p38a mitogen-activated protein kinase/Mapkapk2a signaling regulates zebrafish tristetraprolin in the yolk syncytial layer: A role for tristetraprolin-dependent mRNA degradation in zebrafish early development

by

Sol Gómez de la Torre Canny

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ABSTRACT

p38a mitogen-activated protein kinase/Mapkapk2a signaling regulates zebrafish tristetraprolin in the yolk syncytial layer: A role for tristetraprolin-dependent mRNA degradation in zebrafish early development

by

Sol Gómez de la Torre Canny

The yolk syncytial layer (YSL) is one of the three cell layers that participate in epiboly, the first morphogenetic movement in zebrafish development. The YSL is a site of active transcription and genes expressed in the YSL are essential not only for epiboly, but also for other aspects of zebrafish embryogenesis, underscoring the importance of investigating the regulation of gene expression in this cell layer. To address this question, I used betty boop, an epiboly mutant in zebrafish. betty boop is a mutation in mapkapk2a, the zebrafish homolog of mammalian MAPKAPK2, a protein kinase that is activated by the p38 MAPK signaling pathway. The activity of Mapkapk2a is required in the YSL for epiboly. I identified a novel aspect of the betty boop mutant phenotype that consists in the reduction of mRNA levels of YSL-specific genes. This phenotype prompted me to examine the role of the zebrafish homolog of TTP, a known downstream target of mammalian MAPKAPK2. In mammals, TTP is an RNA-binding protein that promotes mRNA decay. TTP-dependent mRNA degradation is inhibited by MAPKAPK2 phosphorylation. Manipulation of the endogenous activity of Ttp showed that Ttp regulates mRNA levels of YSL-specific genes during epiboly in a Mapkapk2a-dependent manner. Activation of Mapkapk2a outside of the YSL results in dramatic developmental defects and ectopic expression of YSL-specific genes, suggesting that YSL-specific activation of Mapkapk2a inhibits Ttp-dependent mRNA degradation in the YSL. Expression of Mxtx2 in the YSL, one of the YSL-specific mRNA molecules reduced in betty boop mutants, is sufficient to suppress the mutant phenotype. However, expression of Mxtx2 outside of the YSL causes dramatic development defects, suggesting the requirement of a mechanism that restricts the expression of YSL-specific genes to this cell layer, such as the inhibition of Ttp-dependent mRNA decay by
Because Mxtx2 is a transcription factor known to activate a large set of YSL-specific genes, it is likely that p38a/Mapkap2a/Ttp-dependent regulation of mRNA levels of Mxtx2 in the YSL affects the transcriptional activation of other genes that act in a concerted manner to control YSL morphogenesis during zebrafish epiboly.
I would like to thank my advisor Doctor Daniel Wagner for providing me with the opportunity and the means to study the betty boop mutant. It has been a very challenging experience as much as it has been enjoyable.

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and every person I encounter. I would also like to thank my grandmother for her example of physical and emotional strength, and her constant reminding that education is a privilege that we must not take for granted.

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<th>Description</th>
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<tbody>
<tr>
<td>ARE</td>
<td>Adenylate-uridylate rich elements</td>
</tr>
<tr>
<td>ChIP-Seq</td>
<td>chromatin immunoprecipitation sequencing</td>
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<tr>
<td>DEL</td>
<td>deep cell layer</td>
</tr>
<tr>
<td>DMBA</td>
<td>7,12-dimethylbenzanthracene</td>
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<tr>
<td>E-YSL</td>
<td>External yolk syncytial layer</td>
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<td>EVL</td>
<td>enveloping layer</td>
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<td>I-YSL</td>
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<td>K_d</td>
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<td>MAP2K</td>
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<td>MAPK</td>
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<tr>
<td>MO</td>
<td>morpholino oligonucleotide</td>
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<tr>
<td>NES</td>
<td>nuclear export signal</td>
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<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
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<td>WISH</td>
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<td>YSL</td>
<td>yolk syncytial layer</td>
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<td>YSN</td>
<td>yolk syncytial nuclei</td>
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**List of abbreviations of gene names and mutations**

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<td><em>bbp</em></td>
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CHAPTER 1

Introduction

Epiboly is the first coordinated cell movement during zebrafish development. Epiboly is the thinning and the spreading of the embryonic blastoderm to completely engulf a very large yolk cell (Kimmel et al., 1995). Three distinct cell layers participate in this first morphogenetic movement during zebrafish embryogenesis: the enveloping layer, the deep cell layer, and the yolk syncytial layer (Kimmel et al., 1995). In this thesis, we will focus in the morphogenesis of the yolk syncytial layer (YSL). The YSL is a syncytium that lies between the deep cell layer and the yolk mass (Kimmel et al., 1995). The YSL is a site of active transcription. Several genes expressed in the YSL are required for epiboly and for other aspects of zebrafish embryogenesis that include germ layer patterning, organogenesis, and nutrition of the overlying embryo (reviewed by Carvalho and Heisenberg, 2010). The multiple roles that the YSL plays in zebrafish development underscore the importance of studying the regulation of gene expression in this cell layer.

The YSL is a morphological novelty in teleost fishes. Teleost gastrulation was derived from an ancestral form of gastrulation and includes morphological changes such as the presence of the YSL (Collazo, Bolker and Kelly, 1994). This raises the question of what is the gene expression landscape underpinning the generation of this novel embryonic structure and, most importantly, what are the regulatory pathways that have evolved to regulate the expression of genes in the YSL. In my thesis, I examine the role of a conserved p38 MAPK/MAPKAPK2 signaling pathway that regulates the activity of the zebrafish homolog of tristetraprolin (TTP) during epiboly. I propose that the activation of a conserved
p38 MAPK/MAPKAPK2/TTP signaling in the YSL during zebrafish epiboly is a mechanism that contributes to the regulation of mRNA levels in this novel cell layer.

To study the p38 MAPK/MAPKAPK2/TTP signaling axis during epiboly, I use the betty boop maternal-effect epiboly mutant in zebrafish (Wagner et al., 2004). The betty boop<sup>p58cd</sup> mutation is a lesion in mapkapk2a, the gene encoding the zebrafish homolog of the mammalian MAPKAPK2, that causes a defect in YSL morphogenesis (Holloway et al., 2009). In mammals, MAPKAPK2 is activated by the p38 MAPK signaling pathway (Rouse et al., 1994; Freshney et al., 1994). p38 MAPK is one of the four MAPK signaling pathways identified in vertebrates that transduce extracellular stimuli into intracellular responses through the sequential phosphorylation of proteins (reviewed by Cargnello and Roux, 2011). The activity of Mapkapk2a is required in the YSL for zebrafish epiboly (Holloway et al., 2009). However, the downstream targets of Mapkapk2a required for epiboly have not been identified. My work describes a novel aspect of the betty boop mutant phenotype, which is the reduction in the levels of mRNA in the YSL, and identifies the zebrafish homolog of Ttp as a downstream target of Mapkapk2a required during epiboly.

In mammals, TTP is one of the known downstream targets of MAPKAPK2 phosphorylation (Hitti et al., 2006; Chestensen et al., 2004). TTP binds type II AU-rich elements (AREs) at the 3’ untranslated region (3’ UTR) of mRNA molecules (Lai et al, 1999). TTP-binding to specific mRNA molecules promotes their decay by recruiting members of the enzymatic machinery that degrades mRNA in the cytoplasm (Lai et al., 2003; Sandler et al., 2011; Fabian et al., 2013). Phosphorylation of TTP by MAPKAPK2 inhibits mRNA decay (Stoecklin et al., 2004; Marchese et al., 2010). Because TTP is required for the rapid decay of mRNA encoding molecules involved in inflammation such as TNF-α, TTP
deficiency is characterized by a hyperinflammatory phenotype (Taylor et al., 2006). However, the role of TTP is vertebrate development remains largely unexplored. In this thesis, I study the role of zebrafish tristetraprolin (Ttp) in the regulation of mRNA levels of the YSL-specific genes *mxtx2, mxtx1, nrz/bcl2l10, and slc26a1* during epiboly. I was particularly interested in *mxtx2* and *nrz/bcl2l10* because loss of function of these genes using antisense oligonucleotide morpholinos disrupts epiboly (Bruce et al., 2006; Arnaud et al., 2006).

In Chapter 4 of this thesis, I investigate the conservation and the requirement of p38 MAPK/MAPKAPK2 signaling pathway in zebrafish epiboly. I show that the zebrafish homolog of p38 MAPK, p38a, is also required for epiboly; that Mapkapk2a is activated in the YSL; and that the enzymatic function of mammalian MAPKAPK2 is conserved in the context of epiboly.

In Chapter 5 of this thesis, I investigate the role of the p38a/Mapkapk2a/Ttp signaling axis during zebrafish epiboly. I show that *betty boop* mutant embryos have reduced mRNA levels of YSL-specific genes; that p38a/Mapkapk2a signaling regulates mRNA levels of YSL-specific genes during epiboly; and that Ttp regulates mRNA levels of YSL-specific genes in an Mapkapk2a-dependent manner.

In Chapter 6 of this thesis, I study the function of two of the genes of which the mRNA levels are reduced upon the loss of p38a/Mapkapk2a/Ttp signaling: *mxtx2* and *nrz/bcl2l10*. I show that the expression of *mxtx2* must be restricted to the YSL during epiboly and that Mxtx2 function is required in the YSL as a transcriptional activator during epiboly. Also, I show that Nrz/Bcl2l10 function in the YSL is required but not sufficient for epiboly.
mapkapk2a and mapkapk2b are annotated in the zebrafish genome as gene duplicates. A third round of whole genome duplication likely anteceded the teleost clade radiation (Taylor et al., 2003; Postlethwait et al., 2000). Whole genome duplication events generate duplicate genes, which through diverse fates, give rise to novel gene expression patterns and possibly novel protein functions that may have contributed to the generation of morphological diversity both in the embryo and the adult form of teleost fishes (reviewed by Prince and Pickett, 2002). In chapter 7 of this thesis, I investigate the annotated gene duplicates mapkapk2a and mapkapk2b. I show that mapkapk2a and mapkapk2b are gene duplicates; that the expression patterns of mRNA of mapkapk2a and mapkapk2b do not overlap until day 3 post fertilization; and that expression of Mapkapk2b during epiboly disrupts this morphogenetic movement.

I propose a model in which the activation of p38a/Mapkapk2a in the YSL inhibits Ttp-mediated mRNA decay (Figure 1.1). Outside of the YSL, where p38a/Mapkapk2a signaling is not activated, Ttp promotes the degradation of mRNA molecules encoding YSL-specific genes. Ttp directly regulates the mRNA levels of at least two YSL-specific genes: mxtx2, and mxtx1. Mxtx2 transcriptionally activates mxtx1, slc26a1 and nrz/bcl2l10. Because Mxtx2 is a transcription factor that activates a large set of YSL-specific genes (Xu et al., 2012), it is likely that the p38a/Mapkapk2a/Ttp-dependent regulation of Mxtx2 expression in the YSL leads to the transcriptional activation of other genes that act in a concerted manner to control YSL morphogenesis during zebrafish epiboly. This model does not exclude the possibility that nrz/bcl2l10 is also subject to the post-transcriptional regulation of Ttp. However, this hypothesis is only supported by the in silico prediction of nrz/bcl2l10 mRNA as a likely target of TTP binding.
My work shows that YSL-specific activation of a conserved p38a/Mapkapk2a/Ttp signaling axis restricts the expression of YSL-specific genes to this cell layer during zebrafish epiboly. Most notably, because Mtxt2 is a transcription factor that binds cis-acting regulatory sequences of a large set of YSL-specific genes (Xu et al., 2012), p38a/Mapkapk2a/Ttp-dependent regulation of mtxt2 mRNA levels in the YSL may be an important regulatory mechanism of gene expression in this novel embryonic structure.
The protective role that MAPKAPK2-deficiency confers during inflammatory processes (Wang et al., 2002; Shiroto et al., 2005; Kotlyarov et al., 1999; Thomas et al., 2008; Jagavelu et al., 2007; Hegen et al., 2006; Ghasemlou et al., 2010; Johansen et al., 2009), and the striking hyper inflammatory phenotype of TTP-deficient mice (Taylor et al., 2006) underscore the importance of investigating the function of MAPKAPK2 and its downstream effector TTP. A further understanding of the function of these proteins may contribute to the better design of therapies for chronic inflammation. My work shows that zebrafish epiboly is an accessible system to investigate the function of p38a, Mapkapk2a, and Ttp.
CHAPTER 2

Background and Literature review

2.1 - Zebrafish Epiboly

2.1.1 - Epiboly is the first morphogenetic movement during zebrafish development

Epiboly is the thinning and the spreading of the embryonic blastoderm to completely engulf a very large yolk cell (Kimmel et al., 1995; Figure 2.1, Panels A-F). It is the first coordinated cell movement during zebrafish embryogenesis, preceding gastrulation by approximately one hour and twenty minutes (Kimmel et al., 1995). After fertilization, a series of meroblastic cleavages (i.e. a cleavage pattern in which the cleavage furrow does not cut across the yolky cytoplasm) results in a mound of blastomeres that sits on top of a very large yolk cell (Figure 2.1, Panel A). The initiation of epiboly is marked by the protrusion of the yolk towards the animal pole forming a dome (Figure 2.1, Panel B, red arrow indicates the dome of the yolk).

As epiboly progresses, the mound of blastomeres thins to form a multilayered blastoderm that continues to spread over the yolk cell to completely engulf it (Figure 2.1, red bars highlight the progressive thinning of the blastoderm cells). The thinning and spreading of the blastoderm allows for other morphogenetic movements to occur; including gastrulation where cells involute at the blastoderm margin to form the germ layers, and the convergence and extension of cells to extend the embryonic rostrocaudal axis (Kimmel, Warga, and Schilling, 1990; Montero et al., 2005).
Figure 2.1 - Three cell layers undergo epiboly in the zebrafish embryo. Wild type zebrafish embryos at different stages of development (A-E). Developmental stages represented are sphere stage before the initiation of epiboly (A), 30% epiboly (B), 50% epiboly (C), 75% epiboly (D), and the bud stage at the end of epiboly (F). Asterisks in A mark the animal (*) and the vegetal (**) pole of the early embryo. Red arrow in B indicates the dome of the yolk that forms at the beginning of epiboly. Red bars in A-E show the thinning of the blastoderm as epiboly progresses. Crosses in F mark the rostral (+) and the caudal (+++) end of the embryonic axis. Schematic of the longitudinal section of an embryo (G). Three cell layers that coordinately progress through epiboly: the enveloping layer (EVL), the deep cell layer (DEL), and the yolk syncytial layer (YSL). Schematic in panel G was based in Holloway et al., 2009.
2.1.2 - Three cell layers undergo epiboly in zebrafish embryos: the enveloping layer, the deep cell layer and the yolk syncytial layer

The enveloping layer (EVL) is the epithelial-like and outermost monolayer that acts as an embryonic barrier to the environment (Figure 2.1, Panel G; Kimmel et al., 1995). The differentiation and lineage restriction of the EVL occurs early in development (Kimmel, Warga and Schilling, 1990). The EVL forms the superficial layer of the embryonic epidermis (Fukazawa et al., 2010). The deep cell layer (DEL) contains highly motile cells that will form most of the embryo (Figure 2.1, Panel G; Kimmel et al., 1995). The cells of the DEL change their positions relative to each other and relative to the EVL and the yolk syncitial layer (YSL) during epiboly (Warga and Kimmel, 1990). The thinning of the cells in the DEL is thought to occur mainly through a cell behavior called radial intercalation, which is executed by molecules involved in cell adhesion such as Cdh1, PrnPrs1, and Pcdh18a (Kane et al., 2005; Malaga-Trillo et al., 2009; Aamar and David, 2008). The YSL is a syncytium that lies between the DEL and the yolk mass. The YSL also undergoes epiboly, spreading and displacing the anuclear yolk cytoplasmic layer (Figure 2.1, Panel G; Kimmel et al., 1995). Despite their morphological differences, these three cell layers undergo epiboly in a concerted manner likely by executing highly regulated cell behaviors within each layer as well as by maintaining highly regulated interactions amongst these layers. In this thesis, we use the *betty boop* (*bbp*), the maternal-effect epiboly mutant in zebrafish (Wagner et al., 2004) to examine the morphogenesis of the YSL and the protein functions that are required within the YSL to regulate epiboly. The *bbp*<sup>p58cd</sup> mutation is a lesion in *mapkapk2a* (*mk2a*), the gene encoding the zebrafish homolog of the mammalian MAPKAPK2 (MK2, Holloway et al., 2009). The *bbp* mutation causes a striking lethal phenotype of abnormal contractions in
the YSL that precede the lethal constriction of the blastoderm margin at 50% epiboly, leading to lysis of the zebrafish embryo (Holloway et al., 2009).

2.2 - The yolk syncytial layer is an extraembryonic cell layer that is unique to teleost development

2.2.1 - Formation of the yolk syncytial layer in the zebrafish

The yolk syncytial layer (YSL) is a syncytium formed around the ninth and the tenth cell cycle after fertilization (Kimmel and Law, 1985a). Before the formation of the YSL, the blastomeres adjacent to the yolk cell are still connected to the anuclear cytoplasm surrounding the yolk mass (Kimmel and Law, 1985b). The connection between blastomeres and the yolk cell cytoplasm is maintained because zebrafish has a meroblastic cleavage pattern during which the furrow does not cut the yolk mass (Collazos, Bolker and Keller, 1994). The YSL is formed by the collapse of the blastomeres adjacent to the yolk cell, which contribute their nuclei and cytoplasm to the cortical cytoplasm of the yolk cell (Kimmel and Law, 1985). Once the YSL is formed the nuclei of the YSL (YSN) undergo 4 to 5 rounds of nuclear division before exiting the cell cycle (Kimmel and Law, 1985). The YSL persists until larval stages when the yolk is consumed (D’amico and Cooper, 2001). The YSL that is covered by the blastoderm during epiboly is known as the internal YSL (I-YSL), and the rest of the YSL that extends beyond the margin of the blastoderm is known as the external YSL (E-YSL; Figure 2.2; Kimmel et al., 1995).
2.2.2 - The yolk syncytial layer has a dynamic actin and microtubule cytoskeleton that is required for epiboly

The nuclei of the YSL are associated with a network of microtubules, which is required for the progression of epiboly (Solnica-Krezel, and Driever, 1994; Figure 2.2, Panel A). The microtubules of the I-YSL form a dense network underneath the blastoderm, whereas the microtubules emanating from the E-YSL form hair-like structures that spread over the yolk cortical cytoplasm towards the vegetal pole (Figure 2.2, Solnica-Krezel and Driever, 1994). The microtubules of the YSL are required for epiboly as shown by the pharmacological disruption of microtubule dynamics using nocodazole and taxol (Solnica-Krezel and Driever, 1994). However, it is unknow how the growth or the shortening of microtubules contribute to the progression of the blastoderm throughout the different stages of epiboly. Several stresses, as well as chemical and genetic manipulations that alter the homeostasis of the microtubule network in the YSL also disrupt the progression of epiboly. These insults include UV radiation (Strahle and Jesuthasan, 1993) and ethanol exposure (Sarmah et al., 2013). It has also been shown that the manipulation of the levels of pregnenolone through the gain and loss of function of the biosynthetic enzyme Cyp11la alter the microtubules in the YSL and also disrupt epiboly (Hsu et al., 2005). However, whether the microtubule network of the YSL acts as an active motor, maintain structural integrity, or provide positional identity is still a matter of debate.

Another important structural feature of the YSL is the presence of a punctuate band of polymerized actin located vegetally to the margin of the blastoderm (Figure 2.2, Panel A; Cheng et al., 2004). This punctuate actin band is also required for epiboly as shown by the treatment with the depolymerizing agent cytochalasin B which disrupts the punctuate actin
band and the progression of epiboly (Cheng et al., 2006). As is the case with the microtubule network, genetic manipulations that alter the morphology of this punctuated actin band also result in the disruption of epiboly. Loss of function of zebrafish homologs of proteins such as Cofilin 1 (Lin et al., 2010), and Diaphanous and Profilin (Lai et al., 2008), known to regulate actin dynamics in other model systems, disrupts the morphology of the punctuate actin band and alters epiboly. More recent work by Koppen and coworkers (2006) showed that this punctuate actin band is co-localized with activated non-muscle myosin. Based on this observation, Koppen and coworkers proposed that a competent actomyosin contractile apparatus is present during late epiboly. Thus, execution of contraction would promote the progression of the blastoderm cell to completely engulf the yolk cell during late epiboly (i.e. blastopore closure). This hypothesis was further supported by the pharmacological inhibition of the non-muscle myosin based contraction using blebbistatin, which caused a delay in epiboly (Koppen et al., 2006).

