Investigating The Role Of LBH During Early Embryonic Development In Xenopus Laevis

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INVESTIGATING THE ROLE OF LBH DURING EARLY EMBRYONIC DEVELOPMENT IN *XENOPUS LAEVIS*

A Thesis Presented

by

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INVESTIGATING THE ROLE OF LBH DURING EARLY EMBRYONIC DEVELOPMENT IN XENOPUS LAEVIS

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ABSTRACT

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LBH is a highly conserved protein whose role during vertebrate development is relatively under-studied. It was first identified in the mouse as a nuclear protein expressed in the limb bud and heart. It has since been shown to play a role in mammary gland development in the mouse and wing development in the chicken. In collaboration with the Albertson lab, our lab has shown that it is necessary for cranial neural crest cell migration in the zebrafish and in Xenopus laevis. The molecular mechanisms through which it acts are not well understood.

In Xenopus, LBH is a maternally deposited protein. As such, studying its role in early embryonic development has not been feasible through the morpholino-mediated knockdown techniques that prevent translation of target genes. Recently, a technique for degrading endogenous proteins was developed, called Trim-Away. This was developed in mammalian systems and utilizes the E3 ubiquitin ligase Trim21 in conjunction with an antibody against a protein of interest in order to degrade the protein. In order to observe the effects of a knockdown of LBH during early embryonic development, we sought to modify the technique for use in Xenopus.

We injected embryos with mRNA encoding the human form of trim21 along with a monoclonal antibody against LBH that our lab developed (2B8). We tracked
degradation of the protein over time and monitored embryos for any phenotypes arising during early development.

Our results demonstrate that Trim-Away can be utilized in *Xenopus*, although it is not as efficient as the original publication. The LBH depleted embryos display a variety of defects during gastrulation, the process by which the three germ layers are organized during development. These appear to be mainly due to defects in fibronectin fibrillogenesis and mesodermal migration. Our results suggest that LBH may be acting through Wnt signaling, although further work must be done to determine this.
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CHAPTER 1

INTRODUCTION

Gastrulation

Gastrulation is the process by which the three germ layers- ectoderm, mesoderm, and endoderm- are properly organized during early embryonic development. Before migration, these 3 germ layers are organized in a “layer cake” fashion. By the end of gastrulation, the endoderm will be found in the deepest part of the embryo, while the ectoderm will be found superficially with the mesoderm organized in between the first two. In amphibians, this process requires a series of complex cell movements and migration. These include internalization of endomesoderm into the blastocoel cavity, epiboly of the ectoderm, and convergence and extension of the dorsal mesoderm.

Up through the beginning of gastrulation, the Xenopus embryo can be divided into the animal hemisphere and vegetal hemisphere. The animal hemisphere contains a cavity, referred to as the blastocoel, and the vegetal hemisphere contains the cells of the vegetal mass, which contain the majority of the yolk (Figure 1, stage 10).

In Xenopus, gastrulation requires the following sequence of events. First, bottle cells arise on the dorsal side of the equatorial region. These bottle cells will attach themselves onto the blastocoel roof and drive the involution of the endoderm and mesoderm (Gilbert 2010). The further internalization of endoderm and movement of mesoderm is driven by vegetal rotation of the vegetal mass (Winklbauer & Schurfeld 1999). This site of invagination forms the blastopore lip, which eventually expands laterally to form the ring-shaped blastopore present in the vegetal hemisphere of the
embryo. A general schematic for this movement can be seen in Figure 1, adapted from Wen & Winklbauer 2017.

![Figure 1. Tissue movement during gastrulation](modified from Wen & Winklbauer 2017). Shown are mid-sagitally fractured embryos. White-ectoderm, blue-mesoderm, yellow-endoderm.

Once the invagination has started, other cell movements will occur to accommodate the change of the embryo’s geometry triggered by the internalization of the vegetal mass. Ectoderm, covering only half of the embryo at the beginning of gastrulation, needs to expand to cover the entirety of the embryo. This ectodermal epiboly movement is achieved by the thinning and lengthening of tissue. In *Xenopus*, this is achieved through radial interdigitation whereby cells of the deeper layers lengthen and intercalate to decrease the number of cell layers in the tissue (Keller 1980). Oriented cell division also contributes to epiboly although represents a minor contribution (Marsden and DeSimone 2001).

In anurans, the main driver of the mesoderm’s internalization is not the involution of the bottle cells and their migration onto the blastocoel roof, but the convergence and extension of the dorsal tissues, including the dorsal mesoderm and dorsal ectoderm. This convergence and extension movement describes narrowing and lengthening movements.
of the aforementioned tissues which happen by mediolateral intercalation of the cells toward the embryos’ midline (Keller et al. 1985). This movement will allow the mesoderm to cover the blastocoel roof. This mesoderm migrates as a sheet with a leading edge and ultimately meets to form a circular mantle at the animal pole (Figure 1). Incidentally, this movement is responsible for changing the geometry of embryos from a ball of cells to an elongated shape. Altogether, these movements are responsible for the closing of the blastopore which then will become the future vent of the animal (Keller & Shook 2008).

