Chapter Seven

Regulation of COPI Expression

7 Regulation of COPI Expression

In this chapter I will examine some of the questions raised by the observations of the previous section. To that end, I examined the conditions under which COPI subunit expression is maintained. Specifically, I examined the role of several components of the unfolded protein response (UPR) in development, in the normal expression of COPI subunits and in their maintained expression in the COPI mutants. A brief review of unfolded protein response and its role in both stress response and in normal secretory network maintenance is provided.

7.1 Introduction

Loss of COPI function in response to treatment with BFA results in a breakdown of secretory organelle structure, a loss of proper secretion and protein folding, which thus leads to an increase in the build up of unfolded protein. To ensure proper folding of intra-organelle, secretory and transmembrane proteins, the ER hosts a set of specialised protein folding components that promote and assist the proper folding and act to prevent the formation of protein aggregates. During normal secretion these components are lost from the ER through forward movement along the secretory pathway, though they are then transported back to their proper localisation though retrograde action by COPI (Letourneur et al., 1994; Sonnichsen et al., 1996). Effects that alter the delicate balance of ER components can lead to disruptions in protein folding and a build up of harmful protein aggregates. Loss of COPI, which leads to a loss of retrograde transport, causes a loss of ER components

and hence leads to a loss of protein folding and a build up of protein aggregates. Increases in the protein folding load, due to elevations in the synthesis of secretory proteins, can lead to the increase in the requirement for protein folding and hence an increase in unfolded proteins and protein aggregates, as can treatment with small molecules, such as tunicamycin, which blocks *N*-glycosylation of newly synthesised protein (Duksin and Bornstein, 1977; Kawahara et al., 1997). The requirement of eukaryotic cells to adapt to such demands and to avoid the detrimental affects of unfolded protein build up and the accumulation of protein aggregates has led to the evolution of an adaptive response to limit the accumulation of unfolded proteins within the ER. Originally described through the study of the SV40T antigen that was targeted to the ER (Kozutsumi et al., 1988), this signalling response has become known as the Unfolded Protein Response (UPR) (for reviews see (Kaufman et al., 2002; Patil and Walter, 2001)).

The initial characterisation of the components of the UPR was performed in the budding yeast *Saccharomyces cerevisiae*, where genetic screens identified three proteins involved in the transduction of signals from the ER to the nucleus. Three proteins, Ire1p, Hac1p and Rlg1p were identified as playing a vital role in the process of UPR signalling. Ire1p is a transmembrane serine/threonine kinase with three functional domains. The amino-terminal domain resides within the ER lumen, where it acts to sense the level of unfolded protein with the ER (Cox et al., 1993). The accumulation of unfolded protein within the ER causes dimerisation of Ire1p, which results in trans-autophosphorylisation by its cytosolic kinase domain (Shamu and Walter, 1996; Welihinda and Kaufman, 1996). This then causes the activation of Ire1p's, carboxy-terminally located, site specific endoribonuclease, the substrate for which is *Hac1* mRNA. *Hac1* encodes a basic leucine zipper (bZIP) transcription

factor that acts to induce the expression of UPR target genes (Kawahara et al., 1997). *Hac1* is constitutively expressed in an un-translated from, such that, although *Hac1* mRNA is continually present, the encoded Hac1p protein is not detected under normal conditions (Cox and Walter, 1996). The *Hac1* un-translated mRNA contains a 252 nucleotide intron located near the 3' end that, when present, acts to block the translation of the mRNA (Chapman and Walter, 1997; Cox and Walter, 1996; Kawahara et al., 1997). Removal of this intron results in an mRNA encoding a 10-fold more effective transcription factor and an mRNA that is more efficiently translated (Mori et al., 2000; Ruegsegger et al., 2001). In response to activation of the UPR, Ire1p acts directly upon *Hac1* mRNA to cleave at two specific sites (Sidrauski and Walter, 1997). The 5' and 3' *Hac1* mRNA fragments are then joined through the action of the tRNA ligase Rlg1p (Sidrauski et al., 1996). The splice-activated form of *Hac1* mRNA is then translated to produce the Hac1p protein, which is translocated to the nucleus, where it activates the expression of UPR genes through direct binding to the upstream Unfolded Protein Response Element (UPRE) (Cox and Walter, 1996; Kawahara et al., 1997; Mori et al., 1996). The UPRE is both necessary and sufficient for up-regulation in response to the UPR (Mori et al., 1998; Mori et al., 1992). Thus, in yeast, the action of Ire1p in splicing the *Hac1* mRNA regulates activation of the UPR (**Figure 7.1**).