The EVL contacts the surface of the yolk at the blastoderm margin (Figure 2.2, Panel B). Using Fundulus epiboly as a model, classic work from Betchaku and Trinkaus (1978) showed that tight junctions maintained the attachment of the EVL to the YSL (Figure 2.2, Panel B, tight junctions schematized as a red asterisk). More recent work has shown the presence of tight junction components, such as ZO-1 and Claudin E, and their requirement for zebrafish epiboly (Koppen et al., 2006; Siddiqui et al., 2009). Koppen and coworkers (2006) showed a change in the morphology of the EVL cells as they transition from mid-epiboly to late epiboly. During late epiboly, the vegetal border of the EVL cells at the blastoderm margin becomes enriched with polymerized actin. During late epiboly, the vegetal border of the EVL cells at the blastoderm margin tightens, and as epiboly progresses
the EVL cells elongate along the animal-vegetal axis (Koppen at al., 2006). The morphological changes of the EVL, together with the presence of a competent actin-myosin contractile apparatus and of tight junctions between the EVL and the YSL, have led to the most widely accepted hypothesis that a YSL-based contraction actively pulls the EVL towards the vegetal pole during epiboly.

Figure 2.2 – Schematic of the cytoskeleton of the yolk syncytial layer and of the attachment of the yolk syncytial layer to the enveloping layer. Schematic of the YSL cytoskeleton (A). Cells of the blastoderm are depicted as transparent in order to visualize the internal YSL (I-YSL; i.e. portion of the YSL located underneath the blastoderm), the external YSL (E-YSL; i.e portion of the YSL that extends beyond the blastoderm margin), and the particular microtubule networks associated to the nuclei of the E-YSL and the I-YSL. Black bars represent microtubules. Grey bar represent the punctuate polymerized actin band. Arrows indicate the direction of the progression of epiboly. Schematic of the attachment of the EVL to the YSL (B). Longitudinal section at the margin of the advancing blastoderm. The EVL cells are attached to YSL by tight junctions (represented by a red asterisk).
2.2.3 – Transcription in the yolk syncytial layer is essential for zebrafish embryogenesis

The nuclei of the YSL are transcriptionally active, and express several genes that are required not only for epiboly but also for other aspects of zebrafish embryogenesis that include germ layer patterning, organogenesis, and nutrition of the overlying embryo.

The YSL has a similar function to the amphibian Niewkoop-center during dorsal-ventral patterning of the embryo. The expression of the dharma in the dorsal YSL is required for the induction of organizer specific genes in the dorsal blastoderm for dorsal-ventral patterning (Mizuno et al., 1999; Koos and Ho, 1998). Also, expression of the product of the nodal genes squint/ndr1 and cyclops/ndr2 in the YSL and the adjacent blastoderm is required for dorsal mesoderm and endoderm formation (Feldman et al., 1998; Ober and Shuelte-Merker, 1999). Nodal signals in the YSL are required to induce a stable source of the product of squint/ndr1 in the blastoderm margin, that is a prerequisite for mesodermal and endodermal induction (Fan et al., 2007).

The YSL also has a function during organogenesis. For instance, loss of function of the YSL-specific transcription factor Mxtx1 leads to defects in cardiac morphogenesis. Knock down of Mxtx1 using oligonucleotide morpholino inhibited the fusion or myocardial progenitor cells at the midline causing cardia bifida (Sakaguchi et al., 2006).

The YSL in zebrafish persists throughout embryonic development and is the only interface between the developing embryo and the nutrient reserve in the yolk (D’Amico and Cooper, 2001). However, the role in metabolism and nutrient transport from the yolk mass has been mostly investigated in other teleost species such as turbot and trout (Poupad et al., 2000; Sire et al., 2005). The expression patterns of some genes involved in metabolism and
nutrient transport such as creatinine metabolism genes, as well as iron and fatty acid transport, has been examined in the zebrafish (Mudumana et al., 2004; Wang et al., 2007). However, the specific function of these genes within this cell layer and in the context of embryo and larval development has not been studied in detail. The multiple roles that the YSL exerts in zebrafish development underscore the importance of studying the regulation of the transcriptional activity in this cell layer, as well as the importance of understanding the genetic mechanisms underlying the structure and function of this unique cell layer.

2.3 - Evolution of gastrulation and morphological diversity in teleost fishes

2.3.1 - The teleost clade

Teleost fishes include more than 20,000 species that account for approximately half of the existing vertebrates (Postlethwait et al., 2004). This clade rose from a radiation that took place approximately 250 million years ago (Figure 2.3; Lauder and Liem, 1983). Teleost fishes are members of the dominant group within the ray-finned fishes. The ray-finned fishes and the lobe-finned fishes share a common vertebrate ancestor that existed ≈450 million years ago (Janvier, 1996). The lobe-finned fishes constitute a group that includes tetrapods (Metscher and Ahlberg, 1999, Figure 2.3).

2.3.2 - The yolk syncytial layer is a morphological novelty in teleost fishes

The large number of vertebrate species that are included within the teleost clade is mirrored by its morphological, physiological and ecological diversity. However, teleost fishes share a common mode of gastrulation that has been hypothesized as essential to their evolutionary success (Cooper and Virta, 2007; Collazo, Bolker, and Keller, 1994). Teleost
gastrulation was derived from an ancestral form of gastrulation to include morphological and developmental changes such as (i) the execution of a meroblastic cleavage pattern leading to the formation of a cellular blastula and an uncleaved yolk mass, (ii) the presence of the EVL acting as an osmotic barrier and (iii) the absence of bottle cells that initiate the formation of the germ layers (Collazos, Bolker and Keller, 1994). These developmental and morphological transformations are the evolutionary scenario in which epiboly becomes a novel morphogenetic movement preceding teleost gastrulation and the YSL becomes a novel cell layer that has a crucial role during epiboly. This raises the question of what is the gene expression landscape underpinning the generation of this novel embryonic structure and, most importantly, what are the novel gene regulatory pathways that have evolved to regulate the expression of genes in the YSL.

2.3.3 - Whole genome duplication at the origin of the teleost clade

Whole genome duplications provide material for the evolution of new protein functions that can contribute to morphological diversity. Based on the analysis of genomic architecture in different species, it is thought that two whole genome duplication events anteceded the evolution of vertebrates (Figure 2.3; Meyer and Van der Peer, 2005). A third round of whole genome duplication is thought to antecede the teleost clade radiation (Figure 2.3; Taylor et al., 2003; Postlethwait et al., 2000). Whole genome duplication events generate duplicate genes, which, through diverse fates, give rise to novel gene expression patterns and possibly novel protein functions. These genetic innovations may have contributed to the morphological diversity that allowed the expansion of the teleost clade (reviewed by Prince and Pickett, 2002).
There are several models of functional divergence that explain the fate of duplicated genes. Force and coworkers (1999) proposed that duplicate genes could follow paths that include non-functionalization, sub-functionalization and neo-functionalization. Additionally to these three paths, a recent model by He and Zheng (2005) proposed the possibility of genes undergoing a sub-neo-functionalization. Divergence in gene function is a requisite for maintenance of one of the duplicates. Divergence in gene function is acquired by mutations in the coding region as well as in the regulatory regions (Force et al., 1999). Non-functionalization rises through a null mutation of the coding region or through degeneration of the regulatory region. Neo-functionalization is the result of mutations that cause acquisition of a novel function by one of the duplicates while retaining the ancestral function in the other. Sub-functionalization occurs when both of the duplicates undergo mutations that alter their expression patterns to an extent that both duplicates are required to complement the ancestral function. The sub-neo-functionalization model combines an initial sub-functionalization of the ancestral function, followed by the acquisition of a novel function by the gene duplicates (He and Zhang; 2005; Force et al., 1999).

These and many more models propose mechanisms by which duplicate genes are retained. These models need to be complemented with experimental data from model systems of synthetic polyploidization to examine genomic modifications after whole genome duplications, as well as comparative genome analysis between species. Finally, the study of the divergence of the products and the regulatory regions of zebrafish duplicate genes can help us elucidate the actual relationship between whole genome duplication events and the vast morphological diversity in the teleost clade.
Figure 2.3 - Teleost fishes and whole genome duplication events during vertebrate evolution. Phylogeny of vertebrates depicting the ray-finned and the lobe-finned fishes clades. The ray-finned and the lobe-finned clades had a common ancestor that existed approximately 450 million years ago (MYA). Members of the lobe-finned fishes clade include tetrapods. Two whole genome duplication (WGD) events are thought to have occurred at the base of the vertebrate lineage (1R, 2R). One additional WGD is thought to have occurred at the base of the teleost clade, and it is thought to have contributed to the radiation of teleost fishes. The radiation of teleost fishes may have occurred approximately 250 MYA.

2.4 - The p38 MAPK/MAPKAPK2 signaling pathway in vertebrates

2.4.1 - The betty boop mutation is a lesion in the zebrafish homolog of MAPKAPK2

_betty boop_ (bbp) is a maternal-effect epiboly mutant in zebrafish (Wagner et al., 2004). The _bbp<sup>p58cd</sup>_ mutation is a lesion in _mapkapk2a_ (mk2a), the gene encoding the zebrafish homolog of the mammalian Mitogen Activated Protein Kinase Activated Protein Kinase (MAPKAPK2, later on referred to as MK2), that results in a nonsense codon causing the truncation of 40 amino acids of the wild type Mk2a protein (Holloway et al., 2009, Figure 2.4, Panels B and C). The _bbp_ mutation causes a striking lethal phenotype of abnormal
contractions in the YSL that precede the lethal constriction of the blastoderm margin at 50% epiboly, leading to lysis of the zebrafish embryo (Holloway et al., 2009). To confirm the identity of the bbp mutation, I expressed the wild type version of Mk2a in bbp mutant embryos by injecting in vitro transcribed mRNA at the one-cell stage. Injection of full-length mk2a mRNA in bbp mutant embryos suppresses the lethal lysis phenotype (Holloway et al., 2009). Moreover, expression of the wild type version of mk2a mRNA exclusively in YSL suppresses the lethal lysis phenotype demonstrating that the function of Mk2a is only required in the YSL during epiboly.

Another aspect of the bbp mutant phenotype is the aberrant calcium release. Ratiometric analysis of intracellular calcium using the calcium Fura-2 dextran showed that bbp mutant embryos have ectopic flashes of calcium in the YSL throughout early epiboly as opposed to the sustained levels observed in wild type embryos. As epiboly progresses, the intensity and the frequency of the ectopic flashes of calcium increases. Finally, there is a dramatic increase of the calcium concentration at the blastoderm margin in bbp mutants but not in wild type embryos near the onset of the phenotype (Holloway et al., 2009).

Mk2a is protein kinase and the kinase activity of Mk2a is required for epiboly progression. Injection of in vitro transcribed mRNA encoding a kinase-dead mutant of the full-length Mk2a protein does not rescue the bbp mutant phenotype. Also, western blot analysis of extracts of HeLa cells transfected with the full-length-MK2 and with the Bbp-MK2 showed a reduced phosphorylation of HSP27 in extracts from cells transfected with the truncated mutant form without altering total HSP27 protein levels (Holloway et al., 2009).
Figure 2.4 - Human MK2 and zebrafish Mk2a have conserved structural features that are key for their function. Human MK2 (A), zebrafish Mk2a (B) and the Bbp mutant form of Mk2a (C). Both MK2 and Mk2a have the S/T catalytic domain and C-terminal regulatory domain. They also have conserved structural features that include sites of p38 MAPK phosphorylation, a nuclear localization signal (NLS), a nuclear export signal (NES), an auto-inhibitory domain, and the p38 MAPK docking domain. The $bhp^{p58cd}$ mutation causes a premature stop codon that truncates the wild type protein, disrupting the p38a docking domain and the NLS.
2.4.2 - MK2 is a protein kinase activated by the p38 MAPK signaling pathway

MK2 is a protein kinase initially identified as a stress-induced kinase capable of phosphorlating a small heat shock protein in rat and mouse (Zhou et al., 1993; Stokoe et al., 1992a; Stokoe et al., 1992b; Freshney et al., 1994). MK2 is a member of the MAPKAPK subfamily of the calcium/calmodulin-dependent protein kinase superfamily. The MAPKAPK subfamily also includes MAPKAPK3 and MAPKAPK5 (Gaestel et al., 2006).

MK2 has two main domains: a catalytic domain homologous to the calcium/calmodulin-dependent protein kinase and a C-terminal regulatory domain (Figure 2.4, Panel A; Engel, Plath and Gaestel, 1993). The C-terminal regulatory domain has a nuclear localization signal (NLS), a p38 MAPK binding site, a nuclear export signal (NES), and an auto-inhibitory helix domain (Figure 2.4, Panel A; Engel, Kotlyarov, and Gaestel, 1998; White et al., 2007; Zu, Ai and Huang, 1995). Three main sites of p38 MAPK phosphorylation were identified in human MK2: T222 and S272 in the catalytic domain, and T334 in the hinge between the catalytic domain and the C-terminal regulatory domain. An auto-phosphorylation site at position T338 has also been identified (Figure 2.4, Panel A; Ben-Levy et al., 1995).

p38 MAPK was identified as a stress-dependent upstream activator of MK2 and is the only protein kinase known to activate MK2 in vivo (Rouse et al., 1994; Freshney et al., 1994). p38 MAPK is one of the four MAPK signaling pathways identified in vertebrates that also include JNK, ERK1/2 and ERK5 (Cargnello and Roux, 2011). MAPK signaling pathways transduce extracellular stimuli into intracellular responses through the sequential phosphorylation of proteins from the MAP3K level to the MAP2K level, then to the MAPK level (Figure 2.5).
Figure 2.5 - The p38 MAPK signaling pathway transduces extracellular stimuli into an intracellular response. A set of proteins, referred to as activators in this schematic, activates MAPK signaling pathways at the MAP3K level upon extracellular stimuli. Sequential phosphorylation from the MAP3K level to the MAP2K level, and then to the MAPK level relays this signal. At the MAPK level, p38 MAPK can activate a diverse array of downstream targets, one of which being MK2. Some activators and MAP3Ks can be shared by p38 MAPK and the other MAPK signaling pathways (i.e. JNK, ERK1/2, and ERK5). Crosstalk between p38 MAPK and JNK has been reported. However, these relationships are not depicted in this schematic for simplification purposes. This schematic lists the proteins identified in vertebrates that correspond to each level of the p38 MAPK signaling pathway according to Cuadrado and Nebreda, 2010.

p38 MAPK and MK2 are thought to exist as a stable complex in the nucleus in resting state (Ben Levy et al., 1998; Lukas et al., 2004; White et al., 2007). The NLS maintains MK2 in the nucleus at resting state (Engel, Kotlyarov and Gaestel, 1998). Activation by p38 MAPK, and most notably phosphorylation of T334, causes a conformational change that inhibits the interaction of the auto-inhibitory helix domain with the catalytic domain, resulting in the unmasking the NES which mediates the transport of the activated p38 MAPK:MK2 complex to the cytoplasm (Engel, Kotlyarov and Gaestel, 1998; Zu, Ai and Huang, 1995; White et al., 2007). MK2 phosphorylates a set of downstream targets to regulate their function. MK2 downstream targets are involved in a large variety of cellular
processes including mRNA stabilization, transcriptional activation, cell growth and proliferation, epithelial integrity and actin cytoskeleton organization (Table 1.1).

2.4.3 - The role of p38 MAPK/MK2 signaling in vertebrate development

The p38 MAPK/MK2 signaling pathway is required during pre-implantation in cultured mouse embryos. Pharmacological inhibition of p38 MAPK using SB203580 in 2-cell stage mouse embryos reduces the phosphorylation of MK2 and causes developmental arrest at 8-cell and 16-cell stage (Natale et al., 2004). Treatment of cultured mouse embryos with a p38 MAPK inhibitor at different stages between 8-cell and blastocyst results in defects in compaction, as well as disorganization in the cortical actin network, tight junctions, and adherens junction (Natale et al., 2004; Bell and Watson, 2013).

Knockout of p38 MAPK in the mouse is embryonically lethal. Embryonic lethality in p38 MAPK-deficient mice is caused by angiogenic defects in the placenta and the yolk sac (Mudgett at al., 2000; Adams et al., 2000). p38 MAPK-deficient mice also have embryonic blood vessel and myocardium defects that are hypothesized to be secondary to the disruption of extraembryonic angiogenesis (Adams et al., 2000). However, the contribution of MK2 as a downstream target of p38 MAPK angiogenesis in the mouse embryo is unknown.

2.4.4 - The role of p38 MAPK/MK2 in disease

Mouse models of ischemia and reperfusion show that MK2 deficiency provides protection against organ injury. MK2-deficient mice show smaller size of infarction, less severe neurological deficit, and inhibition of cell death in a cerebral ischemia model (Wange et al., 2002). In the case of the myocardial ischemia model, MK2-deficient mice also show smaller size of infarction, better recovery of cardiac function after reperfusion, and smaller number of apoptotic cardiomyocytes (Shiroto et al., 2005).
MK2-deficient mice have an impaired inflammatory response. Unlike wild type mice, MK2-deficient mice do not undergo the endotoxic shock when challenged with purified bacterial lipopolysaccharide (LPS) via intraperitoneal injection (Kotlyarov et al., 1999). This hypoinflammatory phenotype stems from the inability of MK2-deficient mice to produce wild type levels of the pro-inflammatory cytokine TNF-α (Kotlyarov et al. 1999).

Conversely, other murine models have shown that MK2 can exert a protective role during inflammatory conditions. Some of these models include the MPTP mouse model for Parkinson’s disease in which MK2-deficient mice show less severe neuroinflammation and neuronal degeneration (Thomas et al., 2008), the hypercholesterolemic mice model for atherosclerosis in which MK2-deficient have reduced atherosclerosis (Jagavelu et al., 2007), the collagen-induced model of arthritis in which MK2-deficient mice have less severe lesions (Hegen et al., 2006), the spinal cord injury model in which MK2-deficient mice have reduced secondary tissue damage and improved recovery of locomotor control (Ghasemlou et al., 2010), and the DMBA/TPA-induced skin tumorogenesis model in which MK2-deficient mice show fewer tumors and increased number in apoptotic keratynocytes (Johansen et al., 2009). The protective role of MK2-deficiency during inflammatory processes underscores the importance of investigating the function of MK2 and its downstream effectors to improve the specificity of anti-inflammatory therapies.
Table 2.1 - MK2 targets of phosphorylation reported in literature.

<table>
<thead>
<tr>
<th>PROTEIN NAME</th>
<th>SYMBOL</th>
<th>FUNCTION</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>tristetraprolin</td>
<td>TTP</td>
<td>mRNA stability</td>
<td>Chrestensen et al., 2004</td>
</tr>
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<td>polyA-binding proteins 1</td>
<td>PABP1</td>
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<td>hnRNPA0</td>
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<tr>
<td>polyA-specific ribonuclease</td>
<td>PARN</td>
<td></td>
<td>Reinhardt et al., 2010</td>
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<tr>
<td>E2F transcription factor 1</td>
<td>E2F1</td>
<td>Transcriptional activation</td>
<td>Olano et al., 2012</td>
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<tr>
<td>serum response factor</td>
<td>SRF</td>
<td></td>
<td>Heidenreich et al., 1999</td>
</tr>
<tr>
<td>5-lipoxygenase</td>
<td>5-LO</td>
<td>Inflammation</td>
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<td>TSC2</td>
<td>Growth and proliferation</td>
<td>Li et al., 2003</td>
</tr>
<tr>
<td>MDM oncogene</td>
<td>MDM</td>
<td></td>
<td>Weber et al., 2005</td>
</tr>
<tr>
<td>v-akt murine thymoma viral oncogene homolog 1</td>
<td>AKT1</td>
<td></td>
<td>Rane et al., 2000</td>
</tr>
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<td>Epithelial keratins 8, 18 and 20</td>
<td>K8 K18 K20</td>
<td>Epithelial integrity</td>
<td>Menon et al., 2010</td>
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<tr>
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<td>HSP25</td>
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<td>Singh et al., 2003</td>
</tr>
</tbody>
</table>
2.5 - Tristetraproline is an RNA-binding protein that regulates the stability of mRNA molecules

2.5.1 – Tristetraproline-deficient mice develop a hyperinflammatory syndrome

The gene encoding tristetraproline (TTP) was initially cloned and described as an immediate early response gene in murine cell culture, and the transcription of TTP was shown to be activated by several extracellular stimuli that include phorbol esters, serum, and insulin (Lai et al., 1998; DuBois et al., 1990; Varnum et al., 1990). The physiological role of TTP in a whole organism was first studied in TTP-deficient mice. TTP-deficient mice are phenotypically comparable to wild type mice until approximately 10 days after birth. Around this period, TTP-deficient mice become unable to gain weight and develop cachexia as they age. The TTP-deficient phenotype includes other symptoms of inflammation such as arthritis and dermatitis with infiltration of neutrophils and macrophages (Taylor et al., 1996). The functional relationship between TTP and TNF-α was initially established by Carballo and coworkers (1997) who showed that the macrophages of TTP-deficient mice had abnormally elevated levels of TNF-α upon LPS stimulation. Granulocyte macrophage-colony stimulation factor (GM-CSF), a potent factor in the differentiation of macrophages, was identified using the same approach as an additional molecule with increased levels mediating the hyperinflammatory syndrome of TTP-deficient mice (Carballo, Lai and Blackshear, 2000).

