG 3'; reverse 5' TCA CTG CCC AGC TTG GCT GGC ATC CTC AG 3'; for the PDmut forward 5' GCT GCT GCT AAA AAA GCT AAC GCT CAA GAG GTT CCC AAA CTG GAG 3'; reverse 5' CTC TTG AGC GTT AGC TTT TTT AGC AGC AGC ATC TCT GAT GTT GTC AGG 3'; for the PD, 5’ GCT CTA GAT CAT CTC CTC TTC CTC TCT 3’; and for PD-HA, 5’ GCT

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CTA GAT CAG GCA TAG TCT GGG ACG TCA TAT GGA TAT CTC CTC TTC CTC TCT CTG TG 3'. The 5' primer sequence used for the amplification of Xlefty-HA was 5' CGG GAT CCA GAA TGG GTG TCA CTA CCA 3'. The 3' primer sequence for amplification of Xlefty-HA was 5' ACG GGA TCC GTC AGG CAT AGT CTG GGA CGT CAT ATG GAT ATA TTA TAG CGA TAT TG 3'. Myc-tagged Xlefty was generated by amplifying the Xlefty cDNA from pCS2+Xlefty (5' CGG GAT CCA GAA TGG GTG TCA CTA CCA 3' and 5'CGG GAT CCT ATT ATA GCG ATA TTG TCC 3'), digesting the product with BamHI and cloning it into pCS2+MT(Rupp et al., 1994; Turner and Weintraub, 1994). For all HA-tagged constructs, a single HA epitope was inserted at the C-terminus of Xlefty or its truncations. Capped mRNAs for microinjection were synthesized using the SP6 mMessage mMachine (Ambion) for pCS2+Xlefty and its mutated derivatives. For in situ hybridization, RNAs were transcribed with the MAXIscript protocols (Ambion).

Protein quantification and analysis.

The mutated Xlefty mRNAs were microinjected to assess proper translation and stability by western blotting. Whole embryo protein extraction was done following similar methods as described in Yeo and Whitman (Yeo and Whitman, 2001). Following microinjection, ten embryos were harvested at stage 10.5 and homogenized by lysis in 1X lysis buffer (200ul 5X IP buffer [2.5ml Tris-HCL pH 7.5, 1.5ml 5M NaCl, 200ul 0.5M EDTA, 1.0ml 0.5M NaF, 4.7ml 106mM $Na_4P_2O_7$, 100ul 0.1M Na_3VO_4], 100ul mini EDTA-free protease inhibitor solution (Roche), 50ul 10% Triton (FisherSci), 650ul H2O). Proteins were then quantified by using the Modified Lowry Protein Assay Kit as per the manufacturer's directions (Thermo Sci). Using an Xcell Surelock system (Invitrogen), gel electrophoresis of 10µl protein sample was run on an SDS-PAGE gel for

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90 minutes at 125 volts. 10µl of uninjected control embryos served as negative controls. An Xcell blot module (Invitrogen) was used for blotting onto a polyvinylidene flouride (PVDF) transfer membrane (GE Healthcare). Western hybridization was carried out using rabbit anti-HA polyclonal primary antibody (Covance), anti-MYC (Covance) or anti-GAPDH (Millipore) followed by incubation with goat anti-rabbit (Jackson), rabbit anti-mouse (Covance) or mouse anti-rabbit secondary antibodies conjugated with hydrogen peroxidase (Covance). Detection was performed with the ECL Plus Western Blotting Detection System (GE Healthcare).

Co-immunoprecipitation.

Embryos were harvested at stage 10.5 and lysed with 1X lysis buffer extracts and then incubated with 1:1000 primary antibody and rotated overnight in 4C. The following day proteins were incubated for two hours at RT rotating with Protein G Agarose plus slurry beads (Pierce). Samples were eluted by centrifugation for 2-3 minutes at 2,500 x g. This step was repeated several times with the supernatant being removed. Electrophoresis loading buffer (Fisher Sci) was added to the complex bound resin and incubated for 5 minutes at 95^{o}C. The contents were centrifuged and evaluated by SDS-PAGE using standard western blotting procedures (see above).