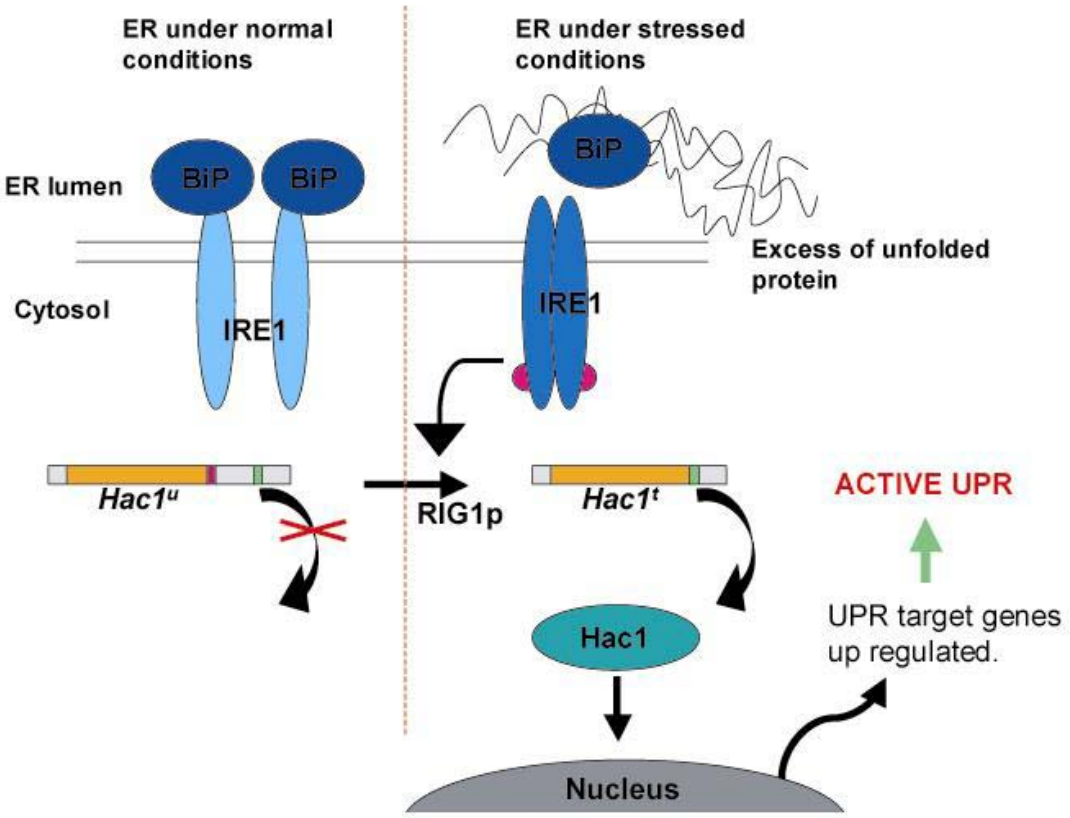

Figure 7.1 UPR activation in yeast.

In yeast, a build up of unfolded protein within the ER prevents inhibitory binding of BiP to IRE1. Under these conditions, IRE1 dimerises and self-phosphorylates, leading to is activation and the removal of the inhibitory intron from *Hac1*. *Hac1* is then spliced, where RIG1p acts to ligate the exons of *Hac1*. This more efficiently translated mRNA is then translated to form the Hac1 protein. This protein then acts to directly up-regulate UPR target genes.

Ire1p and Hac1p act in a linear pathway to active UPR response genes under conditions of ER stress. Ire1p acts to detect the level of unfolded protein within the ER and under conditions of high unfolded protein load, splices *Hac1p* which then up-regulates the UPR response genes. The key factor in Ire1p's ability to sense the level of ER stress is the ER chaperone protein BiP. Bip/GRP78 has long been a marker of UPR activation, since it is rapidly upregulated under conditions of UPR activation (Kaufman, 1999) and acts in the ER to chaperone proteins during proper protein folding (for review see (Ma and Hendershot, 2004)). Under normal conditions, Ire1p exists in a monomeric form through the binding of BiP to its ER lumen exposed surface, but under conditions of ER stress, where there is a build up of unfolded protein, more of the available BiP becomes associated with chaperoning unfolded proteins through binding to the exposed hydrophobic regions (Bertolotti et al., 2000). This reduces the level of BiP available to bind to Ire1p, thus resulting in the oligomerisation of Ire1p. So, the activation of the UPR in yeast is regulated by the ER protein chaperone BiP, which, under normal conditions, acts to prevent oligomerisation and hence activation of Ire1p through direct interaction. However, under conditions of ER stress, where there is a build up of unfolded protein, BiP is engaged in chaperoning the increased unfolded proteins, leaving Ire1p unbound and allowing oligomerisation, trans-autophosphorylation and activation.

The UPR in yeast is a simple linear pathway, signalling increases in unfolded protein load through BiP, Ire1p and Hac1p. However, UPR is more complicated in higher eukaryotic organisms. Though much of the yeast UPR system has been conserved during evolution, there are significant differences in even the conserved components. There are two homologues of yeast IRE1 in mammalian genomes, termed IRE1α and IRE1β (Niwa et al., 1999; Tirasophon et al., 1998; Wang et al.,

1998). Interestingly, Ire1α is expressed in many cells and organs where Ire1β is limited to expression within the epithelial cells of the gut (Urano et al., 2000b). The mechanism of IRE1 action in higher eukaryotes is similar to its action in yeast, oligomerising in conditions of high unfolded protein load due to a reduction in the level of available BiP, and self activating through trans-autophosphorylation. In mammals, the mRNA substrate for Ire1 has been identified as the bZIP transcription factor X-box binding protein 1 (Xbp1) (Calfon et al., 2002; Shen et al., 2001; Yoshida et al., 2001). Activation of Ire1 results in the removal of a 26 nucleotide intron from the *Xbp1* mRNA to generate a potent transcriptional activator similar to *Hac1p* in yeast. However, the loss of both Ire1α and Ire1β does not reduce the transcription activation in response to UPR of several UPR responsive genes (Lee et al., 2002; Urano et al., 2000a; Urano et al., 2000b). However, loss of Ire1α has been reported to result in a transcription defect in mouse fibroblasts, which can be complemented through expression of the spliced *Xbp1* (Lee et al., 2002). Hence, the Ire1 response controls only a subset of UPR genes. In support of this, two other UPR signalling pathways have been characterised, involving the protein ATF-6 (Yoshida et al., 1998) and the protein PERK (Harding et al., 1999; Shi et al., 1998).

