THE EFFECT OF POST-TRANSLATIONAL MODIFICATIONS ON XLEFTY FUNCTION

By

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CHAPTER I

INTRODUCTION

Intercellular communication is absolutely required for proper cell fate specification during early embryogenesis. This process often involves secreted ligands that travel from the source of protein production to responding cells where they bind to their cognate receptors on the cell membrane. This ligand-receptor interaction results in the activation of intracellular signal transduction programs that lead to the expression of a specific collection of genes that are responsible for cell fate determination. Although numerous intercellular signaling pathways have been identified that play a role in cell fate specification, my studies have focused on the Transforming Growth Factor beta (TGFβ) superfamily and specifically two family members, Nodal and Lefty. Nodal signaling has emerged as a crucial and evolutionary conserved dose-dependent inducer of mesendoderm during gastrulation, and is required at later stages for left-right (L-R) axis specification. Many recent data suggest that a precise balance between positive and negative influences is required to generate a proper Nodal activity gradient during embryogenesis. The experiments described in this thesis demonstrate that Lefty, the primary Nodal antagonist, undergoes several post-translational modifications and that some of these modifications are required for Nodal-blocking function. A brief introduction will include information about morphogen activity gradients and the mechanisms that

generate or regulate such gradients. This will be followed by a description of Nodal signaling and the role of Lefty molecules in modulating Nodal activity. Finally, I will end with a brief description on the structure of Lefty-related molecules and describe the types of post-translational processing that Lefty may undergo.

Nodal ligands as morphogens

Morphogen activity gradients

A morphogen is defined as a secreted signaling molecule that patterns a field of cells by activating characteristic sets of target genes in a concentration-dependent manner. Morphogens are generally thought of as being distributed along a gradient with a higher concentration near the source of protein production (Green, 2002). Responding cells then perceive their position within the morphogen gradient in order to determine the appropriate developmental fate. There is developing evidence that morphogen-like characteristics are carried by proteins in the Hedgehog, Wingless, and TGFβ families (Tabata and Takei, 2004). Recently, FGF (Fibroblast growth factor) family members have also been shown to have morphogen-like behaviors. A good example of the concentration-dependent dose response relationship was shown by recent studies on FGF2, where it was demonstrated that different concentrations of the FGF2 protein were able to induce different levels of *Hox-c* expression in spinal motor neurons (Liu et al., 2001). In addition, Dubrulle and Pourquie (2004) have

demonstrated that a gradient of FGF8 protein results in a graded response in the phosphorylation of Akt, a well-known downstream target of FGF signaling (Schlessinger, 2000).

Since the action of a single morphogen can lead to the specification of multiple cell fates, the embryo needs to have in place mechanisms that not only generate but strictly control morphogen gradient formation. Intense research in a multitude of organisms has begun to dissect these mechanisms. One way to control the formation of these gradients would be to regulate the transcription and translation of both the morphogen and its extracellular antagonist. A good example of translational control was demonstrated by the fact that *fgf2* antisense RNA transcripts seem to govern the amount of protein produced from translation of *fgf2* mRNA (Li and Murphy, 2000). The stability or the rate of degradation of the mRNAs encoding these morphogens is another method to control the activity gradient. This mechanism is the result of the binding of trans-acting proteins and RNAs to cis-elements in the mRNA message to either promote or inhibit degradation by RNases (Dibrov et al., 2006). In vertebrate axis elongation, the role of mRNA degradation, in particular *fgf8*, was shown to contribute to formation of the FGF8 protein gradient (Dubrulle and Pourquie, 2004).

Another way to achieve the generation of a morphogen gradient would be to control the amount of extracellular secreted ligand available for effective signaling, which could be done by modulating the level of endocytic clearance from the extracellular compartment (Cadigan, 2002). Experiments using *dynamin* mutant clones in *Drosophila* wing imaginal discs, and overexpression of

dominant negative components of the endocytic pathway in zebrafish, have demonstrated that endocytosis controls the amount of Wingless and FGF8 available for signaling (Scholpp and Brand, 2004; Marois et al., 2006). However, the role of endocytosis in generating the Dpp (Decapentaplegic) gradient in *Drosophila* wing imaginal discs is controversial. Entchev et al. (2000) suggested that Dynamin-mediated endocytosis was required for the long-range movement of Dpp while Belenkaya et al. (2004) reported that Dpp movement was independent of Dynamin function.

Interactions with cell surface proteins, such as heparan sulfate proteoglycans (HSPGs), have been shown to both positively and negatively alter the distance that morphogens move from the source of their production (Ohkawara et al., 2002; Belenkaya et al., 2004; Han et al., 2004; Lin, 2004). This interaction is thought to occur electrostatically, through the basic amino acids of HSPG-interacting proteins associating with the negatively charged heparan sulfate (Irie et al., 2003). For Hedgehog, Wg, and Dpp, HSPGs have been demonstrated to permit the long-range movement of these molecules in *Drosophila* wing imaginal discs (Belenkaya et al., 2004; Takei et al., 2004). The interaction between HSPGs and BMP-4 has been tested in *Xenopus* embryos (Ohkawara et al., 2002). When three basic amino acids (R-K-K) were deleted from the N-terminus of the ligand domain of BMP-4 (Δ-BMP-4), the signaling range of Δ-BMP-4 was increased compared to wild-type BMP-4. This increase was caused by Δ-BMP-4 having a lower capacity to bind heparin compared to the wild-type molecule. It has been postulated, based on the data presented above, that extracellular

“molecular highways” are generated by HSPGs that facilitate the long-range movement of morphogens (Strigini, 2005). This model suggests that morphogens are effectively transferred from one sugar chain to another sugar chain on the large proteoglycans, with a net movement of morphogen down the concentration gradient (Strigini, 2005).

Cleavage and post-translational modification of extracellular signaling molecules

Members of the subtilisin-like proprotein convertase family (SPC) are calcium-dependent serine endoproteases that recognize the R-X-X-R motif found in many intercellular signaling molecules, including TGFβ superfamily members (Nakayama, 1997; Molloy et al., 1999). There are seven vertebrate family members, of which SPC1, SPC4, and SPC6 have been shown to mediate cleavage of both Lefty and Nodal in several transfected cell lines (Ulloa et al., 2001; Beck et al., 2002; Sakuma et al., 2002; Nelsen et al., 2005). The tissue distribution and even the intracellular localization seem to vary among the various SPCs. The prevailing idea used to be that SPCs functioned intracellularly within the source cells to process the precursors of peptide hormones and other proteins into their biologically active forms (Bergeron et al., 2000). Recently, however, several reports suggested that SPCs may also function at the cell surface, or even become secreted into the extracellular milieu and, therefore, act non-cell-autonomously on proproteins secreted from adjacent or nearby cells. For example, endogenous SPC6A has been detected at the surface in adult mouse liver, duodenum, and jejunum (Nour et al., 2005).

Another relevant example came from work by Daniel Constam and colleagues. They demonstrated that, at embryonic day 5.5, mouse *nodal* and *spc1/spc4* were expressed in the epiblast and extraembryonic ectoderm, respectively (Beck et al., 2002). The model arising from these findings is that proprotein cleavage may occur in the extracellular space (Beck et al., 2002). In agreement with this supposition, an artificial system was used to demonstrate that cleavage of mouse Nodal and Lefty could occur by secreted SPCs secreted from COS1 (Beck et al., 2002). In this system, conditioned medium from COS1 cells expressing Lefty and Nodal proproteins were incubated with soluble SPCs, which were secreted into the condition medium by COS1 cells expressing *spc1*, *spc4*, and *spc6* (Beck et al., 2002).

In addition to control being exerted at the level of proprotein cleavage, secondary modifications of the core polypeptide could easily be thought of as modifying interactions with extracellular components and with the cognate receptors. Carbohydrate addition, such as N-linked glycosylation, can affect a myriad of biological processes. It may stabilize the protein against denaturation and proteolysis, influence protein folding, increase protein solubility, and provide structural rigidity to the core protein (Helenius and Aebi, 2004). The presence of N-linked glycans can increase the secretion and biological activity of several TGFβ–related members. For example, both TGFβ1 and TGFβ2 contain several glycosylation motifs in the prodomain and require N-linked glycans for efficient secretion of the ligand into the culture medium (Sha et al., 1989; Brunner et al., 1992; Lopez et al., 1992). Since its discovery, mouse Nodal has been

notoriously difficult to detect when secreted from several mammalian cell lines, but the addition of an artificial glycosylation motif into the ligand domain increased its steady-state level of protein accumulation, signaling strength, and range of signaling compared to the wild-type protein (Le Good et al., 2005). These results suggest that glycosylation may play important functions in controlling secretion and stability of TGFβ molecules.

Nodal signaling in vertebrate embryos

Although Nodal signaling seems to involve a specific complex of TGFβ receptors and cofactors (see below), ligand maturation and initiation of the signaling cascade follow the general rules of all TGFβ molecules. Nodal is initially synthesized as a large pre-proprotein with an N-terminal hydrophobic signal sequence for secretory pathway targeting, a prodomain, and a C-terminal mature ligand domain (Kingsley, 1994). As described above, maturation of the pre-proprotein involves proteolytic cleavage at the dibasic R-X-X-R motif by SPCs to release the mature ligand from the prodomain in the form required to engage the receptor complex for active signaling (Kingsley, 1994; Nakayama, 1997; Cui et al., 1998; Constam and Robertson, 1999; Molloy et al., 1999; Ulloa et al., 2001; Beck et al., 2002; Sakuma et al., 2002; Ben-Haim et al., 2006). Most TGFβ family members contain 7-9 conserved cysteine residues of which six are involved in generating a structure referred to as a “cysteine knot”. One of these cysteines will form a disulfide bond between two monomers to generate the biologically active homodimer or heterodimer (Sun and Davies, 1995). These

primary structure characteristics (dibasic cleavage motif and 7 cysteine residues) are contained within the amino acid residues of mouse Nodal and several of the Xenopus Nodal-related proteins (Xnr), such as Xnrs1, 2, 4, 5, and 6 (Conlon et al., 1994; Jones et al., 1995; Joseph and Melton, 1997; Takahashi et al., 2000).

In order to initiate downstream signaling, the Nodal dimer engages a receptor complex consisting of type I and type II Activin receptors that both possess intracellular serine/threonine kinase domains (Fig. 1.1) (Massague, 1998; Schier, 2003). Biochemical and genetic evidence has demonstrated that, unlike Activin, for example, the activation of Nodal signaling also requires the presence of the EGF-CFC co-receptor (Gritsman et al., 1999; Whitman, 2001; Yeo and Whitman, 2001; Schier, 2003; Dorey and Hill, 2006; Onuma et al., 2006). The downstream result of Nodal-receptor complex interaction is, similar to Activin, however, involving the phosphorylation of either Smad2 or Smad3, which then associates with Smad4 (Schier, 2003). The activated Smad complex becomes translocated to the nucleus in association with a more sequence-selective DNA-binding co-factor, such as FoxH1, where it acts to induce the transcription of Nodal-target genes (Whitman, 2001; Schier, 2003).

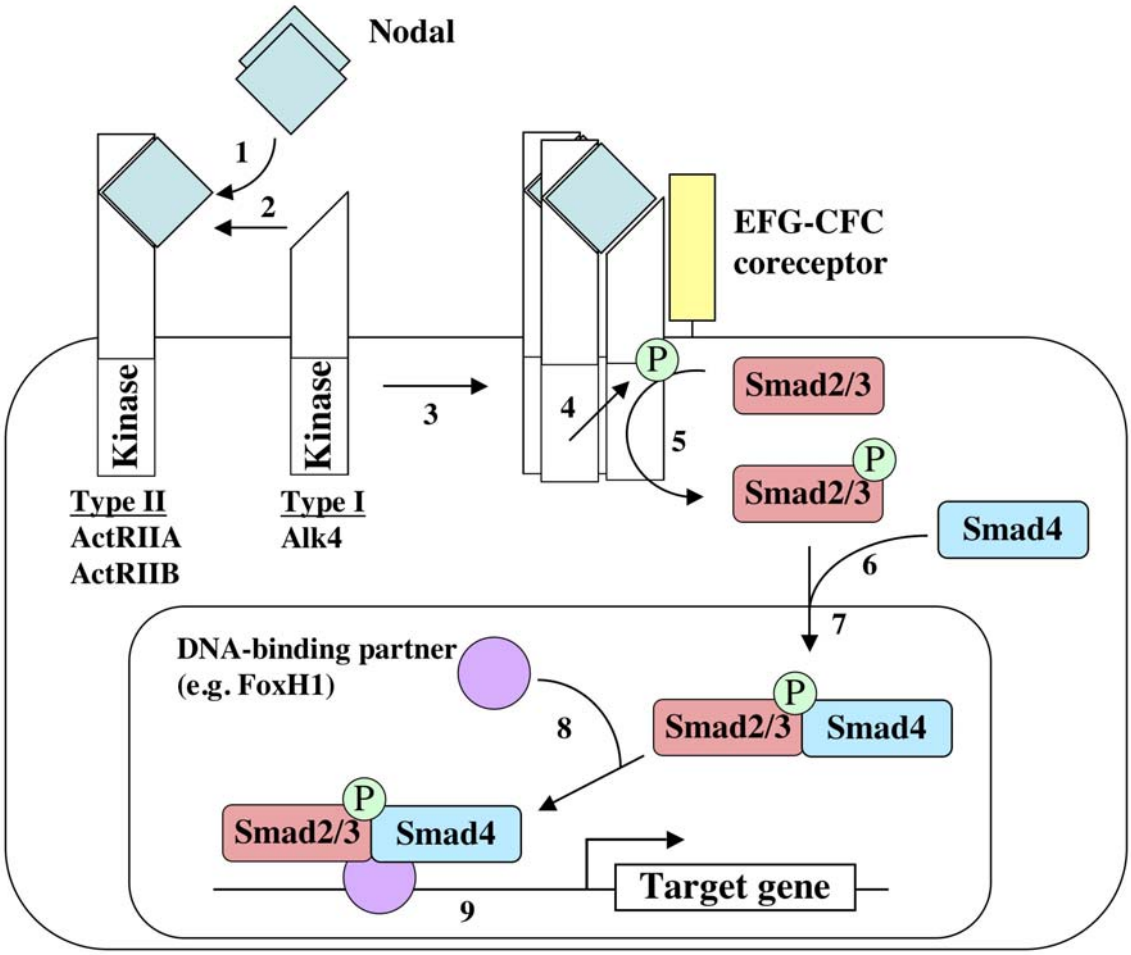

Figure 1.1 Nodal signal transduction pathway. Binding of the Nodal dimer to the ActRIIA/B type II receptor (1) in combination with the Alk4 type I receptor (2) and the EGF-CFC co-receptor leads to formation of an activated receptor complex (3) and phosphorylation of the type I receptor (4). Thus activated, the type I receptor phosphorylates Smad 2 or Smad3 (5), allowing this complex to associate with Smad4 (6). The activated Smad complex translocates to the nucleus (7) and associates with DNA-binding partners, such as FoxH1 (8) resulting in transcription of downstream target genes (9). Modified from Massague, 1998.

There are several proteins that both positively and negatively influence Nodal signaling during embryogenesis. Some of these proteins have a broad effect for the various TGFβ family members, whereas some appear relatively restricted in affecting Nodal/Activin signaling. For example, Smad6 and Smad7 are proteins utilized by both TGFβ and Nodal pathways to attenuate signaling by competitively binding to activated receptor complexes and recruiting E3-ubiquitin ligases to target the receptors for degradation (Kavsak et al., 2000; Suzuki et al., 2002). An additional function of Smad6 and Smad7 includes binding to Smad4 to prevent Smad2/3-Smad4 complex formation (Massague, 1998). Processes that induce the degradation of Smad4 can curtail TGFβ signaling. For example, Ectodermin, a RING-type ubiquitin ligase, is responsible for terminating TGFβ signaling by catalyzing the addition of poly-ubiquitin to Smad4, leading to its proteosome-mediated degradation (Dupont et al., 2005). Proteins that remove phosphate moieties from Smads can also attenuate signaling. Recently, several phosphatases, such as PPM1A and small C-terminal domain phosphatases, have been found to reside in the nucleus and dephosphorylate Smad proteins, leading to termination of signaling (Duan et al., 2006; Knockaert et al., 2006; Lin et al., 2006; Sapkota et al., 2006; Wrighton et al., 2006). On the other hand, proteins that inhibit the function or induce the degradation of such negative regulators of signal transduction can enhance TGFβ signaling. For example, Arkadia, a RING domain E3-ubiquitin ligase, can induce the proteosome-dependent degradation of several negative regulators (Smad7, SnoN, and c-Ski)

of the TGFβ pathway and leads to enhanced signaling (Liu et al., 2006; Nagano et al., 2007).

As compared to the proteins discussed above, which seem to regulate TGFβ pathways, there are several molecules that appear to modulate only Nodal/Activin signaling. Recently, Suri et al. (2005) and Mir et al. (2007) used subtractive hybridization and microarray technologies to discover factors that were down-regulated by Xnr/Activin signaling. Both groups independently identified the transcription factor gene *Xema/Foxi1e* and showed that it was expressed in the animal region, where it functioned to suppress mesendoderm formation. Data gathered using both transactivation domain (VP16) or repression domain (EnR) fusion proteins suggested that Foxi1e either directly or indirectly stimulated the transcription of as yet unknown inhibitors of mesendodermal fates (Suri et al., 2005). The experimental findings to date suggest that it acts as an endogenous suppressor of mesodermalization to force the embryo to overcome a “mesoderm induction threshold”, thus ensuring correct apportioning of tissue fates across the embryo.