Fibronectin (FN) plays a critical role during amphibian gastrulation. It is necessary for proper epiboly (Marsden & Desimone 2001), mesodermal mantle closure (Rozario et al. 2009), and endoderm internalization (Winklbauer & Keller 1996, Ramos & Desimone 1996, Wen & Winklbauer 2017). Fibronectin is an extracellular matrix protein secreted by cells as globular dimers. These dimers bind with integrins on the cell surface (Mao & Schwarzbauer 2005a). More specifically, fibrils form when the FN dimers form puncta at the cell surface then stretch to form multimers with other FN dimers (Davidson et al., 2008). While many other FN binding integrins are present in Xenopus embryo, the integrin α5β1 is necessary and sufficient for FN fibrillogenesis (Ramos et al. 1996, Davidson et al. 2002, Davidson et al. 2006). Integrin α5β1 is present throughout all 3 germ layers of the Xenopus embryo (Joos et al. 1995), but only the cells of the blastocoel roof (ectodermic in nature) are capable of performing fibrillogenesis. It is thought that integrin α5β1 exists in different activation states in the mesoderm and ectoderm during gastrulation in Xenopus (Ramos & Desimone 1996).
Blocking FN from forming a matrix during gastrulation prevents embryos from undergoing epiboly of the blastocoel roof (Fig. 2 Marsden & Desimone 2001). When FN fibrillogenesis is perturbed through blockage of FN-FN binding, fibronectin accumulates at the cell surface and migrating mesendoderm does not adhere as well to the blastocoel roof but advances to close the mantle more quickly (Fig. 3 Rozario et al. 2009). Vegetal cells also secrete FN (Winklbauer 1998). When integrin-FN interaction is blocked, endoderm cell adhesion is not maintained in the embryo and endodermal cells do not migrate \textit{in vitro} (Fig. 2 Wen & Winklbauer 2017). Additionally, blocking the FN RGD cell attachment site prevents endoderm internalization, as evidenced by the failure of the blastopore to close (Winklbauer & Keller 1996, Ramos & Desimone 1996).

FN has been implicated in Wnt signaling pathways, both during gastrulation in \textit{Xenopus} and in \textit{Xenopus} cell culture. Wnt signaling is important in a variety of processes during development and can be divided into canonical (\(\beta\)-catenin activity) and non-canonical (calcium/NFAT and planar cell polarity) signaling. Wnt signaling is involved in the formation and maintenance of the FN matrix of the blastocoel roof through a non-canonical signaling pathway (Dzamba et al. 2009). Specifically, Wnt11 is required for convergent extension and fibrillogenesis (Dzamba et al. 2009). Canonical Wnt signaling is also associated with FN in \textit{Xenopus in vitro}. In \textit{Xenopus} fibroblasts \(\beta\)-catenin appears necessary for FN expression (Gradl et al. 1999). Sequestration of signaling \(\beta\)-catenin through cadherin overexpression as well as inhibition of canonical Wnt signaling with Wnt5a decrease expression of FN (Gradl et al. 1999).
Figure 2. Epiboly of the blastocoel roof (BCR) in *Xenopus laevis* embryos (modified from Marsden & Desimone 2001). (A) Embryo injected with an antibody against FN that is not function blocking. Green shows the FN matrix. Embryo has undergone epiboly and there is one layer of deep cells (B) Embryo injected with a function blocking antibody against FN. No FN matrix is detected. Embryo failed to undergo epiboly of the BCR, evidenced by the multi-layered deep cells. White arrowheads point to site of FN matrix.

Figure 3. Mesendoderm migration in embryos lacking fibrillar FN (modified from Rozario et al. 2009). (A) Representation of sections shown in B & C. (B) Uninjected control embryo shows normal mesendoderm adhesion and migration. (C & D) Embryos lacking fibrillar FN display mesendoderm that does not adhere as well to the BCR, but advances to the BCR more rapidly. bc-blastocoel, bcr-blastocoel roof, me-mesendoderm
Figure 4. Endodermal dissociation in integrin-FN blocked embryos (modified from Wen & Winklbauer 2017). Embryos in which FN is blocked from interacting with integrin with RGD peptides display defects in endodermal cell association.

LBH

LBH is a small, highly conserved protein that we have previously demonstrated is necessary in cranial neural crest cell migration through collaboration with the Albertson lab. The Albertson lab identified a nonsynonymous mutation in LBH between two cichlid fish species with distinct jaw morphologies: Melandia zebra and Labeotrophi fuelleborni (Powder et al. 2014). The mutation exchanged an arginine in position 17 into a glutamine. Morpholino mediated knockdown of LBH in the zebrafish and Xenopus results in defects in the migration of a population of cells essential for craniofacial development, the cranial neural crest (CNC) cells (Fig. 5, Powder et al. 2014). This defect can be rescued by the expression of the wild type form of Xenopus and M.zebra but not the L.fuelleborni form, indicating that the mutation in LF changed the function of LBH protein. We have previously found that the domains 2-29 and 54-63 are necessary for LBH’s function during CNC migration (Weir et al., in prep). Interestingly, the former is poorly conserved overall among species while the latter is extremely well conserved.
While poorly conserved, the domain 2-28 contains an evolutionary conserved Threonine in position 19 (cichlid) or 20 (vertebrates). This Threonine is predicted to be phosphorylated in Xenopus and MZ form of LBH but not LF form. The mutation of this T20 into a phosphomimic restores the capacity of LF-LBH to rescue the migration of morphant CNC, indicating that phosphorylation of Threonine 20 is critical for LBH function during CNC migration (Cousin et al., in preparation). We also showed using immunoprecipitation and LC-MS/MS mass spec that the domain 54-63 binds the serine/threonine phosphatase calcineurin A (CnA) (Weir et al., in prep). We showed that the depletion of CnA leads to CNC migration defect. We also showed that overexpression of a wild type or constitutively active form of calcineurin A is able to rescue CNC migration of LBH morphant embryos but not the phosphatase dead version of it (Weir et al. in prep).