2.5.2 - Tristetraproline binds mRNA molecules that contain AU-rich elements to promote mRNA decay

TTP binds the mRNA encoding mammalian TNF-α through the CCCH zinc finger domain of TTP. Substitution of single cysteine residues for arginine in either one of the two CCCH zinc finger domains of TTP inhibits the binding of TTP to mouse TNF-α mRNA
TTP binds type II AU-rich elements (AREs) at the 3' untranslated region (3' UTR). TTP has been shown to bind a minimal ARE motif of a nonamer consisting of UUAUUUAUU (Figure 2.6; Blackshear et al., 2003). Further analysis using oligonucleotide probes and the RNA-binding domain of TTP identified several variations of the minimal nonamer that change the affinity of this interaction (Brewer et al., 2004).

Upon binding to AREs, TTP promotes mRNA decay by recruiting members of the enzymatic machinery that degrades mRNA in the cytoplasm (Figure 2.6). TTP has been shown to interact with the Ccr4-Not deadenylase complex. The Ccr4-Not complex is the principal mRNA deadenylase in several organisms and is composed of five subunits: Ccr4, Caf1/Pop2, Not1, Not2 and Not3/5 (reviewed by Wahle and Winkler, 2013). Experimental evidence has shown that TTP binds the Not1 subunit that acts as a scaffold for Ccr4-Not complex. TTP interacts via its C-terminal domain with Not1, and Not1 is required for the recruitment of Caf1, one of the two catalytic subunits of the complex that degrades mRNA decay (Sandler et al., 2011). More recently, Fabian and coworkers (2013) have obtained a crystal structure of TTP complexed with the Not1 subunit of Ccr4-Not deadenylase complex via the C-terminal domain of TTP. More importantly, they have shown that the mutation F319A in TTP, that lies in the interface between TTP and Not1, reduces deadenylation in vitro and also increases the stability of an mRNA reporter containing the 3’UTR of TNF-α (Fabian et al., 2013). Deadenylation is the first step in mRNA degradation and it initiates decapping. Deadenylation and decapping are requisite of 3’ and 5’ exonucleolitic decay (reviewed Wilusz et al., 2001). The levels of polyadenylated GM-CSF are higher than the levels of deadenylated GM-CSF in TTP-deficient macrophages (Carballo, Lai and
Blackshear, 2000). Lai and coworkers (1999) also showed a difference in the proportion of polyadenylated/deadenylated species of TNF-α mRNA molecules in TTP-deficient mice. TNF-α and GM-CSF are more stable in TTP-deficient mice (Carballo, Lai and Blackshear, 2000; Lai et al., 1999).

Full-length, as well as the N-terminal domain of TTP alone have also been shown to co-immunoprecipitate with other proteins involved in mRNA degradation such as the decapping enzyme Dcp2, the 5’ to 3’ exonuclease Xrn1, and Ccr4 subunit of the Ccr4-Not deadenylase complex (Lykke-Andersen and Wagner, 2005). Lykke-Andersen and Wagner (2005) also showed that deletion of the N-terminal domain of TTP reduced the mRNA degrading activity of TTP. Taken together, these results have led to the most widely accepted model that TTP binds AREs-containing mRNA molecules and promotes the removal of the polyA tail. Experimental evidence points to role of the Ccr4-Not1 deadenylase complex (Figure 2.6; Sandler et al., 2011; Fabian et al., 2013). Deadenylation promotes decapping, and the subsequent 5’ or 3’ degradation of mRNA by exonucleases (Figure 2.6; Lykke-Andersen and Wagner, 2005; Sandler et al., 2011).

2.5.3 - p38 MAPK/MK2 inhibit the mRNA-destabilizing function of Tristetraprolin

The relationship between MK2 and TNF-α levels was initially identified by the inability of MK2-deficient mice to produce wild-type levels of TNF-α (Kotlyarov et al. 1999). However, Hitti and coworkers (2006) showed that TTP was a likely downstream target of MK2 by the lack of rescue of the hyperinflammatory phenotype when TTP-deficient mice were bred in an MK2-deficient background. The two main residues for MK2-dependent phosphorylation of mouse TTP have been identified at positions S52 and S178 (Chrestensen et al., 2004).
Figure 2.6 - Tristetraprolin binds mRNA molecules that contain AU-rich elements to promote mRNA decay. Schematic representation representation of the structure of mouse tristetraprolin (A). There are three domains in mouse tristetraprolin (TTP): the N-terminal domain (residues 1-95), the CCCH-type Zinc-finger domain (residues 96-160), and the C-terminal domain (residues 161-319). TTP interacts with mRNA molecules via its CCCH-type zinc-finger domain. The C-terminal domain interacts with the Ccr4:Not deadenylase complex. The N-terminal domain interacts with the Xnr1 5' to 3' exonuclease, the Dcp2 decapping enzyme, and the Ccr4 subunit of the Ccr4:Not deadenylase complex. Schematic representation of the interaction between TTP and its canonical target TNF-α (B). The ARE of Tnf-α contains overlapping repeats of the minimal UUAUUAAUUU nonamer to which TTP binds. Upon binding, TTP recruits the mRNA degrading machinery. Most of the experimental evidence points to the association of TTP with the Ccr4-Not deadenylase complex to initially promote deadenylation. Degradation of the polyA tail facilitates the access of decapping enzymes and exonucleases.
Activation of the p38 MAPK pathway has been shown to inhibit mRNA decay. Expression of an activated form of MAP2K6 increases the stability and reduces the deadenylation of a reporter mRNA containing the ARE of IL-8 (Winzen et al., 2008). Similarly, pharmacological inhibition of p38 MAPK increases deadenylation of a mRNA reporter containing the 3’ UTR of COX-2 (Dean et al., 2003).

MK2 phosphorylation of TTP inhibits mRNA decay (Stoecklin et al., 2004; Marchese et al., 2010). There are two mechanisms that have been proposed to mediate the MK2-dependent inhibition of mRNA decay by TTP, one mediated by 14-3-3 binding to TTP and the other mediated by the inhibition of Ccr4-Not deadenylase complex binding to TTP. MK2 phosphorylation of TTP promotes the formation of a TTP:14-3-3 complex (Chrestensen et al., 2004). The binding of 14-3-3 to TTP has been shown to prevent mRNA decay (Stoecklin et al., 2004). In contrast, Marchese and coworkers (2010) have shown that the phosphorylation of TTP by MK2 inhibits the recruitment of the Caf1 subunit proteins of the Ccr4-Not deadenylase complex independently of the participation of 14-3-3.

2.5.4 - The role of TTP in vertebrate development

The role of TTP in vertebrate development remains largely unexplored. In a recent paper, Treguer and coworkers (2013) showed that knock down of the TTP homolog in Xenopus laevis using morpholino oligonucleotides disrupts the morphogenesis of the pronephric tubule (Treguer et al., 2013).

2.5.4 - The role of TTP in disease

The dramatic hyperinflammatory syndrome, mediated by increased TNF-α production in macrophages observed in mice underscores the role of TTP in inflammatory diseases (Carballo, Gilkenson and Blackshear, 1997). One recently examined aspect of the
hyperinflammatory syndrome in TTP-deficient mice is the inflammation and fibrosis of the mitric and aortic valves in the heart, suggesting a potential role of TTP in the pathology of particular heart diseases (Ghosh et al., 2010). Brennan and coworkers (2009) have shown that TTP is downregulated in transformed tumors in different tissues, that downregulation of TTP negatively correlates with advanced stages of breast tumor, and that patients with low levels of TTP have a poorer prognosis after tumor excision. Decreased levels of TTP may promote carcinogenesis and transformation via the stabilization of genes involved in inflammation, angiogenesis, apoptosis, and tissue invasion (reviewed by Ross et al., 2012). In summary, TTP is involved in inflammatory pathologies of different cell types and tissues. Thus, further understanding of the TTP-dependent regulation of mRNA molecules in diverse cell types will contribute to the development of more specific anti-inflammatory therapies and cancer treatments.
CHAPTER 3
Materials and Methods

3.1 - Adult fish and embryo manipulation

Wild-type zebrafish and *betty boop* mutant zebrafish were maintained at Rice University according to approved institutional animal care procedures. Embryos were collected every 30 minutes after fertilization to ensure that clutches of embryos were age-matched. Embryos were reared in E3 embryonic media at 28°C as previously described (Westerfield, 2000). Staging was performed as described by Kimmel and coworkers (1995).

3.2 - Cloning and plasmid construction

All cloning and site-directed mutagenesis primers are listed in Appendix A. Site-directed mutagenesis was performed using the QuickChange II XL kit (Agilent Technologies, Santa Clara, CA). Wild type *p38a* in pCS2+ vector was a gift of Dr. Toshio Hirano (Osaka University, Japan). Dominant negative *p38a* in pCS2+ (DN-*p38a*, T181A/Y183F) was generated by site directed mutagenesis according to Raingeaud and coworkers (1996). Constitutively active *p38a* in pCS2+ (CA-*p38a*, D177A/F328S) was generated by site-directed mutagenesis according to Diskin and coworkers (2004). Phosphomimetic *mk2a* (PM-*mk2a*, T203/T315E) was generated by site-directed mutagenesis according to Engel and coworkers (1995). Mouse MAPKAPK2 was a gift from Dr. Mathias Gaestel (Hanover Medical School, Germany). *zf36/ttp* (Gene ID: 100334443, annotated as *zf36/l1l*) and the RNA-binding domain of *zf36* was cloned into pCS2+ vector using InFusion (Clontech, Mountain View, CA). Non-phosphorylatable *zf36* (S95A/S231A-*ttp/zf36*) was generated
by site-directed mutagenesis according to Clement and coworkers (2011). \textit{mxtx1} and \textit{mxtx2} were described previously (Hirata et al., 2000). \textit{mxtx1} and \textit{mxtx2} for synthesis of riboprobes and \textit{in vitro} transcription of mRNA were a gift from Dr. Ashley Bruce (University of Toronto, Canada). \textit{nrz/bcl2l10}, \textit{mgll} and \textit{kr8} were cloned into a Gateway entry vector pENTR/D-TOPO (Invitrogen, Carlsbad, CA) and then shuttled into pCS2+. \textit{mk2b} was sucloned into Zero Blunt End TOPO (Invitrogen, Carlsbad, CA), and ligated into pCS2+myc. \textit{slc26a1} and \textit{krt4} were described previously (Xu et al., 2012; Fukazawa et al., 2010). \textit{slc26a1} was a gift from Dr. Leonard Zon (Harvard University, MA, United States). \textit{bcl2} was a gift from Dr. Mary Ellen Lane (Rice University, TX, United States).

3.3 - Microinjection of \textit{in vitro} transcribed mRNA and morpholino antisense oligonucleotides

\textit{mRNA} was \textit{in vitro} transcribed using the mMESSAGE mMACHINE SP6 Kit (Ambion, Austin, TX). \textit{mRNA} dilutions of the transcription reaction were made in 0.1M KCl to achieve the desired concentrations for injection. Morpholino antisense nucleotides were obtained from Gene Tools LLC (Oregon, US). \textit{mxtx2} (\textit{mxtx2-MO}, 5’-ATTGAGTATTTTGCAGCTCTTG-3’) was described previously by Bruce and coworkers (2005). Antisense morpholinos were diluted in 1X MBS (88 mM NaCl, 1 mM KCl, 1 mM MgSO4, 5 mM HEPES, 2.5 mM NaHCO3, 0.7 mM CaCl2) to achieve desired concentration for injection. \textit{mxtx2-MO} injectates were heated at 65°C for 10 minutes and vortexed prior to injection to insure a homogeneous concentration. Embryos were injected at one-cell stage within a 30-minute time window after fertilization to ensure uptake of mRNA by the whole embryo. Embryos were injected into the yolk syncytial layer (YSL) at 3 hours
post fertilization. The needle can occasionally cause mechanical damage at the interface between the YSL and the blastoderm during YSL injections causing the developmental delay of embryo. Thus, embryos injected into the YSL were observed at an hour post injection to select damaged embryos to be discarded. Embryos injected at one-cell stage were observed at one-hour post injection to select unfertilized embryos to be discarded. mRNA encoding the red fluorescent protein DsRed was used as control mRNA for one-cell stage and YSL microinjections.

3.4 - Sytox green labeling of YSL nuclei

Embryos were injected into the YSL with 1nl of solution of 0.05 mM Sytox Green in 0.1M KCl, or with 2nl of a solution of 0.05 mM Sytox Green and DN-p38a in vitro transcribed in mRNA to achieve a dose of 500 pg of DN-p38a per embryo.

3.5 - Time-lapse microscopy

Embryos were dechorionated manually using forceps. Embryos for manual dechorionation were pretreated by gently swirling in a solution of 350 µl of pronase in (30 mg/ml) in 12 ml of E3 embryonic rearing media. Embryos were rinsed after pronase treatment to dilute all remaining pronase. Rinsing of the embryos consisted in four sequential transfers to 1.2% agar coated dishes containing E3. Transfer of the embryos was done using a glass pipette under the microscope to prevent mechanical damage to the embryos. Dechorionated embryos were embedded for immobilization in 0.12% low melting temperature agarose dissolved in E3 embryonic rearing media and sealed with a cover inside individual plastic chambers. The bottoms of the plastic chambers were lined with solid 1%
low melting temperature agarose diluted in E3 embryonic rearing media. The top of the plastic chamber was lined with Vaseline to form a seal with an 18x18 mm coverslip to prevent dehydration of the 0.12% low melting temperature agarose embedding media. Four plastic chambers were mounted in a 3”x2” microscope slide. Individual chambers hosted four embryos. Sixteen embryos were imaged in a single experiment. Time-lapse movies were composed of individual frames collected every 5-minutes unless otherwise indicated in the Results section. Images were acquired on a Zeiss Axiovert 200M, and processed with Axiovision Software, Photoshop, ImageJ and Quicktime Pro.

3.6 - Imaging

Still images of zebrafish embryos were acquired on a Leica MZ FL III stereomicroscope and on a Zeiss Axiovert 200M. Images were processed with Leica Application suite, Photoshop, and ImageJ.

3.7 - Calcium chloride injections

Embryos were injected into the yolk syncytial layer with 2 nl of a solution of 21 mM CaCl$_2$ solution buffered with 5 mM HEPES set at a pH of 7.2. Alexa-488 10000 MW was used as a fluorescent tracer to verify the accuracy of the YSL injection. Embryos were immobilized in an agar hole made with an acrylic mold on a 1.2% agar-coated Petri dish. Time-lapse movies were composed of individual frames collected every three seconds over a period of 5 minutes. Individual frames were acquired immediately after the injection.
3.8 - Whole mount in situ hybridization

Whole mount in situ hybridization was performed as described in Schulte-Merker and coworkers (1994) and Thisse and coworkers (1993). Embryos were fixed in 4% paraformaldehyde in phosphate buffered saline. Stained embryos cleared in 2:1 benzylbenzoate:benzylalcohol to facilitate the visualization of YSL-specific staining. Embryos were mounted in GMM (10% methyl salicylate in Canada balsam (Sigma Aldrich, Saint Louis, MO); and in 90% glycerol in phosphate buffered saline. Photomicrographs were acquired using a color AxioCam MRc5 camera (Zeiss) mounted on an Axiovert 200M inverted stereomicroscope (Zeiss).

3.9 - Phylogenetic analysis, synteny analysis and protein alignments

Jukes cantor trees and multiple protein alignments were performed using Geneious 6.1.6. This software was created by Biomatters and is available at http://www.geneious.com. Accession numbers used are listed Appendix B. Synteny analysis was performed using the database available at http://syntenydb.uoregon.edu/synteny_db/ and described in Catchen and coworkers (2009).

3.10 - RT-PCR

RNA was isolated from the indicated stages using the Trizol lysis and purification (Invitrogen, Carlsbad, CA). cDNA was prepared with the ImPromII cDNA synthesis kit (Promega, Fitchburg, WI) with random hexamers. PCR was performed using the primers listed in Appendix A. The PCR was conducted using an annealing temperature of 66.5°C using 20, 25, and 30 cycles. The PCR product was analyzed using gel electrophoresis with ethidium bromide staining. Ornithine decarboxylase 1 primers were used as a loading control.
CHAPTER 4

p38 MAPK/MK2 signaling pathway is required for the morphogenesis of the yolk syncytial layer during zebrafish epiboly

RESULTS

4.1 - The zebrafish homolog of mammalian MK2 is required for yolk syncytial layer morphogenesis.

The bbp<sup>p58cd</sup> mutation is a lesion in mapkap2a (mk2a), the zebrafish homolog of the mammalian MK2 (Holloway et al., 2009). The bbp mutation causes a striking lethal phenotype that displays abnormal contractions in the yolk cell that precede the lethal constriction of the blastoderm margin at 50% epiboly, leading to lysis of the zebrafish embryo (Holloway et al., 2009, Figure 4.1). Expression of the wild type version of mk2a protein exclusively in the yolk syncytial layer (YSL) suppresses the lethal lysis phenotype demonstrating that the function of mk2a is only required in the YSL during epiboly.

To further investigate the morphogenesis of the YSL, I labeled the nuclei of the YSL with Sytox green, and performed time-lapse analysis comparing the progression of epiboly of the blastoderm, and most particularly of the YSL in wild type and bbp mutant embryos. Time-lapse analysis revealed that the nuclei of the YSL are evenly distributed over the dome of the yolk cell in wild type and bbp mutant embryos before the onset of the mutant phenotype (Figure 4.1). However, when the abnormal contractions are evident in the brightfield channel, I observed the retraction of the yolk syncytial nuclei to the location of the contractions (Figure 4.1; Panels B, C and D, black arrows in brightfield image point to site of retraction of YSL nuclei). The abnormal contractions propagate as a wave, and resolve in the
abrupt constriction of the blastoderm margin (Figure 4.1, Panel F, red arrowhead in brightfield image points to site of blastoderm constriction). The wave of contraction in the YSL causes the rupture of the yolk cell and further fragmentation of the nuclei of the YSL (Figure 4.1, Panel H, white arrowhead in fluorescent channel points to fragmented nuclei). Taken together, these data suggest that the abnormal contractions observed in bbp mutant embryos are localized in the YSL, and not in the yolk mass or the blastoderm, and that the bbp mutation causes a defect in the yolk cell morphogenesis.

4.2 - Expression of a dominant negative form of the zebrafish homolog of p38 MAPK recapitulates the phenotype of the loss of function of its downstream target Mk2a

The protein p38 MAPK is the only kinase known to activate MK2 (Rouse et al., 1994; Han et al., 1994). Thus, I wanted to test whether the zebrafish homolog of p38 MAPK (p38a) was also required during epiboly. I constructed a dominant negative form of p38a (DN-p38a) by mutating the conserved threonine and tyrosine residues at the TGY activation loop to alanine and phenylalanine, respectively. This mutation abrogates the ability of the upstream MAP2K3 and MAP2K6 to activate p38 MAPK (Figure 4.5, Raingeaud et al., 1996). I hypothesized that p38a is also required during epiboly to activate Mk2a. Thus, I would expect that expression of DN-p38a in wild type embryos would recapitulate the lysis phenotype of bbp mutant embryos. Consistent with my hypothesis, microinjection of DN-p38a into wild type zebrafish embryos at one-cell stage recapitulated the bbp mutant phenotype (Figures 4.2 and 4.3).
Figure 4.1 - The *betty boop* mutation causes a defect in yolk cell morphogenesis. Montage of a time-lapse analysis of age-matched *bbp* mutant embryos and wild type controls. Selected frames correspond to a 35-minute period depicting the *bbp* mutant phenotype (A-H). Each panel shows the corresponding brightfield (top) and fluorescent channel (bottom) images. Fluorescent channel collects emission of SYTOX green, a vital dye that labels the nuclei of the YSL. Black arrows in the brightfield channel of panels B-E show the sites of abnormal contraction in the YSL. Red arrow in the brightfield channel of panel F shows site of blastoderm constriction. White arrowhead in the fluorescent channel of panel G shows fragmented yolk syncytial nuclei (Holloway et al., 2009).
Figure 4.2 - Distribution of phenotypes caused by the expression of a dominant negative form of p38a in wild type embryos. Percentage of embryos in phenotypic classes scored at 24hpf that result from the microinjection of a concentration series of DN-p38a mRNA (100 pg, 500 pg, and 1 ng) at the one-cell stage into wild type embryos. Graph represents the results of three independent experiments (Control mRNA at one-cell stage in wild type embryos, n=179; 100 pg of DN-p38a mRNA at one-cell stage in wild type embryos, n=175; 500 pg of DN-p38a mRNA at one-cell stage in wild type embryos, n=171; 1 ng of DN-p38a mRNA at one-cell stage in wild type embryos, n=182).