ATF-6 is a constitutively translated ER membrane protein that remains inactive during normal conditions. However, under conditions of increased unfolded protein load the cytosolic domain of ATF-6 is cleaved in a step requiring the site 2 protease (S2P) (Ye et al., 2000), from the ER and translocated to the nucleus where it activates the expression of genes including *BiP* and *Xbp1* (Haze et al., 1999; Lee et al., 2002; Yoshida et al., 2000). Increased transcription of *Xbp1* by ATF-6 provides more substrate for activated Ire1, which then results in a positive feedback loop for the UPR. Further to this, ATF-6 offers a more rapid response to increases in unfolded

protein load, since it requires only cleavage and translocation to function in up-regulating UPR genes, where Ire1 action requires not only splicing of *Xbp1*, but also translation. Interestingly, loss of either Ire1α and Ire1β or ATF-6 alone does not result in a loss of *Xbp1* expression in response to ER stress, suggesting that there is some overlap in the role of these two pathways in the UPR (Lee et al., 2002). Activation of ATF-6 cleavage in response to ER stress is also controlled through the action of BiP (Shen et al., 2002). In normal conditions, BiP binds to the ER lumen domain of ATF-6, preventing its transport to the Golgi complex. However, during periods of increased ER stress, when there is an increase in the presence of unfolded protein, available BiP is concerned with chaperoning the unfolding proteins leaving ATF-6 free to pass into the Golgi complex for proteolytic cleavage.

Activation of the UPR results in a notable reduction in the rate of translation. This slow down helps to protect cells from the fatal effects of unfolded protein build up by preventing the continued production of proteins under conditions that are non-conducive to proper protein folding. The identification of PERK as a transmembrane ER localised eIF2α kinase strongly implicated it as a key signalling element in the attenuation of translation in response to unfolded protein stress (Harding et al., 1999; Shi et al., 1998). Characterisation of PERK demonstrated that the ER luminal domain is considerably similar to the luminal domain of Ire1 and that the cystosolic domain shows the features of a Gcn2 kinase (Harding et al., 1999; Shi et al., 1998). Interestingly, the ER luminal domains of both Ire1 and PERK from both humans and *Caenorhabditis elegans* can be substituted for the luminal domain of yeast Ire1p and still illicit a UPR response (Liu et al., 2000), thus indicating that both PERK and Ire1 act through a common mechanism. So, it is likely that activation of the UPR results in oligomerisation and trans-autophosphorylation, which results in activation of the

Gcn2 kinase domain that acts to phosphorylate the translation initiation factor eIF2α. Cells lacking PERK demonstrate greater levels of Ire1 phosphorylation upon activation of the UPR, indicating that PERK acts to prevent the increase in unfolded protein load. The ability of cycloheximide to partially inhibit this increase in UPR activation supports the role of PERK in blocking translation (Harding et al., 2000b). The activation of PERK in response to accumulation of unfolded protein in the ER is, like Ire1 and ATF-6 controlled by BiP (Bertolotti et al., 2000; Liu et al., 2000). Under normal conditions BiP binds to the ER luminal domain of PERK and prevents oligomerisation, with BiP becoming involved in chaperoning unfolded protein in times of ER stress, leaving PERK free to dimerise and trans-autophosphorylate, activating its cytosolic Gcn2 domain in a mechanism much like that or Ire1. However, approximately one third of UPR induced genes require the phosphorylation of eIF2α by PERK (Scheuner et al., 2001), indicating that PERK does not just function in the repression of translation during UPR. The phosphorylation of eIF2α by PERK results in the preferential translation of ATF4, which in turn results in the up-regulation of UPR genes, including *CHOP* (Harding et al., 2000a; Scheuner et al., 2001). Indeed, cells lacking PERK or eIF2α lack almost all *CHOP* expression, though this is not the case in cells lacking both *Ire1* isoforms (for an overview of UPR in vertebrates see **Figure 7.2**).