In addition to our work on the role of LBH in CNC cell migration, LBH has been shown to be involved in heart, appendicular skeleton, and mammary gland development (Briegel et al. 2005, Conen et al. 2009, Lindley & Briegel 2013, Lindley et al. 2015). It was first identified in the mouse, with expression detected in the promyocardium, foregut, branchial arches, ventral ectodermal ridge, ventral ectoderm of forelimbs and hindlimbs, otic vesicles, oral epithelium, sensory neurons of the dorsal root, and trigeminal ganglions (Briegel & Joyner 2001). Overexpression of LBH in the mouse myocardium results in cardiovascular defects including outflow and inflow tract defects, abnormal septation, abnormal heart positioning, and abnormal ventricular development (Briegel et al 2005). Mice homozygous for an LBH null allele display no defects in embryonic development, but have postnatal defects in mammary development, namely
delayed expansion of mammary epithelium during puberty and a reduced alveolar compartment in parous mice (Lindley & Briegel 2013). Overexpression of LBH in the chick wing prevents proper chondrocyte maturation, vascular invasion, and therefore normal bone formation (Conen et al. 2009). Additionally, this overexpression was shown to repress mRNA expression of chondrocyte hypertrophy regulator Runx2 and VEGF (Conen et al. 2009).

**Figure 5. Defects in CNC cell migration in LBH knockdown embryos** (modified from Powder et al. 2014). (A) non-injected zebrafish (B-D) morphant zebrafish (E) *Xenopus* with control CNC cell graft (F & G) *Xenopus* with morphant CNC cell graft. e-eye, m-mandibular stream, h-hyoid stream, br-branchial stream, cg-cement gland.

LBH has been implicated in canonical Wnt signaling in the mouse and in mammalian cell culture. LBH has 4 putative TCF/LEF binding sites and is responsive to
β-catenin in luciferase reporter assays (Rieger et al. 2010). LBH mRNA expression is induced by Wnt3a and this expression can be blocked with both the canonical Wnt inhibitor DKK1 and β-catenin siRNA (Rieger et al. 2010). In the mouse limb bud, LBH is present in the apical ectodermal ridge and ventral region of the limb bud ectoderm and expands to the dorsal ectoderm when Wnt7a is knocked out (Rieger et al. 2010). Additionally, it is overexpressed in Wnt1 induced mammary tumors, and tumor development can be reduced when LBH is knocked out in this model (Rieger et al. 2010, Ashad-Bishop et al. 2019)

**Trim-Away**

Trim-Away is a relatively new technique of degrading endogenous proteins. It utilizes the E3 ubiquitin ligase trim21 in conjunction with an antibody against the protein of interest in order to degrade said protein (Clift et al. 2017). Trim21 recognizes the FC domain of the antibody, complexes with the antibody and its target, and the entire complex is sent for degradation in the proteasome (Fig. 6). This technique was initially developed in mammalian systems—both mammalian cell lines and mouse oocytes—and was more recently used successfully in zebrafish embryos (Chen et al. 2017). Although *Xenopus laevis* do not possess a trim21 ortholog, we wanted to see if we could employ the Trim-Away system in *Xenopus* using the mammalian form of trim21 in order to investigate the role of LBH in early embryonic development. The lab previously developed a mouse monoclonal antibody (mAb 2B8) against *Xenopus* LBH. The domain 63-84 was identified as the region containing the epitope recognized by 2B8 (Fig. 7).
Figure 6. Mechanism of Trim-Away mediated protein degradation (Clift et al. 2017). Trim21 binds to the FC domain of an antibody in the cytosol. The trim21, antibody, and protein of interest are degraded in the proteasome.

Figure 7. Alignment of LBH protein sequence. Alignment shown between Xenopus, two cichlid species and chicken. Y indicates region containing epitope for 2B8 binding.
CHAPTER 2

METHODS

Eggs and embryos

Xenopus laevis embryos were fertilized in vitro, as described in Cousin et al. 2000. Embryos were staged according to Nieuwkoop & Faber 1967.

Constructs

LBH deletion mutations were produced by all round PCR using LBH-L pCS2+ as a template and either pfu DNA polymerase as described in Cousin et al., 2008 or the Phusion DNA polymerase (NEB) and the In Fusion HD cloning system (CloneTech) as per manufacturer’s guidelines. Human trim21 (Gift from Eric Streiter) was cloned into pCS107 via Gateway cloning system, per manufacturer’s guidelines (Invitrogen).

All other constructs were previously cloned into pCS2.

mRNA transcription

Trim21, Δ63-84, and membrane cherry plasmids were linearized using NotI enzyme (Thermo Scientific #FD0596). Transcription was carried out using SP6 RNA polymerase as described in Cousin et al. 2000. mRNA was re-suspended in DEPC treated water at 0.2ng/nL for injection.

Antibody purification

Supernatant was collected from hybridoma cells grown in a Bioreactor, as per manufacturer’s guidelines (Corning ref. 353137). Sup was incubated with 200uL of high affinity protein A/G beads (Thermo Scientific ref. 20423) overnight at 4°C. Antibody was eluted in 100mM glycine pH 2.8 and neutralized in tris pH 9.5. Following elution, glycine was removed using Zeba spin desalting columns (Thermo Scientific ref. 89882)
or fractions were pooled together in a centricon (Millipore ref. UFC803008) to remove glycine and concentrate antibody.