Figure 4.3 - Expression of a dominant negative form of p38a recapitulates the betty boop mutant phenotype. Representative phenotypes observed at 6hpf of wild type embryos injected with control mRNA (A), of wild type embryos injected with DN-p38a observed (B) and of hbp mutant embryos (C).
4.3 - Expression of a constitutively active form of zebrafish Mk2a in the whole embryo causes a delay in epiboly

To further test the requirement of p38a/Mk2a signaling during zebrafish epiboly, I constructed a constitutively active form of Mk2a (PM-Mk2a) by replacing conserved targets of phosphorylation by p38 MAPK with phosphomimetic glutamic acid residues. These conserved residues correspond to sites in the activation loop (T203) and in the hinge between the catalytic domain and the C-terminal regulatory domain (T315). This double substitution (T203E/T315E) has been shown to be constitutively active (Engel et al., 1995). The activation of an actin-myosin based contraction during late epiboly is one of the proposed molecular mechanisms that allow the progression of the blastoderm towards the vegetal pole to completely cover the yolk cell. Also, based on my observations of aberrant contractility in the YSL of bbp mutant embryos, I initially hypothesized that p38a/Mk2a signaling was required during epiboly to inhibit the activation of a contractile apparatus until late epiboly when it is required. Thus, I would expect that expression of PM-Mk2a in wild type embryos would result in the sustained inhibition of the activation of contractility, and thus in a delay in epiboly similar to the delay observed in wild type embryos treated with the pharmacological inhibitor of actin-myosin based contractions (Koppen et al., 2006). Wild type mutant embryos at one-cell stage were injected with a series of concentrations of PM-mk2a. Wild type embryos injected with doses of 1ng and 3ng/embryo displayed a severe delay in the progression of epiboly while lower doses had little effect (Figure 4.4).
Figure 4.4 - Distribution of phenotypes caused by the expression of a constitutively active form of Mk2a in wild type embryos. Percentage of embryos in phenotypic classes that result from the microinjection of 1ng and 3ng of PM-mk2a at the one-cell stage in wild type embryos. Embryos were scored approximately at 10hpf when age-matched embryos injected with control mRNA had completed epiboly. Graph represents the results of three independent experiments (Control mRNA at one-cell stage in wild type embryos, n=147; 1ng of PM-mk2a mRNA at one-cell stage in wild type embryos, n=174; 3ng of PM-mk2a mRNA at one-cell stage in wild type embryos, n=101).

Time-lapse analysis revealed that PM-mk2a-injected embryos initiate epiboly later than control-injected embryos as shown by the delay in the formation of the dome of the yolk (Fig 3.5, A-C, red arrows indicates the dome of the yolk). PM-mk2a-injected embryos progress through epiboly more slowly than controls (Figure 4.5, A-F, black arrow indicates the leading edge of the blastoderm margin). PM-mk2a-injected embryos complete epiboly on average 3.16h later than control mRNA-injected embryos (n=16).

When compared to controls, the blastoderm of PM-mk2a-injected embryos shows uneven thickness and less compaction of the cells as epiboly progresses. At the highest dose assayed (3ng/embryo), some embryos arrest their development at sphere stage and undergo lysis of the blastoderm (Figure 4.4). Consistent with a delayed epiboly, PM-mk2a-injected embryos observed at 24hpf show classic phenotypes resulting of a delay in epiboly such as bifurcated embryonic axes, tail and yolk extension defects (data not shown). Taken together,
these data suggest that regulated function of mk2a is required for epiboly and that the constitutive activation of Mk2a in wild type embryos disrupts normal epiboly.

**Figure 4.5 - Expression of a constitutively active form of Mk2a causes a delay in the initiation and the progression of epiboly.** Montage of a time-lapse analysis of age-matched wild type embryos injected with control mRNA and with 1 ng of PM-mk2a mRNA. Selected frames were extracted from 10-hour time-lapse from the initiation of epiboly until a few hours after the completion of epiboly (Panels A-F). Each panel shows the brightfield image that corresponds to a developmental stage indicated. Red arrows in panels A-C point to the dome of the yolk. Black arrows in panels D-F are parallel to the advancing edge of the blastoderm margin.

Expression of PM-Mk2a in the whole embryo disrupted epiboly (Figure 4.5). The pharmacological inhibition of an actin-myosin-based contraction during late epiboly, reported by Koppen and co-workers (2006), did not seem to severely affect the blastoderm. However, time-lapse analysis revealed that expression of PM-Mk2a in the whole embryo caused aberrant cell morphologies and movements in the cells of the DEL. However, because
Mk2a function is only required in the YSL to rescue the *bbp* mutant phenotype, and because competent contractile apparatus of F-actin band and activated myosin is localized on the YSL (Cheng et al., 2004; Koppen et al., 2006), I decided to investigate if the delay in epiboly observed in PM-*mk2a* injected wild type embryos was caused by the activation of Mk2a outside of the YSL, or by postulated p38a/Mk2a-dependent inhibition of an actin-myosin based contraction in the YSL.

**Figure 4.6** - Expression of a constitutively active form of Mk2a in the yolk syncytial layer of wild type embryos does not alter epiboly. Phenotypes observed at 10hpf of wild type embryos injected into the YSL with control mRNA (A) and with PM-*mk2a* (B). Phenotypes observed at 24hpf of wild type embryos injected into the YSL with control mRNA (C) and with PM-*mk2a* mRNA (D).
To do this, I compared the phenotypes resulting of the expression of PM-Mk2a exclusively in the YSL with the phenotypes resulting from the expression of PM-Mk2a in the whole embryo. Wild type embryos injected with a dose as high as 3ng/embryo of PM-*mk2a* into the YSL progress through epiboly at the same rate as age-matched wild type embryos injected with control mRNA in the YSL (Figure 4.6, Panels A and C). At 24hpf wild type embryos injected with a dose as high as 3ng/embryo of PM-*mk2a* into the YSL have the same phenotype as wild type embryos injected with control mRNA (Figure 4.6, Panel D and E; Figure 4.7). Taken together, these data suggest that the YSL can tolerate activation of Mk2a, whereas activation of Mk2a outside of the YSL disrupts epiboly.

**Figure 4.7** - Distribution of phenotypes caused by the expression of a constitutively active form of Mk2a in the yolk syncytial layer of wild type embryos. Percentage of embryos in phenotypic classes that result from the microinjection of control mRNA and of 3ng of PM-*mk2a* in the YSL of wild type embryos. Embryos injected with 3ng of PM-*mk2a* were observed over the course of the first 12hpf to compare their progression through epiboly to age-matched embryos injected with control mRNA. Graph represents the results of three independent experiments (Control mRNA in the YSL of wild type embryos, n=129; 3ng of PM-*mk2a* mRNA in the YSL of wild type embryos, n=123).
4.4 - Expression of a constitutively active form of zebrafish p38 MAPK causes a novel lysis phenotype

p38 MAPK has many identified targets besides MK2 (Cuadrado and Nebreda, 2010; De Nadal et al., 2011). To test the hypothesis that in zebrafish p38a acts through downstream targets other than Mk2a during epiboly, I constructed a mutant form of p38a by substituting the conserved residues D177 to alanine, and F328 to serine (CA-p38a). This double amino acid substitution D177A/F328S has been previously shown to act as a constitutively active form (Diskin et al., 2004). If p38a acts only through Mk2a during epiboly, I would expect that expression of CA-p38a in wild type embryos would recapitulate the phenotype caused by the expression of PM-Mk2a.

Wild type embryos at the one cell-stage were injected with in vitro transcribed CA-p38a at three different doses of 100 pg, 250 pg, and 1 ng per embryo. When compared with age-matched wild type embryos injected with control mRNA, the lowest dose of 100 pg of CA-p38a per embryo did cause a delay in epiboly in approximately 27% of the injected embryos. However, microinjection of doses ≥ 250 pg of CA-p38a in wild type embryos caused a novel phenotype of arrest and lethal lysis during mid-epiboly (Figure 4.8).

Time-lapse analysis revealed that, in contrast to control-injected embryos, CA-p38a-injected embryos do not initiate epiboly, as shown by the absence of both the doming of the yolk and the thinning of the blastoderm margin (Figure 4.9, Panels A, red arrowhead indicates dome of the yolk in control injected embryos). When control-injected embryos are approximately at 50% epiboly, a localized twitching in the yolk cell is observed. From this point on, a wave of contraction propagates and resolves in the herniation and lysis of the yolk cell (Figure 4.9, Panels B-H, black arrowheads indicate location of contraction and herniation
on the yolk cell). This novel yolk cell phenotype is concomitant to a progressive clearing of the overlying blastoderm (Figure 4.9, clearing of the blastoderm is most evident on Panels G to I). The CA-p38a lysis phenotype was reproducible and dose-dependent (Figure 4.8). The CA-p38a lysis phenotype is different than the arrest and lysis observed at the higher doses of PM-mk2a, in which the lysis of the blastoderm precedes the lysis of the yolk cell (data not shown).

![Figure 4.8 - Distribution of phenotypes caused by expression of a constitutively active form of p38a in wild type embryos.](image)

Figure 4.8 - Distribution of phenotypes caused by expression of a constitutively active form of p38a in wild type embryos. Percentage of embryos in phenotypic classes that result from the microinjection of 100 pg, 250 pg and 1 ng of CA-p38a at one-cell stage in wild type embryos. Embryos were scored approximately at 10hpf when age-matched embryos injected with control mRNA had completed epiboly and observed throughout epiboly to verify the lysis phenotype. Graph represents the results of three independent experiments (Control mRNA at one-cell stage in wild type embryos, n=103; 100 pg of CA-p38a mRNA at one-cell stage in wild type embryos, n=119; 250 pg of CA-p38a mRNA at one-cell stage in wild type embryos, n=101; 1 ng of CA-p38a mRNA at one-cell stage in wild type embryos, n=107).
Figure 4.9 - Expression of a constitutively active form of p38a causes a novel lysis phenotype. Montage of a time-lapse analysis of age-matched wild type mutant embryos injected with 250 pg of CA-p38a mRNA and DsRed fluorescent control mRNA. Selected frames were extracted form time-lapse and correspond to a 40-minute time frame spanning the lysis phenotype observed in CA-p38a-injected wild type embryos (Panels A-I). Red arrowhead in Panel A points to the dome of the yolk formed in the control injected wild-type embryo at approximately 50% epiboly. Black arrowheads point to sites of contraction and herniation on the yolk cell.

4.5 - Expression of mouse MK2 in betty boop mutant embryos suppresses the mutant phenotype

Our manipulations of p38a and mk2a suggested that p38 MAPK/MK2 signaling was conserved in the context of zebrafish epiboly. Also, zebrafish Mk2a and mammalian MK2s show a high degree of conservation in domains and structural features (Figure 2.4). Thus, I wanted to test the hypothesis that the function of mouse MK2 was conserved during zebrafish epiboly. If the function of mouse MK2 is conserved during epiboly, then I would expect that microinjection of Mk2 would rescue the bbp mutant phenotype in a similar fashion to mk2a. Microinjection of 250 pg of Mk2 mRNA rescued all bbp mutant embryos to a wild type-like phenotype (Figure 4.10). However, Mk2 did not rescue the bbp mutant phenotype as efficiently as mk2a. While microinjection of mk2a rescues all bbp mutant
embryos to a wild type-like phenotype at a concentration of 25 pg/embryo, microinjection of equivalent amounts of Mk2 only rescued approximately 31% of the bhp mutant embryos (Figure 3.11).

Figure 4.10 - Expression of mouse Mk2 rescues the betty boop mutant phenotype. Representative phenotypes of wild type embryos injected with control mRNA (A), of bhp mutant embryos injected with 25 pg of zebrafish mk2a (B), and of bhp mutant embryos injected with 250 pg of mouse Mk2 (C) observed at approximately 30hpf.

Figure 4.11 - Distribution of phenotypes caused by expression of zebrafish Mk2a and mouse MK2 in betty boop mutant embryos. Percentage of embryos in phenotypic classes that result from the microinjection of 25 pg of mk2a, 25 pg of Mk2, 250 pg of Mk2, and 1 ng of Mk2. Embryos were scored approximately at 24hpf. Graph represents the results of three independent experiments (Control mRNA at one-cell stage in wild type embryos, n=167; 25 pg mk2a mRNA at one-cell stage in wild type embryos, n=82; 25 pg of Mk2 mRNA at one-cell stage in wild type embryos, n=87; 250 pg of Mk2 mRNA at one-cell stage in wild type embryos, n=82; 1 ng of Mk2 mRNA at one-cell stage in wild type embryos, n=99).
DISCUSSION

4.6 - p38a/Mk2a signaling is activated in the yolk syncytial layer during epiboly

The different epiboly phenotypes caused by the constitutive activation of Mk2a in the YSL versus whole embryos (Figures 4.5 and 4.6) and the fact that Mk2a function is required in the YSL (Holloway et al., 2009) suggest that p38a/Mk2a signaling is activated in a YSL-specific manner during epiboly. However, p38a and mk2a are ubiquitously expressed (Holloway et al., 2009; Hsu et al., 2001). This raises the question of how this cell layer-specific activation of p38a/Mk2a signaling during epiboly is achieved.

As discussed in the introduction, sets of different proteins are activated upon extracellular stimuli, first at the MAP3K level then at the MAP2K level, to activate p38 MAPK (Figure 2.5). Extracellular stimuli activate many proteins such as CDC42, RAC1, TRAF2 and TRAF3, which in turn activate the MAPK signaling module at the MAP3K level. Once active, MAP3Ks phosphorylate MAP2Ks, leading to the activation of p38 MAPK (reviewed by Cuadrado and Nebreda, 2010). Thus, YSL-specific expression of upstream signaling components may indicate a mechanism for YSL-specific activation of p38 and MK2. We searched the Zebrafish Model Organism Database (ZFIN, http://zfin.org/) for the expression patterns of the annotated zebrafish homologs of (i) the proteins activated by extracellular stimuli, (ii) the MAP3Ks and (iii) the MAP2Ks involved in the activation of the p38 MAPK signaling pathway. This database compiles expression patterns from large scale whole mount in situ hybridization (WISH) screens (Thisse et al., 2001; Thisse and Thisse, 2004), as well as data from curated publications. We found that the zebrafish homologs of
proteins directly activated by extracellular stimuli, including \textit{traf2a}, \textit{traf2b}, \textit{traf3}, \textit{traf6}, \textit{cdc42}, \textit{rac1a}, \textit{rac1b}, \textit{rac1l}, are either ubiquitously expressed or their expression pattern is currently unknown. Similarly, the annotated MAP3K homologs in zebrafish \textit{taok2a}, \textit{taok2b}, \textit{map3k16}, \textit{map3k3}, \textit{map3k4}, \textit{map3k5}, \textit{map3k9}, \textit{zak1} and \textit{map3k8}, as well as the annotated MAP2K homologs \textit{map2k6}, \textit{map2k4a} and \textit{map2k4b} are either expressed ubiquitously or have an expression pattern that is currently unknown.

One possible mechanism for the YSL-specific activation of p38a/Mk2a is via the zebrafish homologs of the members of the GADD45 family of proteins: GADD45\(\alpha\), GADD45\(\beta\), GADD45\(\gamma\). Members of the GADD45 family of proteins have been shown to mediate a non-canonical activation of p38 MAPK in T-cells (reviewed by Ashwell, 2006). The GADD45 family of proteins has also been shown to interact with, and to activate MAP3K4 (Takekawa and Saito, 1998; Miyake and Saito, 2007). More recently, GADD45e and MAP3K4 have been shown to regulate gonadal sex determination via p38 MAPK (Warr et al., 2012). The annotated zebrafish homologs are listed in Table 3.1. \textit{gadd45ab}, \textit{gadd45ba}, \textit{gadd45bb} and \textit{gadd45g} are expressed during epiboly (Thisse and Thisse, 2004). Only \textit{gadd45ba}, \textit{gadd45bb} and \textit{gadd45g} are expressed in the YSL (Thisse and Thisse, 2004).

Table 4.1 - Expression patterns of the zebrafish homologs of the members of the GADD45 family of proteins.

<table>
<thead>
<tr>
<th>HUMAN GADD45 FAMILY MEMBER</th>
<th>ZEBRAFISH HOMOLOG</th>
<th>EXPRESSION IN EPIBOLY</th>
<th>DESCRIPTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>GADD45C</td>
<td>\textit{gadd45aa}</td>
<td>-</td>
<td>N/A</td>
</tr>
<tr>
<td>GADD45(\alpha)</td>
<td>\textit{gadd45ab}</td>
<td>+</td>
<td>Unspecified</td>
</tr>
<tr>
<td>GADD45(\beta)</td>
<td>\textit{gadd45ba}</td>
<td>+</td>
<td>EVL, YSL, embryonic axis</td>
</tr>
<tr>
<td>GADD45(\beta)</td>
<td>\textit{gadd45bb}</td>
<td>+</td>
<td>Blastoderm margin, YSL</td>
</tr>
<tr>
<td>GADD45(\gamma)</td>
<td>\textit{gadd45g}</td>
<td>+</td>
<td>Anterior axial hypoblast, YSL</td>
</tr>
</tbody>
</table>
4.7 - Map3k4-dependent activation of p38a/Mk2a in the yolk syncytial layer is required for morphogenesis of the yolk syncytial layer

In addition to the recapitulation of the bbp mutant phenotype, some wild type embryos injected with DN-p38a escaped the bbp-like lysis during epiboly and recapitulated the phenotype of the maternal effect mutant pollywog (Figure 4.12, Panels C and D; Wagner et al., 2004). pollywog (pwg) is a maternal-effect mutant that was identified as a lesion in map3k4, the zebrafish homolog of one of the MAP3Ks upstream of the p38 MAPK signaling pathway (Holterhoff and Wagner, unpublished data). pwg mutant embryos have patches of fragmented nuclei on the surface of the yolk cell, as well as loose yolk granules in the yolk extension (Figure 4.12, Panels C and D, black arrows point to patch of fragmented nuclei, red arrowheads point to loose yolk granules in yolk extension). As shown in Figure 4.1, the wave of contraction in bbp mutant embryos causes the fragmentation of YSL nuclei (Figure 4.1, Panel H, white arrowhead in fluorescent channel), suggesting that the pwg phenotype is also caused by the contraction and rupture of the YSL similar to the one observed in bbp mutant embryos but occurring at a later time in development.

4.8 - Conservation of p38 MAPK/MK2 signaling during epiboly

We have shown that the function of both p38a and Mk2a are required during epiboly in the YSL for its morphogenesis. We have also shown that mouse Mk2 can rescue the bbp mutant phenotype, suggesting that the mammalian enzymatic function is conserved in epiboly. Thus, I speculate that it is likely that the function of at least one of the mammalian downstream targets may also be conserved in epiboly. However, the YSL is a morphological
innovation in the teleost clade (Collazo, Bolker and Keller, 1994). These observations suggest the cooption of p38a/Mk2a signaling. In this scenario, the p38a/Mk2a-signaling pathway is a conserved developmental module that acts as the cytoplasmic transducer of a yet unknown development signal. p38a/Mk2a signaling activates at least one of its downstream effectors conserved in mammals and in zebrafish. The question ahead of us is which downstream effector (or effectors) of p38a/Mk2a signaling has diversified its ancestral function to contribute to the generation of a novel embryological structure such as the YSL in the teleost clade.

Figure 4.12 - Expression of a dominant negative form of p38a phenocopies the pollywog mutant phenotype. Phenotypes observed at approximately 30hpf in an uninjected wild type embryo (A), a wild type embryo injected with control mRNA at one-cell stage (B), an uninjected pwg mutant embryo (C) and a wild type embryo injected with DN-p38a mRNA at one-cell stage (D). Each panel shows the overlayed images of the bright field and fluorescent channels. The fluorescent channel, pseudo-colored in green, collects emission for SYTOX green, a vital dye that labels the nuclei of the YSL. In C and D, black arrows point to patches of opaque yolk and fragmented yolk syncytial nuclei and red arrowheads point to loose yolk granules in the yolk extension. Image from panel D is representative of an experiment where 8/8 wild type embryos injected with DN-p38a that have a pwg-like mutant phenotype have fragmented yolk syncytial nuclei and 3/3 wild type embryos injected with DN-p38a that have a wild type-like phenotype have normal yolk syncytial nuclei.
CHAPTER 5

p38a/Mk2a signaling inhibits the mRNA destabilizing function of the zebrafish homolog of tristetraprolin in the yolk syncytial layer during zebrafish epiboly

RESULTS

5.1 – betty boop mutant embryos have reduced expression of mRNA of yolk syncytial layer-specific genes

In mammalian systems, MK2 has been shown to have a role in the post-transcriptional regulation of pro-inflammatory cytokines such as TNF-α (reviewed by Gaestel, 2006). In zebrafish, the function of Mk2a is required in the YSL (Holloway et al., 2009). To test the role of Mk2a in the post-transcriptional regulation of genes during epiboly, I compared the expression of YSL-specific genes in wild type and bbp mutant embryos. In particular, I examined the expression of mxtx2 and nrz/bcl2l10 since the knockdown of these two YSL-specific genes leads to a premature lysis phenotype that resembles the bbp mutant phenotype (Arnaud et al., 2006; Bruce et al., 2005). We used whole mount in situ hybridization (WISH) to assay the mRNA expression of four YSL-specific genes that are detectable before the onset of the bbp mutant phenotype: mxtx1, mxtx2, slc26a1 and nrz/bcl2l10. When compared to wild type embryos, bbp mutant embryos lost mRNA expression of all four genes assayed: among bbp mutant embryos, 53/53 lost expression of mxtx1, 46/46 lost expression of mxtx2, 65/65 lost expression of nrz/bcl2l10 and 54/54 lost expression of slc26a1 (Figure 5.1, Panels A-H). To determine if the loss of mRNA expression is restricted to the YSL, I used WISH to examine the expression EVL-specific
genes that are detectable before the onset of the \emph{bbp} mutant phenotype. Interestingly, the expression of mRNA of EVL-specific genes remained unchanged: among \emph{bbp} mutant embryos, 10/10 maintained the expression of \emph{krt4}, 15/15 maintained the expression of \emph{krt18} and 18/18 maintained the expression of \emph{mgll} (Figure 5.1, Panels I-N). Taken together, these data suggest that the reduction in the levels of mRNA in \emph{bbp} mutant embryos is localized to the YSL.