Morphological studies

To begin characterizing the role of our mutated constructs in the embryo, the phenotype of the microinjected embryos over-expressing our study mRNA was assessed. Embryos were scored at gastrula (stage 10-11), neurula (stage 18-20) and later organogenesis stages (stage 36/38) using an MZ75 Leica Stereomicroscope. Embryos were fixed with 1X MEMFA (MEMFA salt 10X to make up 1X, 10% formaldehyde, H_2O) overnight at 4^{o}C and washed with PBS-T the following day. Embryos were placed

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on an agarose-covered Petri dish and microphotographs were taken with a Leica MZ 16F fluorescent stereomicroscope mounted with a Leica DFC 425C color camera. At stage 10-11 the embryos were scored for blastopore formation. Microinjected embryos were compared with uninjected controls to determine whether proper formation and development of the blastopore was occurring. At neurula stages the embryos were scored for proper neural fold fusion. At later stages, embryos were anesthetized with 0.01% benzocaine in culture media (1X MMR) and scored.

***In situ* hybridization.**

In situ hybridization was carried out on *Xenopus* embryos as per the Early Development of *Xenopus* Embryo lab manual (Hazel L. Sive, 2000). Briefly, embryos were devitellinated and the blastocoel was pierced with a 16-gauge needlepoint (BD syringe). Embryos were transferred to a 5ml screw-cap glass vial (Fisher Sci) and fixed in MEMFA overnight at 4^{o}C. Embryos were the stored the following day in ethanol at -20^{o}C. For hybridization, baskets were used for the subsequent steps. Embryos were permeabilized with Proteinase K followed by incubation overnight at 60^{o}C in hybridization buffer containing probes at a concentration of probes for Nodal-dependent markers Goosecoid (Gsc) and Brachyury (Bra) were made by the suggested method in the *Xenopus* lab manual (Hazel L. Sive, 2000). Embryos were then rinsed with fresh hybridization and SSC buffer and treated with RNase A and RNase T. Embryos were washed in MAB buffer and then incubated overnight at 4^{o}C in MAB containing 2% BMB blocking reagent with a 1/2000 dilution of the affinity-purified anti-digoxigenin antibody coupled to alkaline phosphatase. The following day the embryos were washed in MAB for five 1hr washes. The last wash was replaced with 4.5 ul/ml NBT and 3.5ul/ml BCIP. Staining took approximately five hours for our probes after which we refixed our

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embryos in 4% paraformaldehyde overnight and stored them the following day in PBS-T. For microphotographs, uninjected embryos served as controls and embryos were placed in an agarose-covered Petri dish and microphotographs were taken with a Leica MZ 16F fluorescent stereomicroscope mounted with a Leica DFC 425C color camera.

Embryo secretion assay.

An *in vivo* secretion assay was developed to provide a method to test the secretory properties of Xlefty and its mutated derivatives. Embryos at the 4-cell stage were microinjected in the dorsal animal hemisphere blastomeres with mRNA encoding Xlefty or its mutant forms. The dorsal animal hemisphere blastomeres were targeted because cells from this region are fated to become the tissue that lines the roof of the blastocoel cavity (Moody, 1987). These cells then directly secrete protein products into their surrounding microenvironment including the blastocoel cavity. The blastocoel, a space within the embryo, forms during the blastula stage of development in *Xenopus*. During embryogenesis the blastocoel grows in size and is at its largest volume prior to gastrulation (stage 8-9). At stage 8-9, a small opening was made at the animal pole with fine tipped forceps to expose the blastocoel to the secretion-conditioned medium. At this point, the embryos are incubated in calcium-magnesium free media (CMFM) for 3 hours to allow for secretion. The CMFM prevents the opening in the embryo from healing, which would seal off the inner layer of cells responsible for secretion. The secreted protein products are then secreted into the conditioned medium and harvested at the end of the incubation period of 3 hours. At the end of the incubation time, the conditioned medium was collected and lysis buffer was added. Additionally the embryos are harvested, lysed, spun and the supernatant is collected. To test for debris in the secretion medium and to ensure that the result represented true secretion by the cells lining the

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blastocoel roof, GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) (Millipore), a non-secreted protein found only in the nucleus and cytoplasm and not secreted, was tested as a control in the conditioned medium.

Cell culture secretion assay.