**Injections**

Embryos were injected at the one-cell or eight-cell stage in 1xMBS 3% ficoll. At the one-cell stage injections were done sequentially, with 1ng of trim21 mRNA then 0.5ng or 1ng of LBH Δ63-84 in the case of rescue experiments. 26.8ng, 36.6ng, 70.2ng, or 46.8ng of 2B8 were then injected, depending on the preparation used. At the eight-cell stage, embryos were injected in 4 either the 4 vegetal blastomeres or 4 animal blastomeres with 250 pg trim21 mRNA, 125 pg nuclear cherry mRNA, and 8-11.7 ng 2B8 in each blastomere. Embryos were kept at 14°C overnight following injections, then moved to 18°C the next morning.

**Whole mount in situ hybridization**

Whole mount *in situ* hybridization was carried out as described in Harland 1991. Probes were generated using digoxigenin-rUTP label described in Cousin et al., 2000. Images were taken using a Zeiss stereomicroscope Lumar-V12 equipped with a digital AxioCam HRc color camera and the Axiovision software.

**Cell transfection and protein extraction**

Embryos were extracted in 1xMBS 1% tritonX100 Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific ref. 78444) and 5mM EDTA, at a volume of 20uL/embryo. Yolk was removed by a centrifugation of 15,000g for 30 minutes. Supernatant was processed. Tissue culture cells were transfected using X-tremeGENE HP DNA transfection reagent (Roche ref. 06366236001) or PEI (Polysciences ref. 24765-1). After 24–48 hours, cells were rinsed and either extracted directly or frozen at -80°C.
and extracted later. For direct extraction, reducing laemmli was added to the cells and the sup passed through a 23gauge needle.

**Western blot and antibody usage**

SDS-PAGE, transfer, and western blot were carried out as in (Khedgikar et al. 2017). The primary antibodies used were: mAb 2B8, anti-trim21 (CST # #92043S) anti-Flag M2 (SigmaF3165), anti-D (abm G191), GAPDH (Millipore MAB374), and rpn1 (Khedgikar et al. 2017). In the case of successive blots on the same membrane, HRP in the secondary antibody was rendered inactive with 10% H₂O₂ in water for 10 minutes at room temperature and re-blocked as before.

**Animal caps and immunofluorescence**

Animal caps were dissected from stage 11 embryos as described in (Khedgikar et al. 2017). They were fixed in MEMFA for 1 hour at room temperature or overnight at 4°C. They were then washed 3x5 minutes in PBS 0.1% Tween and blocked in PBS 0.1% Tween 1% BSA for 1 hour at room temperature or overnight at 4°C. Following block, there were incubated with α-fibronectin mAb 4H2 (Ramos et al. 1996) overnight at 4°C, washed 3x5 minutes in PBS 0.1% Tween, incubated in α-mouse 488, washed 3x20 minutes in PBS 0.1% Tween, and mounted in Vectashield (Vector Laboratories #H-1000) on cover slides. Images were taken using Axiovert 200M.

**Single cell migration assay**

The dorsal blastopore lip from 5 embryos stage 10 was dissected using an eyebrow knife. The 5 lips were placed together in calcium/magnesium free media and left for 1 hour at room temperature to allow cells to dissociate. Cells were then plated on a 96 well plate that was coated with fibronectin at 10ug/mL, in DAN BSA. A time-lapse
movie was set up using BZ-X710 to track migration. Images were taken every 3 minutes. Fiji Trackmate was used to create tracing to track single cells.

**Concanavalin A purification**

5 or 10 embryos were extracted per case as outlined under “protein extraction,” and the extract was incubated with 20uL of Concanavalin A bound agarose beads (Vector Laboratories #AL-1003) overnight at 4°C. After incubation, a portion of the extract was taken and added to reducing laemmli as a sample of the non membrane-associated proteins. Beads were washed 3x with 1xMBS 1% tritonX100 and 1x with 1xMBS and eluted in 50 or 100 uL reducing laemmli.
CHAPTER 3

RESULTS

Characterization of *Xenopus laevis* LBH

Western blot was performed on embryos collected at different developmental stages (2-21) to see when LBH was expressed in *Xenopus* during early embryonic development. The presence of LBH at stage 2 indicates that LBH is a maternally deposited protein, as the embryo does not begin translating its own protein until the mid blastula transition, around stage 8 (Fig. 8). LBH is expressed throughout early embryonic development (Fig. 8). There are two bands detected for LBH; one is around 15kDa and is present at all stages shown. The second is slightly heavier and is only present from st.2-st.12.5. This slightly larger band may represent some post-translational modification or alternative splicing.

![Western blot image](image-url)

**Figure 8.** LBH expression during early embryonic development. Western blot performed on protein extracted from wild-type *Xenopus laevis* at various stages. Blot for rpn1 serves as a loading control.

Trim-Away is amenable for use in *Xenopus laevis*

In order to test whether Trim-Away could be used in *Xenopus*, we injected embryos with purified 2B8 (26.8-70.2ng) and mRNA encoding the human trim21 (1ng)
at the one-cell stage. Embryos injected with both trim21 mRNA and 2B8 will be referred to as “Trimmed” or “Trim-Away LBH” embryos. In the mouse and mammalian cell culture (Clift et al. 2017) as well as the zebrafish (Chen et al. 2019), near complete protein degradation was achieved in one hour. We achieved protein degradation in our trimmed embryos approximately 1 day post injection, around st.9 (Fig. 9). We injected different amounts of 2B8 in order to see if increasing the amount of 2B8 would help achieve a more rapid or more complete degradation. Injecting more 2B8 did not appear to achieve either of these aims. Expression of endogenous LBH in our embryos was controlled by Western blot using mAb 2B8 and blotting against housekeeping genes GAPDH or Rpn1 were used as loading controls. This degradation is maintained through the early tailbud stage (st.24).