\subsection*{5.2 - p38a/Mk2a signaling regulates levels of mRNA of yolk syncytial layer-specific genes during epiboly}

We have previously shown that microinjection of mRNA encoding a dominant negative form of the p38 MAPK zebrafish homolog, DN-p38a, in wild type embryos recapitulates the premature lysis phenotype of \emph{bbp} mutant embryos suggesting that \emph{p38a} is also required during epiboly (Holloway et al., 2009, Figure 5.3) Thus, I hypothesized that expression of DN-p38a in wild type embryos would also recapitulate the reduction of mRNA levels of YSL-specific genes. In contrast to wild type embryos microinjected with control mRNA (Figure 5.2, Panels A-D), wild type embryos injected with DN-p38a have reduced mRNA levels of the YSL-specific genes assayed (Figure 5.2, Panels E-H): among wild type embryos injected with DN-p38a, 16/30 have reduced levels of \emph{mxtx1}, 19/31 have reduced levels of \emph{mxtx2}, 27/31 have reduced levels of \emph{nrz/bcl2l10} and 33/33 have reduced levels of \emph{slc26a1}. These results show that expression of DN-p38a not only recapitulates the \emph{bbp} mutant lysis, but also recapitulates the reduction of mRNA levels of YSL-specific genes observed in \emph{bbp} mutant embryos. These results suggest that p38a is required to regulate the levels of mRNA of YSL-specific genes, and that loss of one or more of these YSL-specific genes causes the premature lysis seen in \emph{bbp} mutant embryos.
Figure 5.1 – _betty boop_ mutant embryos have reduced mRNA levels of yolk syncytial layer-specific genes but not of enveloping layer-specific genes. Wild type embryos (A-D) and _bbp_ mutant embryos (E-H). _mxtx1, mxtx2, nrz/bcl2l10_ and _slc26a1_ are four YSL-specific genes of which the mRNA is expressed at approximately 50% epiboly in wild type embryos. Age-matched _bbp_ mutant embryos have reduced mRNA levels of these cell layer-specific genes. Wild type embryos (I-K) and in _bbp_ mutant embryos (L-N). _krt4, krt18_ and _mgll_ are three EVL-specific genes of which the mRNA is expressed at approximately 50% epiboly in wild type embryos. Age-matched _bbp_ mutant embryos maintain the expression of these layer-specific markers.
To further test the requirement of the p38a/Mk2a pathway for the control of the mRNA levels of YSL-specific genes, I expressed the constitutively active Mk2a (PM-Mk2a). I hypothesized that expression of the PM-Mk2a would lead to increased levels of YSL-specific genes. Interestingly, whole-embryo expression of PM-Mk2a caused the ectopic expression of mRNA encoding YSL-specific genes (Figure 5.3, Panels E-H). An examination of the embryos at higher magnification revealed that mRNA encoding YSL-specific genes is expressed in other layers of the early embryo including the DEL and EVL (Figure 5.3, Panels I-L). Among the wild type embryos injected with PM-\textit{mk2a}, 19/27 had ectopic expression of \textit{mxtx1}, 10/27 had ectopic expression of \textit{mxtx2}, 15/28 had ectopic expression of \textit{nrz/bcl2l10} and 6/29 had ectopic expression of \textit{slc26a1}. These data suggest that Mk2a is required during epiboly to control the levels of YSL-specific mRNA and that activation of \textit{mk2a} outside of the YSL is sufficient to increase levels of mRNA of YSL-specific genes.

**Figure 5.2 - Expression of a dominant negative form of p38a in wild type embryos causes a reduction in the levels of mRNA of yolk syncytial layer-specific genes.** Wild type embryos injected with control mRNA (A-D) and wild type embryos injected with 250 pg of DN-\textit{p38a} (E-H). \textit{mxtx1}, \textit{mxtx2}, \textit{nrz/bcl2l10} and \textit{slc26a1} are four YSL-specific genes of which the mRNA is expressed at approximately 50% epiboly in wild type embryos. Wild type embryos injected with DN-\textit{p38a} have reduced levels of mRNA of these YSL-specific genes.
We have previously shown that microinjection of a PM-mk2a mRNA in wild type embryos causes a severe delay in the initiation and the progression of epiboly (Figure 4.5). Thus, it is likely that the disruption in the progression of epiboly is caused by the expression of YSL-specific genes in DEL and EVL cells. In fact, epiboly delay is highly correlated with ectopic expression of \textit{mxtx1} and \textit{mxtx2}. Among the wild type embryos injected with PM-mk2a that are delayed in epiboly, 18/19 had ectopic expression of \textit{mxtx1} and 8/10 had ectopic expression of \textit{mxtx2}. These observations suggest that the ectopic expression of YSL-specific genes is deleterious for normal embryogenesis. Moreover, these results suggest that the p38a/Mk2a pathway acts to control the expression of mRNA of YSL-specific genes.

![Image](image.png)

**Figure 5.3 - Expression of a constitutively active form of Mk2a in wild type embryos causes the ectopic expression of yolk syncytial layer-specific genes.** Wild type embryos injected with control mRNA (A–D) and with 1 ng of a constitutively active form of Mk2a (PM-mk2a, E–L). \textit{mxtx1}, \textit{mxtx2}, \textit{nrz/bcl2l10} and \textit{slc26a1} are four YSL-specific transcripts expressed at approximately 50% epiboly in wild type embryos. Wild type embryos injected with PM-mk2a display ectopic expression of YSL-specific genes. Higher magnification images (I–L) show that expression of mRNA of YSL-specific genes in wild type embryos injected with PM-mk2a is localized to clusters of DEL and EVL cells.
5.3 - *zfp36l1l* is the zebrafish homolog of the mammalian RNA-binding protein tristetraprolin

Many potential targets of MK2 activation have been identified in the literature (reviewed by Gaestel, 2006). Moreover, expression of mouse MK2 in *bbp* mutant embryos rescues the mutant phenotype, suggesting that the enzymatic function of mammalian MK2 is conserved in the context of zebrafish epiboly. The conserved function of MK2 during epiboly supported the hypothesis that function of homologs of known mammalian downstream targets of MK2 may also be conserved in the context of epiboly. The loss of gene expression in the YSL pointed to ZFP36 ring finger protein, also known as tristetraprolin (TTP/ZFP36/TIS11, later on referred as TTP) as a potential target of MK2. TTP binds AU-rich elements (AREs) in the 3’ untranslated region (3’UTR) of target mRNA molecules to promote their degradation (reviewed by Sandler and Stoecklin, 2008). Phosphorylation by MAPKAPK2 inhibits the mRNA-degrading activity of TTP (Clement et al., 2001; Chrestensen et al., 2004). Thus, I wanted to test the hypothesis that the function of the zebrafish homolog of mammalian TTP was required during epiboly to control the stability of the mRNA of YSL-specific genes.

Before directly testing this hypothesis, I identified the zebrafish homolog of human and mouse TTP as zinc finger protein 36 CCCH-type-like 1-like, Zfp36l1l, (Gene ID: 100334443, Zfin gen ID sb: cb81) initially by conducting a BLAST search using well-annotated members of the CCCH tandem zinc-finger proteins family. To further examine this putative homology, I constructed a phylogenetic tree using the amino acid sequences of TTP/ZFP36/TIS11, BRF1/ZFP36L1/TIS11b and BRF2/ ZFP36L2/TIS11d homologs in different vertebrate species including mouse (*Mus musculus, Mm*), human (*Homo sapiens*, *Hs*), and...
Hs), chicken (*Gallus gallus, Gg*) and Xenopus (both *Xenopus laevis, Xi*; and *Xenopus tropicalis, Xt*, where sequences are available). The amino acid sequence of human ZFP8 was used as an outlier. The accession numbers of the protein sequences used in the construction of this phylogenetic tree are listed in Appendix A. The phylogenetic tree (Figure 5.4) shows that there are zebrafish homologs for each one of the members of the CCCH tandem zinc-finger protein family. Zfp36l1l groups with the TTP/ZFP36/TIS11 homologs annotated in Xenopus, human and mouse (Figure 5.4). The same tree shows that Zfp36l1l is the only zebrafish homolog of the mammalian TTP/ZFP36/TIS11 and that Zfp36l2 is the only zebrafish homolog of the mammalian BRF2/ZFP36L2/TIS11d. However, the mammalian BRF1/ZFP36L1/TIS11b has two zebrafish homologs annotated as gene duplicates zfl36l1a and zfp36l1b (Figure 5.4). Thus, the gene annotated as zfp36l1l encodes for the homolog of TTP/ZFP36/TIS11, and I will refer to it from now on as *ttp*.

Multiple protein alignment shows that full-length Ttp has modest homology with the mammalian TTP amino acid sequences (Figure 5.5, Panel A). However, Ttp has extensive homology in the vicinity of the conserved MK2 phosphorylation sites and within the RNA-binding domain (Figure 5.5, Panel A and B; Chrestensen et al., 2004; Lai et al., 1999). Taken together, these results show that *zfp36l1l/ttp* encodes for the zebrafish homolog of the mammalian TTP and that the amino acid sequence of the Ttp is highly conserved in functionally important regions. *ttp* is expressed ubiquitously during early development but becomes enriched in neural tissues and mesoderm later in development (Thisse et al., 2001).
Figure 5.4 - Phylogenetic analysis of the CCCH tandem zinc-finger family of proteins in vertebrates. Phylogenetic tree was constructed using the protein sequences of annotated vertebrate homologs of TTP/ZFP36/TIS11, BRF1/ZFP36L1/TIS11b, and BRF2/ZFP36L2/TIS11d. The human ZFP8 was used as an outlier. Accession numbers of the protein sequences used in the construction of this phylogenetic tree are listed in Appendix B. Vertebrate species include Mus musculus, Mm; human, Homo sapiens, Hs; chicken, Gallus gallus, Gg; Xenopus including Xenopus laevis, Xl and Xenopus tropicalis, Xt; zebrafish, Danio rerio, Dr.

5.4 - Ttp is required to control the stability of the mRNA of yolk syncytial layer-specific genes in a Mk2a-dependent manner

Phosphorylation of mammalian TTP by the p38 MAPK/MK2 signaling pathway promotes the stability of mRNA molecules that are targets of TTP-dependent degradation (Chrestensen et al., 2004; Clement et al., 2011). To test the hypothesis of the Mk2a-dependent regulation of zebrafish Ttp during epiboly, I created a non-phosphorylatable
mutant Ttp by substituting the conserved residues subject to Mk2a phosphorylation for alanine (S95A/S231A-Ttp). We hypothesized that expression of S95A/S231A-Ttp would cause the reduction in the levels mRNA of YSL-specific genes, similar to the reduction observed in bhp mutant embryos and wild type embryos injected with DN-p38a.

**Figure 5.5 - Multiple amino acid sequence alignment of mouse, human and zebrafish homologs of tristetraprolin.** Matrices show the percentage identity between the full-length proteins (A) and RNA-binding domain only (B) of the murine, human and zebrafish homologs. Amino acid alignments of key structural features of TTP/ZFP36 including the two MK2 phosphorylation sites (residues S52/S178 in mouse, S66/S95 in human and S95/S231 in zebrafish) and the RNA-binding domain (C). Numbering of residues corresponds to zebrafish Ttp. Ellipsis indicates omission of amino acids in between regions of interest. Asterisks indicate amino acids target of MK2 phosphorylation.

In contrast to wild type embryos microinjected with control mRNA (Figure 5.6, Panels A-D), wild type embryos injected with S95A/S231A-ttp showed a reduction in the levels of mRNA of the YSL-specific genes assayed (Figure 5.6, Panels E-H). Among the
wild type embryos injected with S95A/S231A-ttp, 6/10 have reduced expression of \(mxtx1\), 10/22 have reduced expression of \(mxtx2\), 9/10 have reduced expression of \(nrz/bcl2l10\) and 12/31 have reduced expression of \(sle26a1\). Taken together, these results suggest that activation of Ttp by Mk2a is required to maintain the stability of mRNA of YSL-specific genes.

![Figure 5.6](image)

**Figure 5.6 - Expression of a non-phosphorylatable form of zebrafish Ttp in wild type embryos causes a reduction in the levels of mRNA of yolk syncytial-specific genes.** Wild type embryos injected with control mRNA (A-D) and with 750 pg of mRNA encoding a nonphosphorylatable mutant from of zebrafish Ttp (S95A/S231A-ttp, E-H). \(mxtx1\), \(mxtx2\), \(nrz/bcl2l10\) and \(sle26a1\) are four YSL-specific genes of which the mRNA is expressed at approximately 50% epiboly in wild type embryos.

To further test the role of zebrafish Ttp in the stability of the mRNA of YSL-specific genes during epiboly, I constructed a Ttp mutant that consists only of the RNA-binding domain (RBD-Ttp). The RBD mutant of TTP promotes the stabilization of ARE-mRNA reporters in tissue culture (Lykke-Andersen and Wagner, 2005). We predicted that if Ttp controls the stability of target mRNA molecules during epiboly, expression of the RBD-Ttp in \(bbp\) mutant embryos should recover the expression of mRNA of the YSL-specific genes assayed. Among the \(bbp\) mutant embryos injected with RBD-ttp, 19/75 recovered the expression of \(mxtx1\) and 48/81 recovered the expression of \(mxtx2\). Expression of RBD-Ttp in
bbp mutant embryos did not recover the expression of slc26a1 or nrz/bcl2l10 (Figure 5.7).

Taken together, these results suggest that Ttp is required to control the stability of the mRNA of YSL-specific genes during epiboly in an Mk2α-dependent manner.

Figure 5.7 - Expression of the RNA-binding domain of Ttp in betty boop mutant embryos recovers the expression of mxtx1 and mxtx2 in the yolk syncytial layer. bbp mutant embryos injected with control mRNA (A-D) and bbp mutant embryos injected with 1 ng of mRNA encoding the RNA-binding domain of Ttp (RBD-ttp, E-H). mxtx1, mxtx2, nrz/bcl2l10 and slc26a1 are four YSL-specific genes of which the mRNA is expressed at approximately 50% epiboly in wild type embryos.

DISCUSSION

5.5 - Conservation of p38α/Mk2α/Ttp signaling and regulation of the expression pattern of mxtx2 contribute to the generation of morphological novelty

In Chapter 3, I showed evidence of the conservation of p38α/Mk2α signaling as well as the conservation of the enzymatic function of mouse MK2 during epiboly. In this chapter I have examined the role of the zebrafish homolog of the known downstream target of the mammalian MK2. In mammals, phosphorylation of TTP by MK2 inhibits the TTP-dependent degradation of ARE-containing mRNA molecules (Clement et al., 2011; Ronkina et al.,
2007; Hitti et al., 2006; Chrestensen et al., 2004). Our results suggest that Ttp can regulate the stability of YSL-specific genes in a p38a/Mk2a-dependent manner in zebrafish, and one of the most interesting targets of this regulation is mxtx2.

Mxtx2 is transcription factor expressed in the YSL and it is essential for epiboly (Hirata et al., 2000; Bruce at al., 2005; Wilkins et al., 2008). Mxtx2 is one of the four known members of the Mix/Bix sub-family of paired-like homeobox genes in zebrafish (Pereira et al., 2012). Seven genes of this family are found in Xenopus laevis, four in zebrafish and only one in mammals and chicken (Pereira et al., 2012), suggesting the expansion of this family in Xenopus and zebrafish. Mxtx2, and its close relative Mtxx1, are the more divergent members of this family (Pereira et al., 2012), suggesting the diversification in their function. A recent analysis of the transcriptional targets of Mtx2 in the YSL of zebrafish showed that approximately 43% of the known YSL genes are bound by Mtx2; thus, suggesting the prominent role of Mtx2 in the regulation of gene expression within this teleost-specific cell layer (Xu et al., 2012).

One exciting interpretation is that the p38a/Mk2a/Ttp signaling is a conserved signaling axis, which acts as a developmental module, regulating the cell layer-specific expression pattern of a divergent transcription factor such as Mxtx2. Mxtx2 can regulate a transcriptional network of its own, altering the gene expression of this cell layer, to contribute to the morphogenesis of this novel embryonic structure. Thus, the sub- or neo-functionalization of a member of the Mix/Bix sub-family of paired-like homeobox genes such as mxtx2, as well as the recruitment of a conserved the p38a/Mk2a/Ttp signaling pathway to a developmental process (co-option), may act as building blocks of morphological novelty.
5.6 - p38a/Mk2a-dependent inhibition of the mRNA-destabilizing function of tristetraprolin is a mechanism for cell layer-specific gene expression

Expression of a constitutively active form of Mk2a (PM- Mk2a) in wild type embryos at one-cell stage severely affects epiboly, whereas expression of PM-mk2a exclusively in the YSL of wild type embryos does not alter epiboly progression (Figures 4.5 and 4.6). Based on these data, I posited the hypothesis that p38a/Mk2a is activated only in the YSL, and that activation of Mk2a outside of the YSL disrupts epiboly. The results of this chapter add evidence to this hypothesis in that expression of PM- Mk2a outside of the YSL causes the ectopic expression of YSL-specific genes (Figure 5.3), and in that ectopic expression is correlated with the phenotype of delay in epiboly observed in wild type embryos microinjected with PM-mk2a (Figure 4.5). The expression of ttp is ubiquitous, yet its mRNA-destabilizing function is suppressed in the YSL. Thus, activation of p38a/Mk2a only in the YSL would allow the YSL-specific stability of mxtx2 and mxtx1.

5.7 - Brief analysis of the in silico prediction of targets is tristetraprolin-dependent mRNA degradation

TTP most commonly binds type II AREs (Lai, Kennington and Blackshear, 2002; Lai, Kennington and Blackshear, 2003; Lai et al., 1999; Lai et al., 2000; Raghvan et al., 2001). Type II AREs have a minimal motif that consists of the nonamer UUAUUUAUU, and the increased number of this nonamer at the 3’UTR of mRNA molecules also increases the rate of their degradation (Zubiaga et al., 1995). We used the bioinformatics tool AREScore (Spasic et al., 2012) to survey putative targets of ARE-dependent mRNA degradation.
AREScore uses the REFSEQ mRNA accession numbers, or any 3’UTR sequence in FASTA format to produce a score based on the number of AUUUA pentamers and their relative position. Mirroring our results from WISH analysis (Figure 5.1), I was interested in looking ARE-containing mRNA molecules in the EVL and the YSL. I used the gene expression database by stage and anatomy term found at the Zebrafish Information Network database. This database compiles expression patterns from two large-scale WISH screens (Thisse et al., 2001; Thisse and Thisse, 2004), as well as data from curated publications. I identified genes expressed in the YSL and the EVL at a selected developmental time window between sphere stage and 75% epiboly that is relevant to the phenotypes caused by our genetic manipulations of p38a, Mk2a, and Ttp function. We used the REFSEQ mRNA accession numbers for these genes to collect their annotated 3’UTRs, and included in our analysis 3’UTRs larger than 100bp. My analysis included a total of 170 YSL-specific genes and 168 EVL-specific genes. I manually identified the count of isolated AUUUA pentamers and count of isolated UUAUUUAUU nonamers. I also ran the selected EVL and YSL-specific genes through the AREScore program (Spasic et al., 2012).