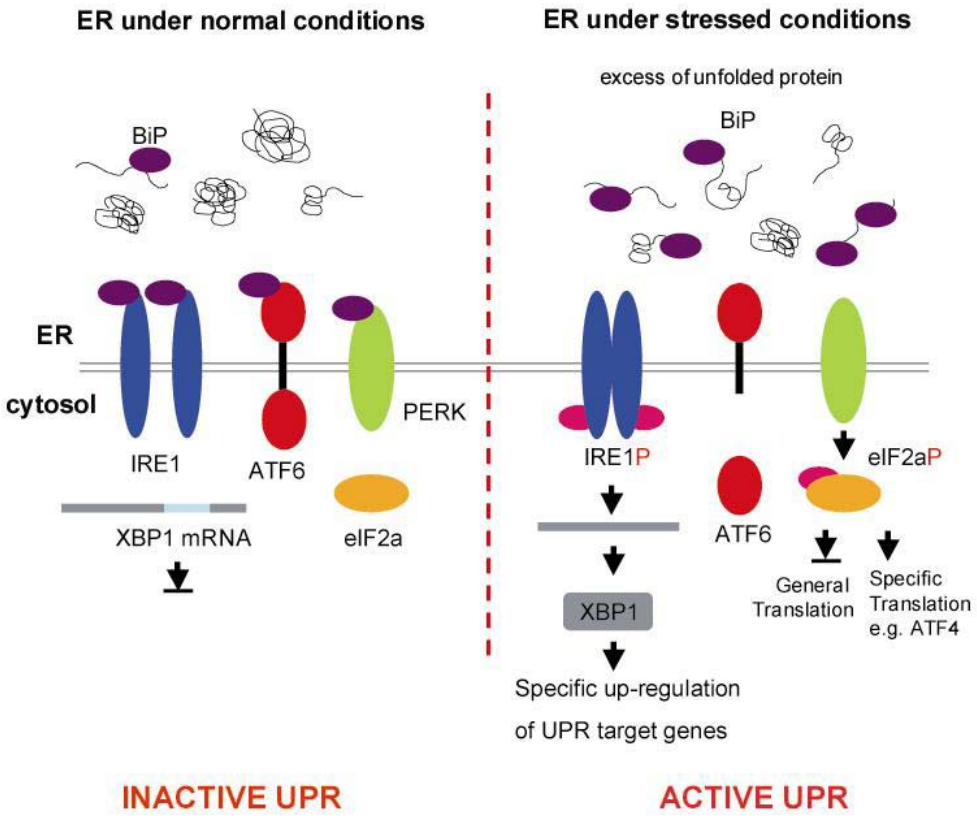

Figure 7.2 Activation of the UPR in higher organisms.

Build up of unfolded protein in higher eukaryotes results in a loss of BiP mediated inhibition of IRE1, ATF-6 and PERK. Under these conditions, IRE1 dimerises, causing self-phosphorylation, which in turn leads to the activatory slicing of *Xbp1* and the efficient translation of functional XBP1 that directly acts in up-regulating UPR target genes. ATF-6 cleaves, releasing the cytosolic tail that then acts to up-regulate UPR target genes, including *Xbp1*. Activation of PERK results in the phosphorylation of eIF2α, which in turn acts to down-regulate translation, relieving the protein load, but also results in the specific translation of certain genes e.g. *ATF4*.

The ultimate fate of cells that are unable to resolve the unfolded protein load after entering the UPR is death through apoptosis. Extended treatment of cells with tunicamycin results in the activation of the ER membrane caspase-12, causing apoptosis (Harding et al., 2000b). This decision is most likely made after the cell has entered cycle arrest, when the UPR has resulted in a sufficient decrease in translation of Cyclin D1 to result in arrest at G1 phase (Brewer and Diehl, 2000). The first cell death promoting factor to be identified was CHOP, a pro-apoptotic transcription factor (Friedman, 1996; Zinszner et al., 1998). The induction of *CHOP* is complicated; over-expression of Ire1 is able to induce its expression, with expression of a dominant negative Ire1 resulting in some loss of expression (Wang et al., 1998). However, a lack of Ire1 does not result in complete loss of *CHOP* expression upon activation of the UPR where loss of PERK results in a much more dramatic loss of *CHOP* (Scheuner et al., 2001). Such evidence suggests that *CHOP* expression may be controlled by PERK activation with Ire1 able to induce expression, possibly though interactions that result in PERK activation. Though CHOP has a key role in inducing the expression of apoptosis genes, it is not required for apoptosis. Cells lacking PERK lack almost all CHOP, but are hypersensitive to ER stress and enter apoptosis more readily than normal cells, thus indicating that other factors are involved in UPR induced apoptosis.

Much information concerning the role of the UPR under normal physiological conditions has been defined through the use of *C. elegans*. The UPR in *C. elegans* involves the same systems as described above, utilising the signalling molecules Ire1, ATF-6 and PERK (Calfon et al., 2002; Shen et al., 2001). Loss of either *ire-1* and *pek-1*, the *C. elegans* homologues of *Ire1* and *PERK* respectively, causes no discernable difference when compared to wild type animals. However,

loss of both *ire-1* and *pek-1* together resulted in developmental arrest and intestinal necrosis at the L2 stage in animals raised under normal type conditions (Shen et al., 2001). This phenotype in the intestinal cells, which demonstrate an increase in the synthesis of secreted protein, suggests that the UPR may function during development to meet the increased stresses placed on cells that require an increase in protein folding and secretion as part of their development. Recent work has demonstrated that the UPR, specifically *ire-1* but not *pek-1* and *atf-6*, are necessary for the proper movement of AMPA-type glutamate receptor subunits through the secretory pathway in neurons (Shim et al., 2004). In animals lacking *ire-1* and raised under normal conditions, the GLR-1 subunit is retained within the ER. In support of this, the same study demonstrated that *Xbp1* is up-regulated in neurons during normal development. Thus suggesting that the UPR plays an essential role during normal development in *C. elegans*.