![Western blot](image)

**Figure 9. Time course of Trim-Away mediated degradation of LBH in Xenopus.** Western blot performed on protein extracted from wild-type and Trim-Away LBH embryos at various stages. Embryos were injected at the one-cell stage with 1ng trim21 mRNA and 46.8ng 2B8. GAPDH serves as a loading control. Ig G shows presence of 2B8 in embryo extract.
Additionally, we injected embryos with either trim21 mRNA or 2B8 alone to control for any potential off-target effects (Fig. 10). Presence of trim21 was detected by blotting with α- trim21 antibody and presence of 2B8 was detected by blotting with an antibody against mouse immunoglobulin light chain. In Trimmed embryos, the amounts of trim21 and 2B8 present are less than the trim21 or 2B8 injected controls, respectively. This is due to the fact that the entire complex of trim21-antibody-target gets degraded, rather than just the target alone (Fig. 6). As another control, to see if the phenotypes seen in Trimmed LBH embryos were indeed due to the loss of LBH, we expressed a Trim-Away resistant LBH mutant. This mutant lacks the domain 63-84 of LBH, which is the region that contains the epitope for 2B8 (Fig. 7). The mutant is flag-tagged and its presence is recognized by an α-D antibody (Fig. 10).

**Figure 10. Controls for Trim-Away experiments.** Western blots performed on protein extracted from st. 12 wild-type, Trim-Away LBH, trim21 mRNA, and 2B8 injected embryos. Embryos were injected with 1ng trim21 mRNA, 36.6ng of 2B8, and/or 1ng LBH Δ63-84 (flag-tagged) at the one cell stage. Rpn1 serves as loading control.
**LBH depleted embryos display defects in gastrulation**

Our Trim-Away LBH embryos displayed abnormalities beginning at mid-gastrula stage (st. 11). The embryos cleave normally and initiate gastrulation with normal appearance of the blastopore lip. However, the embryos are delayed mid-way through gastrulation, as evidenced by significantly bigger blastopores as compared to sibling control embryos (Fig. 11). These Trimmed embryos are not only delayed, but fail to complete gastrulation. Embryos grown beyond gastrulation display a “boat” phenotype typical of embryos that fail to complete gastrulation and therefore neurulation (Fig. 12). Control embryos injected with trim21 mRNA alone display a very mild delay in the closing of the blastopore, but otherwise complete gastrulation normally. Control embryos injected with mAb 2B8 alone display a moderate gastrulation defect as compared to the Trimmed embryos. These embryos are delayed in gastrulation, as evidenced by their larger blastopores like the Trimmed embryos. However, the 2B8 injected embryos eventually recover and develop into normal tailbud stage (Fig. 12).

We can achieve a partial rescue of the Trimmed gastrulation with the antibody resistant form of LBH. In order to try to rescue the loss of LBH, we injected 100pg-1ng of mRNA encoding LBH Δ63-84. This mediated the gastrulation delay; rescue embryos were significantly more advanced in the closing of their blastopores than Trimmed embryos, although the rescue did not return the embryos to normal, wild-type blastopore closure (Fig. 11). Rescue embryos grown to tailbud stage successfully closed their blastopores (Fig. 12). This partial rescue indicates that the phenotype observed in our Trimmed embryos is indeed due to the loss of LBH.
Figure 11. LBH depleted embryos display defects arising mid-way through gastrulation. Representative images of st.12 embryos; vegetal view showing blastopore. Embryos were injected at the one cell stage with 1ng trim21 mRNA, 1ng Δ63-85 LBH as a rescue, and 46.8ng 2B8. Average diameter of blastopore in embryos st.12; n=71 for non-injected, n=72 for trim21, n=73 for 2B8, n=85 for Trim-Away LBH, and n=65 for rescue. Two tailed student’s T-test, ***p<0.001
Figure 12. Embryos grown through tailbud stage display defects typical of failure to complete gastrulation. Embryos were injected at the one cell stage with 1ng trim21 mRNA, 1ng Δ63-85 LBH as a rescue, and 46.8ng 2B8. 87% of Trim-Away embryos display this “boat” phenotype at the tailbud stage, n=78

LBH depleted embryos have defects in mesoderm

Since one of the important events in gastrulation is the internalization and migration of mesodermal cells, we first looked to see if mesoderm is induced in LBH depleted embryos. Using in situ hybridization, we checked for the presence of general mesodermal marker brachyury and dorsal mesodermal marker chordin. Both brachyury and chordin are expressed in Trimmed embryos (Fig. 13). Brachyury has a normal expression pattern in Trimmed embryos, as a ring surrounding the blastopore. Chordin expression was expanded medioventrally in Trimmed embryos, with 70% of Trimmed embryos displaying this phenotype. 2B8 injected embryos display a slight expansion of chordin expression, although it was not observed in as many embryos.
Figure 13. Mesoderm is induced in LBH depleted embryos. *In situ* hybridization using probes against brachyury and chordin. Embryos fixed in MEMFA at st. 12 for brachyury and st. 10.5 for chordin. Expansion of chordin expression seen in 76% of Trim-Away LBH embryos.

Knowing that mesoderm is induced and patterned relatively normally in Trimmed embryos, we next tested the ability of dorsal mesodermal cells to migrate in a single cell migration assay. Briefly, dorsal blastopore lips of trimmed and non-injected embryos
were dissected out as in Cousin 2019 CSHL. After dissociation in a Calcium/magnesium free media, cells were seeded on glass bottom dishes coated with 10ug/ml of fibronectin and the cell movements tracked using time-lapse microscopy and FIJI software. The cells from Trimmed embryos travel significantly further than wild type cells (Fig. 14B&C, movie 2). Generally, LBH depleted cells travel with more persistence, traveling mostly in one direction and traveling far from their point of origin. Wild type cells do not travel far from their point of origin and tend to change direction frequently (Fig. 14A&C, movie 1). LBH depleted cells also travel at a significantly greater velocity than wild-type cells (Fig. 14D).