My results show that 126/170 YSL-specific mRNA molecules and 126/168 EVL-specific mRNA molecules have predicted AREs in their 3’UTR region, suggesting that these two cell layers have overall similar proportions of AREs. I selected five of the canonical targets of TTP binding and ran them through the AREScore program: cyclooxygenase-2, (COX-2), tumor necrosis factor α (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin 3 (IL-3) and interleukin 6 (IL-6). The scores for these well-known targets of TTP fluctuate between 7.1 for IL-6 and 21.5 for COX-2. Using this range as a reference, I classified mRNA molecules with a score \( \geq 7.1 \) as high scoring. When
comparing the scores produced by AREScore of EVL and YSL-specific genes I found that approximately 19% of the YSL-specific genes and 13% of the EVL-specific are high scoring. However, ≈19% of the high scoring EVL-specific genes have a nonamer count of ≥1, while ≈33% of the YSL-specific genes have a nonamer count of ≥1. This shows that there is a larger proportion of high scoring mRNA molecules with higher nonamer counts (i.e. more likely targets of Ttp) in the YSL that in the EVL. When comparing YSL-specific genes with those from EVL, I found that approximately ≈10% of the YSL genes specific genes have a nonamer count ≥1 and that ≈4% of EVL-specific genes have a nonamer count ≥1. Taken together, the results of this simple analysis show that, although the EVL and YSL have approximately the same content of predicted AREs, the YSL has a larger number of mRNA molecules that contain AREs of high affinity for TTP binding. Brewer and coworkers (2004) used nucleotide fragments with different ARE motifs to measure TTP:RNA-binding affinity. They examined the effect of adenylate and urydilate residues, their proportions, and their relative positions in the binding affinity of the nucleotide fragment to the RNA-binding domain of TTP. They identified four sequences of strong affinity that possess dissociation constants between 3nM and 6.4nM. These sequences of strong affinity include the minimal nonamer of UUAUUAUUU. Other sequences of strong and intermediate affinities have been found and are described in Table 5.1. We examined the 3’UTR sequences of the YSL-specific genes that I assayed using WISH analysis to look for ARE motifs with strong and intermediate affinity described by Brewer and coworkers (2004). We also compared the count of these ARE motifs in the canonical mammalian TTP targets, TNF-α; and in the highest scoring AREScore-predicted mammalian TTP target, cyclooxygenase 2 (encoded by Ptgs2).
Table 5.1 - Sequence analysis of the 3’ UTRs of selected zebrafish and mouse mRNA molecules.

<table>
<thead>
<tr>
<th>AREScore score</th>
<th>mxtx1</th>
<th>mxtx2</th>
<th>scl26a1</th>
<th>nrz/bcl2l10</th>
<th>Tnf</th>
<th>Ptgs2</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUUUAUUUAUUUA</td>
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<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
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<tr>
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<td></td>
</tr>
<tr>
<td>UUAAUUAAUU</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>5</td>
<td>1</td>
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<tr>
<td>(High affinity, (K_d=3.0 \text{nM}))</td>
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<td></td>
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<td>0</td>
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</tr>
<tr>
<td>UAUUUAU</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>2</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>(Intermediate affinity, (K_d=19 \text{nM}))</td>
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3’UTR length in base pairs

<table>
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<tr>
<th>mxtx1</th>
<th>mxtx2</th>
<th>scl26a1</th>
<th>nrz/bcl2l10</th>
<th>Tnf</th>
<th>Ptgs2</th>
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<tr>
<td>866</td>
<td>407</td>
<td>562</td>
<td>769</td>
<td>778</td>
<td>2236</td>
</tr>
</tbody>
</table>

The murine TNF-\(\alpha\) and COX-2 (encoded by \(Ptgs2\)) are both canonical and high scoring AREScore-predicted TTP targets, and they both have high affinity and intermediate affinity AREs. The 3’UTR of TNF-\(\alpha\) and COX-2 have more intermediate affinity ARE motifs than the zebrafish 3’UTR of the four genes examined. The 3’UTR of TNF-\(\alpha\) has more high affinity AREs than the 3’UTR regions of all the zebrafish genes examined. COX-2, the highest scoring predicted TTP target according to AREScore, has more high affinity ARE motifs than \(mxtx1\) and \(scl26a1\), but not more high affinity ARE motifs that \(mxtx2\) or \(nrz/bcl2l10\). As shown in Table 4.1, the 3’UTR of \(mxtx2\) and \(nrz/bcl2l10\) contain both high and intermediate affinity AREs. The 3’UTR of \(mxtx1\) contains only intermediate affinity AREs and the 3’UTR of \(scl26a1\) contains neither high nor intermediate affinity AREs. From these 3’UTR region sequence analysis I speculate that (i) \(mxtx2\) and \(nrz/bcl2l10\) are likely targets of TTP-dependent mRNA degradation, (ii) \(mxtx1\) is a less likely a target of TTP-dependent mRNA degradation than \(mxtx2\) and \(nrz/bcl2l10\) and (iii) \(scl26a1\) is probably not a direct target for TTP-binding.
CHAPTER 6

Reduction of mRNA levels of mtxx2 and nrz/bcl2l10 in the YSL during epiboly contribute to the morphogenesis defect in betty boop mutant embryos

RESULTS

6.1 – Reduction of the mRNA levels of yolk syncytial layer-specific genes causes the lysis of betty boop mutant embryos

Wild type embryos injected with a non-phosphorylatable mutant of zebrafish ttp (S95A/S231A-ttp) recapitulate the bbp mutant phenotype (Figure 6.1, Panel B). 25%-43% of the wild type embryos injected with S95A/S231A-ttp showed a bbp-like phenotype during mid-epiboly. A portion of the escapers from the bbp-like lysis was delayed in epiboly and their blastoderm was unable to completely cover the yolk cell, causing the lysis of the embryos at the site of the exposed yolk between 10hpf to 12hpf (Figure 6.2). Another portion of the escapers from the bbp-like mutant lysis showed a pwg-like phenotype at 24hpf. These embryos had thick yolk extensions, loose yolk granules, opaque yolk mass, defects in the morphogenesis of anterior structures and of the somites (Figure 6.1, Panel D). These results link the molecular phenotype of reduced mRNA levels of YSL-specific genes and the morphological phenotypes caused by the manipulation of p38a, Mk2a and Ttp activity, suggesting that the phosphorylation of zebrafish Ttp by the p38a/Mk2a signaling pathway is required to maintain the stability of specific transcripts within the YSL and that the loss of these YSL-specific genes caused the premature lysis phenotype observed in bbp mutants.
Figure 6.1 - Expression of a non-phosphorylatable mutant form of tristetraprolin in wild type embryos recapitulates the *betty boop* and *polywog* mutant phenotypes. Representative phenotypes of wild type embryos injected with control mRNA observed at 6hpf (A) and 24hpf (C). Representative phenotypes of wild type embryos injected with 750 pg a non-phosphorylatable mutant of Ttp (S95A/S231A-ttp) observed at 10hpf (B) and at 24hpf (D).

Figure 6.2 - Distribution of phenotypes caused by expression of a non-phosphorylatable form of tristetraprolin in wild type embryos. Percentage of embryos in phenotypic classes scored at 24hpf that result from the microinjection of a concentration series of S95A/S231A-ttp (100 pg, 500 pg and 1 ng) at the one-cell stage into wild type embryos. Graph represents the results of two independent experiments (Control mRNA at one-cell stage in wild type embryos, n=149; 100 pg of S95A/S231A-ttp/zfp36 mRNA at one-cell stage in wild type embryos, n=176; 500 pg of S95A/S231A-ttp/zfp36 mRNA at one-cell stage in wild type embryos, n=181; 1 ng of S95A/S231A-ttp/zfp36 mRNA at one-cell stage in wild type embryos, n=167).
6.2 - Mxtx2 is required in the yolk syncytial layer for morphogenesis

bbp mutant have reduced levels of mRNA of mxtx2 in the YSL (Figure 5.1). mxtx2 encodes a transcription factor that is required in the YSL for epiboly. Loss of function of Mxtx2 using mxtx2 morpholino antisense nucleotides (mxtx2-MO) results in a mid-epiboly lysis phenotype similar to the bbp mutant phenotype (Bruce et al., 2005; Wilkins et al., 2008). We verified the reproducibility of the mxtx2 morphant phenotype. To do this, I microinjected different doses of mxtx2-MO in wild type embryos. As previously shown by others (Bruce et al., 2005; Wilkins et al., 2008), wild type embryos injected with mxtx2-MO at one-cell stage displayed a mid-epiboly lysis phenotype that resembles the bbp mutant phenotype (Figure 6.3). Approximately 53% of the wild type embryos injected with 1ng of mxtx2-MO undergo a bbp-like lysis. Interestingly, approximately 18% of the wild type embryos injected with mxtx2-MO at the 1ng/embryo dose also display a pwg-like mutant phenotype. As discussed in Chapter 4, the pwg phenotype may also be a result of contraction and rupture of the YSL. Microinjection of mxtx2-MO exclusively in the YSL of wild type embryos also recapitulated the bbp and the pwg mutant phenotypes (data not shown).

I wanted to test the hypothesis that loss of function of mxtx2 in the YSL mediated the bbp mutant lysis. If so, I would expect that supplementing the function encoded by mxtx2 function in bbp mutant embryos would suppress the mutant phenotype. Microinjection of mxtx2 mRNA exclusively in the YSL of bbp mutant embryos, at doses between 100 pg/embryo and 1ng/embryo inhibited the premature lysis phenotype of bbp mutant embryos (Figure 6.4). Whereas by 9hpf all bbp mutant embryos microinjected with control mRNA had lysed (Figure 6.5, Panel A), bbp mutant embryos injected with 500 pg of mxtx2 progressed past 50% epiboly and showed a wild type like phenotype at 24hpf (Figure 6.5,
Panels B and D). Taken together, these results suggest that loss of at least \textit{mxtx2} in the YSL causes the \textit{bbp} mutant phenotype.

![Figure 6.3 - Distribution of phenotypes caused by microinjection of \textit{mxtx2} morpholino antisense oligonucleotides at the one-cell stage in wild type embryos.](image)

**Figure 6.3 - Distribution of phenotypes caused by microinjection of \textit{mxtx2} morpholino antisense oligonucleotides at the one-cell stage in wild type embryos.** Percentage of embryos in phenotypic classes scored at 24hpf that result from the microinjection of a concentration series of \textit{mxtx2}-MO (250 pg, 500 pg and 1 ng) into one-cell stage wild type embryos. Graph represents the results of 2 independent experiments (Control 0.1M KC1 at one-cell stage in wild type embryos, n=74; 250 pg of \textit{mxtx2}-MO at one-cell stage in wild type embryos, n=43; 500 pg of \textit{mxtx2}-MO at one-cell stage in wild type embryos, n=74; 1 ng of \textit{mxtx2}-MO at one-cell stage in wild type embryos, n=76).

6.3 - \textit{Mxtx2} is a transcriptional activator of \textit{mxtx1}, \textit{slc26a1} and \textit{nrz/bcl2110}

As previously shown, expression of RBD-Ttp in \textit{bbp} mutant embryos recovers the expression of \textit{mxtx1} and \textit{mxtx2} mRNA in the YSL of \textit{bbp} mutant embryos (Figure 5.7). Moreover, Mtx2 has been shown to be a transcription factor required for expression of a subset of YSL-specific and is required for epiboly (Xu et al., 2012; Bruce et al., 2005). We hypothesized that Ttp regulates the stability of \textit{mxtx2} in the YSL, and that Mtx2 acts to activate the transcription of one or more of the YSL-specific assayed. If so, I would expect that expression of Mtx2 in the YSL of \textit{bbp} mutant embryos would recover the reduced
levels of mRNA of the YSL specific-genes assayed. We would also expect that microinjection of *mxtx2*-MO in wild type embryos, which disrupts the function of *mxtx2* (Bruce et al., 2005), would result in the loss of the mRNA of one or more of the YSL-specific genes assayed. Expression of *mxtx2* in the YSL of *bbp* mutant embryos recovered the expression *slc26a1, mtxtx1* and *nrz/bcl2110* (Figure 6.6, Panels A-F). Among the *bbp* mutant embryos injected with *mxtx2* in the YSL: 72/102 recover the expression of *mtxtx1* in the YSL, 19/40 recover the expression of *nrz/bcl2l10* in the YSL, and 28/75 recover the expression of *slc26a1* in the YSL. Conversely, among the wild type embryos injected with *mxtx2*-MO, 23/28 have reduced levels of *mtxtx1*, 62/62 have reduced levels of *nrz/bcl2l10*, and 31/32 have reduced levels of *slc26a1* (Figure 6.6, Panels G-L). These results suggest that Mtxx2 is a transcriptional activator of *mtxtx1, slc26a1* and *nrz/bcl2l10*.

![Figure 6.4 - Distribution of phenotypes caused by the expression of Mtxx2 in the yolk syncytial layer of betty boop mutant embryos. Percentage of embryos in phenotypic classes scored at 24hpf that result from the microinjection of a concentration series mtxx2 mRNA (100 pg, 500 pg and 1 ng) into the YSL of bbp mutant embryos. Graph represents the results of 3 independent experiments (Control mRNA in the YSL of bbp mutant embryos, n=169; 100 pg of mtxx2 mRNA in the YSL of bbp mutant embryos, n=208; 500 pg of mtxx mRNA in the YSL of bbp mutant embryos, n=200; 1 ng of mtxx2 mRNA in the YSL of bbp mutant embryos, n=169).](image-url)
6.4 - Expression of Mtxt2 outside of the yolk syncytial layer disrupts epiboly

Expression of Mtxt2 exclusively in the YSL of bbp mutant embryos rescues the bbp mutant phenotype (Figure 6.5). Expression of a constitutively active form of Mk2a outside of the YSL causes ectopic expression of mxtx2, which is highly correlated with a severe delay in epiboly (Figure 5.3). I wanted to test the hypothesis that expression of mxtx2 outside of the YSL disrupts zebrafish epiboly. If so, I would expect that wild type embryos microinjected with mxtx2 at one-cell stage would display a delay in epiboly progression. I microinjected in vitro transcribed mxtx2 mRNA into wild type embryos at one-cell stage at three different doses (100 pg, 500 pg and 1 ng/embryo). Expression of Mtxt2 in wild type embryos caused an epiboly arrest. By 9hpf, when embryos injected with control mRNA are approximately at 90% epiboly, the majority of wild type embryos injected with mxtx2 were arrested at sphere
stage or displayed an incipient doming of the yolk (Figure 6.7, Panel B). Careful observation of the arrested embryos demonstrated that the blastoderm of most of the arrested embryos clears progressively, before it lyases by the sloughing off of blastoderm cells (Figure 6.7; Panel B, red arrowhead points mxtx2-injected embryos as it lyases, Panel D).

A smaller portion of the embryos remains severely delayed in epiboly and show phenotypes of arrest and profound developmental delay in epiboly at 24hpf (Figure 6.7, Panel E). Less severely affected embryos show classic phenotypes resulting from a delay in epiboly such as bifurcated embryonic axes, tail and yolk extension defects (Figure 6.7, Panel F). Microinjection of a dose as low as 100 pg/embryo into wild type embryos at one cell-stage is sufficient to cause the lysis of approximately 60% of the injected embryos (Figure 6.8).

I also microinjected 1ng of mxtx2 mRNA exclusively into the YSL of wild type embryos to further test the hypothesis that expression of mxtx2 outside of the YSL disrupts zebrafish epiboly. Microinjection of 1ng of mxtx2 mRNA in one-cell stage caused the lysis of approximately 87% of the wild type embryos injected. If expression of Mxtx2 outside of the YSL disrupts epiboly, I would expect that expression of Mxtx2 into the YSL of wild type embryos should not lead to the severe defects observed when Mxtx2 is expressed outside of the YSL. Indeed, the phenotype of wild type embryos injected with 1ng of mxtx2 in the YSL was indistinguishable from that of wild type embryos injected with control mRNA (Figures 6.9 and 6.10). Taken together, these results suggest that the expression of Mxtx2 must be restricted to the YSL during epiboly.
Figure 6.6 – Mxtx2 transcriptionally activates mxtx1, nrz/bcl2l10, and slc26a1. bbp mutant embryos injected with control mRNA (A-C) and in bbp mutant embryos injected with mxtx2 (D-F). Wild type embryos injected with 0.1M KCl (G-I) and wild type embryos injected with 1 ng of mxtx2-oligonucleotide morpholino (mxtx2-MO, J-L). mxtx1, nrz/bcl2l10 and slc26a1 are three YSL-specific genes of which the mRNA is expressed at approximately 50% epiboly in wild type embryos.
Figure 6.7 - Expression of Mtx2 in wild type embryos at one-cell stage causes an arrest in epiboly. Representative phenotypes of wild type embryos injected with control mRNA observed at 9hpf (A) and 24hpf (C). Representative phenotypes of wild type embryos injected at one-cell stage with mtx2 mRNA observed at 9hpf (B) and 24hpf (D, E and F). Red arrows in panel B points the blastoderm of a wild type embryo injected with mtx2 as it lyses. Panel D correspond to lysed embryos. Panel E corresponds to arrested and severely delayed embryos. Panel F correspond to embryos with lesser defects.
Figure 6.8 - Distribution of phenotypes caused by the expression of Mxtx2 in wild type embryos at one-cell stage. Percentage of embryos in phenotypic classes scored at 24hpf that result from the microinjection of a concentration series mxtx2 mRNA (100 pg, 500 pg and 1 ng) into wild type embryos at one-cell stage. Graph represents the results of 2 independent experiments (Control mRNA at one-cell stage in wild type, n=47; 100 pg of mxtx2 mRNA at one-cell stage in wild type embryos, n=69; 500 pg of mxtx2 mRNA at one-cell stage in wild type embryos, n=59; 1 ng of mxtx2 mRNA at one-cell stage in wild type embryos, n=71).

Figure 6.9 - Expression of Mxtx2 into the yolk syncytial layer of wild embryos does not affect epiboly. Representative phenotypes of wild type embryos injected into the YSL with control mRNA observed at 10hpf (A) and 24hpf (C). Representative phenotypes of wild type embryos injected into the YSL with 1ng mxtx2 mRNA observed at 9hpf (B) and 24hpf (D).
6.5 - Nrz/Bcl2110 is required but not sufficient for the morphogenesis of the yolk syncytial layer during epiboly

*bhp* mutant embryos have lost the expression of *nrz/bcl2l10* in the YSL (Figure 5.1). We have also shown that Mxtx2 regulates the transcription of *nrz/bcl2l10* (Figure 6.6). Nrz/Bcl2l10 is a member of the Bcl-2 family of protein in zebrafish and disruption of the function of Nrz/Bcl2l10 using morpholino antisense nucleotides causes an epiboly phenotype that resembles the *bhp* mutant phenotype (Arnaud et al., 2006). Thus, I wanted to test whether the reduction in the levels of *nrz/bcl2l10* contributed to the *bhp* mutant phenotype. If so, I would expect that expression of Nrz/Bcl2l10 in the YSL would suppress the *bhp* mutant phenotype. To test this hypothesis, I microinjected *in vitro* transcribed mRNA of *nrz/bcl2l10* into the YSL of *bhp* mutant embryos. By 10hpf, approximately 80% of the *bhp* mutant embryos injected with 1ng of *nrz/bcl2l10* in the YSL have progressed past 50%-75% epiboly without undergoing the *bhp* mutant lysis (Figure 6.11, Panel C, black arrow indicates the margin of the blastoderm). At this time point all *bhp* mutant embryos injected with fluorescent control mRNA in the YSL have lysed, and age-matched wild type embryos injected with fluorescent control mRNA in the YSL have completed epiboly and have begun forming the embryonic axis (Figure 6.11, Panels A and B, respectively). Thus, whereas the *bhp* mutant lysis was inhibited in *bhp* mutant embryos injected with *nrz/bcl2l10*, these embryos display a severe delay in epiboly that is accompanied by a delay in the formation of the embryonic axis.

Careful observation demonstrated that approximately 45% of embryos injected with *nrz/bcl2l10* do not undergo the *bhp* mutant lysis but are unable to completely engulf the yolk
cell, which causes the lysis of the yolk cell at the open blastopore a few hours later (Figure 6.12). By 24hpf, 32% of the bhp mutant embryos injected with nrz/bcl2l10 in the YSL escaped the bhp mutant lysis but did not develop normally. The majority of the surviving bhp mutant embryos injected with nrz/bcl2l10 in the YSL resemble the pwg mutant (Figure 6.11, Panel G, asterisks indicate pwg-like embryos, black arrow points to the open blastopore of a bhp mutant embryo). Expression of Nrz/Bcl2l10 reduced the severity of the yolk morphogenesis defect in bhp mutant embryos, as it suppressed the aberrant contractility at 50% epiboly of bhp mutant embryos.

![Figure 6.10 - Distribution of phenotypes caused by the expression of Mtx2 into the yolk syncytial layer of wild type embryos.](image)

It is noteworthy that expression of equivalent levels of Nrz/Bcl2l10 in the YSL of wild type embryos does not disrupt normal development (Figure 6.11, Panels D, F and H). Thus, it is likely that the defects observed in bhp mutant embryos injected with nrz/bcl2l10 were not a result of the overexpression of Nrz/Bcl2l10 but a result of its inability to rescue
other aspects of the *bbp* mutant phenotype. Taken together, these results suggest that the function *nrz/bcl2l10* is required, but not sufficient by itself, in the YSL during epiboly for normal morphogenesis.