In this section, the role of the UPR during development of the zebrafish will be examined, specifically, the role of the UPR in relation to coatomer expression and function during development. In this section I will put forward evidence to suggest that the UPR is not simply a stress response, but a general mechanism functioning during normal development to meet the increased rate of secretion and translation in specific tissues as they develop and differentiate. I will also attempt to link the UPR to expression of coatomer, proposing the UPR as a possible mechanism for both the normal expression of coatomer during development as well as the maintained expression of coatomer under conditions of COPI loss of function.

7.2 Expression of COPI in Undifferentiated Notochords

COPα mRNA is maintained in the undifferentiated notochord of *sny* embryos at 28 hpf, as is *ehh*. The early notochord marker *ehh* is also maintained within the undifferentiated notochord of *sly* embryos, which lack the laminin γ1 chain, thus *ehh* is maintained simply due to a lack of proper notochord development rather than the lack of any specific gene function. To examine the possibility that *COPα*, and other COPI subunits, may be maintained due to the specific loss of COPI function in the COPI mutants or due to the failure to fully differentiate a proper notochord, the expression of *COPα* and *ehh* was examined in *sly* embryos, which fail to differentiate notochord but still have a functional COPI complex.

Expression of *ehh* is maintained specifically within the notochord of both *sny* and *sly* embryos, demonstrating that the notochord fails to differentiate properly in both mutants. However, *COPα* expression is maintained only in the notochord of *sny* mutants, indicating that it is the specific loss of COPI function that causes the maintenance of *COPα* expression in COPI mutants beyond the normal temporal limitations (**Figure 7.3**).

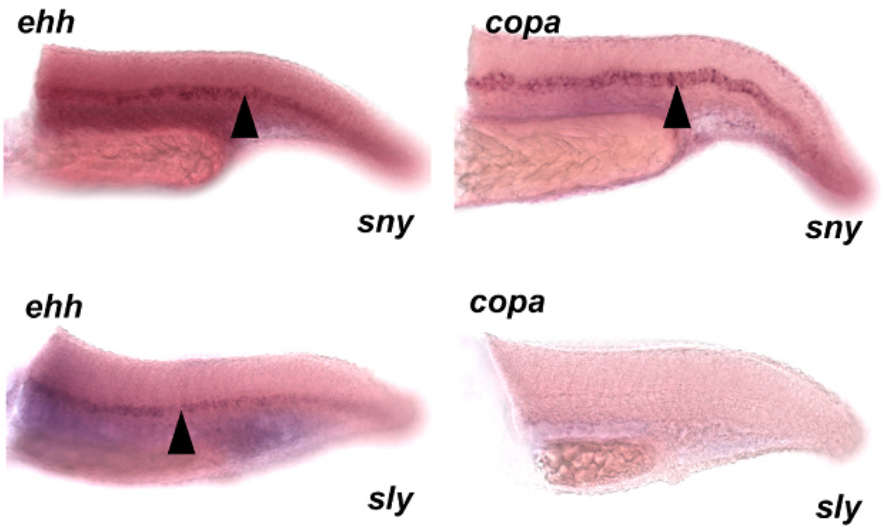

Figure 7.3 Expression of *COPα* and *ehh* in *sny* and *sly* embryos.

Lateral view of fixed, 24hpf tails, anterior to the left, dorsal to the top.
ehh is maintained in the undifferentiated notochords of both *sny* and *sly* mutants at 28 hpf. *COP*α is maintained only in the undifferentiated notochords of *sny* embryos. Thus, *COP*α is maintained only in notochords lacking COPI function, and is not maintained just as a results of lack of notochord differentiation.

7.3 Identification of UPR Components in Zebrafish

Using publicly available EST and genomic information, combined with cDNA sequence for UPR components from mouse, full length cDNA sequence was generated for the zebrafish components of the UPR. In this way, a single IRE1 homologue was identified as being 2813 bases long, predicting a protein of 931 aa that shows 58% identity to mouse IRE1α and 52% identity to mouse IRE1β. Full length cDNA sequence was generated for zebrafish ATF-6, PERK and BiP of 1797, 3006 and 2347 bases respectively. These encoded proteins of 560, 952 and 650 aa that show 41%, 58% and 89% identity to the mouse AFT-6, PERK and BiP respectively. The identified zebrafish *Xbp1* sequence was 1696 and 1675 in its inactive and active form respectively. These encoded proteins of 263 and 383 aa demonstrating identities of 56% and 47%, relating to the inactive and active forms respectively.

7.4 Activation of the UPR During Development and in COPI Mutants

Using the generated full length cDNA sequence for *BiP*, primers were designed to amplify a 1357bp fragment from wild type cDNA. From this, an *insitu* riboprobe for *BiP* was synthesised. This probe was then used to examine the expression of *BiP* in staged wild type embryos; at approximately 4 cell stage, shield stage, tail-bud stage, 5 somite stage, 12 somite stage, 24 hpf and in the three coatomer mutants; *sny*, *hap* and *dop* at 28hpf. *BiP* is considered to be one of the earliest induced components of the UPR and the encoded protein is a major

controller of activation of the UPR. As such, *BiP* is one of the most commonly used makers of UPR activation and in this instance, *BiP* expression is used to examine where and when the UPR is active.