**Defects in gastrulation in LBH depleted embryos are largely due to contributions from the animal hemisphere**

To further elucidate the basis of the defects in gastrulation seen in our Trimmed embryos, we fixed embryos at st. 11-12 and bisected them along their sagittal plane. This allows us to observe the state of the ectoderm, mesoderm, and endoderm. Trimmed embryos have defects in each of these three tissues. Mesoderm in Trimmed embryos appeared to migrate farther onto the blastocoel roof as compared to their control siblings, which is consistent with the increased migration seen in our single-cell migration assay (Fig. 15B’). Endoderm in Trimmed embryos was also abnormal. Many of these embryos have cavities in the endoderm mass and appear to have lost tissue cohesion, as seen by the presence of single endoderm cells and general loose appearance of the mass (Fig. 15B’’). The cavity seen in the endoderm of the Trimmed embryo may be due to a rip caused by the mesoderm migrating too quickly while the endoderm fails to internalize. In terms of ectoderm, the Trim-Away LBH embryos have a thickened blastocoel roof, which is indicative of defects in epiboly.
Figure 14. Mesodermal cells from Trim-Away LBH embryos are more migratory than wild-type. (A) Still image from single-cell migration assay of wild-type mesodermal cells. (B) Still image from single-cell migration assay of Trimmed mesodermal cells. Tracings were done with FIJI Trackmate (C) Average distance traveled by cells from the dorsal blastopore lip in non-injected compared to Trim-Away LBH embryos. Two-tailed student’s T-test shows increased distance traveled is significant, p<0.001, (D) Average velocity of cells from the dorsal blastopore lip in non-injected compared to Trim-Away LBH embryos. Two-tailed student’s T-test shows increased velocity is significant, p<0.001 (C) and (D) were calculated using manual tracking plug-in with Fiji.
Figure 15. Trimmed embryos display defects in ectoderm, mesoderm, & endoderm. (A) and (B) Representative sagittal sections of embryos fixed in paraformaldehyde at st. 12. (A) Non-injected. (B) Trim-Away LBH. Embryos were injected at the one cell stage with 1ng trim21 mRNA and 46.8ng 2B8 (A’) Close up of blastocoel roof from non-injected. (A’’) Close up of endoderm from non-injected (B’) Close up of blastocoel roof from Trim-Away LBH. (B’’) Close up of endoderm from Trim-Away LBH Yellow double arrow shows width of mesodermal mantle. Red double arrowhead shows thickness of blastocoel roof.
Since our embryos display defects in both animal and vegetal hemisphere derivatives, we wanted to sort out the contributions from each of these areas. To do so, we injected embryos at the 8-cell stage in either the 4 animal blastomeres or 4 vegetal blastomeres with 125 pg of mRNA encoding nuclear cherry, 8-11.7 ng of mAb 2B8 and 250 pg of trim 21 mRNA per cell. Here, the 4 animal blastomeres primarily contribute to the ectoderm derivatives while the 4 vegetal blastomeres contribute to the endoderm and mesoderm derivatives (Fig. 16). In the non-injected and vegetal injected embryos, the dorsal mesoderm has just begun migrating. In the animal injected embryo, the dorsal mesoderm appears to have migrated further and the ventral mesoderm also appears to have started to migrate. The animal injected embryos display the same failure in the thinning of the blastocoel roof and increased mesodermal migration as our 1-cell stage injected Trimmed embryos (Fig. 16B). The vegetal injected embryos display some dissociation of endodermal cells, but otherwise do not recapitulate the phenotypes seen in the 1-cell stage injected Trimmed embryos (Fig. 16C). This suggests that the defects in gastrulation in Trimmed embryos are largely ectoderm derived.

Figure 16. Defects in gastrulation in Trimmed embryos are largely due to contributions from the animal hemisphere. (A-C) Representative sagittal sections of embryos fixed in paraformaldehyde at st. 10.5. Embryos were counterstained with Hoechst 33342 to visualize cell nuclei. Blastomeres injected with 250pg trim21 mRNA, 125pg nuclear cherry mRNA, and 11.7 ng 2B8. (A) Non-injected control embryo (B) Embryo injected in all 4 animal blastomeres (C) Embryo injected in all 4 vegetal blastomeres

**LBH depleted embryos have defects in the FN matrix of the blastocoel roof**

The defects in epiboly and mesoderm migration are similar to the gastrulation defects observed when fibrillogenesis in the blastocoel roof is perturbed (Marsden &
Desimone 2001, Rozario et al. 2009). In order to observe the fibronectin matrix of the blastocoel roof, we injected embryos in one cell at the 2-cell stage with trim21 mRNA, 2B8, and membrane cherry. We then dissected animal caps at stage 11.5/12 and fixed them in MEMFA. The fibronectin matrix was detected by immunofluorescence using the mAb 4H2 against fibronectin and α-mouse 488 and nuclei were counterstained using HOESCHT 33342 (Fig. 17). Trimmed cells express the membrane cherry, while wild-type cells do not. Wild-type cells display a normal FN matrix deposition- a dense network of fibers expanding across cells. Trimmed cells assemble fewer fibers and those that are assembled appear longer and are localized more around the cell surface, which is a pattern similar to an earlier stage embryo (Dzamba et al. 2009). This defective FN matrix supports the defects observed in epiboly and mesodermal migration (Marsden & Desimone 2001, Rozario et al. 2009).