![Figure 6.11](image)

**Figure 6.11 - Expression of Nrz/Bcl2l10 in the yolk syncytial layer of betty boop mutant embryos partially rescues the betty boop mutant phenotype.** Representative phenotypes of *bbp* mutant embryos injected with control mRNA in the YSL observed at 10hpf (A) and 24hpf (E). Representative phenotypes of *bbp* mutant embryos injected with 1ng of *nrz/bcl2l10* in the YSL observed at 10hpf (C) and 24hpf (G). In C, black arrows point to the margin of the blastoderm. In G, the asterisk indicates *bbp* mutant embryos injected with *bcl2* that display a *pwg*-like phenotype. Representative phenotypes of wild type embryos injected with control mRNA in the YSL observed at 10hpf (B) and 24hpf (F). Representative phenotypes of *bbp* mutant embryos injected with 1ng of *nrz/bcl2l10* in the YSL observed at 10hpf (D) and 24hpf (H).
Figure 6.12 - Expression of Nrz/Bcl2l10 in the yolk syncytial layer of betty hoop mutant embryos and wild type embryos. Percentage of embryos in phenotypical classes scored at 24hpf that result from the microinjection of 1ng of nrz/bcl2l10 in the YSL of bbp mutant embryos and wild type embryos. Graph represents the results of three independent experiments (1ng of control mRNA in the YSL of bbp mutant embryos, n=195; 1ng of nrz/bcl2l10 mRNA in the YSL of bbp mutant embryos, n=199; 1ng of control mRNA in the YSL of wild type embryos, n=146; 1ng of nrz/bcl2l10 mRNA in the YSL of wild type embryos, n=186).

6.6 - The function of Nrz/Bcl2l10 in the yolk syncytial layer is homologous to the function of the ubiquitously expressed Bcl2

One striking aspect of the bbp mutant phenotype is the fragmentation of nuclei of the YSL (Figure 4.1). Before I had identified the loss of nrz/bcl2l10 in bbp mutant embryos, I hypothesized that bbp mutant embryos executed a premature apoptosis because nuclear fragmentation is a hallmark of apoptosis. This prompted me to test the role of the zebrafish homolog of one of the members of the anti-apoptotic Bcl-2 family of proteins, Bcl2, in the YSL of bbp mutant embryos (Kratz et al., 2006). Similarly to Nrz/Bcl2l10, Bcl2 expression in the YSL partially rescues the bbp mutant phenotype. By 10hpf, when all bbp mutant embryos injected with control mRNA in the YSL had lysed (Figure 6.13, Panel A), approximately 80% of the bbp mutant embryos injected with bcl2 in the YSL have progressed between 50% and 75% epiboly without undergoing the bbp mutant lysis (Figure
6.14, Panel C, black arrows indicate the margin of the blastoderm). However, when compared with age-matched wild type embryos injected with control mRNA in the YSL (Figure 6.14, Panel B), which by 10hpf have already completed epiboly and had begun forming an embryonic axis, bbp mutant embryos injected with bcl2 have a blastoderm of uneven thickness and are severely delayed in the formation of the embryonic axis.

Careful observation demonstrated that a portion of escapers of the bbp mutant lysis was unable to completely engulf the yolk cell, which resulted in the lysis of the yolk cell at the open blastopore a few hours later. By 24hpf, half of the bbp mutant embryos injected with bcl2 in the YSL escaped lysis but did not develop normally (Figure 6.13). Because of the severe delay, approximately 30% of the embryos injected with bcl2 in the YSL display phenotypes characteristics of a delay in epiboly such as the presence of an open blastopore, bifurcated tail, kinked axis and short yolk extension at 24hpf (Figure 6.13, Panel G, black arrow points to the open blastopore of a bbp mutant embryo). Interestingly, approximately 21% of the bbp mutant embryos injected with bcl2 in the YSL display a phenotype similar to the pwg mutant phenotype (Figure 6.13, Panel G, asterisks indicate pwg-like embryos).

In summary, bcl2 reduces the severity of the yolk morphogenesis defects in bbp mutant embryos. Noteworthy, expression of equivalent levels of bcl2 in the YSL of wild type embryos does not disrupt normal development (Figure 6.13, Panels D, and H). Thus, it is likely that the defects observed at 24hpf in bbp mutant embryos injected with bcl2 are not a result of the overexpression of Bcl2, but a result of its inability to rescue other aspects of the bbp mutant phenotype. Taken together, these results suggest that Nrz/Bcl2l10 has a function that is homologous to Bcl2 in the YSL.
Figure 6.13 - Expression of Bcl2 in the yolk syncytial layer of betty boop mutant embryos and wild type embryos. Percentage of embryos in phenotypical classes scored at 24hpf that result from the microinjection of 1ng of bcl2 in the YSL of bbp mutant embryos and wild type embryos. Graph represents the results of three independent experiments (1ng of control mRNA in the YSL of bbp mutant embryos, n=107; 1ng of bcl2 mRNA in the YSL of bbp mutant embryos, n=107; 1ng of control mRNA in the YSL of wild type embryos, n=110; 1ng of bcl2 mRNA in the YSL of wild type embryos, n=147).
Figure 6.14 - Expression of Bcl2 in the yolk syncytial layer of betty boop mutant embryos partially rescues the betty boop mutant phenotype. Representative phenotypes of bbp mutant embryos injected with control mRNA in the YSL observed at 10hpf (A) and 24hpf (E). Representative phenotypes of bbp mutant embryos injected with 1ng of bcl2 in the YSL observed at 10hpf (C) and 24hpf (G). In C, black arrows point to the margin of the blastoderm. In G, the asterisk indicates bbp mutant embryos injected with bcl2 that display a pwg-like phenotype. Representative phenotypes of wild type embryos injected with control mRNA in the YSL observed at 10hpf (B) and 24hpf (F). Representative phenotypes of bbp mutant embryos injected with 1ng of bcl2 in the YSL observed at 10hpf (D) and 24hpf (H).
6.8 - Nrz/Bcl2l10 controls calcium release during epiboly

Nrz/Bcl2l10 is a member of the Bcl2-family of proteins expressed in the YSL during epiboly and somitogenesis (Arnaud et al., 2006). More recently, Nrz/Bcl2l10 has been shown to have a role in the control of cytoskeletal dynamics through the regulation of calcium dynamics but not through apoptosis-dependent mechanisms (Popgeorgiev et al., 2011). bhp mutant embryos also have a defect in calcium release (Holloway et al., 2009). In Chapter 5, I showed that nrz/bcl2l10 expression in the YSL is reduced in bhp mutant embryos. In this chapter I showed that expression of Nrz/Bcl2l10 and Bcl2 reduced the severity of the yolk morphogenesis defect in bhp mutant embryos. Both Nrz/Bcl2l10 and Bcl2 have been previously shown by others to control the release of calcium from the endoplasmic reticulum through binding of their BH4 domain to the inositol 1,4,5-triphosphate receptors, IP3R (Chen et al., 2004; Rong et al., 2009; Popgeorgiev et al., 2011). I have also found that calcium release evokes aberrant contractility in the YSL. Time-lapse analysis during mid-epiboly, which corresponds to the approximate timing of the onset of the bhp mutant phenotype, revealed that injection of a calcium containing buffered solution into the YSL of wild type embryos elicited a wave of contraction that propagated from the site of injection towards the vegetal pole within a few seconds after injection (Figure 6.15, Panel A). This sudden wave resolved in a ring-like constriction of the yolk near the blastoderm margin approximately three minutes post injection (Figure 6.15, Panel A; n=7). Three different time points during mid-epiboly were assessed (30%, 50% and shield), and they all led to the formation of the ring-like structure close to the margin of the blastoderm. However, during late epiboly stages such as 75% epiboly, whereas the wave of contraction is evident, the ring-like structure forms further from the blastoderm margin than during mid-epiboly (Figure 6.15, Panels B-D).
Taken together, these results suggest that calcium can induce contractility in the yolk cell, and that the elicited response varies throughout the course of epiboly.

Finally, I have performed some experiments to confirm that Nrz/Bcl2l10 does not function in the YSL though an apoptosis-related mechanism. Microinjection of a p53 morpholino into the YSL, a reagent commonly used in zebrafish to prevent activation of apoptosis (Robu et al., 2007) did not rescue the bbp mutant phenotype (data not shown). Similarly microinjection into the YSL of bbp mutant embryos of in vitro transcribed mRNA of mdm2, a negative regulator of p53 (Montes de Oca Luna et al., 1995), did not rescue the bbp mutant phenotype (data not shown). Taken together, our work and work by others suggest that loss of Nrz/Bcl2l10 function in bbp mutants leads to the uncontrolled release of calcium in the YSL and contributes to the bbp mutant lysis.

Figure 6.15 - Microinjection of a buffered solution containing CaCl$_2$ into the yolk syncytial layer evokes contractility in wild type embryos. Montage of a time-lapse movie depicting the first 90 seconds after the injection a buffered solution containing 21 mM CaCl$_2$ (A). The black asterisk depicts the site of the injection. Still images extracted from time-lapse depicting the formation of a ring-like constriction. Images were extracted at three minutes post injection of a buffered solution containing 21 mM CaCl$_2$ into the YSL of wild type embryos at 30% epiboly (B), 50% epiboly (C), shield stage (D), and 75% epiboly (E).
RESULTS

7.1 - *mk2a* and *mk2b* are gene duplicates of an ancestral *mk2*

MK2, MK3 and MK5 comprise the MAPKAPK (MK) sub-family of structurally related protein kinases (reviewed by Gaestel et al., 2006; Cargnello and Roux, 2011). *mk2a* and *mk2b* are annotated in the zebrafish genome as gene duplicates. To further examine the relationship between zebrafish Mk2a, zebrafish Mk2b and the members of the MK sub-family of proteins in zebrafish and in other vertebrates, I constructed a phylogenetic tree (Figure 7.1) using the sequences of zebrafish Mk2a and Mk2b as well as the sequences for MK2s, MK3s and MK5s of vertebrates species, including mouse (*Mus musculus*, *Mm*), human (*Homo sapiens*, *Hs*), chicken (*Gallus gallus*, *Gg*) and Xenopus (both *Xenopus laevis*, *Xl* and *Xenopus tropicalis*, *Xt* where sequences were available). I used Drosophila Mk2 amino acid sequence as an outlier.

The phylogenetic tree (Figure 7.1) shows that zebrafish Mk2a and Mk2b group with vertebrate MK2 orthologs. However, zebrafish Mk2b forms a separate branch from the cluster that groups zebrafish Mk2a, human MK2, murine MK2 and Xenopus Mk2. Also, the MK3s and MK5s orthologs of different vertebrates species cluster together. These results show that zebrafish Mk2a and Mk2b share an ancestral Mk2, and that zebrafish Mk2b is more closely related to MK2s than to MK3 and MK5 orthologs (Figure 7.1). If *mk2a* and *mk2b* were gene duplicates that originated from an additional whole genome duplication
event in teleost fishes, I would expect *mk2a* and *mk2b* to be located in different chromosomes. Indeed, *mk2a* is located in the zebrafish chromosome 11 (Dr11) while *mk2b* is located in the zebrafish chromosome 8 (Dr8). Also, I would expect to find conservation of the spatial order of genes within the chromosome in the vicinity of *mk2a* and *mk2b* when comparing the zebrafish chromosomes 8 and 11. To investigate this aspect, I performed a syntenic analysis using Synteny Database (available at [http://syntenydb.uoregon.edu/synteny_db](http://syntenydb.uoregon.edu/synteny_db), Catchen et al., 2009). Using this tool, I searched for paralogous gene pairs (*i.e.* genes within a genome that derive from a duplication event) and orthologous gene pairs (*i.e.* genes in different species that derive from an ancestral gene after a speciation event) of *mk2a*. I analyzed windows of 50 and 100 genes that have been previously shown to have the highest probability of producing regions of chromosomal synteny due to gene duplication rather than chance alone. The results are presented in Figure 7.2. I found three additional pairs of paralogous genes to *mk2a/mk2b* within a syntenic cluster between Dr11 and Dr8, including *fmoda/fmodb*, *csf1a/csf1b* and *rbm39a/rbm39b*. Also, I found 30 additional pairs of orthologous genes in addition to *mk2a/MK2* within the syntenic cluster between the Dr11 and the mouse chromosome 1 (Mm1). In addition to ortholog pair *mk2a/MK2* and paralog pair *mk2a/mk2b* found in the chromosomal regions of Dr8, Dr11 and Mm1 examined, I found the ortholog pair *fmoda/Fmod* and paralog pair *fmoda/fmodb*. The regions of conserved synteny between Dr8, Dr11, and Mm1, together with the phylogenetic analysis of proteins of the MK family in vertebrates suggest that *mk2a* and *mk2b* are in fact gene duplicates.
Figure 7.1 - Phylogenetic analysis of members of the structurally related MAPKAP family of proteins in vertebrates. Phylogenetic tree was constructed using the protein sequences of annotated vertebrate homologs of MAPKAPK2, MAPKAPK3, and MAPKAPK5; as well as the annotated duplicates Mapkapk2a and Mapkapk2b. The Drosophila melanogaster Mk2 (Dm Mapkapk2) was used as an outgroup. Accession numbers of the protein sequences used in the construction of this phylogenetic tree are listed in Appendix B. Vertebrate species include Mus musculus, Mm; human, Homo sapiens, Hs; chicken, Gallus gallus, Gg; Xenopus including Xenopus laevis, Xl and Xenopus tropicalis, Xt; zebrafish, Danio rerio, Dr; and Drosophila, Drosophila melanogaster, Dm.
Figure 7.2 - Synteny analysis of zebrafish and mouse chromosomes containing *mk2a*, *mk2b* and *Mk2*. Schematic representation of the regions of synteny in the vicinity of *mk2a* in the zebrafish chromosome 11 (Dr11), of *mk2b* in the zebrafish chromosome 8 (Dr8) and of *Mk2* in the mouse chromosome1 (Mm1) obtained using Synteny Database ([http://syntenydb.uoregon.edu/synteny_db](http://syntenydb.uoregon.edu/synteny_db)). Genes in each chromosome are represented by colored boxed and gene names are indicated. Open boxes represent the number of genes on the chromosome between genes of orthologous and paralogous pairs. Gene size is not drawn to scale. Black lines connect orthologous pairs in the syntenic regions between Mm1 and Dr11. Green lines connect paralogous pairs in the syntenic regions between Dr11 and Dr8. *Mk2/mk2a* and *mk2a/mk2b* are connected with red lines. Similarly to *Mk2/mk2a* and *mk2a/mk2b*, *Fmod/fmoda* and *fmoda/fmodb* are orthologous and paralogous pairs, respectively, that are found within the syntenic regions of the three chromosomes (also connected with red lines).
7.2 - mk2a and mk2b have non-overlapping expression patterns during embryogenesis

The analysis of the relationship between members of the MK sub-family and the analysis of synten in the chromosomal regions in the vicinity of mk2a, mk2b and Mk2 suggest that mk2a and mk2b are gene duplicates of an ancestral mk2. The gene expression pattern of mk2b is currently unknown, thus I wanted to know whether mk2b is expressed during embryogenesis. Also, I wanted to know how the expression pattern of mk2b compares with the one of mk2a. Stage-specific reverse transcription polymerase chain reaction (RT-PCR) was used to analyze the expression patterns of mk2a and mk2b in whole wild type zebrafish embryos and larvae (Figure 7.3). mk2a is strongly expressed in the ovary and oocytes of female fish, consistent with the identification of this gene as maternal factor required for early development in the zebrafish. mk2a is also expressed throughout embryogenesis, from sphere stage to 5dpf. In contrast, mk2b expression was detected starting at 3dpf and onwards. Taken together these results show that mk2a and mk2b have non-overlapping expression patterns in developmental stages earlier than 3dpf, raising the possibility of the sub-functionalization of mk2a and mk2b, or the neo-functionalization of either mk2a or mk2b, with respect to the ancestral mk2

7.3 - Several conserved amino acid residues in Mk2b are divergent from Mk2a and tetrapod homologs of MK2

The non-overlapping expression patterns of mk2a and mk2b in early developmental stages raise the possibility that the function of the ancestral mk2 has been retained but partitioned between mk2a and mk2b (sub-functionalization). Another possibility is that either mk2a or mk2b have acquired a novel function with respect to the ancestral mk2. Because the
function of \( mk2b \) in zebrafish development has not been previously described, I first examined the amino acid sequence of Mk2b to identify the conservation or divergence of amino acid residues and structural features with respect to zebrafish Mk2a and other vertebrate homologs. I performed a multiple alignment using the protein sequences of MK2s in different species of the tetrapod lineage including mouse MK2, \textit{Xenopus laevis} Mk2, \textit{Xenopus tropicalis} Mk2, and human MK2. Figure 7.4 shows a matrix summarizing the percentage identity (i.e. the percentage of amino acid residues that are identical between two proteins) between the full-length, as well as between specific regions of tetrapod MK2s. Whereas the N-terminal proline rich domain is highly variable even between more closely related sequences such as the mouse and human MK2, the catalytic and the C-terminal regulatory domains are highly conserved.

![Figure 7.3 - Temporal expression pattern of \( mk2a \) and \( mk2b \). Stage-specific RT-PCR showing the expression pattern of \( mk2a \) and \( mk2b \) in the ovary and oocytes of female fish and in embryos and larvae at the following stages of development: sphere stage, 30\% epiboly, shield, bud, 10-somites, 20-somites, 1, 2, 3, 4 and 5dpf. Ornithine decarboxylase 1 (\textit{odc1}) was used as a loading control.](image-url)
Figure 7.4 - Analysis of sequence identity between MK2 homologs in tetrapods. Protein sequences of human (*Homo sapiens, Hs*), mouse (*Mus musculus, Mm*), and Xenopus (*Xenopus tropicalis, Xt*, and *Xenopus laevis, Xl*) MK2 homologs were aligned. Alignments were performed using the full-length protein, the N-terminal proline-rich domain, the kinase catalytic domain and the C-terminal regulatory domain.

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I extracted the consensus sequence from the alignment of tetrapod MK2 homologs that identifies residues that are highly conserved or highly variable across tetrapod species. I aligned the vertebrate consensus sequence to the sequences of zebrafish Mk2a and Mk2b. Figure 7.5 shows a matrix summarizing the percentage identity of the catalytic and the C-
terminal regulatory domains between the tetrapod consensus MK2, the zebrafish Mk2a and Mk2b protein sequences. The matrix shows that these domains are more conserved, with respect to the tetrapod MK2 consensus sequence, in Mk2a that in Mk2b.

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Figure 7.5 - Analysis of sequence identity between the consensus sequence of the tetrapod MK2 homologs and zebrafish Mk2a and Mk2b. The sequences of human (Homo sapiens, Hs), mouse (Mus musculus), and Xenopus (Xenopus tropicalis, Xt, and Xenopus laevis, Xl) MK2 homologs were aligned. Alignments were performed using the kinase catalytic domain and the C-terminal regulatory domain.

Further analysis of the catalytic domain, identified a total of thirteen amino acid residues in Mk2b that have diverged from highly conserved residues in both the tetrapod MK2 consensus and in Mk2a sequences. Some of these divergent residues are depicted in Figure 7.6.

Among the thirteen divergent amino acid residues, residue 255 is a change from a conserved alanine to a proline in Mk2b, introducing a proline into an α-helix in the vicinity of one of the p38 MAPK phosphorylation sites (Underwood et al., 2003, Figure 7.6). We also identified a total of 6 amino acid residues in Mk2b that have diverged from highly conserved residues in the C-terminal region of the tetrapod consensus MK2 sequence and of Mk2a.
Figure 7.6 - Sequence alignment between the consensus sequence of the tetrapod MK2 homologs and zebrafish Mk2a and Mk2b. The amino acid sequences for consensus sequence of the tetrapod MK2, zebrafish Mk2a and Mk2b were aligned. Tetrapod consensus sequence was obtained from the alignment of *Xenopus laevis* Mk2, *Xenopus tropicalis* Mk2, mouse MK2 and human MK2. X in the consensus sequence of tetrapod MK2 stands for a variable amino acid. Amino acid alignments show regions in the vicinity of residue divergent in Mk2b. Red box underscores the Mk2b residue and its corresponding position. Ellipsis indicates omission of amino acids in between regions of interest. Asterisks indicate amino acids phosphorylated by p38 MAPK.

Q320 is a divergent amino acid residue in Mk2b that has changed from a conserved proline adjacent to conserved p38 MAPK phosphorylation site to a glutamine (Ben-Levy et al., 1995). p38 MAPK is a proline-directed kinase, and thus the proline residue is required at the position +1 from the threonine of its downstream targets (Roux and Blenis, 2004). Q325, Q328 and Q331 are three residues that are highly conserved across tetrapod species but are divergent in Mk2b. These residues are located in the auto-inhibitory helix domain of MK2 (Zu, Ai, and Huang, 1995; Underwood et al., 2003). Residues 325, 328, and 331 have changed from conserved positively charged arginine and lysine residues (Figure 7.6) to neutral glutamine. The auto-inhibitory helix is thought to interact with the catalytic domain to promote an auto-inhibitory conformation that is released upon the phosphorylation of a threonine residue at the hinge between the catalytic domain and the C-terminal regulatory
A359 is another residue that is highly conserved across tetrapod species but that is divergent in Mk2b. Residue 359 is a change from a conserved arginine or lysine in the K/R-K/R-(X)_{10}-K/R-K/R-K/R bipartite nuclear localization signal that overlaps with the p38 MAPK binding site (Meng et al., 2002).