Nodal, an inducer of mesendoderm

The formation of the three primary germ layers, ectoderm, mesoderm, and endoderm, is a critical patterning event of early embryogenesis, which sets the stage for all future inductive events. Early tissue grafting experiments by Nieuwkoop and others showed that the mesoderm induction process results from signals that emanate from the vegetal region to specify the overlying equatorial

region (Harland and Gerhart, 1997). Subsequently, heterochronic tissue grafts suggested that the endogenous mesoderm-inducing factor was a maternally supplied protein or RNA (Harland and Gerhart, 1997). At the time of these experiments, the only inducer that fulfilled this requirement was Activin (Asashima et al., 1990; Slack, 1990; Smith et al., 1990). The subsequent discovery of *Vg1* mRNA, localized to the vegetal hemisphere in *Xenopus* embryos, suggested that this TGFβ molecule was the long-sought-after inducer (Weeks and Melton, 1987; Pondel and King, 1988). Further experiments, however, cast doubt on the role of both Activin and Vg1 in patterning the early embryo. Whereas overexpression of many TGFβ members induced mesoderm, injection of *Vg1* RNA did not (Tannahill and Melton, 1989) and strong inducing properties of the Vg1 ligand were only seen when it was fused to a BMP prodomain region (Thomsen and Melton, 1993). Moreover, the endogenous ligand domain of Vg1 could not be detected in embryos (Tannahill and Melton, 1989). Recently, Janet Heasman and coworkers have made clear progress toward resolving the role of Vg1 in embryonic patterning. They discovered a new isoform of Vg1, which contained a serine instead of a proline at amino acid position 20. This version had mesoderm inducing properties when overexpressed in *Xenopus* embryos without the need to resort to the chimeric prodomain/ligand approach referenced above (Birsoy et al., 2005; Birsoy et al., 2006). Evidence against Activin being an endogenous mesoderm inducer came from genetic studies in mouse, in which *activin βA* and *activin βB* have been mutated using homologous recombination techniques. These homozygous null

mutant mice progressed normally through embryogenesis and contained normally patterned mesoderm (Matzuk et al., 1995). The role of Activin in patterning the early embryo has been further clouded by two recent reports from the laboratory of James Smith (Piepenburg et al., 2004; Ramis et al., 2007). In the first report, morpholino-mediated knockdown was concluded to show that endogenous Activin was involved in mesoderm induction, as rt-PCR assays showed that there was a reduction in the expression of mesodermal marker genes within the embryo (Piepenburg et al., 2004). Later experiments using microarray analysis of embryos injected with the same morpholino targeted against *activin* (described above) suggested that Activin signaling functioned to control cell division (Ramis et al., 2007). It is puzzling why the same morpholino used in the same lab would generate data suggesting different functions of Activin.

The initial evidence that Nodal was the endogenous mesendoderm inducer came from a mouse mutant, 413.d, that carries a retroviral insertion-mediated inactivation of the *nodal* locus (Zhou et al., 1993; Conlon et al., 1994). The homozygous mutant mice fail to form a primitive streak and lack most mesoderm and endoderm. Following the isolation of Nodal in mice, Nodal-related proteins have been identified in all vertebrate species. In *Xenopus*, six nodal-related proteins (Xnrs1-6) have been isolated, of which Xnrs1, 2, 4, 5, and 6 have been shown to possess mesoderm-inducing activities (Jones et al., 1995; Smith et al., 1995; Joseph and Melton, 1997; Takahashi et al., 2000). Xnr3 is more divergent, as it lacks the last of the seven cysteines and has a serine instead of a glycine

between the second and third cysteines (Ezal et al., 2000). Probably, the most important function for Xnr3 is its role in regulating convergent and extension movements in *Xenopus* (Yokota et al., 2003). *Squint (sqt)*, *cyclops (cyc)*, and *southpaw (spaw)* are the three *nodal*-related genes known in zebrafish (Erter et al., 1998; Sampath et al., 1998; Long et al., 2003). In gain-of-function studies, mouse Nodal, Cyc, Sqt and Xnrs1, 2, 4, 5, and 6 can dose-dependently induce mesoderm when overexpressed in *Xenopus* animal caps (Jones et al., 1995; Joseph and Melton, 1997; Erter et al., 1998; Sampath et al., 1998; Takahashi et al., 2000). Consistent with the loss-of-function data from mouse, null mutations in *cyc* and *sqt* produce a highly mesoderm-deficient embryo (Feldman et al., 1998). Similarly, loss-of-function experiments in *Xenopus* revealed the conserved mesoderm-inducing activities of Xnrs. Overexpression of Xnr-specific inhibitors, such as cleavage-mutant Xnr2, a Nodal-specific truncation version of the secreted antagonist Cerberus-short, as well as Lefty, resulted in reduction or elimination of mesendodermal derivatives (Osada and Wright, 1999; Agius et al., 2000; Cheng et al., 2000; Onuma et al., 2002). Altogether, these data firmly establish the role of Nodal/Xnr signaling in the dose-dependent formation of mesendoderm in vertebrate embryos.

Nodal signaling in left-right axis formation

Vertebrates exhibit a stereotypical and conserved left-right asymmetry in, for example, the differential lobation of the lungs, the placement of visceral organs and the morphogenesis of the cardiovascular system (Wright, 2001; Hamada et

al., 2002). In zebrafish, anatomical asymmetries have been identified in the forebrain region, such as the diencephalon and parapineal gland (Gamse et al., 2003; Long et al., 2003). Deviations from the normal asymmetric placement of the internal organs, which is referred to as situs solitus, can lead to randomization of organ placement (heterotaxia) or complete reversal of organ symmetry (situs inversus). Although situs inversus is not harmful, heterotaxia can have severe medical consequences, such as cardiovascular connection abnormalities and atrial and ventricular septal defects. (Ramsdell, 2005). Such congenital heart defects occur in approximately 90% of individuals exhibiting heterotaxic phenotypes (Ramsdell et al., 2006).

Initially, vertebrate embryos are bilaterally symmetric but various species-specific mechanisms have been discovered that break this embryonic symmetry (Raya and Belmonte, 2006). As a result of early breaking of embryonic symmetry, at around tailbud stage (the equivalent of 2-3 somites in mouse), there is a transient expression of a “left-side gene cassette” (*nodal*, *lefty* and *Pitx2*) in the left lateral plate mesoderm (LPM). This conserved feature is likely a central event leading to asymmetric morphogenesis (Lowe et al., 1996; Wright, 2001). During neurula stage in *Xenopus*, the expression of *Xnr1* is observed as bilaterally symmetrical domains flanking the posterior notochord (Lowe et al., 1996). By a mechanism that is not at all well understood, *Xnr1* expression then becomes activated asymmetrically with higher levels on the left LPM and an extremely low level on the right side (Nakamura et al., 2006). Recent work in the Wright lab has demonstrated that planar tissue communication is required for a

rapid posterior-to-anterior (P-to-A) expansion of the *Xnr1* expression domain. Subsequently, *Xnr1* expression becomes rapidly suppressed in a P-to-A manner (Ohi and Wright, 2007). In the left LPM, the expression domain of *Xenopus lefty* (*Xlefty*) spatially mirrors that of *Xnr1* but with a temporal delay in accordance with its activation as a direct target of Nodal signaling (Ohi and Wright, 2007).

As described in detail below, Xlefty inhibits Xnr signaling in both mesendoderm induction and during L-R axis formation. In Chapter III, I will provide evidence that Xlefty undergoes N-linked glycosylation. Nevertheless, my assays showed that glycan addition did not influence either the ability of Xlefty to regulate Xnr signaling during mesoderm induction or the movement of Xlefty through embryonic tissues of blastula-stage embryos. Since carbohydrates are a source of negative charges and may interact with basic domains on extracellular proteins (Janosi et al., 1999), it is a possible that glycans can influence the speed of movement of Xlefty during stages of L-R axis formation in *Xenopus*. If "molecular highways" (described above) exist in the left LPM of *Xenopus* embryos, there is a possibility that N-linked glycans may affect the path that Xlefty travels within the embryo.

Negative feedback regulation of Nodal signaling by Lefty

Lefty-related proteins have been identified in chordates ranging from the very primitive *Ciona* to higher vertebrates like mouse and human. Although several biological functions have been proposed for Xlefty (Chapter IV), the general consensus is that a principal activity of Lefty is to antagonize Nodal signaling.

Lefty molecules are thought to limit the strength, range of signaling, and duration of Nodal signaling during mesendoderm induction, thereby ensuring the formation of the proper amount and type of mesendoderm within the embryo (Branford and Yost, 2002; Chen and Schier, 2002; Feldman et al., 2002; Cha et al., 2006). The importance of Lefty molecules in regulating Nodal signaling has been demonstrated by loss-of-function and gain-of-function experiments in mice, zebrafish, and frogs. For example, mice deficient for *lefty2* resulted in expansion of the primitive streak and excess formation of mesoderm (Meno et al., 1999). This phenotype can be partially rescued by reducing the gene dosage of *nodal*, consistent with the idea that overproduction of mesoderm in *lefty2* mutants resulted from increased Nodal signaling (Meno et al., 1999). Experiments performed by overexpressing Lefty proteins in zebrafish produced a phenotype that closely resembled *cyc;sqt* double mutants or maternal-zygotic *oep* mutants, a mutation in the Nodal pathway-required EGF-CFC co-factor (Meno et al., 1999). *Xenopus* Lefty was isolated by Abby Cheng, a former graduate student in the Wright laboratory, who showed that Xlefty overexpression suppressed Xnr signaling in mesoderm induction experiments (Cheng et al., 2000). Subsequently, Young Cha, another former lab member, demonstrated the importance of Lefty by morpholino-mediated knockdown of the Xlefty protein. These embryos showed massive expansion in the expression of Xnr-regulated genes. For example, *Xbra* expression in morphant gastrula stage embryos often encompassed the entire animal hemisphere (Cha et al., 2006).

Initially, Lefty-related proteins were thought to inhibit Nodal signaling at the level of membrane-bound Activin type II receptors (ActRII), as overexpression of membrane-bound ActRIIA, as well as the extracellular domain of ActRIIB, suppressed the antagonistic effects of Lefty (Meno et al., 1999; Thisse and Thisse, 1999). Subsequent studies, however, suggested a different mechanism of inhibition. Using biochemical extracts prepared from *Xenopus* embryos, and cultured cells that overexpressed various components of the Nodal signaling pathway, there are now data supporting the hypothesis that Lefty antagonizes Nodal signaling by binding either the EGF-CFC cofactor or the Nodal ligand dimer, thereby blocking the Nodal-receptor interaction (Chen and Shen, 2004; Cheng et al., 2004; Tanegashima et al., 2004).

Structure of Lefty-related proteins

Lefty-related proteins are a highly divergent subclass within the TGFβ superfamily (Meno et al., 1996; Thisse and Thisse, 1999; Cheng et al., 2000). Compared to canonical TGFβ members, Lefty molecules have several unique structural features (Fig. 1.2). First, most TGFβ proteins contain a single proteolytic cleavage site in the proprotein, which generates a mature domain that is 110-140 amino acid residues (Kingsley, 1994). Lefty molecules contain two cleavage sites (CS1 and CS2), such that proteolytic cleavage of the proprotein could generate cleaved ligand-like domains of either 220 or 290 amino acids (Thisse and Thisse, 1999; Cheng et al., 2000; Sakuma et al., 2002). Second, the carboxy terminus of most TGFβ-related ligands is CX_1CX_1 while that in Lefty-

related proteins are extended to CX_1CX_{8-13}. Third, Lefty molecules lack the large α-helix and the fourth cysteine residue, both of which are involved in ligand dimerization. Thus, Lefty molecules are thought to function as monomers. Indeed, when secreted from *Xenopus* animal caps, mouse Lefty 1 and Lefty 2 have so far been detected biochemically as monomers, consistent with these structural predictions (Sakuma et al., 2002).

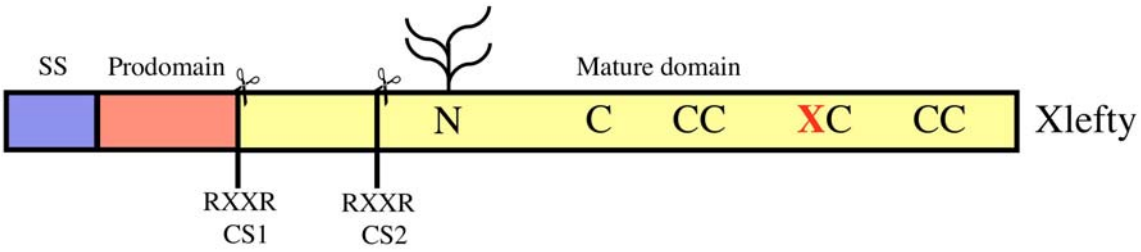

Figure 1.2 Structure of the Xlefty pre-proprotein. Xlefty is synthesized as a large pre-proprotein, which can undergo several post-translational modifications during its maturation. The signal sequence (SS) targets the proprotein to the secretory pathway. There are two potential cleavage sites (CS1 and CS2) in the proprotein that would be expected to generate long and short isoforms depending on cleavage site usage. The one N-linked glycosylation motif ( ) is present in both long and short isoforms. The mature domain is missing the cysteine that is involved in dimerization. C, cysteine; R, arginine; X, any amino acid

All TGFβ-related proproteins undergo proteolytic cleavage in order to generate the active ligand (Kingsley, 1994; Nakayama, 1997; Cui et al., 1998; Constam and Robertson, 1999; Molloy et al., 1999; Ulloa et al., 2001; Beck et al., 2002; Sakuma et al., 2002; Ben-Haim et al., 2006). As stated above, proteolytic cleavage at CS1 and CS2 would be expected to generate long ($Xlefty^{L}$) and short ($Xlefty^{S}$) isoforms depending upon the cleavage site used. Such differential cleavage has of Lefty has been detected in transfected overexpressing cultured cell lines (Meno et al., 1996). When mouse Lefty 1 was secreted from 293T and BALB/3T3 cell lines, proteolytic processing at CS1 and CS2 occurred in a cell type-dependent manner (Sakuma et al., 2002). The reason for differential cleavage of Lefty is not yet understood, but this issue is returned to in Chapter IV. Lefty molecules contain a single consensus site for N-linked glycosylation in the mature domain, and in Chapter III, I present my studies on the characterization and functional evaluation of N-linked glycosylation and proprotein cleavage in *Xenopus* tissues.

The prevalent idea used to be that TGFβ molecules were cleaved at a single SPC motif within the proprotein to allow the release of the ligand for receptor engagement. Recently, however, several reports have suggested that a second cleavage event could influence the biological activity of these secreted molecules. I will describe some of the experimental findings illustrating that cleavage at a second site in the prodomain increases the signaling activity of BMP-4. The idea of using different cleavage sites to regulate the activity of a TGFβ molecule is important because Lefty molecules contain two cleavage sites

that could regulate protein turnover or Nodal-blocking function (discussed in Chapter IV). As a prototypical member of the TGFβ superfamily, BMP-4 is fully expected to require proprotein cleavage to release the active ligand for productive engagement with receptors. In vitro assays demonstrated that *Xenopus* BMP-4 can be processed by SPC1 (Furin), SPC4, SPC6, and SPC7 (Cui et al., 1998). In these in vitro BMP-4 cleavage assays, Jan Christian and colleagues noticed cleavage occurring at an unexpected site in the BMP-4 prodomain (Cui et al., 1998). From a subsequent series of experiments, they determined that BMP-4 experienced an ordered cleavage event that influenced both the strength and range of signaling in *Xenopus* tissues (Cui et al., 2001). The first cleavage occurs at the optimal SPC1/Furin site (Site 1; S1) in the proprotein, which separates the ligand from the prodomain. The second cleavage event takes place at a minimal furin site (Site 2; S2) in the prodomain. Cleavage at S2 is thought to disrupt the non-covalent ligand/prodomain interaction, and the ligand is released for productive receptor binding. By generating an S2 cleavage site mutant, it was determined that preventing cleavage at S2 caused a marked decrease in the secretion of the mature ligand domain (Cui et al., 2001). The decrease in secretion was discovered to be the result of targeting the ligand domain within the producing cells for rapid degradation via the lysosomal/proteosomal pathways (Degnin et al., 2004). Based on the above data, Degnin et al. (2004) suggested that the basal level of BMP signaling is caused by cleavage of BMP-4 at S1 and tissues that required higher levels of signaling cleaved BMP-4 at S2. This hypothesis was supported

by analyzing mice carrying a targeted mutation that prevented cleavage at S2, which led to defects being detected in tissues that required the highest level of BMP signaling (Goldman et al., 2006).

Nodal and Lefty constitute a reaction-diffusion system

The appropriate level of Nodal signaling is a central determination of proper pattern formation during mesendoderm formation and L-R axis specification. Currently, Lefty is thought to act over a long distance to inhibit Nodal signaling. For example, experiments utilizing both zebrafish and *Xenopus* showed that localized injections of *lefty* RNA into the animal hemisphere could suppress Nodal signaling many cell diameters away, at the marginal zone (Branford and Yost, 2002; Chen and Schier, 2002). It has also been demonstrated that green fluorescent protein (GFP)-tagged mouse Lefty 2, when electroporated into chicken embryos, was able to travel further away from its source cells than was a similarly introduced mouse Nodal-GFP fusion protein (Sakuma et al., 2002). It has been suggested that the relationship between Nodal and Lefty resembles that of the two hypothetical molecules in the classical “reaction-diffusion system”, as originally proposed by Turing (1990). Using a series of mathematical equations, this model describes the generation of complex tissue patterning within the embryo as a self-buffering system between a short-range morphogen and its long-range antagonist.

There are several principal interactions that are postulated to occur between the activator and the inhibitor in the reaction-diffusion model. First, the

morphogen induces its own production, as well as activating the expression of the inhibitor. The antagonist then functions to short-circuit the auto-activation of the activator. Finally, the long-range antagonist acts to restrict the range of short-range positive feedback loop of the activator. All of the current loss-of-function and gain-of-function data, including effects on target genes, suggest that Nodal and the feedback antagonist Lefty interact in such a self-regulating system.