Figure 17. LBH depleted embryos fail to properly form a fibronectin matrix in the blastocoel roof. (A) Immunofluorescence of fibronectin in animal cap. Embryos were injected in one cell at the two cell stage with 250pg membrane cherry mRNA, 250pg trim21 mRNA, 11.7ng 2B8. Caps were counterstained with Hoechst 33342. (B-D) Magnified view of regions indicated by yellow arrowheads. Images taken at 20x magnification using Apotome.
**LBH depleted embryos have reduced expression of cytoplasmic β-catenin**

Since LBH is implicated in the canonical Wnt signaling pathway, we wanted to see if Wnt signaling was perturbed in our Trimmed embryos. To do so, we looked at the presence of β-catenin at the membrane and in the cytoplasm of our Trimmed embryos. In addition to its transcription factor role, at the plasma membrane, β-catenin is involved in cellular adhesion (Kim et al. 2013). Separating the membrane-associated pool from the nuclear/cytoplasmic pool allows us to separate in what context we’re observing β-catenin. We extracted embryos at st. 12 and separated membrane bound β-catenin from cytoplasmic β-catenin by concanavalin A purification. We then blotted for β-catenin, using GAPDH and Rpn1 as loading controls. In our Trimmed embryos, cytoplasmic β-catenin is noticeably reduced (Fig. 18 A&B). There is a small reduction in membrane-bound β-catenin in Trimmed embryos in 2 of the three replicates (Fig. 18 C&D), though it is minimal compared to the drastic reduction we see in the cytoplasmic/nuclear β-catenin.

**Wnt signaling and LBH in vitro**

To briefly try to gain further understanding of where LBH may factor into Wnt signaling, we overexpressed LBH with a number of different Wnt related factors in 293T cells. We then extracted protein and looked at the amount of LBH present (Fig. 19). Thus far, this experiment has only been repeated twice, and in the second experiment LBH expression in the control-RFP was slightly low. Consistent with previous studies, overexpression of canonical Wnt inhibitor DKK decreases LBH expression (Rieger et al. 2010). Expression of Wnt11, which is implicated in canonical Wnt signaling during axis specification (Tao et al. 2005, Cha et al. 2008) and non-canonical Wnt signaling in
fibronectin matrix organization (Dzamba et al. 2009) appears to decrease LBH expression. β-catennin appears to slightly increase LBH expression. Curiously, both GSK3, which promotes β-catenin degradation, and dominant negative GSK3 appear to increase LBH expression.

Figure 18. Presence of β-catenin is decreased in LBH depleted embryos. (A) Western blot performed on protein extracted from st. 12 non-injected and Trimmed LBH embryos, after membrane associated proteins removed by concanavalin A purification. (B) Quantification of band intensity for β-catenin, normalized to GAPDH. Average of three biological replicates.
Figure 19. Overexpression of LBH with components of Wnt signaling pathways. Western blot of 293T cells transfected with 0.5ug of LBH and 0.5ug of RFP, Dkk, β-catenin, Wnt11, GSK3, or dominant negative GSK3. Cells were collected 24 hours post transfection. GAPDH serves as a loading control.
CHAPTER 4
DISCUSSION

Delay in degradation of LBH

Degradation of LBH in *Xenopus* takes longer than degradation of proteins in previously published systems (Clift et al. 2017, Chen et al. 2019). This may be due to a number of factors, either due to trim21, the antibody, or the embryo itself.

Factors related to trim21: We used mRNA encoding trim21, so the delay in degradation of LBH may be related to the time it takes to translate enough trim21. In order to account for this, in the future we could try to inject purified trim21 protein instead. Additionally, we used the human form of trim21. As a mammalian protein, trim21 is more active at higher temperatures, rather than the 14-20°C at which our *Xenopus* embryos are raised. Using Trim-Away in *Xenopus* with other proteins of interest would help answer whether the delay is as simple as an issue of temperature.

Factors related to 2B8: It is possible that the constant of association (Ka) for 2B8 mAb is rather low (the typical range for mouse mAb is $10^7$ to $10^{12}$). This would affect the speed at which 2B8 binds to LBH, therefore delaying degradation. Additionally, 2B8 binding to LBH may be affected by post translational modifications found only in the maternal pool of LBH. It may be worth using Trim-Away in cell culture to degrade an overexpressed LBH in order to see if the degradation delay is specific to maternal LBH, rather than just the Trim-Away technique in *Xenopus*.

Since Trim-Away otherwise utilizes endogenous protein degradation machinery, it is possible that the early *Xenopus* embryos are deficient in a critical enzyme necessary for the degradation of the LBH-2B8 complex. For example, trim21 mediated degradation
requires the AAA ATPase VCP/p97 (Hauler et al., 2012). It is possible that one of the factors necessary for LBH degradation is missing during early development. In order to further elucidate the causes behind this delay, we will need to use Trim-Away in *Xenopus* with antibodies against more proteins of interest.

**Phenotypes seen in injection of 2B8 alone**

The gastrulation defect that we see in our 2B8 alone injected embryos may be due to steric encumbrance. LBH is a small protein of about 15kDa. The antibody, on the other hand, is about 150kDa. The binding of this much larger protein to LBH may interfere with the function of LBH. However, this simple physical limitation is not as severe as the degradation of LBH in the embryo. Interestingly, the expanded chordin phenotype was also observed in the 2B8 injected embryos, although it did not appear quite as robust.