Mouse Embryonic Fibroblast (MEF) cells were cultured on DMEM (Dulbecco's Modified Eagle's Medium) (Gibco, Invitrogen) containing FBS (fetal bovine serum) (Invitrogen), and supplement (Invitrogen) on petri culture dishes (Fisher Sci). When cells were observed to be 75-80% confluent, cells were harvested by trypsinization (trypsin, Invitrogen) and transformation was carried out using the Nucleofection method (Lonza). After nucleofection the cells were cultured in a humidified incubator at 37^{o}C, 5% $C0_2$ (Fisher Sci) for 24 or 48 hours respectively. At both time points conditioned medium and cells were harvested by first removing the conditioned medium and adding 200ul 1X lysis buffer, followed by treating the cells with 200ul lysis buffer and incubating them for 20 minutes at room temperature. Cells were then harvested by lifting the cells with a cell scraper (Invitrogen). Cells were then spun at 7000 Gs in a microcentrifuge at 4^{o}C to remove all cell debris. Protein samples were stored at -80C until further analysis was warranted.

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ABSTRACT

THE XENOPUS LEFTY (XLEFTY) PRODOMAIN NEGATIVELY REGULATES XLEFTY ACTIVITY AND IS NECESSARY FOR PROPER XLEFTY SECRETION

By

ADRIAN AMELIO VASQUEZ

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Advisor: Dr. William Branford

Major: Biology

Degree: Master of Science

In vertebrates, the TGFβ superfamily of secreted peptides are stringently regulated since they are responsible for multiple cell processes and behaviors that give rise to the proper patterning of embryonic tissues. Examples of members of the TGFβ superfamily are Nodal and Lefty. Dysregulation of these proteins can lead to many diseases and developmental syndromes in humans.

Lefty functions by antagonizing Nodal an essential organizer signal that patterns dorsal mesoderm and the embryonic axes. Loss of Lefty expression results in excess Nodal signaling which has been shown to cause several perturbations including metastatic cancer. Although studies have clearly shown that Lefty antagonizes Nodal signaling, there is a lack of understanding of the regulatory mechanism of Lefty itself. Previous studies have shown that proteolytic cleavage of the prodomain (PD) from mature Lefty (Mat-Lefty) is necessary for Lefty activity.

Here we present studies carried out using the *Xenopus laevis* embryo that demonstrate that PD over-expression causes exogastrulation, a phenotype also resulting from a loss of *Xenopus* Lefty (Xlefty) function. Furthermore, when the PD and Xlefty

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are co-expressed, the effects of Xlefty over-expression are rescued. Our biochemical studies also showed that the PD interacts with Mat-Xlefty but a PD mutated (PDmut) molecule does not. The sites mutated in the PDmut are evolutionarily-conserved residues that mediate the interaction between the prodomain and the mature ligand in other TGFβ proteins making our results consistent with previously observed behavior of TGFβ prodomains but new to Lefty. Our study also shows that the PDmut is unable to rescue the effects of a Xlefty over-expression phenotype unlike the un-mutated PD.

In addition we show here that mutations of the PD affect the secretion of these Xlefty-mutated derivatives. Taken together, these results suggest that the PD negatively regulates Xlefty activity by interacting with Mat-Xlefty and cleavage of the PD releases regulation allowing proper secretion and function. This new insight into the regulatory role of the Xlefty PD provides potential therapeutic value to address dysfunctional Nodal signaling. Furthermore, our secretion studies of the PD and Xlefty revealed that Xlefty is secreted but the PD is not. The Xlefty-mutated derivatives, including a PD Less, Xlefty, Xlefty cleavage mutants and the PDmut, resulted in no secreted products. These secretion results open a Pandora's box and further studies are warranted to elucidate the mechanism of PD regulation of Xlefty and whether it occurs in an intracellular or extracellular context.

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AUTOBIOGRAPHICAL STATEMENT

ADRIAN AMELIO VASQUEZ

My pursuit of the knowledge of biology has existed since my formative years growing up in the biologically diverse country of Belize; I always dabbled in making mock laboratories and intensely studied the creatures that abounded in the nearby tropical forests and seas. Growing up in this "natural lab" within a multi-cultural society fueled my scientific creativity that I am now putting to use in the laboratory. I intend to establish research-learning centers and collaborate with academic bodies in countries that are under-represented in the sciences creating portals to promote a global appreciation and preservation of science with an indigenous flavor. I would achieve this via several objectives such as establishing a nexus of science and technology based initiatives where I could collaborate on novel research or curriculum development on a vast array of biological topics such as molecular ecology and cellular developmental biology. I am also pivoting towards budding young scientists to agitate and motivate their scientific interests and provide a roadmap for their career objectives.

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