Aims of the dissertation

The broad goal of my Ph.D. thesis research project was to examine how post-translational modifications influence the ability of Xlefty to regulate Nodal signaling during mesendoderm induction in *Xenopus laevis*. Currently, the published reports characterizing how proprotein cleavage of Lefty affects its ability to regulate Nodal signaling have come from heterologous experiments, which could lead to incorrect findings. In these experiments, mouse Lefty and the various cleavage mutants were co-expressed with Nodal and a Nodal-regulated luciferase reporter plasmid in *Xenopus* animal caps. Therefore, my studies were carefully designed to analyze proteolytic cleavage of Xlefty in the homologous tissue context of *Xenopus* and at the appropriate developmental time. In Chapter III of this dissertation, I describe biochemical and embryological assays that demonstrate the role of post-translational modifications in regulating Nodal signaling. Some major conclusions from my studies are that Xlefty undergoes both proteolytic cleavage and N-linked glycosylation. Proprotein

cleavage of Xlefty is required to inhibit Nodal signaling while glycosylation does not inhibit Xnr signaling in mesoderm induction assays and does not alter the movement of Xlefty through embryonic tissues. Because the proteolytic cleavage characteristics of Xlefty occurred at CS1 and CS2 when secreted from oocytes but cleavage only happened at CS1 when embryonic tissues were assayed, a major conclusion is that future experiments should utilize embryonic cells to investigate the mechanisms that regulate the biochemical processing of secreted proteins. In chapter IV, I present a synopsis on the significance of my findings and the direction of future experiments.

CHAPTER II

MATERIALS AND METHODS

Embryo manipulations

Embryos were obtained by in vitro fertilization of eggs from hormonally induced *Xenopus laevis* females (Kay and Peng, 1991). Embryos were dejellied in 1% thioglycolic acid in 1X Steinberg solution (1X SS; 4.6 mM Tris-HCl pH 7.4, 58 mM NaCl, 0.67 mM KCl, 0.34 mM $Ca(NO_3)_2$, 0.83 mM $MgSO_4$) and subsequently cultured in 1X SS. Embryos were staged according to Nieuwkoop and Faber (1967).

Embryo injections

Fertilized embryos were transferred to 5% Ficoll/1X SS and injected using a Narashige gas driven microinjector. Depending on the blastomere size, the injection volume ranged from 1 nl to 10 nl (10 nl for the one-cell stage, 2.5 nl for the 4-cell stage, and 1 nl for the 32-cell stage). Injected embryos were allowed to recover at room temperature in 5% Ficoll/1X SS until stage 9 and then transferred to 0.1X SS for the remainder of the culture period.

Capped RNAs were synthesized using SP6 mMessage mMachine (Ambion) from the following linearized plasmids: CS2+Xnr2 (Jones et al., 1995); CS2+Xlefty (Cheng et al., 2000); CS2+Zebrafish Lefty1 (Thisse and Thisse, 1999); SP64TL-Mouse Lefty1, pSP64TL-Mouse Lefty2; (Sakuma et al., 2002);

CS2+Xnr5 (Takahashi et al., 2000); CS2+nLacZ. The following plasmids (generated for the studies performed in this thesis; for detail see below) were linearized to make synthetic RNA: CS2+Xnr2^{NGM}; CS2+Xnr5^{G}; CS2+Xlefty^{mcs1}; CS2+Xlefty^{mcs2}; CS2+Xlefty$^{mcs1/2}$; CS2+Xlefty^{mcs1myc}; CS2+Xlefty^{mcs2myc}; CS2+Xlefty$^{mcs1/2myc}$; CS2+Xlefty^{HA}; CS2+Xlefty^{NGM-A}; CS2+Xlefty^{NGM-S}; CS2+Xlefty^{myc}; CS2+Xlefty$^{NGM-Amyc}$.

Xenopus oocyte isolation and injection

Xenopus oocytes were isolated and defolliculated according to Sive et al. (2000). Oocytes were cultured in O-R2 (pH 7.8: 82.5 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, and 5 mM HEPES) supplemented with 0.5 mg/ml bovine serum albumin (BSA), 100 units/ml penicillin (Specialty Media), and 100 μg/ml streptomycin (Specialty Media). For protein expression, a 10 nl volume was used to inject 30-50 ng of synthetic RNA per oocyte. After injection, the oocytes were allowed to recover for three hours at room temperature in O-R2/BSA/penicillin/streptomycin described above. Subsequently, five oocytes were transferred to a single well (prerinsed with O-R2 plus 15 mg/ml BSA) in a 96-well plate containing 50 μl of Labeling Medium. Labeling Medium consisted of 250 μCi [^{35}S]methionine and [^{35}S]cysteine (Promix; >1000mCi/mmol; GE Healthcare catalogue # AGQ0080) dissolved in O-R2/BSA/penicillin/streptomycin described above. A 10 mg/ml stock concentration of tunicamycin (Sigma) was prepared by dissolving in dimethyl sulfoxide (DMSO). To inhibit N-linked glycosylation, 10 nl was used to inject of 2 ng of tunicamycin per oocyte and

followed by culturing in O-R2/BSA/penicillin/streptomycin plus 2 μg/ml tunicamycin (Colman, 1984). After tunicamycin injection, oocytes were cultured with no agitation for 2 hours before RNA injection. Supernatants were collected by carefully removing as much conditioned medium as possible (approximately 40 μl) with the P200 pipetmen after overnight incubation at 19ºC. Samples were stored at -20ºC for further analysis. Sample buffer (4X: 40 % glycerol, 0.1M 2-mercaptoethanol, 25 mM EDTA, 10 % SDS, 0.125 M Tris pH 6.8, 0.05 % bromophenol blue) was added to 15 μl of conditioned medium, boiled for three minutes, microcentrifuged for 30 seconds, and resolved using precast 10% Bis-Tris NuPAGE SDS-PAGE gels (Invitrogen; catalogue # NP0301Box) and NuPAGE MOPS SDS running buffer (Invitrogen; NP0001)

Protein isolation from whole embryos

For analysis of proteins secreted from embryonic tissues, embryos were injected with the indicated RNAs and cultured to stage 10-10.5 in 1 X SS. Ten whole embryos were lysed by pipetting up and down in 300 μl of lysis buffer (pH 7.4: 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 μM leupeptin, 1 μM pepstatin, 1 mM PMSF). Lysates were microcentrifuged at 14,000 rpm at 4ºC for 5 minutes. Following microcentrifugation, soluble protein (located between the insoluble protein pellet and the pellicular layer; approximately 200 μl) was transferred to a new 1.5 ml tube. Following the addition of sample buffer (see above), one-half embryo equivalent of soluble protein was loaded per lane.

Protein secretion from embryonic tissues

Animal halves were isolated by cutting along the equator with a surgical knife to separate the animal hemisphere from the vegetal hemisphere, and animal caps were isolated using the Gastromaster® dissector with 400 μm size square loop tips. After tissue isolation, 12 animal halves or 25 animal caps were cultured per well in a 96-well plate containing 40 μl of calcium magnesium-free medium (7.5 mM Tris-HCl pH 7.6; 88 mM NaCl,1 mM KCl, 2.4 mM $NaHCO_3$) containing 0.1 mg/ml BSA at room temperature (Sives et al., 2000). Supernatants (approximately 35 μl) were collected with a P200 pipetmen after three hours for western blot analysis.

Enzymatic removal of N-linked glycans

PNGase F (New England Biolabs, catalogue # P0704S) digestion was performed on conditioned medium secreted from *Xenopus* oocytes and embryonic tissues. Conditioned medium (30 μl from oocytes and 20 μl from animal halves and animal cap) was performed according to the manufacturer protocol prior to gel electrophoresis. Briefly, protein was denatured in 1X glycoprotein denaturation buffer (0.5% SDS and 2% β-mercaptoethanol) for 10 minutes at 100ºC. Next, the solution was allowed to cool to room temperature. 10X G7 reaction buffer (0.5 M sodium phosphate, pH 7.5) was diluted to a final concentration of 1X, and 10% NP-40 was diluted to a final concentration of 1%. Finally, 1 μl of PNGase F (500 units) was added and the reaction was incubated at 37ºC for 1 hour.

MAPK assay

In order to stimulate activation of endogenous MAPK, whole embryos were wounded by rapidly cutting the embryo into quarters along the animal-vegetal axis with a surgical knife at stage 9 and cultured for 15 minutes. Animal caps were isolated using the Gastromaster® dissector with 400 μm size square loop tips at stage 9 and cultured for 15, 60, and 120 minutes. Three whole embryo equivalents (12 quarters) were lysed by rapidly pipetting up and down with a P200 pipetmen in 100 μl of lysis buffer (10 mM Tris–HCl pH 7.5, 100 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 1 μM leupeptin, 1 μM pepstatin, 1 mM PMSF, 50 mM NaF, 10 mM $Na_2P_2O_7$, 0.5 mM Na_3VO_4) and 15 animal caps were lysed by a similar pipetting method in 30 μl of lysis buffer. Lysates from whole or quartered embryos were microcentrifuged at 14,000 rpm at 4ºC for 5 minutes. Soluble protein (located between the pellet and pellicle; approximately 70 μl) was transferred to a new tube and stored at -20ºC for further analysis. Sample buffer was diluted to a final concentration of 1X and boiled for three minutes. One-half embryo equivalent of soluble protein was loaded per lane for western blot analysis. Sample buffer (10 μl) was added to lysates from animal caps and microcentrifuged at 14,000 rpm at 4ºC for 5 minutes to pellet insoluble protein. 7.5 animal cap equivalents of soluble protein were loaded per lane.

Western blots

Embryonic lysates and secreted proteins were resolved using precast 10% Bis-Tris NuPAGE SDS-PAGE gels and NuPAGE MOPS SDS running buffer

(described earlier). Proteins were transferred to Immobilon-P PVDF (Millipore; catalogue # IPVH00010) and were blocked for 2 hours in 1X Tris-buffered saline (TBS; 100 mM Tris-HCl pH 7.6 and 150 mM NaCl) containing 5% non-fat dry milk (NFDM; Kroger brand) and 0.1% Tween 20 (Fisher; catalogue # BP337). Primary and secondary antibodies were incubated in 1X TBS containing 1% NFDM and 0.1% Tween 20. Membranes were washed 4 times in 1X TBS plus 0.1% Tween 20 for 15 minutes after primary and secondary antibody incubation.

The following antibodies were diluted 1/2000 in 1X TBS containing 1% NFDM and 0.1% Tween 20: anti-myc (9E10; Vanderbilt University Antibody Core), anti-HA (12CA5; Vanderbilt University Antibody Core), anti-phospho-p44/42 (phospho-ERK1/2; Cell Signaling Technology catalogue # 9101) and anti-p44/42 (ERK1/2; Cell Signaling Technology catalogue # 9102). Horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG (Santa Cruz Biotechnology catalogue numbers sc-2314 and sc-2004, respectively) were diluted 1/2000 and used as secondary antibodies. SuperSignal West Pico Chemiluminescent Substrate (Pierce) was used to detect Horseradish peroxidase on immunoblots.

In vitro translation and N-linked glycosylation

Canine Pancreatic Microsomal Membranes (Promega; catalogue # L4610) were used for in vitro translation and N-linked glycosylation analysis. Capped mRNA was synthesized using SP6 mMessage mMachine (Ambion). 250 ng of *Xlefty* RNA was used to prime rabbit reticulocyte lysate system. Promix

(described above) was used for metabolic labeling. The reaction was performed according to manufacturer protocol.

Animal cap assays

One-cell stage embryos were injected with RNA. Vitelline membranes were removed from stage 8.5/9 embryos. Animal caps were explanted at stage 8.5/9 using the Gastromaster® 400 μm tip and cultured in 1 X SS. Explanted animal caps were collected at sibling stage 10.5, and flash frozen in dry ice/ethanol for RT-PCR analysis.

RT-PCR

Total RNA from three whole embryos or 25 animal caps was isolated using TRIzol (Invitrogen) according to manufacturer protocol. cDNA synthesis was performed with 2 μg of total RNA using 200 units of SuperScript II (Invitrogen; catalogue # 15596-018) and 5 nmoles of Oligo $d(T)_{16}$ (Applied Biosystem). After cDNA synthesis, the volume of the reaction was adjusted to 80 μl by adding 60 μl of distilled water. Each set of reactions included a control reaction without reverse transcriptase. PCR reactions were performed with 4 μl from the cDNA synthesis reaction and contained 0.75 units of Taq polymerase (Fisher), 0.25 mM of each dNTP, and 0.2 μM of gene-specific primers. PCR products were trace-labeled with 2 μCi of [α-^{32}P]-dATP (GE Healthcare catalogue # AA0004, ~3000Ci/mmol) . After an initial 5 minute denaturation step at 95°C, the reactions

cycled for 1 minute at 94ºC, 1.5 minutes at 55ºC and 1 minute at 72ºC. After 24 to 28 cycles (see Table 2.1 for cycle number), a final extension step was carried out for 5 minutes at 72ºC.

Table 2.1 PCR primers and cycle number

Gene name		Sequences	Cycle number
goosecoid	F	ACAACTGGAAGCACTGGA	28
	R	TCTTATTCCAGAGGAACC	
chordin	F	CCTCCAATCCAAGACTCCAGCAG	26
	R	GGAGGAGGAGGAGCTTTGGGACAAG	
noggin	F	AGTTGCAGATGTGGCTCT	27
	R	AGTCCAAGAGTCTCAGCA	
Xbra	F	GGATCGTTATCACCTCTG	28
	R	GTGTAGTCTGTAGCAGCA	
odc	F	GGAGCTGCAAGTTGGAGA	24
	R	TCAGTTGCCAGTGTGGTC	

Whole-mount immunostaining and Red-Gal staining

Whole-mount immunostaining was performed using albino embryos. Embryos were co-injected with *Xlefty*^{myc} or *Xlefty*$^{NGM\text{-}Amyc}$ RNA and a lineage-tracer RNA that encodes a nuclear-targeted β-galactosidase into one marginal blastomere of a 32-64 cell-stage embryo. Vitelline membranes were carefully removed from stage 9 embryos. Embryos were formaldehyde fixed in MEMFA (0.1 mM MOPS pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$, 3.7 % formaldehyde) for 1 hour, and washed three times in 1X phosphate-buffered saline (PBS). βgal was detected with Red-Gal (Research Organics; catalogue # 1364C) by incubating at room temperature in reaction buffer (1X PBS, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 1 mg/ml Red-Gal; Sive et al., 2000) for approximately 30 minutes. Next, embryos were washed three times in 1X PBS and post-fixed in MEMFA for one hour. Embryos were washed three times in 1X PBS containing 0.2% Triton-X100 (PBST) and blocked in two solutions for 1 hour each: (i) PBST containing 2% milk and 2 mg/ml BSA and (ii) PBST contain 10% donkey serum, 2 % milk, and 2 mg/ml BSA. Anti-myc antibody (9E10; Vanderbilt University Antibody Core) was diluted to 1:3000. Alkaline phosphatase-linked anti-mouse secondary antibody (Jackson ImmunoResearch, catalogue # 715-055-151) was reconstituted in 500 μl of sterile water. 500 μl of glycerol was added for a final concentration of 50% glycerol. The secondary antibody was then diluted to 1:1500. Primary and secondary antibodies were diluted in blocking buffer containing 10% donkey serum and incubated overnight at 4ºC. Embryos were washed 10 times in PBST for 30 minutes after primary and

secondary antibody incubation. BM purple (Roche; catalogue # 11442074001) signal was terminated after approximately 30 minutes. In comparative experiment, all parallel-processed samples were stopped at the same time.

For bisection, embryos were collected 1, 2, 3, and 4 hours post-injection. Embryos were MEMFA-fixed for one hour at room temperature and washed three times in 1X PBS. Red-Gal staining and MEMFA post-fix were performed as described above. Embryos were equilibrated in 0.3 M sucrose in 1X PBS for 5 minutes and then embedded in 1X PBS/0.3 M sucrose/2% low melting point agarose. Embryos were bisected through the patch of Red-Gal marked clone. The bisected embryos were removed from the agarose, washed for 5 minutes in 1X PBS/0.3 M sucrose and then washed three times for 5 minutes in 1X PBST. Antibody incubations and washes were performed as described above.

DNA constructs

Xlefty^{HA}

The QuickChange Site-Directed Mutagenesis Kit (Stratagene; catalogue # 200519) was used to add a *NheI* site 4 amino acids downstream of cleavage site 2 with the following primers: Nhe-Xlefty-top 5′-CACCGACCTGTCAACAATGGAGCTAGCGGAGCCAGAGTTAGTGTGTAT-3′; Nhe-Xlefty-bottom 5′-ATACACACTAACTCTGGCTCCGCTAGCTCCATTGTTGACAGGTCGGTG-3′ (*NheI* site underlined). The following oligonucleotides were annealed and

inserted into *NheI* digested CS2+Xlefty^{NheI}: 5′-CTAGCGGATATCCATATGATGTGCCAGATTATGCAGGATATCCATATGATGTGCCAGATTATGCAGGAG-3′; 5′-CTAGCTCCTGCATAATCTGGCACATCATATGGATATCCTGCATAATCTGGCACATCATATGGATATCCG (HA epitope tag underlined).

Xlefty^{NGM-A} and Xlefty^{NGM-S}

The QuickChange Site-Directed Mutagenesis Kit (Stratagene) was used to mutate the N-linked glycosylation site “NRT” (asparagine arginine threonine) to either “ART” (NGM-A; alanine arginine threonine) or “SRT” (NGM-S; serine arginine threonine) with the following primers: NGM-A-Top 5′-TTGAAAGATGGCACCGCCAGAACCTCCCTGGTG-3′; NGM-A-Bottom 5′-CACCAGGGAGGTTCTGGCGGTGCCATCTTTCAA-3′; NGM-S-Top 5′-TTGAAAGATGGCACCAGCAGAACCTCCCTGGTG-3′; NGM-S-Bottom 5′-CACCAGGGAGGTTCTGCTGGTGCCATCTTTCAA-3′ (mutated N-linked glycosylation underlined).