7.4.1 Expression of BiP mRNA During Development

High levels of *BiP* at the 4 cell stage indicates that *BiP* is maternally expressed. This expression continues through to shield stage at which point *BiP* is expressed ubiquitously. By tail-bud, *BiP* is still ubiquitous, though at very low levels. However, by this stage there is a specific up-regulation of expression within the chordamesoderm. This chordamesoderm/notochord localised up-regulation continues through to 12 somites. Elevated levels of expression are also detected in the developing brain and hatching gland during somitogenesis. By 24 hpf expression is returned to low levels of ubiquitous expression throughout the embryo with the exception of the most posterior tip of the developing notochord. Thus, the expression of *BiP*, a widely used marker of UPR activation, closely resembles the expression of the coatomer subunits α, β, β', ε, δ, γ2 and ζ2 (**Figure 7.4**).

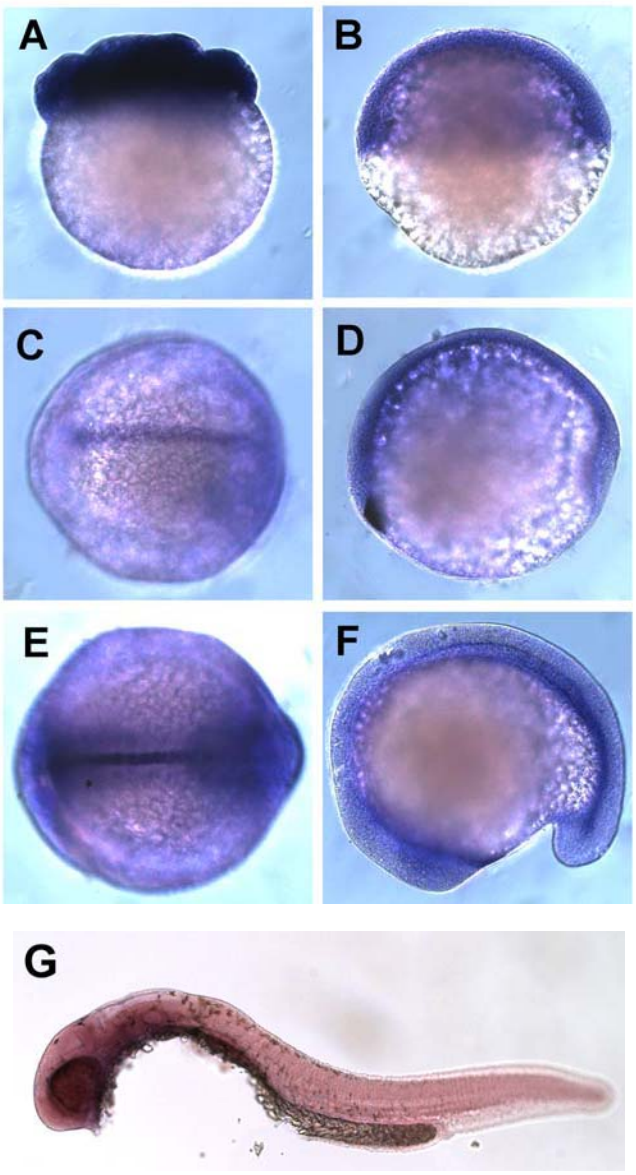

Figure 7.4 Staged expression profile of the UPR marker *BiP*.

At (**A**) 32-cell, (**B**) shield, (**C**) tailbud dorsal view, (**D**) tailbud lateral view, (**E**) 5-somite dorsal view, (**F**); and lateral view, anterior to the left, of 14 somite and (**G**) 24 hpf. (**A**) High level of expression at 32 cell stage demonstrates that *BiP* is provided maternally. At shield stage (**B**) expression is ubiquitous and at relatively high levels. By tailbud (**C** and **D**) stage, the ubiquitous expression is lower with the exception of specific up-regulation within the chordamesoderm. This is maintained through 5 somite (**E**) and 12 somite stages (**F**), with noticeably high expression in the developing brain at these stages. By 24 hpf (**G**) notochord specific expression has been extinguished and up-regulation is confined to the developing brain.

7.4.2 Expression of BiP mRNA in Coatomer Mutants

Comparison of *BiP* expression in both mutant and wild type sibling *sny*, *hap* and *dop* mutants demonstrated that, as observed with the majority of the COPI subunits, the chordamesoderm/notochord expression of *BiP* is maintained in 28 hpf *sny*, *hap* and *dop* embryos. In 28 hpf sibling wild type embryos, the chordamesoderm specific up-regulation is extinguished (**Figure 7.5**).