**Gastrulation defects**

The inability of Trimmed embryos to complete gastrulation is due to a combination of defects both in the mesoderm and the blastocoel roof. As mentioned earlier, the increased mesodermal movement along the blastocoel roof is consistent with defects in the fibronectin matrix of the blastocoel roof (Rozario et al. 2009). What’s interesting, however, is that LBH depleted mesodermal cells migrate further and more quickly on a fibronectin coated plate than wild-type cells. This increased movement is independent of the fibrillar network on the BCR. The increased mesodermal migration that we see, then, is not exclusively due to the lack of the fibronectin matrix. *In vitro*, it has been demonstrated that an optimal receptor-ligand (integrin-FN) concentration dictates the speed at which cells migrate along their substrate (Palecek et al. 1997). Cells expressing low levels of integrin α5β1 migrate more quickly on a substrate of higher FN
concentration while cells expressing high levels of integrin $\alpha 5\beta 1$ migrate more quickly on a substrate of lower FN concentration (Palecek et al. 1997). LBH may affect either integrin expression or their activation level. Integrin $\alpha 5\beta 1$ is an important FN receptor involved in cell migration speed in vitro (Palecek et al. 1997) and convergent extension movements (Davidson et al. 2006). In *Xenopus*, it has been shown that integrin $\alpha 5\beta 1$ adopts various activity level depending on the germ layer, which in turns dictates what kind of function it performs. In the ectoderm, integrin $\alpha 5\beta 1$ has a “lower activation” level which allow it to perform FN fibrillogenesis. In the mesoderm, integrin $\alpha 5\beta 1$ has different activation levels which allows those cells to migrate. Its expression in Trimmed LBH embryos is worth exploring. Conducting immunofluorescence against integrin $\alpha 5\beta 1$ in Trimmed LBH vs. non-injected embryos would be a good first step in exploring this potential connection. Additionally, it would be useful to look at $\alpha 5\beta 1$ by Western blot.

It would also be worth exploring cadherins in our Trimmed embryos. We observed defects in cellular adhesion in both mesoderm and endoderm. In our animal injected embryos, mesoderm that migrated onto the BCR was observed falling away (Fig. 16B). Endoderm in Trimmed embryos also appeared to lose cellular adhesion, with its loose appearance and single cells. We could fix Trimmed LBH and non-injected embryos at various gastrula stages and conduct immunofluorescence on C-cadherin, which is important for both FN matrix assembly in the BCR (Dzamba et al. 2009) and endoderm migration (Wen & Winklbauer 2017).

**Potential Wnt signaling links**

Our results suggest that LBH may play a role in Wnt signaling, however further work must be done to determine this. Dkk1 is a canonical Wnt inhibitor that has been
shown to decrease Wnt3a mediated LBH expression in 293T cells (Rieger et al. 2010). Interestingly, knock down of Dkk1 results in an expansion of chordin expression ventrally (Cha et al. 2008), similar to our LBH knockdown. Wnt7a has been shown to restrict LBH to the ventral ectoderm in the mouse limb bud (Rieger et al. 2010). Perhaps LBH plays a role in maintaining ventral identity? Although our results do not show any issues in dorsal-ventral axis specification (for example, the dorsal blastopore lip appears normally at st. 10.5), per se, we only achieve a strong knockdown mid-way through gastrulation. If we can achieve a more rapid knockdown, perhaps through the use of purified trim21 protein rather than trim21 mRNA, it would be worth looking at the Wnt target genes involved in dorsal-ventral axis specification by in situ hybridization or QPCR.

Wnt 11 has previously been shown to be necessary for FN matrix assembly in *Xenopus* (Dzamba et al. 2009). This matrix assembly was also shown to be dependent on tension in the blastocoel roof, with FN present at cell edges in embryos where tissue tension was disrupted (Dzamba et al. 2009). Our embryos display a similar phenotype, in which FN appears more localized around cell edges than across cells as a matrix. We noticed that the Trim-Away embryos were often softer than their non-injected counterparts, suggesting a decrease in tissue tension.

The decreased expression of β-catenin we see in our Trimmed embryos is particularly interesting. Previous research has placed LBH as a target of canonical Wnt signaling (Rieger et al. 2010). Here, we see that β-catenin decreases in response to LBH depletion. Specifically, the signaling β-catenin is decreased, rather than the cadherin/membrane associated β-catenin. This would seemingly place LBH upstream of
β-catenin in canonical Wnt signaling. Combining our results with the work done in Rieger et al. 2010 suggests that LBH may be part of a feedback loop regulating Wnt signaling. Additionally, β-catenin has been shown to regulate fibronectin expression in *Xenopus* fibroblasts (Gradl et al. 1999). To further test this link between β-catenin and LBH, we could inject β-catenin mRNA into our Trimmed LBH embryos to see if we can rescue any of the phenotypes we see as a result of LBH depletion. In a complementary experiment, we could knock down β-catenin with morpholinos against β-catenin and inject LBH mRNA in order to see if we can rescue the phenotypes that arise as a result of β-catenin knockdown.

In this study, we have demonstrated that Trim-Away is amenable for use in *Xenopus*. More work is necessary for determining if it is possible to achieve more rapid protein degradation, as observed in other model systems. Additionally, we have uncovered another role for LBH in embryonic development. LBH is necessary during gastrulation in amphibians and plays a role in fibronectin matrix organization as well as mesodermal migration. Its function during gastrulation may be Wnt related, however more work must be conducted to uncover this potential connection.
Figure 20. Model for LBH role during gastrulation