Xlefty^{mcs1}, Xlefty^{mcs2}, and Xlefty$^{mcs1/2}$

The QuickChange Site-Directed Mutagenesis Kit (Stratagene) was used to mutate cleavage sites (CS1 and/or CS2) from “R-X-X-R” to “G-V-D-G” with the following primers: CS1-Top 5′-ATGCTGCACAATCACAGAGAGGGGGTGGATGGATCACTGCCCAGCTTGGCTGGC-3′; CS1-Bottom 5′-

GCCAGCCAAGCTGGGCAGTGATCCATCCACCCCCTCTCTGTGATTGTGCAG CAT-3′; CS2-Top 5′-

ATCATGAACGTTCCAGAAAGGGGAGTCGACGGACCTGTCAACAATGCCAGA GTT-3′; CS2-Bottom 5′-

AACTCTGGCATTGTTGACAGGTCCGTCGACTCCCCTTTCTGGAACGTTCATG AT-3′ (mutated cleavage site underlined).

Xlefty^{myc}, Xlefty^{NGMmyc}, Xlefty^{mcs1myc}, Xlefty^{mcs2myc} and Xlefty$^{mcs1/2myc}$

The open reading frame of Xlefty, Xlefty^{NGM} and the various cleavage mutants were PCR amplified with the following primers: Xlefty-5′-myc 5′-CGCGGATCCATGGGTGTCACTACCAAATCTTTG-3′; Xlefty-3′-myc 5′-CGCGGATCCTATGATAGCGATATTGTCCATTGT-3′. The PCR product was digested with *BamHI* and subcloned into CS2+MT digested with *BamHI*.

Xnr2^{NGM}

The QuickChange Site-Directed Mutagenesis Kit (Stratagene) was used to mutate the N-linked glycosylation site “NET” to “AET” (NGM) with the following primers: Xnr2-NGM-5′ 5′-GCCTGTCCTATTCCTTTAGCTGAAACCTTCAAGCCAACG-3′; XNR2-NGM-3′ 5′-CGTTGGCTTGAAGGTTTCAGCTAAAGGAATAGGACAGGC-3′ (mutated glycosylation site underlined).

Xnr5^{G}

The QuickChange Site-Directed Mutagenesis Kit (Stratagene) was used to add N-linked glycosylation site “NGT” with the following primers: Xnr5-N-5′ 5′-TGCCCGATTCCACTGAATGAGACCTTCAAGCCAACAAA-3′; Xnr5-N-3′ 5′-GTTTGTTGGCTTGAAGGTCTCATTCAGAGGAATCGGGCA-3′ (N-linked glycosylation motif underlined).

CHAPTER III

THE EFFECT OF POST-TRANSLATIONAL MODIFICATIONS ON XLEFTY FUNCTION

Introduction

During metazoan embryogenesis, intercellular signaling molecules in the hedgehog, Wnt and TGFβ families are used reiteratively and in combination to initiate various developmental programs (Freeman and Gurdon, 2002; Tabata and Takei, 2004). Especially in early embryogenesis, the regulated transcription of the genes encoding these signaling molecules, as well as the level of activity of their extracellular antagonists, and other factors, are involved in the generation of morphogen activity gradients that lead to the spatially ordered specification of cell fates. There are several mechanisms that assist in shaping morphogen gradients. A good example of the effect of post-translational modification is seen in the addition of cholesterol to proteins in the Hedgehog family, which regulate the range of spreading of this factor through tissue in several systems (Li et al., 2006; Su et al., 2007). For FGF8, the rate of clearing via endocytosis controls the amount of extracellular protein available for signaling as well as the distance the protein is able to travel from the source of production (Scholpp and Brand, 2004). It is easy to imagine how receptor availability and extracellular antagonists are powerful dynamic regulators of morphogen gradients (Smith and Harland, 1992; Sasai et al., 1994; Bouwmeester et al., 1996; Goodrich et al., 1996; Meno et al., 1996; Meno et al., 1999; Thisse and Thisse, 1999; Cheng et al., 2000;

Tanegashima et al., 2000; Larrain et al., 2001; Branford and Yost, 2002; Chang et al., 2003; Harms and Chang, 2003; Zhang et al., 2004). The preferential intracellular degradation of mRNA for a signaling molecule gene can contribute to formation of a morphogen gradient. For example, progressive clearance of *fgf8* mRNA is translated into a gradient of FGF8 protein that appears to be required for proper axis elongation during vertebrate embryogenesis (Dubrulle and Pourquie, 2004).

The Nodal ligand binds a receptor complex that includes Activin type 1 and type 2 receptors (Whitman, 2001), together with an EGF-CFC family co-receptor (Shen et al., 1997; Ding et al., 1998; Zhang et al., 1998; Dorey and Hill, 2006; Onuma et al., 2006). Lefty, a divergent member of the TGFβ family whose transcription is directly regulated by Nodal signaling, is one of the principal extracellular feedback inhibitors of Nodal signaling (Meno et al., 1996; Meno et al., 1999; Thisse and Thisse, 1999; Cheng et al., 2000; Tanegashima et al., 2000; Branford and Yost, 2002; Cha et al., 2006). The current understanding is that Lefty antagonizes Nodal signaling by binding to either the EGF-CFC cofactor directly, or by physically interacting with the Nodal ligand, thereby blocking Nodal-receptor interaction and inhibiting downstream signal transduction (Chen and Shen, 2004; Cheng et al., 2004; Tanegashima et al., 2004).

Calcium-dependent serine endoproteases of the subtilisin-like proprotein convertase family (SPCs) recognize the consensus R-X-X-R motif found in many intercellular signaling molecule proproteins, including Nodal and Lefty (Nakayama, 1997; Molloy et al., 1999). SPCs, of which there are seven distinct

mammalian family members, have been localized to the intracellular secretory network as well as having been detected in association with the extracellular matrix. SPC-mediated cleavage releases the active ligand during the maturation of TGFβ proteins (Kingsley, 1994; Nakayama, 1997; Cui et al., 1998; Constam and Robertson, 1999; Molloy et al., 1999; Ulloa et al., 2001; Beck et al., 2002; Sakuma et al., 2002; Ben-Haim et al., 2006). In principle, therefore, SPCs may work in both a cell-autonomous (i.e., within the proprotein producing cell) and non-cell-autonomous manner from adjacent cells (Nakayama, 1997; Molloy et al., 1999). Although mammalian Lefty molecules have been shown to undergo proteolytic cleavage by SPC1, SPC4, and SPC6 in several transfected cultured cell lines, the endogenous SPC enzyme(s) that is involved in the proteolytic processing of Lefty *in vivo* is currently not known (Ulloa et al., 2001; Beck et al., 2002; Sakuma et al., 2002).

There is much evidence that cleavage mutants (which cannot undergo proprotein processing) of various TGFβ molecules are either not secreted or are biologically inactive (Lopez et al., 1992; Hawley et al., 1995; Osada and Wright, 1999; Sun et al., 1999; Yeo and Whitman, 2001; Eimon and Harland, 2002; Onuma et al., 2002; Sakuma et al., 2002; Onuma et al., 2005). Recently, however, several reports have suggested that uncleaved proproteins retain some signaling function. In one example, Eimon and Harland (2002) demonstrated that overexpressed cleavage-resistant Xnr2 was capable of inducing the expression of mesodermal genes in *Xenopus* embryos, although this activity was weaker than normal protein, and has so far not been detected by other groups

(Osada and Wright, 1999; Onuma et al., 2002; Hashimoto-Partyka et al., 2003; Onuma et al., 2005). The reason for this discrepancy remains unclear. A similar activity was reported for a non-cleavable form of mouse Nodal, and in this case, the proprotein has been suggested to be able to bind and signal through Activin receptors to induce significant expression of *BMP4*, *furin/spc1* and *spc4* (Ben-Haim et al., 2006). Yet another report demonstrated that affinity purified human Lefty A proprotein could activate the MAP kinase pathway in P19 mouse embryonic carcinoma cells when added to the culture medium (Ulloa et al., 2001).

The *Xenopus* Lefty proprotein has two potential cleavage sites that would be expected to produce a long (Xlefty^{L}) or short (Xlefty^{S}) isoform by cleavage at site 1 (CS1) or site 2 (CS2), respectively. When tested in 293T and BALB/3T3 cell lines, CS1 and CS2 cleavage of mouse Lefty 1 seemed to depend on the cell type (Meno et al., 1996). Expression of mouse Lefty 1 and Lefty 2 in *Xenopus* tissues (animal caps and oocytes) and COS-7 cells resulted in the detection of the short isoform for Lefty 1 and the long isoform for Lefty 2 (Sakuma et al., 2002).

Several TGFβ family members contain at least one N-linked glycosylation consensus sequence in the proprotein that may affect the secretion, protein stability and biological function of the ligand domain. Specifically, TGFβ1 and TGFβ2 require the addition of N-linked glycans at three sites in the prodomain for efficient secretion of the ligand into the culture medium (Brunner et al., 1992; Lopez et al., 1992). In another example, the modification of mouse Nodal by

inserting an N-linked glycosylation motif that is found in five of the six *Xenopus* Nodal-related factors and absent from mouse Nodal led to a greatly increased ligand stability (Le Good et al., 2005). The presence in Lefty molecules of a single N-linked glycosylation motif in the mature region that might influence intrinsic protein stability or Nodal-blocking function is an issue that we address in this thesis.

As stated above, Sakuma et al. (2002) and Ulloa et al. (2001) assayed the cleavage of mammalian Lefty in *Xenopus* tissues or in several mammalian cultured cell lines, which may not be the appropriate tissue context to study the processing of this important intercellular antagonist, especially when considering how its activity in specifying the patterning of embryonic tissue. Therefore, we decided to examine the single *Xenopus* Lefty molecule in a homologous tissue context within the developing *Xenopus* embryo, and during a period when *Xlefty* is normally expressed. We report here on our assays of how proprotein processing and N-linked glycosylation affected protein stability, biological function and movement through embryonic tissues. We found that Xlefty undergoes cleavage at CS1 and CS2 when secreted from *Xenopus* oocytes, but that only Xlefty^{L} is detected when produced from embryonic tissues. The use of cleavage mutants to direct the production of either the long or short isoform showed in a mesoderm induction assay context that Xlefty^{L} is the inhibitory isoform. Additionally, this is the first report that demonstrates that vertebrate Lefty molecules are secreted as glycosylated proteins, but our assays suggest

that glycosylation does not alter the gross biological function or the movement of Xlefty through blastula stage embryonic tissue.

Results

Lefty molecules are secreted as glycoproteins

Lefty molecules contain a single N-linked glycosylation consensus sequence in the mature domain that could affect intrinsic protein stability or post-secretion clearance, its biological effectiveness as a Nodal antagonist, and/or the range of movement through embryonic tissue. To determine if core glycosylation of the proprotein could occur, in vitro translation of *Xlefty* RNA was performed in the presence and absence of canine pancreatic microsomal membranes. When *Xlefty* was used as a template in the absence of microsomal membranes, a signal band of approximately 38 kDa was produced (Fig. 3.1). When the reaction was carried out in the presence of microsomal membranes, two bands were detected. The lower band had a migration equivalent to that of Xlefty produced in the absence of microsomal membranes, while the slower migrating and distinct appearance of the second band indicated post-translational modification, likely glycosylation (Fig. 3.1).

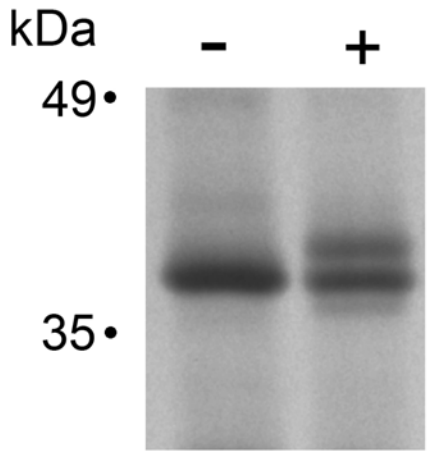

Figure 3.1 Post-translational modification of Xlefty. TNT reaction primed with 250 pg of *Xlefty* RNA in the absence (-) and (+) presence of canine pancreatic microsomal membranes.

In order to determine if the glycosylation motif is functional *in vivo*, RNAs encoding *Xenopus* Lefty, zebrafish Lefty 1, mouse Lefty 1 and mouse Lefty 2 were injected into *Xenopus* oocytes and the resulting conditioned medium was analyzed by SDS-PAGE. Radiolabeled Lefty molecules detected in the conditioned medium migrated slower than predicted and as diffuse bands, a typical behavior of glycoproteins (Fig. 3.2A). When tunicamycin was injected into the oocytes prior to the RNA, the secreted proteins migrated faster and as sharper bands. The presence of glycosylation was confirmed by treating the Lefty-containing media samples with a deglycosylase, PNGase F, prior to gel electrophoresis (Fig. 3.2A). These data demonstrated that vertebrate Lefty molecules were glycosylated when secreted from *Xenopus* oocytes. The tunicamycin result showed that, unlike TGFβ1 and TGFβ2, Lefty molecules do not require the presence of N-linked glycans for efficient secretion into the medium.

Mouse Lefty 2 contains two potential cleavage sites, with cleavage reported to occur only at CS1 in *Xenopus* animal caps and COS-7 cells. Furthermore, I the same studies, animal cap expression of mouse Lefty 2 carrying a mutant CS1 resulted in the detection of only the proprotein, suggesting that CS2 is strongly refractory to cleavage (Sakuma et al., 2002). We wanted to determine if both CS1 and CS2 were utilized for proteolytic processing of the Xlefty proprotein. Injection of *Xlefty* RNA into *Xenopus* oocytes resulted in equal amounts of both long and short isoforms (Xlefty^{L} and Xlefty^{S}, respectively) in the conditioned medium (Fig. 3.2).

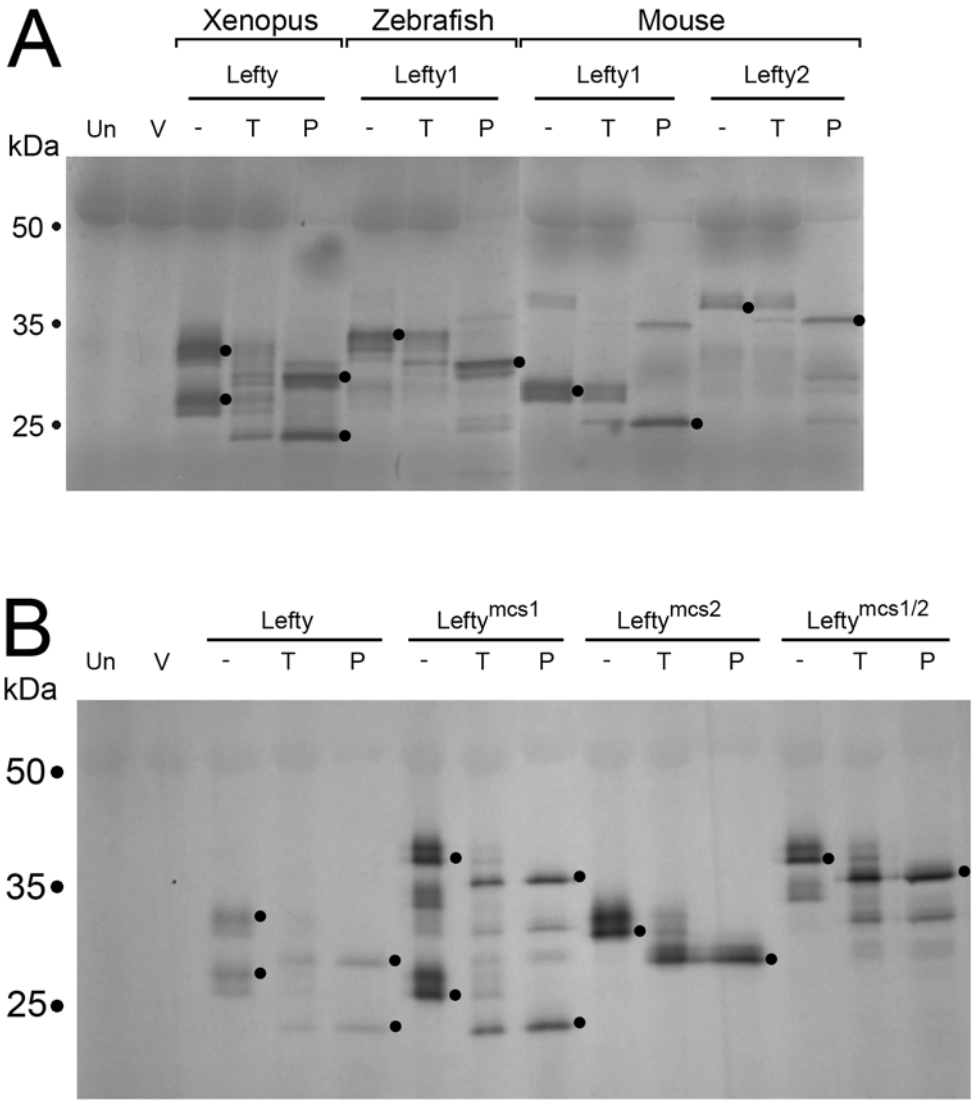

Figure 3.2 Glycosylation of Lefty molecules. A, B: Medium was analyzed from *Xenopus* oocytes injected with the indicated RNAs, metabolically labeled with [^{35}S]-methionine/cysteine. Black dots, major glycosylated and deglycosylated bands. Un, uninjected oocytes; V, vehicle (1% DMSO); (-), oocytes not injected with tunicamycin, or conditioned medium not treated with PNGase F; T, tunicamycin; P, PNGase F.