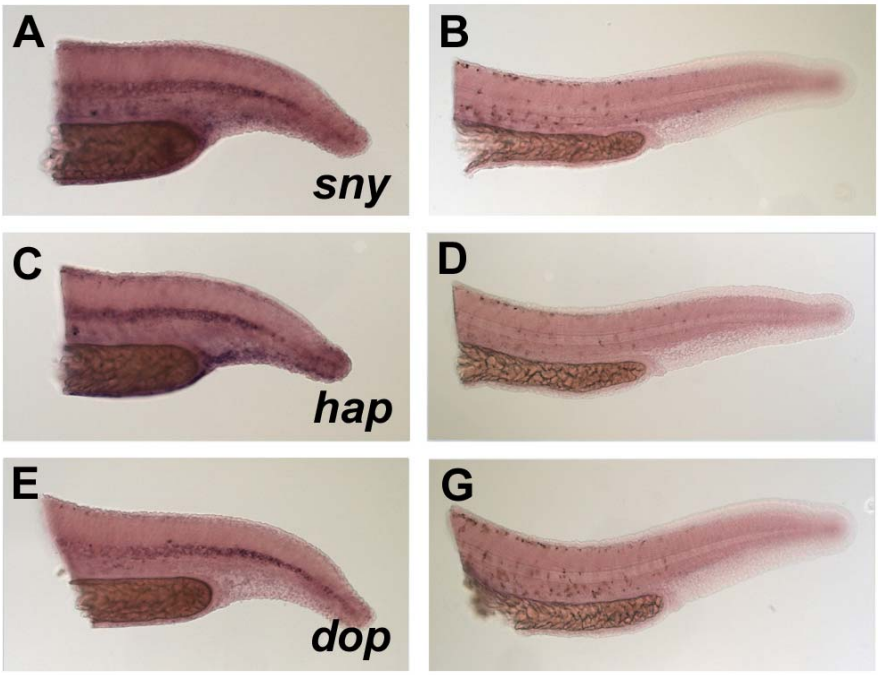

Figure 7.5 Expression profile of *BiP* in mutant and wild type embryos at 28 hpf.

Lateral view of fixed, 24hpf tails, anterior to the left, dorsal to the top. Tails taken from mutant embryos (**A**, **C**, **E**) and wildtype siblings (**B**, **D**, **F**).
At 28 hpf, the Expression of *BiP* is maintained within the undifferentiated notochord of the COPI mutants *sny* (**A**), *hap* (**C**) and *dop* (**E**). The chordamesoderm/notochord specific expression of *BiP* is shut down in the properly differentiated notochord wild type siblings of *sny* (**B**), *hap* (**D**) and *dop* (**F**).

7.5 Morpholino Knock-Down of UPR Components

To examine the role to the UPR in development and in both the normal developmental expression of COPI and the abnormally maintained expression within COPI mutant embryos, MO's were designed against IRE1, ATF-6, PERK, BiP and XBP1. Using the generated cDNA sequences, MO's were directed against the ATG start of translation. Comparable doses of standard control MO (not shown) demonstrated no phenotype and resembled wild type uninjected embryos of equal stage.

7.5.1 Signalling Components of the UPR

Injection of 8ng of either an ATG targeted IRE1 or an ATG targeted ATF-6 resulted in comparable phenotypes. Both resulted in minor shortening along the A-P axis and a slight curling of the most posterior tail at 24 hpf. Neither IRE1 nor ATF-6 results in any observable defect in any other developmental process at this stage. In stark contrast to this, injection of 8ng of both ATF-6 and IRE-1 results in an obvious shortening of the embryo along the A-P axis at 24 hpf. At 24 hpf there are minor observable defects in notochord differentiation though there is not a complete failure to differentiate. By 48 hpf, embryos injected with both IRE1 and ATF-6 MO show a dramatic loss of notochord differentiation. These double injected embryos bear slight resemblance to the mutants *sly, hap* and *dop* at this stage. By 48 hpf, the double injection embryos show an even more dramatic shortening of the A-P axis, failure in notochord differentiation, with associated 'U' shape defects in somite

formation, major loss of melanophores, observable defects in the developing brain and the beginnings of wide spread necrosis in the majority of injected embryos. Despite the similarity of this phenotype to the COPI mutants, it was noticeably less severe, especially given that ATG targeted MOs should remove both zygotic and maternal transcripts (

Figure 7.6).

Since only one IRE1 isoform was isolated in zebrafish, a MO designed against the ATG of XBP1 was designed and used, since XBP1 is vital for IRE1 signalling and loss of XBP1 translation would block signalling from any uncharacterised IRE1 isoforms. Injection of 8ng of XBP1 resulted in a much more dramatic phenotype than either IRE1 or ATF-6 alone or IRE1 and ATF-6 combined. At 24 hpf, XBP1 injected embryos have a stark shortening along the A-P axis, loss of notochord differentiation combined with the associated 'U' shaped somite defect and major defects within the developing brain and head. In this way, XBP1 injected embryos also show similarity with COPI mutants, since the observed notochord defect resembles that observed in *sly, hap* and *dop*. However, the observed phenotype is more severe than that observed in the COPI mutants, since the COPI mutants show no obvious neural defects (**Figure 6.7**). Co-injection of 8ng of ATF-6 along with 8ng of XBP1 results in embryos demonstrating major defects in head structures, with considerable defects in somite structure, lacking any form of differentiated notochord and showing no discernable extension along the A-P axis. These defects result in a grossly amorphous embryo, which is further compounded by the beginnings of cell death throughout the embryo.