Y343 is the sixth divergent amino acid residue in the C-terminal regulatory domain of Mk2b. Residue 343 is a change from a conserved serine to a tyrosine in the nuclear export signal (Engel, Kotlyarox and Gaestel, 1998).

This analysis allowed us to identify at least 7 divergent residues in Mk2b that are likely to disrupt structural features previously shown as required for MK2 signaling transduction and that are conserved in Mk2a.

7.4 - The expression of Mk2b causes a delay in epiboly

The identification of several amino acid residues in Mk2b that were divergent from the conserved residues in human MK2, mouse MK2, Xenopus Mk2 and zebrafish Mk2a prompted us to examine the function of mk2b using zebrafish epiboly as a platform to compare the function of mk2b to the function of mk2a. A concentration series of in vitro transcribed mk2b mRNA was injected into wild type embryos at one-cell stage. Overexpression of mk2b in wild type embryos at doses of 500 pg/embryo and higher caused a noticeable delay in the initiation and progression of epiboly (Figure 7.7).

Time-lapse analysis revealed that wild type embryos injected with 1ng of mk2b initiate epiboly later than wild type embryos injected with control mRNA (Figure 7.8, Panel A; red arrowheads indicate the dome of the yolk). The blastoderm of wild type embryos
injected with \textit{mk2b} is thicker than the blastoderm of embryos injected with control mRNA (Figure 7.8, Panel B). When compared with wild type embryos injected with control mRNA, embryos injected with \textit{mk2b} show a lag in the epiboly of the DEL with respect to that of the EVL (Figure 7.8, Panels B and C; red arrows are parallel to the advancing edge of the blastoderm margin of the EVL, black arrows are parallel to the advancing edge of the DEL). PM-\textit{mk2b}-injected embryos complete epiboly on average 3.25h later than control mRNA-injected embryos. Thus, I can conclude that overexpression of \textit{mk2b} in wild type embryos disrupts epiboly.

\textbf{Figure 7.7 - Distribution of phenotypes caused by the expression of Mk2b in wild type embryos.} Percentage of embryos in phenotypic classes that result from the microinjection of \textit{mk2b} mRNA at one-cell stage in wild type embryos. Embryos were scored approximately at 10hpf when age-matched embryos injected with control mRNA had completed epiboly. Graph represents the results of two independent experiments (Control mRNA at one-cell stage in wild type embryos, \textit{n}=72; 100 pg of PM-\textit{mk2b} mRNA at one-cell stage in wild type embryos, \textit{n}=71; 500 pg of PM-\textit{mk2b} mRNA at one-cell stage in wild type embryos, \textit{n}=141; 1 ng of PM-\textit{mk2b} mRNA at one-cell stage in wild type embryos, \textit{n}=122).
DISCUSSION

7.5 - Divergence in amino acid residues in Mk2b from highly conserved residues in Mk2a and tetrapod homologs of MK2

As described earlier, we identified at least 7 divergent residues in Mk2b that could disrupt key conserved structural and functional features of MK2 (Figure 7.6). Nevertheless, it is difficult to predict how these divergent residues will act in a concerted manner. Moreover, it is difficult to predict how changes in individual amino acids can compensate for gain or loss of the conserved signaling activity of MK2 and Mk2a. For example, if p38 MAPK is unable to phosphorylate Mk2b, which is thus retained in the nucleus, could the disruption in the nuclear localization signal act as a compensatory mechanism to maintain it in the cytoplasm? What level of activity does Mk2b have compared to Mk2a when the...
phosphorylation of two out of three of the main phosphorylation sites may be disrupted?
These are just a few of the fascinating questions that the study of structural and biochemical aspects of Mk2a, Mk2b and Bbp may help answer.

To address these questions, future work could first focus on the structural determination of Mk2a and Mk2b. There are crystal structures available for MK2 in complex with p38 MAPK (ter Haar et al., 2007), in complex with a low molecular weight complex inhibitor (Hilig et al., 2007), in complex with staurosporine and ADP (Underwood et al., 2003) and in the unphosphorylated state (Meng et al., 2002). Solving the crystal structures of Mk2a and Mk2b and comparing them to the available crystal structures of MK2 could help us understand how the divergent residues affect the conformation of Mk2b, particularly the interaction of the auto-inhibitory domain helix with the catalytic domain, which is thought to control MK2 ability to phosphorylate downstream targets (Zu, Ai and Huang, 1995; Undergwood et al., 2003). Further understanding of the relationship between the amino acid sequences, the structure and the function of Mk2a, Mk2b and MK2 would provide with valuable information for the design of new inhibitors of MK2.

Another area of future work is the study of the sub-cellular localization of Mk2b. Nuclear targets of MK2 include SRF (Heidenreich et al., 1999), E47 (Neuefeld et al., 2000) and ER81 (Janknecht, 2001). Cytoplasmic targets include HSP27 (Stokoe et al., 1992), LSP1 (Huang et al., 1997), 5-lipoxygenase (Werz et al., 2000) and tyrosine hydroxylase (Thomas et al., 1997). The sub-cellular localization of Mk2b could determine the subset of targets, nuclear or cytoplasmic, that are accessible to Mk2b. Identifying the repertoire of MK2, Mk2a and MK2b in different cellular contexts, sub-cellular compartment and even cell types would contribute to the understanding of events downstream of MK2. This can also impact the
design of anti-inflammatory therapies directed to inhibit the interaction with particular downstream targets, or the inhibition of particular downstream targets, to create therapies more specific to the diverse array of inflammatory conditions.

7.6 - Assessing the sub-functionalization and neo-functionalization of *mk2a* and *mk2b*

Wild type embryos injected with a constitutively active form of *mk2a* (PM-*mk2a*) and wild type embryos injected with *mk2b* mRNA have phenotypes of delay in the initiation and the progression of epiboly (Figure 7.9). The DEL and the EVL of wild type embryos injected with PM-*mk2a* seem to be equally affected by the delay in epiboly. In contrast, the EVL of wild type embryos injected with *mk2b* seems to be less affected by the delay in epiboly than the DEL. The difference in the morphological phenotypes as a result of the microinjection of *mk2a* and *mk2b* during epiboly foreshadows a divergence in the function of these two kinases. However, this hypothesis needs to be further tested at the molecular and biochemical level.

Our recent work on the role of Mk2a in the regulation of mRNA stability via Ttp can provide a useful assay to examine the divergence in the function of Mk2b. This could be achieved, for instance, by testing the ability of Mk2a to recover the expression of mRNA transcripts in *bbp*. However, this genetic analysis should be complemented with the quantification of the enzymatic activity of Mk2a, Mk2b and MK2. In addition to this, the investigation of the interactions of Mk2a, Mk2b and MK2 with known downstream targets or with scaffolding proteins could be informative of changes in the specificity of the signal transduction event. Also, comparison of the endogenous sub-cellular localization, or the sub-cellular localization in tissue culture could help us identify changes in the
compartmentalization and the dynamics of the interaction of Mk2a, Mk2b and MK2 with p38 MAPK, as well as with context-specific downstream targets. The results of these experiments will help us answer the question of the functional divergence of Mk2a, Mk2b, and the mammalian MK2.

**Figure 7.9 - Expression of constitutively active Mk2a and of Mk2b in wild type embryos disrupt epiboly.** Selected frames of a time-lapse analysis of age-matched wild type embryos injected with 1ng of PM-mk2a mRNA and control mRNA (A, C and E); and with 1ng of mk2b (B, D, and F). Expression of both Mk2a and Mk2b cause a delay in epiboly. However, expression of Mk2b also causes a lag in the progression of the DEL with respect to the EVL.

One remaining question is what are the roles of Mk2a and Mk2b during larval stages. The bulk of our work has focused on the requirement of Mk2a during early development, however we have not examined the requirement of Mk2a in other aspects of larval, or even adult fish physiology. We have shown that Mk2b expression starts at 3dpf (Figure 7.3). Thus, it is necessary to examine the function of Mk2a and Mk2b later in development to determine whether mk2a and mk2b are duplicates that together complement the ancestral function (i.e. sub-functionalization).
8.1 – Conclusions

8.1.1 – Co-option of the p38 MAPK/MAPKAPK2 signaling pathway to generate morphological novelty

As eloquently highlighted by Francois Jacob (1977) in the lecture “Evolution and Tinkering”, one fascinating aspect of morphological diversity is the underlying chemical and molecular unity across different phyla. Further understanding of molecular principles of development in different model organisms supports the hypothesis that the use of highly conserved developmental modules to regulate divergent regulatory networks leads to morphological novelty. Pires da Silva and Sommer (2012) enumerate at least seven signaling pathways that are highly conserved in vertebrate and invertebrate development, thus regulating a diversity of animal forms.

As I have discussed across the chapters of this thesis, our findings fit a model in which a conserved p38a/Mk2a signaling pathway is activated only in the YSL (refer to Chapter 4). p38a/Mk2a activation in the YSL leads to the inhibition of Ttp, and is likely a mechanism to achieve cell layer-specific mRNA expression (refer to Chapter 5). We have shown that expression of Mxtx2 outside of the YSL is toxic for development, thus that mxtx2 must be spatially restricted to the YSL (refer to Chapter 6). These results show that the conserved p38a/Mk2a-dependent regulation of Ttp is a mechanism for YSL-specific expression of mxtx2. Interestingly, Mxtx2 is a transcription factor has been shown to regulate a large portion of YSL-specific genes (Xu et al., 2012). Thus, Mxtx2 likely activates a
regulatory network that contributes to the morphogenesis of a novel embryonic structure such as the YSL of teleost fishes (Figure 8.1).

8.1.2 - Epistatic relationships among Ttp, mxtx1, mxtx2, slc26a1 and nrz/bcl2l10: preliminary model

I have shown that expression of the RNA-binding domain of Ttp can recover the YSL-specific expression of mxtx2 and mxtx1 reduced in bbp mutant embryos, but not the expression of slc26a1 or nrz/bcl2l10. I have also shown that expression of Mxtx2 in the YSL of bbp mutant embryos can recover the expression of mxtx1, slc26a1, and nrz/bcl2l10 mRNA in the YSL. Similarly, microinjection of mxtx2 MO in wild type embryos causes a reduction in the expression of mxtx1, slc26a1, and nrz/bcl2l10 mRNA. Taken together, these results contribute to the formulation of a model in which Ttp directly controls the stability of at least mxtx2 and mxtx1 (Figure 8.1).

Mxtx2 is a teleost specific transcription factor that is expressed exclusively in the YSL and that is required for epiboly (Bruce et al., 2005; Wilkins et al., 2008). Mxtx2 likely activates a large portion of YSL specific genes at a transcriptional level (Xu et al., 2012). Our experimental data suggest that Mxtx2 in its turn activates mxtx1, slc26a1, and nrz/bcl2l10 at a transcriptional level (Figure 8.1). Using chromatin immuno-precipitation sequencing analysis (ChIP-Seq), Xu and coworkers (2012) showed that Mxtx2 directly binds slc26a1 and mxtx1. Our experimental results concur with these findings. However, the results of their ChIP-Seq analysis did not show nrz/bcl2l10 as target of Mxtx2 binding (Xu et al., 2012). This could be explained by the Mxtx2-dependent regulation of a transcriptional activator of nrz/bcl2l10.
My current model does not exclude the possibility that \textit{mxtx1}, \textit{slc26a1}, and \textit{nrz/bcl2l10} mRNAs are also subject to post-transcriptional regulation by Ttp in addition to the transcriptional regulation by Mxtx2. \textit{In silico} prediction of AREs using the bioinformatics tool AREScore (Spasic et al., 2012) show that the 3’ UTR of \textit{mxtx1}, \textit{mxtx2}, \textit{nrz/bcl2l10}, and \textit{slc26a1} likely contain these motifs in their 3’UTR (Table 5.1). In addition to the AREScore-based \textit{in silico} prediction, I further analyzed the 3’ UTR region of these genes and found that \textit{mxtx2} and \textit{nrz/bcl2l10} have the minimal nonamer found in type II AREs that are bound by TTP. \textit{mxtx1}, \textit{mxtx2}, and \textit{nrz/bcl2l10} also have variations of this nonamer that have been shown to have high and intermediate binding affinity for TTP in an \textit{in vitro} assay (Table 5.1; Brewer et al., 2004). Out of the four mRNA molecules that I assayed using whole mount \textit{in situ} hybridization, \textit{slc26a1} is the less likely TTP target according to \textit{in silico} predictions and 3’UTR sequence analysis (Table 5.1). Thus, reduction in the levels of mRNA of \textit{slc26a1} in \textit{bbp} mutant embryos can be explained by loss of function of its transcriptional activator Mxtx2. To sum up, I propose that \textit{mxtx1} and \textit{nrz/bcl2l10} are subject of both post-transcriptional regulation by Ttp and transcriptional regulation by Mxtx2; and that \textit{slc26a1} is only transcriptionally regulated by Mxtx2 (Figure 8.1). To further strengthen this model it will be necessary to test the direct interaction of Ttp with \textit{mxtx1}, \textit{mxtx2}, \textit{slc26a1} and \textit{nrz/bcl2l10} mRNA using immunoprecipitation of Ttp, RNA-extraction from the precipitate, and RT-PCR analysis (Emmons et al., 2008).
Figure 8.1 – Model of the role of p38a/Mk2a-dependent regulation of Ttp in YSL morphogenesis. YSL-specific activation of p38a/Mk2a signaling inhibits Ttp activity. Inhibition of Ttp-dependent mRNA decay in the YSL allows expression of mRNA of YSL-specific genes in the YSL, while reducing the levels of mRNA of YSL-specific genes outside of the YSL. Mxtx2 is a transcription factor unique to zebrafish that is expressed in the YSL and that activates a regulatory network that controls YSL morphogenesis. Black arrows and bars represent relationships established using experimental evidence. Red bars represent relationships established using *in silico* prediction.
8.2 - New perspectives in the investigation of p38 MAPK/MK2/TTP-dependent mRNA degradation

8.2.1 - The yolk syncytial layer is a novel cell type to study TTP-dependent mRNA degradation

A recent review by Brooks and Blackshear (2013) compiled a list of the mRNA molecules that have been reported in literature as targets of TTP degradation. Most of these studies were performed in mouse macrophages and dendritic cells, or in cancer cells. The study of the role of zebrafish TTP in the yolk syncytial layer (YSL) provides a novel cell type to investigate the function of TTP. There are several reasons that make the YSL an interesting model to investigate the molecular mechanism of TTP-dependent mRNA degradation:

i. The YSL is a syncytial cell that is transcriptionally active. It expresses both zebrafish specific genes and conserved mammalian genes. Also, I have shown with experimental evidence and in silico prediction that the YSL is a cell type populated with mRNA molecules that are likely TTP targets.

ii. Some YSL-specific mRNA molecules are present during specific time window. For example, mxtx2 is detected only during mid-epiboly (Hirata et al., 2000). YSL-specific mRNA molecules with tightly regulated temporal expression patterns during development may be useful as reporters of the manipulation of TTP-dependent degradation.

iii. The YSL has sub-cellular structures such as an the endoplasmic reticulum and mitochondria (Popgeorgiev et al., 2011), as well as dynamic microtubule arrays
(Solnica-Krezel and Driever, 1994), polymerized actin-based structures (Cheng et al, 2004), and a non-muscle contractile apparatus (Koppen et al., 2006). These structures can be used as an output to measure cellular responses to the manipulation of TTP-dependent mRNA degradation.

iv. The YSL is part of a whole developing organism, and the regulation of the structure and function of the YSL is required for embryogenesis. Thus, the YSL might be an *in vivo* model system that is more complex than tissue cell culture.

v. *In vitro* transcribed mRNA can be microinjected exclusively in the YSL. We have shown that this technique is a rapid assay to test the results of the manipulation of protein activities involved in the regulation of mRNA decay.

I have used a developmental genetics approach to investigate the p38a/Mk2a/Ttp-dependent mRNA degradation in the YSL, and our work shows that zebrafish epiboly has great potential as a platform to investigate this process. To achieve this potential my work should be further complemented with well-established biochemical techniques that identify and quantify the direct binding of TTP to mRNA molecules that encode YSL-specific genes, as well as techniques that quantify the levels of their adenylation. In addition to this, the development of robust assays of calcium dynamics, ER stress, and cytoskeleton dynamics in the YSL can contribute to a further understanding of the role of mRNA degradation in particular cellular processes.
8.2.2 - Complementing the *in silico* approach for the discovery of new and physiologically relevant TTP targets.

There is a large gap to bridge between *in silico* and *in vivo* approaches to identify targets of TTP-dependent mRNA degradation. For example, Yang at coworkers (2003) measured the mRNA decay rates of 5245 human genes in human hepatocarcinoma cells. They found that the median half-life of the mRNA molecules measured was of ten hours, and they identified fast decaying mRNA molecules as those with a half-life of less than two hours. However, only 10-15% of the genes containing AREs are fast decaying mRNA molecules. This highlights the fact that the presence of AREs is not an absolute criterion to identify TTP targets.

TTP has been shown to interact with diverse proteins of the mRNA degradation machinery (Figure 2.6). The rate of mRNA degradation is likely to be controlled not only by TTP binding alone; but also by the binding of TTP to other proteins that may or may not also bind mRNA. Thus, the regulatory mechanisms of these complex protein:protein and protein:mRNA interaction may partially explain why not all type II ARE-containing mRNA molecules are subject to TTP-dependent degradation.

As shown by my work, zebrafish epiboly can be used as model system that is amenable to the genetic manipulation of protein activities. I can successfully manipulate the function of proteins by microinjection of *in vitro* transcribed mRNA encoding their dominant negative and constitutively active forms, as well as by the microinjection of morpholino antisense oligonucleotides. Thus, using zebrafish epiboly we may be able to regulate the activity of TTP binding partners and other RNA interacting proteins to dissect their contribution to TTP-dependent mRNA regulation. Further understanding and dissection
of these complex protein:protein and protein:mRNA interactions may contribute to the
design and the improvement of new tools for the in silico prediction of TTP targets.

8.3 - Study of TTP-dependent mRNA degradation in inflammation, and
immunity of zebrafish

Mouse models have shown that MK2 deficiency has a protective role in inflammatory
pathologies. In contrast to the hypoinflammatory phenotype of MK2 deficient mice, TTP-
deficient mice develop a hyperinflammatory syndrome. These results show that MK2 and
TTP have a prominent role in the initiation and the resolution of the inflammatory response.
Whereas the bulk of this work is focused on the requirement of p38a/Mk2a signaling to
regulate TTP-dependent mRNA degradation during early development, zebrafish can also be
used to study inflammation and immunity. Zebrafish has several advantages as a model for
inflammation and immunity (reviewed by Novoa and Figueras, 2012). Zebrafish rely on
innate immunity for the first three weeks after fertilization, before maturation of adaptive
immunity. Thus, the zebrafish model provides the opportunity to separate the contributions of
the adaptive and innate responses. In addition to this, several in vivo tools for the
examination of the inflammatory and immune responses have been developed (reviewed by
Tobin and coworkers, 2012).

MK2-deficient mice do not show their hypoinflammatory phenotype unless exposed
to bacterial, inflammatory, or carcinogenic insults. Thus, it is necessary to develop assays
that test the response of bbp mutant larvae and adult fish to these challenges to assess the role
of Mk2a zebrafish in inflammation. Our findings that expression of Mxtx2 rescues the bbp
mutant phenotype will allow homozygous mutants to survive past embryogenesis, and to be
used as an Mk2a-deficient model to assess potential inflammatory phenotypes in zebrafish larvae and adult fish. The conservation of Mk2a and Ttp in the inflammatory response in zebrafish could greatly contribute to the study of inflammatory pathologies because zebrafish is a whole organism-based model that is amenable to large-scale chemical screens.

I have also shown that mk2a and mk2b are gene duplicates. mk2a is expressed maternally and throughout embryogenesis, and mk2b expression starts to be detected at day 3 post fertilization, around the time that immune cells first differentiate in the embryo. One potential implication of this finding is that mk2a and mk2b may have redundant functions later in development, and most notably in the context of inflammation and immunity (Figure 8.2)

![Diagram](image)

**Figure 8.2 – Conservation of p38 MAPK/MK2/TTP signaling axis in diverse cellular contexts.** In mammals, TTP-dependent mRNA degradation is essential for the activation of the inflammatory response via the regulation of the mRNA levels of Tnf-α and other proinflammatory cytokines. In zebrafish epiboly, TTP-dependent mRNA degradation is essential for the regulation of YSL morphogenesis via the regulation of the mRNA levels of mxtx2 and other YSL-specific genes. One remaining question is whether or not the gene duplicates mk2a and mk2b are involved in the regulation of Ttp-dependent mRNA degradation of the zebrafish homologs of Tnf-α and other proinflammatory cytokines to regulate the inflammatory respose in zebrafish.
References


preventing chromosome fusion during nuclear division in yolk syncytial layer. J. Biol. Chem. 286, 9514–9525.


## SITE DIRECTED MUTAGENESIS

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Appendix A

Primers
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Appendix B

Protein sequences used in the construction of phylogenetic trees

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