This finding suggested that cleavage occurred at CS1 and CS2 with equal efficiency for wild-type Xlefty. To test for the cleavage efficiency at each CS, we used site-directed mutagenesis to disrupt the protease recognition site at one or both cleavage sites. Injection of *Xlefty*^{mcs1} RNA (mutant cleavage site 1; encoding an uncleavable CS1) resulted in detection of Xlefty^{S} and the proprotein, together with minor amounts of unknown and perhaps spurious cleavage products. Although only a functional CS2 was available in this proprotein, the presence of substantial proprotein in the conditioned medium indicated that CS2 cleavage was inefficient. This result can be interpreted as suggesting that cleavage at CS1 is required for further processing at CS2, a situation similar to that noted for BMP-4, as described in more detail in the Discussion (Cui et al., 2001). Conversely, injection of *Xlefty*^{mcs2} RNA (in which only CS1 is functional) resulted in detection of Xlefty^{L} without appreciable proprotein, suggesting that CS1 cleavage was extremely efficient (Figure 3.2B). Injection of double-cleavage-site-mutant *Xlefty*$^{mcs1/2}$ RNA resulted in secretion of the proprotein and a small proportian of unknown cleavage products (Figure 3.2B). Proprotein cleavage is therefore not required for Xlefty secretion, which differs from reports showing that cleavage is required for secretion of TGFβ1 and Derrière (Lopez et al., 1992; Eimon and Harland, 2002).

The initial steps of N-linked glycosylation occur in the endoplasmic reticulum with further maturation in the Golgi complex (Helenius and Aebi, 2001). SPC1, SPC4, and SPC6 have been localized to the trans-Golgi network and secretory granules, as well as at the cell surface and secreted to the extracellular matrix

(Molloy et al., 1999; Bergeron et al., 2000; Beck et al., 2002; Tsuji et al., 2003; Nour et al., 2005). Although the effect of N-glycosylation has not yet been directly assessed on the structure of TGFβ family proteins, experiments with synthetic peptides have demonstrated that N-linked glycans can induce a compact β-turn in the vicinity of the carbohydrate addition (O'Conner and Imperiali, 1998; Helenius and Aebi, 2001). Therefore, there is a possibility that the presence or absence of N-linked glycans could generate a protein conformation that is unsuitable for SPC-mediated cleavage. In order to test if glycosylation affected cleavage at CS1 and CS2, tunicamycin was injected into the oocytes prior to the RNAs encoding the various cleavage mutants. Although the secreted proteins migrate at different rates, the similarity of the cleavage patterns generated from the various cleavage mutant proteins in the presence or absence of tunicamycin suggests that glycosylation does not alter CS1/CS2 cleavage characteristics (Figure 3.2B). Again, PNGase F treatments confirmed the presence of glycosylation. In addition, the similarity of migration of the cleavage products detected in the presence of tunicamycin or with PNGase F treatment suggested that the previous tunicamycin treatment blocked the majority of N-linked glycan addition.

We next examined the cleavage and glycosylation characteristics of Xlefty secreted from embryonic tissues. We analyzed conditioned media from the dissociated cells from entire animal halves (the animal hemisphere cut away at the equator) or standard animal caps. Since there are no Xlefty antibodies available to detect either the endogenous (which is in any case likely expressed

at difficult-to-detect low levels) or overexpressed normal protein, an HA epitope-tagged version was utilized for these experiments. When overexpressed in whole embryos, Xlefty^{HA} (containing a tag placed 4 aa after CS2; see Chapter II) induced an embryonic phenotype similar to that caused by wild-type Xlefty at equivalent RNA doses (not shown), and the HA tag did not interfere with secretion or stability of Xlefty (not shown), demonstrating that HA tagging at this location did not affect its biological function. When expressed in *Xenopus* embryonic tissues, Xlefty^{HA} was secreted as a glycosylated protein, as confirmed by PNGase F treatment (Figure 3.3) and only Xlefty^{L} was detected. In similar experiments with myc-tagged versions of Xlefty or the various cleavage mutants, Xlefty^{L} and the proprotein were detected in cell extracts from whole embryos, or in the conditioned media from dissociated animal halves (Fig. 3.4). Similar to data gathered from oocyte secretion assays, the proprotein was efficiently secreted from animal halves (Fig. 3.4B). These results indicate that Xlefty is glycosylated in embryonic tissues, and that Xlefty^{L} is the only isoform of the protein that accumulates to detectable levels either in homogenized whole embryos extracts, or secreted from embryonic cells.

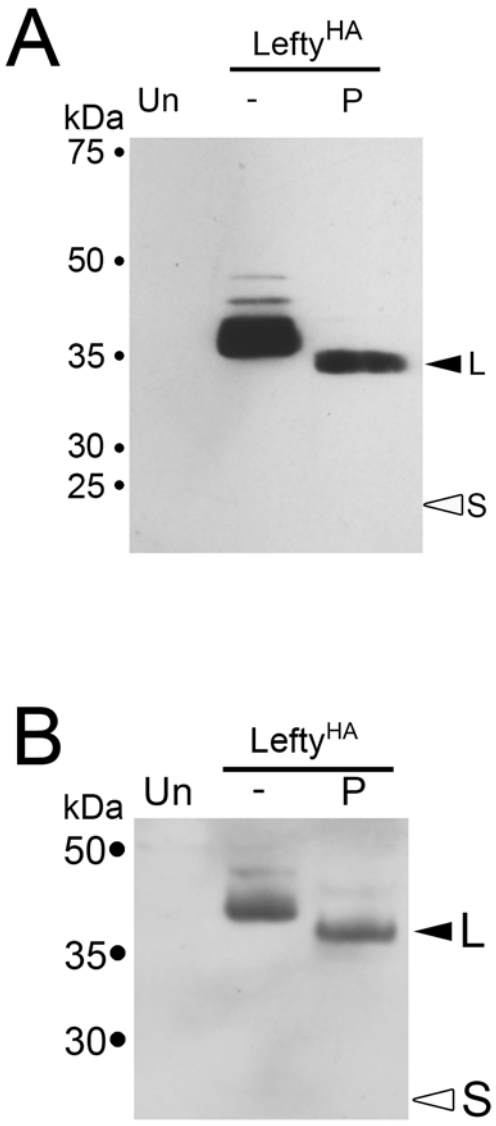

Figure 3.3 Secreted Xlefty is a glycoprotein. A,B: Western blot of conditioned medium from dissociated animal halves (A) and animal caps (B) isolated from embryos injected at the one-cell stage with 2.5 ng of *Xlefty*^{HA} RNA. Un, uninjected embryos; (-), conditioned medium not treated with PNGase F; P, PNGase F; L, Xlefty^{L} (filled arrowhead); S, Xlefty^{S} (open arrowhead).

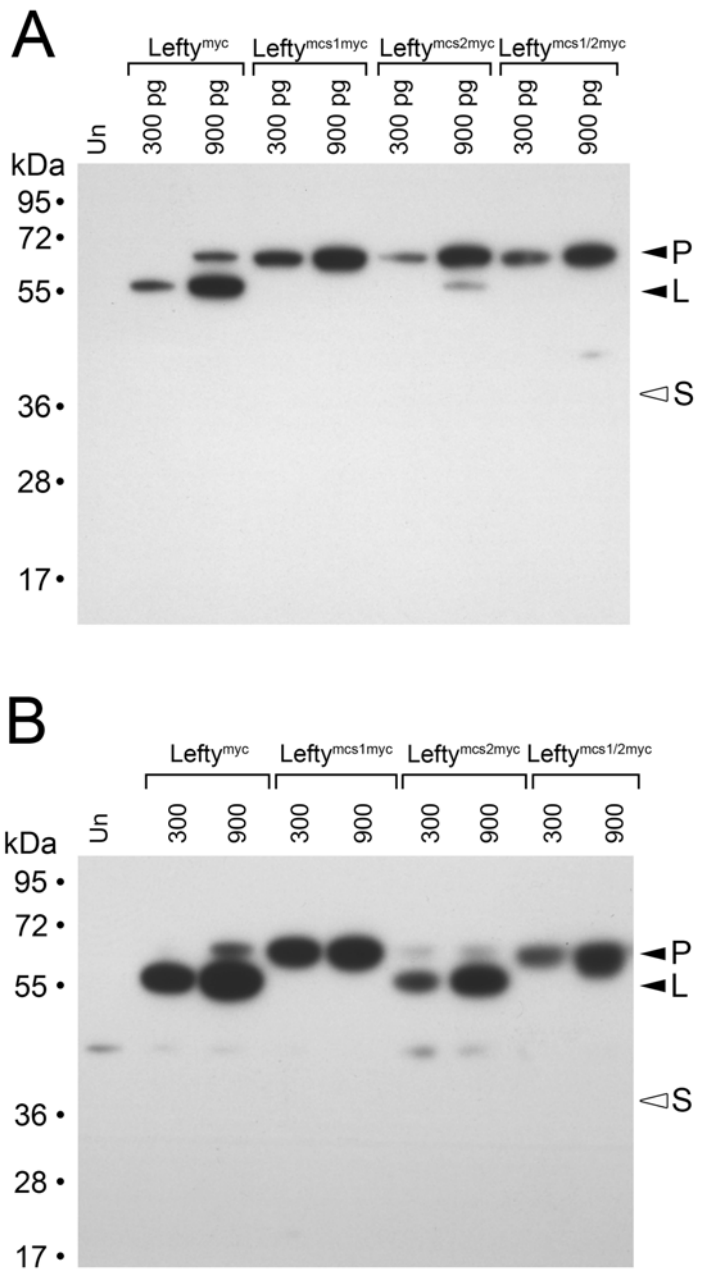

Figure 3.4 Xlefty^{S} not detected in whole embryos or conditioned medium. Embryos were injected at the one-cell stage with 300 or 900 pg of RNA encoding myc-tagged "wild-type" Xlefty or the various cleavage mutants. Western blot analysis of cell extracts from whole embryos (A) or conditioned medium from animal halves (B). Filled arrowheads, proprotein (P) and Xlefty^{L} (L) open arrowhead, Xlefty^{S} (S). Un, uninjected.

Cleavage of Xlefty required for Xnr antagonism

Xlefty inhibits the expression of organizer-specific and mesendodermal genes that are induced in isolated animal caps by *Xenopus* Nodal-related factors such as Xnr2 (Cheng et al., 2000). To determine if Xlefty^{L}, Xlefty^{S} and the proprotein differ in their capacity to inhibit Xnr signaling, we co-expressed normal Xlefty or its various cleavage mutants with Xnr2. For wild-type Xlefty, a 1:1 ratio of *Xlefty*:*Xnr2* RNA resulted in the induction of mesodermal markers (*gsc*, *chd*, and *Xbra*) at a level similar to or only slightly down-regulated compared to *Xnr2* alone. A higher 10:1 *Xlefty*:*Xnr2* RNA ratio led to suppressed organizer-specific and pan-mesodermal marker gene expression (Fig. 3.5). Co-injection of RNAs encoding Xlefty that should produce only the proprotein (*Xlefty*$^{mcs1/2}$) or a proprotein that can be cleaved only to Xlefty^{S} (*Xlefty*^{mcs1}) were incapable of blocking Xnr2-mediated induction of mesodermal markers, even at the 10:1 ratio (Fig. 3.5A, B). In contrast, RNA that encoded a protein only capable of cleavage to Xlefty^{L} inhibited Xnr2 function as efficiently as did wild-type Xlefty (Fig. 3.5C). The difference in Xnr-blocking ability is not explained by different translation efficiencies, as the level of protein produced from injected myc-tagged RNAs encoding Xlefty and the various cleavage variants were similar in whole embryos or when secreted from animal halves (Fig. 3.4). These results suggest that cleavage of Xlefty proprotein to Xlefty^{L} is required to block Xnr signaling, a finding consistent with the requirement of proprotein cleavage of mouse Lefty 1 and mouse Lefty 2 to block Nodal signaling in luciferase assays (Sakuma et al., 2002).

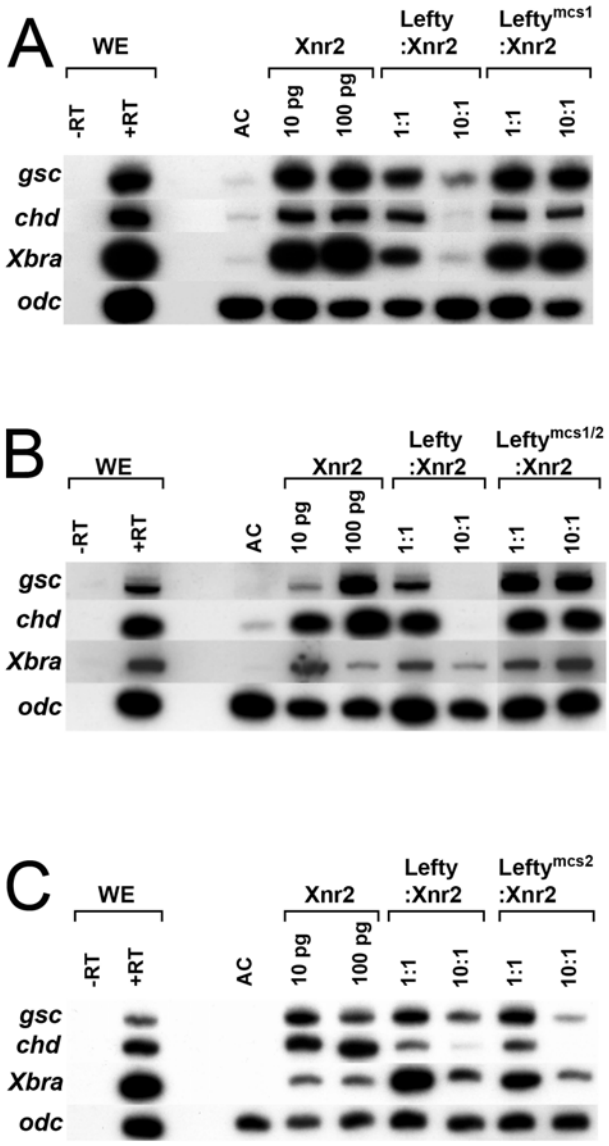

Figure 3.5 Inhibition of Xnr2 by Xlefty^{L}. A: One-cell stage embryos were injected with RNA (pg/embryo indicated) encoding Xnr2 plus or minus Xlefty or Xlefty^{mcs1}, the latter to enforce Xlefty^{S} production. Animal caps were analyzed at stage 10.5 for organizer-specific (*goosecoid* and *chordin*) or pan-mesodermal markers (*Xbra*). *Odc*, loading control. **B, C:** Similar analysis of one-cell stage embryos overexpressing uncleavable Xlefty proprotein (B, *Xlefty$^{mcs1/2}$* RNA used) or proprotein that enforces Xlefty^{L} production (C, from *Xlefty^{mcs2}* RNA) on Xnr2-mediated mesoderm induction. WE, whole embryo plus (+) and minus (-) reverse transcriptase (RT); AC, uninjected animal cap; 1:1, 10 pg of each RNA; 10:1, 100 pg of RNA encoding Xlefty or cleavage mutant protein, and 10 pg of *Xnr2* RNA.

No dorsal-ventral difference in Xlefty processing

Tanegashima et al. (2000) demonstrated that Xlefty was unable to inhibit the Xnr1-mediated induction of mesodermal markers in animal caps, while it could completely suppress the Nodal-induced expression of *chordin* in ventral marginal zone explants. One interpretation of their data is that SPC enzyme activity could vary throughout the embryo. We have been interested in the idea that a dorsal-to-ventral gradient of Xlefty-processing activity in the gastrula-stage embryo might be set up as a consequence of the reported dorsal-to-ventral gradient of Nodal signaling (Lee et al., 2001). We assayed Xlefty processing in dissociated animal hemispheres from embryos in which *Xlefty*^{myc} RNA was targeted to either both dorsal, or both ventral, blastomeres at the 4-cell stage. The animal half conditioned medium (removed at St. 8.5 to 9, cultured for 3 hours; approx. equivalent of sibling stage 10-10.5) and whole Stage 10-10.5 embryo extracts were analyzed by western blot. There was equivalent accumulation of Xlefty^{L} from embryos producing Xlefty from either the dorsal or ventral side (Fig. 3.6).

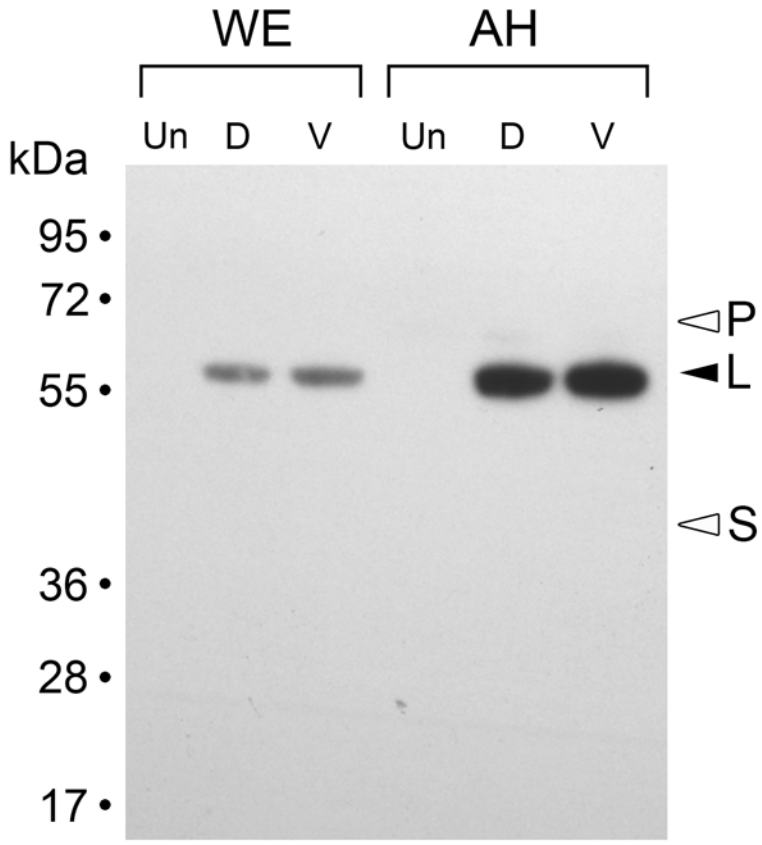

Fig. 3.6 Absence of dorsal-ventral difference in Xlefty processing. Western blot, cell extracts from embryos (WE) and conditioned medium from dissociated animal halves (AH) from embryos injected in either both dorsal (D) or ventral (V) blastomeres at the 4-cell stage with 150 pg/cell of *Xlefty*^{myc} RNA. The completeness of processing to Xlefty^{L} at this 300 pg total RNA dose is similar to that seen at 300 pg/embryo in Figure 3.4. Un, uninjected embryos; L (solid arrowhead), Xlefty^{L}; proprotein (P) and Xlefty^{S} (S) open arrowhead.

Xlefty does not induce prolonged MAPK activation

Despite the demonstration above that proprotein cleavage seems to be required for Xlefty activity, at least as related to Nodal signaling, we were interested in following up, but this time in a homologous embryonic tissue, a previous report that human Lefty proprotein showed some biological activity as an inducer of the MAP kinase pathway in P19 mouse embryonic carcinoma cells (Ulloa et al., 2001). To test if either Xlefty or the full-length proprotein caused prolonged MAPK activation, we assayed for intracellular diphosphorylated ERK1/2 (dpERK) in whole embryos and animal caps injected with *Xlefty* and *Xlefty*$^{mcs1/2}$ RNAs. Uninjected embryos showed no detectable dpERK at stage 9, consistent with previous reports (LaBonne and Whitman, 1997; Christen and Slack, 1999). However, as expected, wounding (cutting the embryo into quarters) resulted in robust ERK activation (LaBonne and Whitman, 1997; Christen and Slack, 1999; Kuroda et al., 2005). Injection of 100 pg of either *Xlefty* or *Xlefty*$^{mcs1/2}$ RNAs did not induce ERK activation in whole embryos. In animal caps isolated from uninjected embryos, surgical removal of the animal cap, which induces a wounding response, resulted in detection of dpERK, but this activation was short-lived, becoming barely detectable after 120 minutes. In animal caps removed from embryos that were injected with *Xlefty* or *Xlefty*$^{mcs1/2}$ RNAs, the steady-state level of dpERK became progressively reduced at a rate similar to that in animal caps isolated from uninjected embryos (Fig. 3.7). These results suggest that neither Xlefty, nor the forcibly overexpressed uncleavable

proprotein, are capable of inducing prolonged activation of the MAPK pathway in the homologous context of the *Xenopus* embryo.

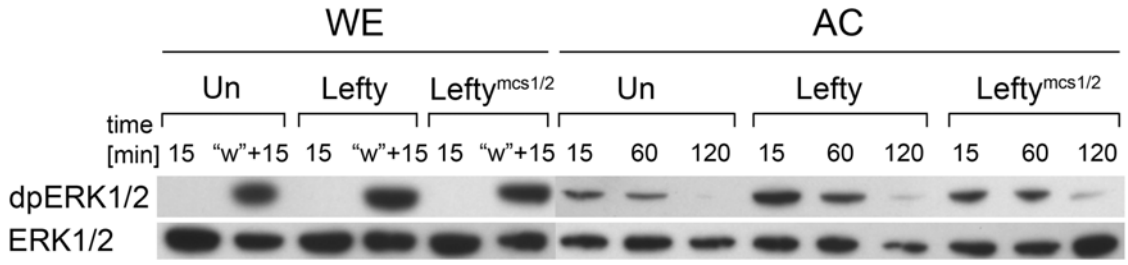

Figure 3.7 Xlefty does not induce prolonged MAPK activation. Western blot of cell extracts from embryos and animal caps injected with 100 pg of either *Xlefty* or *Xlefty*$^{mcs1/2}$ RNA, using antibodies against diphospho-ERK1/2 or total ERK1/2. Time after wounding ("w"; quartering of embryos) or animal cap isolation is given in minutes. Un, uninjected; WE, whole embryo; AC, animal cap.

Glycosylation not required for Xlefty stability

Several reports suggest that the addition of carbohydrate moieties affect the secretion, stability and biological activity of TGFβ family molecules. For TGFβ1 and TGFβ2, blocking N-linked glycan addition by either mutating asparagine residues, or adding tunicamycin to the culture medium, resulted in no detectable proprotein or ligand being secreted from COS, 293S or CHO cells (Sha et al., 1989; Brunner et al., 1992; Lopez et al., 1992). In addition, tunicamycin treatment resulted in the intracellular accumulation of the TGFβ1 proprotein (Sha et al., 1989). Active Nodal ligand has been notoriously difficult or impossible to detect in conditioned medium from COS1 or 293T cells (Constam and Robertson, 1999; Le Good et al., 2005), but a modified version created by the insertion of an artificial N-linked glycosylation site into the ligand region (the glycosylation motif was the same as that in Xnr1/Xnr2/Xnr3) increased the stability of mouse Nodal (Le Good et al., 2005). Given that the above reports suggested that glycosylation was playing a role in secretion and/or stability of TGFβ-related molecules, we next determined if glycosylation influenced Xlefty secretion and stability. In the context of either wild-type or Myc epitope-tagged Xlefty, the latter having a similar level of function to the wild-type protein as explained below, the asparagine in the N-linked glycosylation motif (NRT) was mutated to either alanine ($Xlefty^{NGM-A}$) or serine ($Xlefty^{NGM-S}$) to prevent N-linked glycan addition (Brunner et al., 1992; Carter et al., 2005). The equivalent migration characteristics of untagged versions of either $Xlefty^{NGM-A}$ or $Xlefty^{NGM-S}$ in the presence or absence of tunicamycin showed that these mutations blocked

addition of all N-linked glycans (Fig. 3.8A). Dissociated animal halves were used to test the stability of myc-tagged Xlefty compared to myc-tagged Xlefty^{NGM-A}. As judged by the equivalent accumulation of Xlefty^{myc} and Xlefty$^{NGM-Amyc}$, glycosylation does not alter the stability or secretion of Xlefty from embryonic tissues (Fig. 3.8B). Also, data gathered from mesoderm induction assays suggest that the glycosylation mutant is an effective blocker of Xnr2, which argues for no difference in protein stability compared to wild-type (Fig. 3.9).

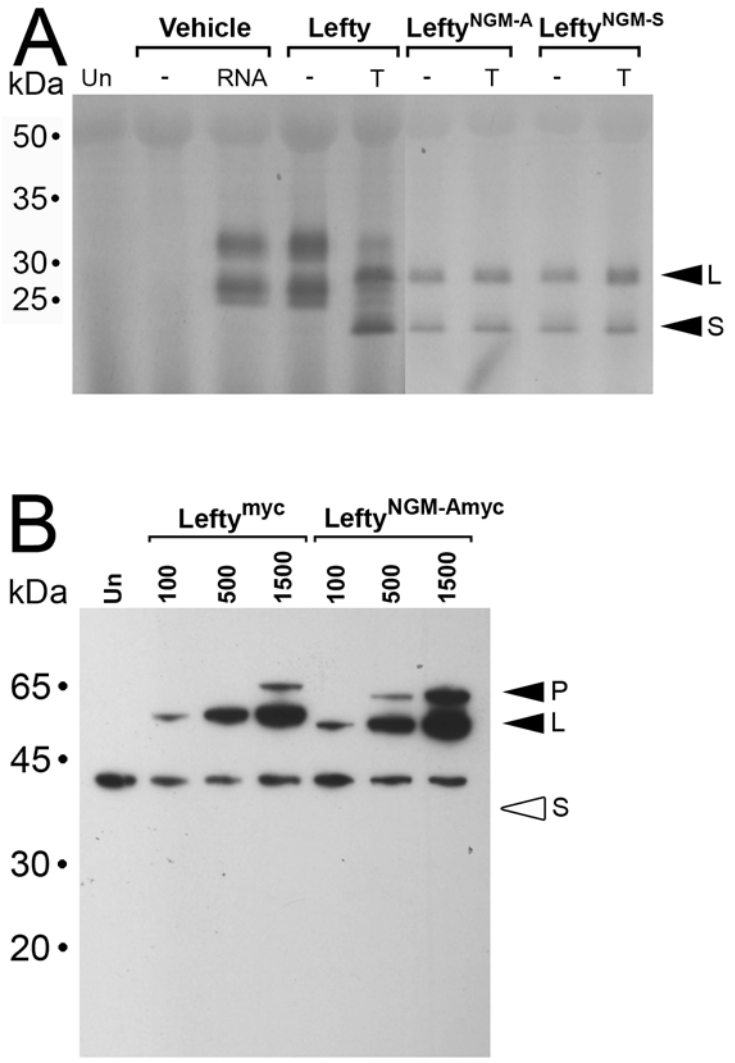

Fig 3.8 Glycosylation not required for protein stability. A: *Xenopus* oocytes were injected with RNA encoding Xlefty or either non-glycosylatable mutant, Xlefty^{NGM-A} or Xlefty^{NGM-S}. Arrowheads, Xlefty^{L} and Xlefty^{S}. **B:** Western blot, conditioned medium from animal halves from embryos injected at the one-cell stage with 100, 500, or 1500 pg of *Xlefty*^{myc} or *Xlefty*$^{NGM-Amyc}$ RNA. Filled arrowheads, proprotein and Xlefty^{L}; open arrowhead, Xlefty^{S}. Un, uninjected oocyte or embryo; Vehicle, 1% DMSO; (-) conditioned medium from oocytes not injected with tunicamycin; T, tunicamycin; L, Xlefty^{L}; S, Xlefty^{S}; P, proprotein.

Glycosylation of Xlefty not required for Xnr antagonism

Artificial glycosylation of mouse Nodal greatly increased its signaling activity compared to the wild-type ligand (Le Good et al., 2005). Since the stability of Xlefty secreted from embryonic tissue was not altered by glycosylation, we tested if glycosylation affected its ability to block Xnr signaling. Untagged versions of *Xlefty* or *Xlefty*$^{NGM-A}$/*Xlefty*$^{NGM-S}$ RNAs were co-injected with *Xnr2* RNA at the one-cell stage, and the induction of mesodermal markers in animal caps was analyzed. Similar to wild-type Xlefty, Xlefty^{NGM-A} and Xlefty^{NGM-S} efficiently blocked Xnr2-induced mesodermal gene expression, and we conclude that glycosylation does not alter the efficacy of Xlefty as an antagonist of Xnr signaling in mesoderm induction assays (Fig. 3.9) in which the distribution of *Xlefty* RNA amongst all animal cap cells effectively removes any potential effect from the range of movement of the inhibitor (a “range-independent” assay).

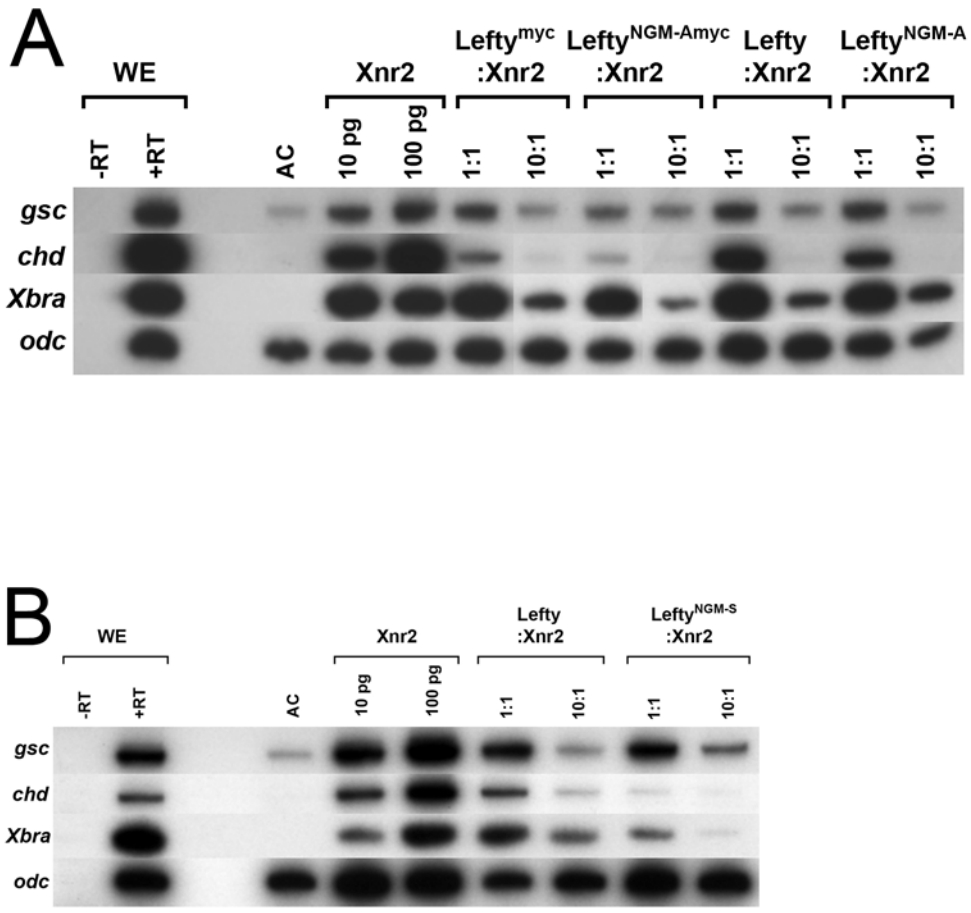

Fig 3.9 Glycosylation is not required to suppress Xnr2-mediated mesoderm induction. A: Mesoderm induction assay for effectiveness of non-glycosylated Xlefty. Marker expression was assayed at stage 10.5 in animal caps explanted from embryos injected with *Xnr2* (pg/embryo indicated) with or without RNA encoding Xlefty, Xlefty^{NGM-A}, Xlefty^{myc} or Xlefty$^{NGM-Amyc}$. **B:** Animal caps were isolated from embryos injected with *Xnr2* (pg/embryo indicated) plus or minus *Xlefty* or *Xlefty*$^{NGM-S}$ RNA. WE, whole embryo plus (+) and minus (-) reverse transcriptase (RT); AC, uninjected animal cap. 1:1, 10 pg of each RNA; 10:1, 100 pg of RNA encoding untagged or tagged versions of Xlefty, Xlefty^{NGM-A} or Xlefty^{NGM-S} and 10 pg of RNA encoding Xnr2.

Glycosylation does not alter the movement through embryonic tissues

Interaction with heparan sulfate proteoglycans (HSPGs) has been suggested to regulate the movement of BMP-4 and Dpp in *Xenopus* embryonic tissue and *Drosophila* imaginal discs, respectively (Ohkawara et al., 2002; Belenkaya et al., 2004; Han et al., 2004). Since glycosylation of Xlefty did not alter protein stability in embryonic tissues or its biological activity in antagonizing mesoderm induction, we next tested for an effect on the movement of Xlefty. For range of movement experiments, we utilized myc-tagged versions of Xlefty and Xlefty$^{NGM\text{-}A}$, which antagonize Xnr2 signaling as effectively as the untagged proteins (Fig. 3.9). *Xlefty^{myc}* or *Xlefty$^{NGM\text{-}Amyc}$* were co-injected with *LacZ* RNA, encoding nuclear-targeted β-galactosidase as a lineage tracer, into a single marginal blastomere of 32-64 cell-stage albino embryos. The embryos were analyzed at later stages by Myc immunostaining. In uninjected and control-injected embryos (untagged *Xlefty* RNA), no specific staining was detected. In embryos injected with either *Xlefty^{myc}* or *Xlefty$^{NGM\text{-}Amyc}$*, external observation of whole embryos showed a specific immunostaining reaction that appeared discontinuous and localized to the interstitial spaces between cells, as opposed to a smoothly distributed signal (Fig. 3.10A-D). The distance that Xlefty^{myc} and Xlefty$^{NGM\text{-}Amyc}$ moved from the source of production was assessed by counting the maximal observable number of cell widths of visible signal from the edge of the clone of producer cells (measured on the vegetal aspect, in the direction of the vegetal pole; single measurement per embryo; Table 3.1). Among three independent injection experiments, the inferred range of movement away from the source cells, as

assessed at stage 9, was similar between wild-type and non-glycosylated Xlefty. We also collected embryos at 1, 2, 3, and 4 hours after injection that were then bisected through the patch of marked producer cells prior to Myc immunostaining in order to observe the internal signal (Fig. 3.10E-F). The signal was now intracellular as well as in the interstitial spaces, but the distal-most point of signal from the marked clone of producer cells still seemed to be marked by an extracellular or interstitial signal. As with the surface Myc staining, there was no difference at any time point in the distance that wild-type Xlefty and Xlefty^{NGM} moved from the source cells.

Fig 3.10 Range of Xlefty movement is not altered by glycosylation. A-H: Embryos co-injected with 250 pg of *LacZ* RNA plus or minus 150 pg of tagged or untagged RNAs encoding Xlefty and Xlefty^{NGM-A} and stained for Red-Gal and then Myc-immunostained. A-D: Dashed line, boundary of Red-Gal lineage-label-marked producer cell clone. Broken arrow, direction of vegetal pole. Specific Myc immunodetection signal is purple (arrowhead). **A-D:** Embryos were visualized externally after staining in whole-mount. Embryos were uninjected (A), or received 150 pg untagged *Xlefty* RNA (B), 150 pg *Xlefty^{myc}* RNA (C), or 150 pg *Xlefty$^{NGM-Amyc}$* RNA (D). **E-H:** Embryos were bisected through the patch of Red-Gal staining before myc immunostaining. Embryos were uninjected (E) or received 150 pg untagged *Xlefty* RNA (F), 150 pg *Xlefty^{myc}* RNA (G), or 150 pg *Xlefty$^{NGM-Amyc}$* RNA (H).

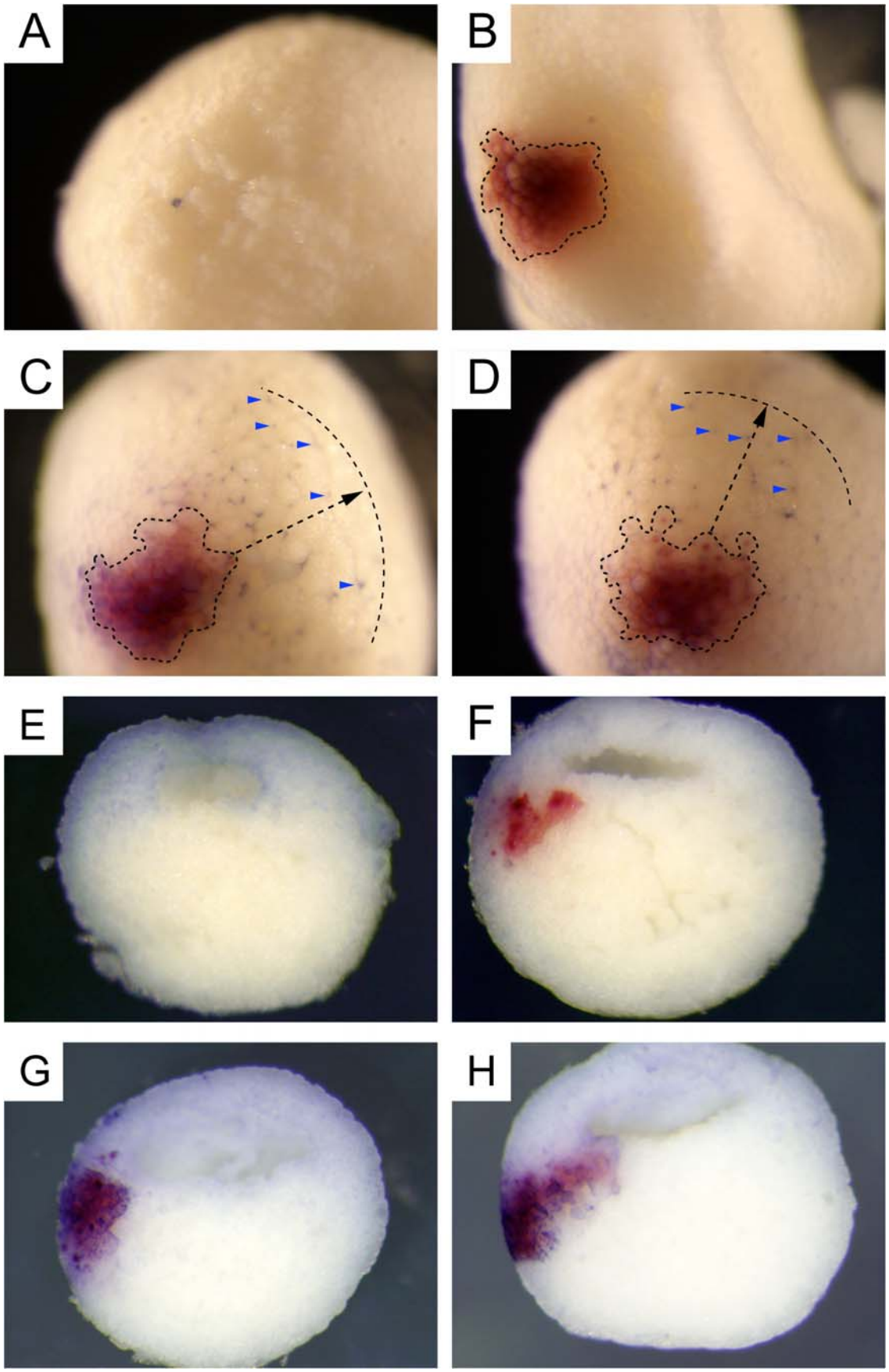
A
B
C
D
E
F
G
H

Table 3.1 Range of movement is not altered by glycosylation

RNA	1			2			3		
	n	average cells	p	n	average cells	p	n	average cells	p
Xlefty^{myc}	17	6.65 ± 2.2	0.103	21	5.28 ± 1.6	0.225	8	6.75 ± 2.1	0.613
Xlefty$^{NGM\text{-}Amyc}$	16	5.40 ± 2.0		17	6.00 ± 1.9		9	7.33 ± 2.5	

150 pg of either *Xlefty*^{myc} or *Xlefty*$^{NGM\text{-}Amyc}$ RNAs were co-injected with 250 pg of RNA encoding nuclear-localized β-galactosidase into one marginal blastomere of a 32-64 cell-stage embryo. Cell widths were calculated as described in the text. Three independent experiments (1, 2, and 3) were performed. n, number of embryos scored.

Discussion

In this study, we demonstrated that various vertebrate Lefty molecules were secreted as glycoproteins from oocytes and that Xlefty was glycosylated when secreted from embryonic cells. While all vertebrate Lefty proteins contain a single N-linked glycosylation motif in the mature region, our data suggest that, at a gross level, glycosylation does not influence protein stability, Nodal-blocking function, or its movement through embryonic tissues.

We showed that Xlefty is capable of being cleaved to produce both long and short isoforms in oocytes, although efficient cleavage at CS2 may be regulated by cleavage at CS1. Xlefty^{L} was the only isoform detected, however, when produced from embryonic cells and seems to be the inhibitory isoform in mesoderm induction assays. These findings lead to the conclusion that future biochemical experiments should be sure to employ the Xlefty^{L} isoform in, for example, determining which region(s) of Xlefty binds to either the Nodal ligand or xCR1-3 to antagonize Nodal signaling (Chen and Shen, 2004; Cheng et al., 2004; Tanegashima et al., 2004). Additionally, determining the binding constants for the interaction of Xlefty^{L} with xCR1-3 or Xnr ligands, by producing stable Xlefty^{L} from an Xlefty^{mcs2} construct, may determine the level to which each mechanism contributes to the antagonism of Nodal signaling.

Although the movement characteristics of mouse Lefty 2 and mouse Nodal have been analyzed by expressing EGFP-tagged proteins in chick mesoderm, the movement of Xlefty^{L} in relation to the various Xnrs should be compared in *Xenopus* tissues, where localized injections can be performed to determine how

Xlefty shapes the Xnr morphogen gradient (Sakuma et al., 2002). Since Nodal signaling is important for both mesendoderm induction and proper establishment of the Left-Right (L-R) axis during embryogenesis, understanding how Xlefty shapes the Nodal morphogen gradient during mesendoderm formation might also be relevant to its role in modulating Nodal signaling during L-R specification (Wright, 2001; Schier, 2003). It remains possible that in our range of movement experiments, we are only detecting a fraction of the Xlefty gradient in regions where enough protein accumulates to generate a detectable immunostaining signal, while lower levels are in fact functional beyond these domains. A similar problem exists with the published studies on EGFP-tagged TGFβ-related molecules (Entchev et al., 2000; Teleman and Cohen, 2000; Sakuma et al., 2002; Belenkaya et al., 2004; Williams et al., 2004; Nakamura et al., 2006), which must also rise above a detection threshold that is currently unknown with respect to the level of the ligand that induces downstream gene pathways. This issue is further complicated because the tag location in principle cannot distinguish between the active or inactive ligands or indeed the proprotein forms. Relevant to this point, at the doses used for our range of movement assays, the animal half secretion experiments show that the great majority of the overexpressed Xlefty is being cleaved to the Xlefty^{L} form, as the proprotein is not detected at this RNA level (Fig. 3.8).

Cleavage of Xlefty is required to block Xnr signaling but not secretion

Similar to other TGFβ molecules, Xlefty required proprotein cleavage to block Nodal signaling. In P19 mouse embryonic carcinoma cells, however, human Lefty A proprotein has been reported to activate the MAPK pathway (Ulloa et al., 2001). Mechanistically, this result might be explained by the fact that Lefty molecules have been shown to associate physically with EGF-CFC family members (Chen and Shen, 2004; Cheng et al., 2004; Tanegashima et al., 2004) and that overexpression of FRL1/xCR1 in *Xenopus* tissues can activate MAPK (Yabe et al., 2003; Yokota et al., 2003). Thus, Xlefty engagement of EGF-CFC co-receptors might lead to MAPK activation. Additionally, cross-talk between Nodal and FGF signaling pathways, which is known to occur during mesendoderm specification, may involve physical interactions between EGF-CFC, Xlefty and the FGF and Nodal ligands (Mizoguchi et al., 2006; Poulain et al., 2006). In our experiments in the context of the *Xenopus* embryo, however, we detect no evidence of prolonged activation of ERK1/2 by Xlefty or the proprotein. This discrepancy may be related to the different experimental designs. Ulloa et al. (2001) incubated P19 mouse embryonic carcinoma cells with affinity-purified human Lefty A generated from conditioned medium from transfected human embryonic kidney 293 cells expressing *leftyA*. Western blot analysis was used to confirm that human Lefty A was contained in the eluate, but the purity of the eluted protein was not verified, and it is hard to rule out the additional possibility that a protein capable of MAPK activation was co-purified.

Xlefty^{L} and Xlefty^{S} were produced at approximately equivalent levels when normal (i.e., untagged) Xlefty was produced from oocytes. However, the lack of Xlefty^{S} accumulation to significant levels when embryonic tissues were analyzed may indicate that the short isoform is a clearance intermediate that is much less efficiently eliminated when secreted from oocytes, perhaps related to the absence of an appropriate SPC produced by oocytes. Alternatively, the secondary structure of Xlefty, or the inherent protease sensitivity at each site, may differ between oocytes and embryonic tissues, such that CS2 cleavage does not occur in embryonic tissues. A general conclusion from our studies, therefore, despite the numerous reported experiments using *Xenopus* oocytes to infer the mechanisms regulating the biochemical processing of secreted proproteins (Dale et al., 1989; Thomsen and Melton, 1993; Jones et al., 1996; Cui et al., 1998; Cui et al., 2001; Eimon and Harland, 2002; Degnin et al., 2004), is that only embryonic cells should be used for future studies of Xlefty.

BMP-4 undergoes an ordered cleavage process that affects the strength and range of signaling during gastrulation stages in *Xenopus*. Cleavage at site 1 (S1) separates the ligand from the prodomain and subsequent cleavage within the prodomain (at S2) seems to be required in order to disrupt non-covalent prodomain/ligand interactions, which releases the BMP-4 ligand for productive receptor engagement (Cui et al., 2001). When the BMP-4 ligand remained prodomain-associated, the complex seems to be targeted for efficient degradation via the lysosomal/proteosomal pathway (Degnin et al., 2004). Therefore, Degnin et al. (2004) suggested that tissue-specific cleavage at S2

might contribute to the spatiotemporal regulation of BMP-4 activity. This hypothesis was validated by mutating the cleavage motif at S2 in the endogenous locus of mice, which led to defects being detected in those tissues that require the highest levels of BMP signaling (Goldman et al., 2006).

Because Xlefty^{S} did not accumulate to detectable levels when secreted from embryonic tissues, and overexpression of an engineered Xlefty^{S}-only-expressing proprotein did not block Xnr2-mediated mesoderm induction, we hypothesize that the ordered cleavage of Xlefty, first at CS1 to release the active ligand, and then at CS2 as a post-secretion clearance mechanism, might regulate turnover of Xlefty in the extracellular milieu. Post-secretion CS2 cleavage, from SPC produced by the same or from nearby cells, has a precedent in the numerous reports of extracellular cleavage of TGFβ-related molecules. For example, mouse Lefty and Nodal can be cleaved by SPCs secreted from COS-1 cells (Beck et al., 2002). Moreover, the idea that proprotein-processing enzymes can act non-cell-autonomously is supported by the detection of mouse *nodal* and *spc1/spc4* expression in non-overlapping tissue regions during embryogenesis (Beck et al., 2002). Another relevant example is the detection of endogenously produced SPC6 at the cell surface in adult mouse liver, duodenum and jejunum (Nour et al., 2005). A post-secretion, clearance activity of SPC at CS2 on Xlefty^{L} might not be detectable in our secretion assays (i.e., there was no stabilization, or increased level, of Xlefty^{L} from the CS2 cleavage site mutant over the wild-type protein in, for example, Fig. 3.4B) because movement of Xlefty^{L} into the

culture medium and away from the cell reduces its probability of interaction with secreted or, in particular, cell-surface-localized SPC.

Glycosylation does not alter Xlefty stability, function or movement through tissue

As described in the Introduction of this chapter and in Chapter I, studies of several TGFβ molecules suggest that glycosylation functions to increase the stability of the proprotein/ligand and/or aid in secretion from the producing cells (Sha et al., 1989; Brunner et al., 1992; Lopez et al., 1992; Le Good et al., 2005). When the single N-linked glycosylation motif in Xlefty was mutated, the steady-state level of protein accumulated was similar between Xlefty and Xlefty^{NGM}. In mesoderm induction assays, N-linked glycosylation was not required for Xlefty to antagonize Xnr2 signaling. We cannot yet rule out a role for glycosylation affecting the biological function of Xlefty, as we still might not have performed the signaling or movement assays at an appropriate time. For example, glycosylation of Xlefty may assist in regulating Nodal/Xnr1 signaling during L-R specification.

Vg1 and xBMP-4 have been shown to undergo N-linked glycosylation, but we are uncertain if such a modification is a general mechanism for the stabilization of the ligand domain, or if this effect is specific for a subset of the TGFβ family (Dale et al., 1989; Degnin et al., 2004). We are currently testing the role of glycosylation in regulating the protein stability and signaling strength of the *Xenopus* Nodal-related proteins.

CHAPTER IV

SUMMARY AND FUTURE AIMS

Members of the TGFβ superfamily are key controllers of a variety of cellular processes that include cell differentiation, proliferation, apoptosis, motility and adhesion. A broad range of experiments on the Nodal-related proteins has demonstrated that they are functionally conserved between vertebrates and essential for mesendoderm specification and L-R axis formation. During my thesis research, I investigated the role of post-translational modifications of Xlefty, the primary Nodal antagonist, and how these modifications affect its ability to inhibit Nodal signaling during mesendoderm induction in *Xenopus*. Specifically, I wanted to determine why Xlefty contained two potential cleavage sites in the proprotein. Are both Xlefty^{L} and Xlefty^{S} produced in embryonic tissues? If so, do Xlefty^{L} and Xlefty^{S} have the same potency as antagonists of Nodal signaling? Moreover, I wanted to determine if the glycosylation motif in the mature domain of Xlefty was functional allowing the addition of N-linked sugars to the core protein, as well as to investigate the biological function of this modification.

By performing my experiments in *Xenopus laevis*, which is amenable to both biochemical analysis of proteins and various embryological manipulations, I was able to show that Xlefty undergoes proprotein cleavage and N-linked glycosylation in *Xenopus* tissues. Furthermore, I presented data that shows that

proteolytic cleavage of the proprotein to Xlefty^{L} is required for inhibition of Nodal signaling. At the end of this chapter, I will switch my focus somewhat and present preliminary data demonstrating that N-linked glycosylation does not affect the signaling strength of two Nodal-related proteins, Xnr2 and Xnr5. Since many studies have demonstrated that the function of Lefty and Nodal-related proteins are evolutionarily conserved, my results of Xlefty add significantly to the knowledge of how post-translational modifications modulate Nodal activity. Moreover, my findings strongly support the idea that analysis of biochemical processing of secreted ligands should be performed in the appropriate tissues and at the relevant developmental time.

Post-translational modifications of Xlefty

Proprotein cleavage of Xlefty

When I began my thesis research studies in the Wright laboratory, a previous student, Abby Cheng, had isolated and performed the initial characterization of Xlefty as a feedback inhibitor antagonist of Nodal signaling (Cheng et al., 2000). Proteolytic cleavage of the Xlefty proprotein could generate two isoforms, which might have differed properties with respect to the antagonizing of Nodal signaling. The central issue addressed in my thesis research was the determination of how biochemical processing (proprotein cleavage and glycosylation) of Xlefty influenced its ability to regulate Nodal signaling.

When Xlefty was produced in *Xenopus* oocytes, proprotein cleavage occurred at both CS1 and CS2, although CS2 processing occurred at a low efficiency in the absence of CS1 cleavage. This result could be interpreted as indicating that CS2 cleavage is regulated by cleavage at CS1. Such sequential cleavage would be a situation reminiscent of BMP-4, as described in Chapter I and Chapter III. But in the case of BMP-4, which is an inducer, the second cleavage disrupts a ligand/prodomain complex that allows for secretion of the ligand to activate downstream signaling. In the case of Xlefty, my data suggest that Xlefty^{L} is the functional blocker and that Xlefty^{S} (produced by CS2 cleavage following CS1) might be part of a clearance mechanism to fine-tune the level of Lefty, and thus Nodal signaling present in the embryo. We only detected Xlefty^{L}, which is generated from cleavage at CS1, when we assayed embryonic cells isolated at a stage when Xlefty is normally expressed. The reason why we did not detect Xlefty^{S} when secreted from embryonic cells might be explained by inappropriate or extremely high SPC protease activity in oocytes, which leads to improper CS2 cleavage. Furthermore, Xlefty^{S} might be more stable in the oocyte situation, i.e., the components of the clearance machinery may not be present in the oocyte. Alternatively, SPCs may be unable to access CS2 when Xlefty is produced in embryos, which could be caused by different secondary branching of the sugar side chains on Xlefty when produced in oocytes versus embryonic cells. The idea that glycans can mask CS2 seems unlikely, as we do not detect Xlefty^{S} when Xlefty$^{NGM\text{-}Amyc}$ was secreted from embryonic cells. Future studies should be designed to ascertain the basic secondary structure of the N-linked glycans

attached to Xlefty and determine if the secondary carbohydrate branching is the same when Xlefty is secreted from oocytes or embryonic tissues. Although the Wright lab is not in a position to pursue these experiments, a combination of high-performance liquid chromatography and mass spectrometry could be used for the structural analysis of these carbohydrate moieties. Further biochemical analysis should include assays to determine whether Xlefty^{S} is produced at detectable levels in any other specific stage of development, such as, for example, tailbud stages when Xlefty functions to modulate Nodal signaling during L-R axis formation. This experiment could be performed by injecting pCSKA-Xlefty^{myc}, a plasmid utilized to drive expression of Xlefty^{myc} after gastrulation, into the left four blastomeres of 8-cell stage embryos. After the embryos develop to tailbud stages, the careful isolation of left LPM tissue from uninjected and plasmid injected embryos for western blot analysis could show if Xlefty^{S} accumulates at the stages, suggesting regulated cleavage at different stages could alter the effectiveness of Xlefty as a Nodal antagonist.

Data in Chapter III illustrated that proprotein cleavage of Xlefty was required to inhibit mesendoderm induction initiated by Xnr signaling, which is consistent with the requirement for cleavage of mouse Lefty 1 and Lefty 2 in order to block Nodal signaling (Sakuma et al., 2002). Unlike mouse Lefty 1, in which both long and short isoforms effectively inhibited Nodal signaling when expressed in *Xenopus* animal caps, injection of *Xlefty^{mcs1}* RNA designed to be able to produce only Xlefty^{S} could not block Xnr2-mediated induction of mesodermal marker

genes. The most parsimonious explanation for this result is that Xlefty^{S} is never produced in embryonic tissues.

While Xlefty^{S} may not play a role in mesoderm induction, we do not know if the short isoform functions during L-R specification stages to negatively influence Nodal signaling. If Xlefty^{S} is produced during tailbud stages, there is a possibility that Xlefty^{S} associates with xCR2, the only EGF-CFC family member expressed during stages of L-R axis specification, to terminate Xnr1 signaling in the left LPM (Dorey and Hill, 2006; Onuma et al., 2006). Future analysis should include tissue-grafting assays pioneered by Yuki Ohi, a former graduate student (Ohi and Wright, 2007). In this assay, LPM tissue overexpressing normal Xlefty, when transplanted into the left LPM of host embryos, was able to suppress Xnr1 signaling and block the anteriorward progression of *Xnr1* expression (Ohi and Wright, 2007). A similar experimental design could be performed but now with tissue overexpressing Xlefty^{S} from a plasmid encoding Xlefty^{mcs1}, a cleavage mutant version that should be capable of producing the short isoform.

The endogenous SPC(s) responsible for proteolytic cleavage of vertebrate Lefty molecules is/are currently not known. There are, however, data to suggest that SPC1, SPC4, and SPC6 mediate proprotein cleavage of Lefty in several transfected cell lines (Ulloa et al., 2001; Beck et al., 2002; Sakuma et al., 2002). While these *in vitro* assays are useful in determining which SPCs can cleave Lefty in an overexpressed, and perhaps non-physiological situation. These assays may not be relevant to the endogenous enzyme produced and functional in the normal embryonic environment and stage in which Lefty and Nodal

operate. The problem of studying proteolytic cleavage of TGFβ molecules in a non-physiological assay is illustrated by the fact that Activin was cleaved by recombinant but not endogenous SPC4 (Cui et al., 1998; Birsoy et al., 2005). In the experiment utilized in determining that Activin was not an enzymatic substrate for endogenous SPC4, *spc4* mRNA was depleted by injecting antisense oligonucleotides into *Xenopus* oocytes, which were transferred to recipient *Xenopus* females (Birsoy et al., 2005). Following fertilization, embryos were injected with a RNA encoding an epitope-tagged version of Activin. Western blot analysis of Activin processing activity was performed on blastocoel fluid and was compared between normal (not injected with oligonucleotides targeted against *spc4*) and *spc4* depleted embryos. In order to establish a full detailed mechanistic understanding of the way in which Lefty inhibits Nodal signaling activity, future analyses would therefore be expected to include experiments to determine which SPC(s) is the endogenous enzyme responsible for Xlefty cleavage. In one approach, antisense phosphorothioate oligonucleotides, which are resistant to nucleases, will be injected into *Xenopus* oocytes to deplete specific *spc* mRNAs and then transferred to recipient females for fertilization. Western blot analysis of conditioned medium would then be used to compare the cleavage products of Xlefty produced from *spc*-deficient embryos and embryos not injected with spc oligonucleotides. This type of experiment has already been performed in *Xenopus* to analyze some of the biological substrates of SPC4 and should be relatively straightforward (Birsoy et al., 2005).

Xlefty is N-glycosylated

The addition of N-linked glycans to core proteins is an energy and time consuming process (Jones et al., 2005). The process involves the ATP-dependent generation of the dolichol-phosphate acceptor, to which seven sugars are added on the cytoplasmic face of the endoplasmic reticulum (ER) (Helenius and Aebi, 2004). This dolichol-heptasaccharide precursor undergoes a topological flipping such that the sugar residues are now facing the lumen of the ER. The seven sugar precursor is extended to 14, and this “full precursor” is transferred as a core oligosaccharide unit onto the nascent polypeptide chain (Helenius and Aebi, 2001). The basic oligosaccharide is then extensively modified by removing and adding sugar residues in the ER and Golgi complex (Helenius and Aebi, 2001). The modification of the N-linked glycans results in three general categories of oligosaccharides (high-mannose, hybrid, and complex glycans) with distinct functions (Helenius and Aebi, 2001). For example, high-mannose glycans can be further modified to mannose-6-phosphate, a moiety that functions to target proteins to the lysosome (Helenius and Aebi, 2001).

As stated in Chapter I and Chapter III, the presence of N-linked glycans can affect numerous biological processes. The presence of a conserved N-linked glycosylation site in the mature domain of Lefty raised the possibility that carbohydrate moieties influence protein secretion, biological activity and/or movement through embryonic tissues. There are several reports of glycosylation effects on the TGFβ family, such as TGFβ1, TGFβ2, and Nodal. All vertebrate

Lefty molecules contain a single N-linked glycosylation motif in the mature domain. Furthermore, the placement of this motif is conserved at approximately 20 amino acids downstream of CS2. For this reason, I wanted to ask whether N-linked glycans could be added to Xlefty as well as to define the biological role for this modification. In the case of TGFβ1 and TGFβ2, blocking the addition of N-linked glycans by either mutating asparagine residues or adding tunicamycin to the culture medium resulted in a block to secretion and led to intracellular accumulation of the proprotein (Sha et al., 1989; Brunner et al., 1992; Lopez et al., 1992). It is possible that N-linked glycans are required for proper protein folding and that non-glycosylated TGFβ1, which does not reach the normal conformation, is retained in the ER for eventual degradation (Helenius and Aebi, 2004). Furthermore, N-linked glycans have been shown to positively influence several biological attributes of mouse Nodal. For example, the insertion of an artificial glycosylation site (to mimic the site found in several Xnr ligands, as described in more detail below) into mouse Nodal resulted in increased steady-state protein levels when secreted from cultured cell lines, and signaling strength in activating Nodal-response genes in zebrafish embryos compared to the wild-type protein (Le Good et al., 2005).

The data presented in Chapter III demonstrate that Xlefty was N-glycosylated when secreted from animal halves, but that glycosylation did not influence the steady state level of protein accumulation, Nodal-inhibitory function during mesoderm induction, or long-range movement through *Xenopus* blastula stage tissues. Future analysis should include assays to determine if sugars residues

alter the intrinsic protein half-life of Xlefty in its normal embryonic context, which could be done by pulse-chase experiments. These experiments are not trivial to perform in *Xenopus*, because it is well known that these large embryos contain massive stores of RNAs and amino acids deposited during oogenesis. The difficulty arises from the principles of the pulse-chase type assay. In this analysis, there is a depletion of a precursor (amino acids or RNAs) pool, which is followed by a short labeling time with radioactive precursor. Next, the non-radioactive amino acids or RNAs are added to reconstitute the large pool. The amount of labeled protein or RNA is followed in time, and how it decays or becomes converted into specific cleavage products, is then determined. While radioactive amino acids can be incorporated into *Xenopus* embryos to a level that allows detection, the specific activity of the labeled protein is often low and variable, and the amount that needs to be added results in the post-pulse being difficult to attain properly.

As mentioned above, Xlefty is thought to function as a long-range feedback inhibitor of Xnr1 during the process of L-R axis specification in *Xenopus* embryos and the glycosylation state may positively or negatively influence its range of movement through the left LPM. This issue could be addressed using the techniques of Ohi and Wright (2007) by transplanting LPM tissue overexpressing *Xlefty*^{myc} or a glycosylation mutant version of Xlefty (*Xlefty*^{NGMmyc}) [which are known to have equal functions as Xlefty; they antagonize Xnr signaling as effectively as the untagged proteins as measured by the suppression of Xnr2-mediated induction of mesodermal marker genes] into the left LPM of host

embryos. After the embryos reach tailbud stage, they could be fixed at various time points for immunohistochemical analysis with an anti-myc antibody. The result of this experiment might determine how glycosylation influences the movement of Xlefty through the LPM. Furthermore, the route that Xlefty travels within the embryo during tailbud stages is unknown. Based on the low resolution histological analysis of neurula and tailbud stage embryos, the LPM consists of more than one cell layer sandwiched between the overlying ectoderm and the adjacent endoderm (Hausen and Riebesellm, 1991). There could be several potential paths that Xlefty could utilize for its long-range movement through the embryo to attenuate Nodal signaling. For example, Xlefty could travel through the interstitial space between cells in the plane of the LPM. Alternatively, there could be space between the LPM and endoderm (or ectoderm) through which Xlefty travels. Glycans present on Xlefty may influence that course and speed of movement though the LPM or embryonic tissues at these later stages.

Xlefty is Nodal-specific antagonist

While the general consensus is that a principal conserved function of Lefty is a feedback inhibitor of Nodal signaling during mesendoderm induction and L-R patterning in vertebrate embryos, there are a few reports that suggesting that Lefty molecules can function in other signaling contexts. Furthermore, there are data to suggest that signaling pathways other than Nodal can induce the transcription of *lefty*.

Initially, mouse Lefty 1 was isolated from a subtractive hybridization screen trying to identify genes that were regulated by the transcription factor Oct-3 in P19 embryonic carcinoma cells (Meno et al., 1996). Subsequently, a second isoform was discovered in mouse by similarity-based methods (Meno et al., 1997). Concurrent with these studies, Oulad-Abdelghani et al. (Oulad-Abdelghani et al., 1998) also isolated mouse *lefty 1* as a retinoic acid-induced gene in P19 embryonic carcinoma cells (Oulad-Abdelghani et al., 1998).

Prior to defining the function of Lefty molecules as an inhibitor of Nodal, Lefty was thought to be an anti-BMP molecule. This idea came from experiments overexpressing $BMP^{prodomain}$-$Lefty^{mature}$ chimeric proteins in *Xenopus* animal caps, which led to a neuralized phenotype (Meno et al., 1997). However, when normal Xlefty was overexpressed in animal caps, there was no induction of neural markers (Cheng et al., 2000). These conflicting results might be explained by results from Daniel Constam and colleagues. They demonstrated that the prodomains of TGFβ-related factors can influence the stability of the mature domains (Constam and Robertson, 1999). Thus, it is possible that the prodomain from the chimeric protein mediates an inappropriate association with the ligand domain of endogenous BMPs, generating a complex that was unable to initiate downstream signaling. This neuralized phenotype was the result of diminished BMP signaling, which directed animal caps down the default neural induction program (Kuroda et al., 2005).

Recently, another function has been ascribed to Lefty molecules. Overexpressed Human Lefty molecules (both the proprotein and the mature

domain) could apparently induce the MAP Kinase pathway in P19 mouse embryonic carcinoma cells (Ulloa et al., 2001). However, in my experiments that directly addressed this issue by overexpressing Xlefty in *Xenopus* tissue, I could not detect MAPK activation. The reason for this inconsistency is not known but could relate to the way Lefty was produced in their assay. Ulloa et al. (2001) incubated P19 mouse embryonic carcinoma cells with affinity-purified human Lefty A generated from conditioned medium from transfected human embryonic kidney 293 cells expressing *leftyA* and while western blot analysis confirmed the presence of human Lefty A in the eluate, its purity even after partial "affinity" purification was not verified. It is, therefore, possible that a protein capable of MAPK activation was co-purified.

Despite these few reports that Lefty might be a multifunctional protein (anti-BMP and inducer of MAPK), the overwhelming weight of data being to work as a Nodal-inducible feedback antagonist of the Nodal auto-regulatory signaling loop, is in agreement with the primary role of Lefty is that of a Nodal antagonist.

Does glycosylation regulate Xnr signaling?

In the previous sections of this thesis, I have been discussing how post-translational modifications influence the ability of Xlefty to modulate Xnr signaling. In this section, I begin to change the focus from the antagonist and move onto explorations of the effect of N-linked glycosylation on the activities of the inducers themselves; specifically the Xnr ligands. As discussed in Chapter I and Chapter III, when mouse Nodal was created by inserting an artificial

glycosylation site into the ligand domain (a site present in five of the six Xnrs), it had increased signaling strength in zebrafish embryos and stability in culture cells (Le Good et al., 2005). The placement of the glycosylation motif is conserved (five amino acids downstream of the third cysteine residue) in Xnr 1, 2, 3, 4, and 6, but is absent from in Xnr5 (Table 4.1). If the data from Daniel Constam and colleagues are correct, sugar residues present on the Xnr ligands should increase the intrinsic protein stability and signaling strength of these powerful inducers compared to the non-glycosylated version (Le Good et al., 2005).

Table 4.1 Alignment of amino acid sequences of Nodal-related proteins

Nodal-related protein	Amino acid sequence
Xnr1	C P I P L N E T F K P T
Xnr2	C P I P L N E T F K P T
Xnr3	C A V P Q N E T E N A T
Xnr4	C P S P V N E S V K P N
Xnr5	C P I P L D E N F K P T
Xnr6	C P I P L N E S F K P T
Nodal	C P N P V G E E F H P T
Cyclops	C P N P L G E E L R P T
Squint	C P T P V D E T F T P T
Southpaw	C P S P L D E T Y N P T

Alignment of Xnr1 residues 339-350 and the corresponding sequences from the Nodal-related proteins from *Xenopus*, mouse, and zebrafish. Putative N-glycosylation sites are boxed in yellow.

For simplicity, I selected two Xnrs (Xnr2 and Xnr5) for these studies. In order to test the biological function of these N-linked glycans, I used site-directed mutagenesis to remove the glycosylation motif in Xnr2 and add the N-E-T consensus site to Xnr5 (the N-linked site is at the same location as Xnr1/2/4/6). Using mesoderm induction assays, we compared the activity of wild-type Xnrs to the mutant versions (Xnr2 glycosylation mutant, $Xnr2^{NGM}$; glycosylated version of Xnr5, $Xnr5^{G}$). My preliminary experimental results show that N-linked glycans do not influence the signaling strength of Xnrs in this range-independent assay, as the distribution of RNAs amongst all animal cap cells effectively removes any potential effect from the range of movement of the inducer (Fig. 4.1). Using similar experiments described in Chapter III, future analysis will include assays to determine if glycosylation affects intrinsic protein stability and the movement of these ligands through blastula stage tissues.

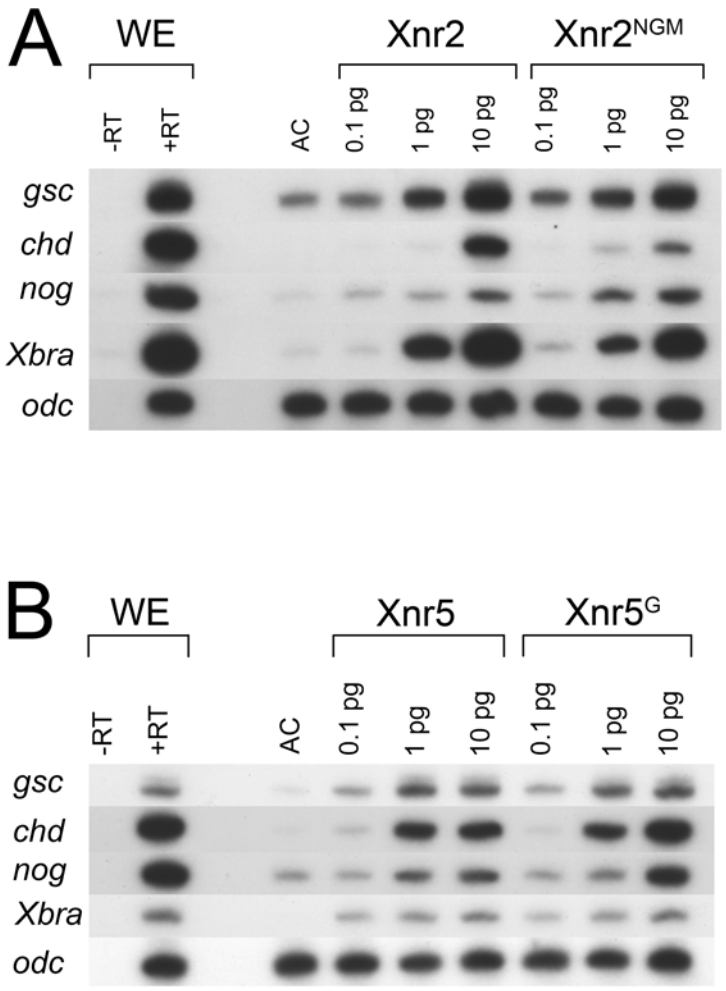

Fig 4.1 Glycosylation does not alter the signaling strength of Xnr2 and Xnr5. A, B: One-cell stage embryos were injected with RNA (pg/embryo indicated) encoding Xnr2, Xnr2^{NGM} (mutated glycosylation site), Xnr5, or Xnr5^{G} (inserted glycosylation site). Animal caps were analyzed at stage 10.5 for mesodermal marker gene expression. WE, whole embryo plus (+) and minus (-) reverse transcriptase (RT); AC, uninjected animal cap.

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