Injection of an ATG targeted MO against PERK produced a phenotype comparable to that observed in XBP1 injected embryos. Though in the case of

PERK, defects are obvious at a 4ng dose. In both cases there is a loss of proper notochord differentiation, the associated 'U' shape defect in somite structure and a loss of extension along the A-P axis. However, in the case of PERK, the observed phenotype in head and neural structures is less pronounced. Co-injection 8ng of ATF-6 with 4ng of PERK resulted in a more dramatic phenotype, generating embryos lacking almost all A-P extension and with severe malformations of the head, as well as lacking any notochord differentiation. Injection of 8ng of XBP1 with 4ng of PERK was more severe than co-injection of ATF-6, though the phenotypes were comparable. Co-injection of XBP1, ATF-6 at 8ng and PERK at 4ng resulted in mass necrosis and embryo death by 24 hpf. In the small percentage of embryos that do survive, there is a complete loss of proper morphology and structure. Injection of 4ng of IRE1 and ATF-6 and 2ng of PERK results in noticeable necrosis and a loss of both proper neural and notochord development. At this level of knockdown, the majority of embryos survive to 24 hpf but die by 36 hpf (**Figure 7.7**).

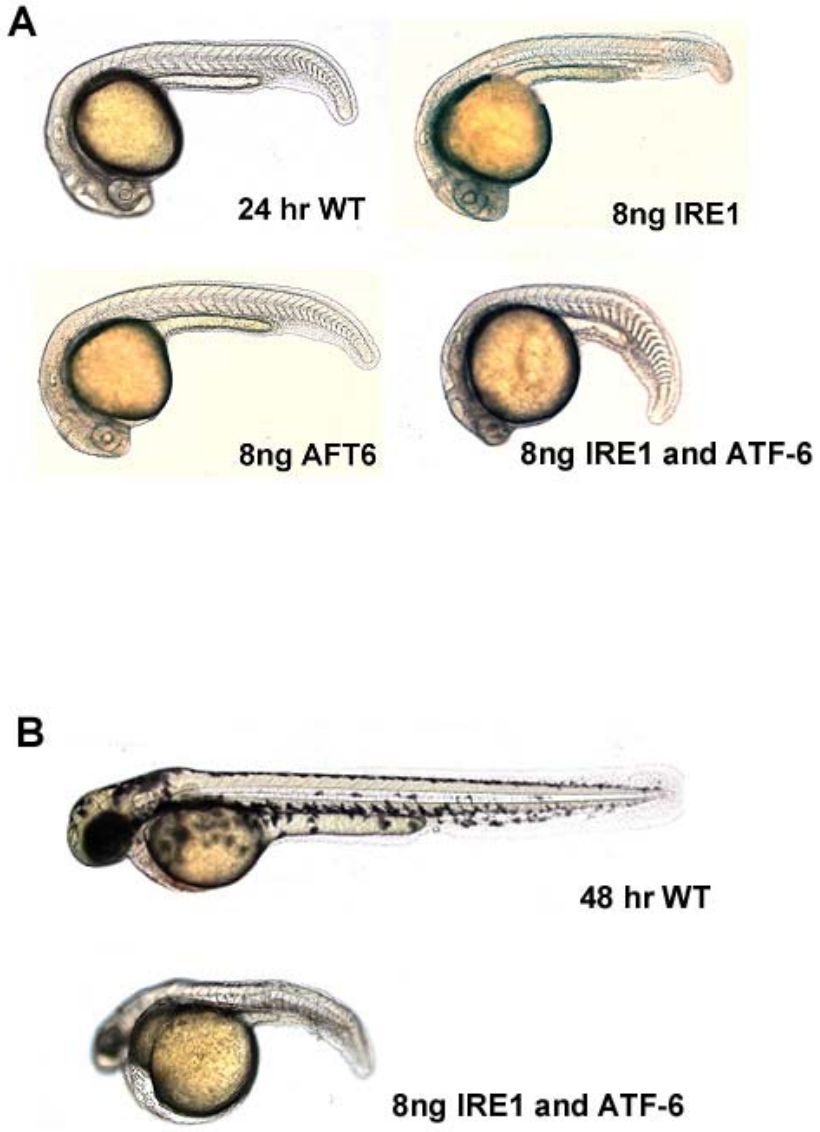

Figure 7.6 Embryos injected with ATG targeted MO's for IRE1 and ATF-6.

Lateral view, anterior to the left, dorsal to the top, of live 24 hpf embryos.
(**A**) 24 hpf WT embryos (top left) compared to embryos injected with 8ng IRE1 (top right), 8ng ATF-6 (bottom left) and IRE1 and ATF-6 combined (bottom right). Both IRE1 and ATF-6 alone result in no obvious defects, where combined injection results in an obvious A-P axis shortening, marked lack of notochord differentiation and minor neural defects.
(**B**) 48 hpf WT embryo (top) compared to 48 hpf embryos injected with 8ng of both IRE1 and ATF-6 (bottom). By 48 hpf, A-P axis reduction is more dramatic, somites have developed in a 'U' shape and there is no discernable notochord differentiation. In addition, neural defects are more obvious, with embryos displaying smaller heads and melanophores have failed to develop normally.

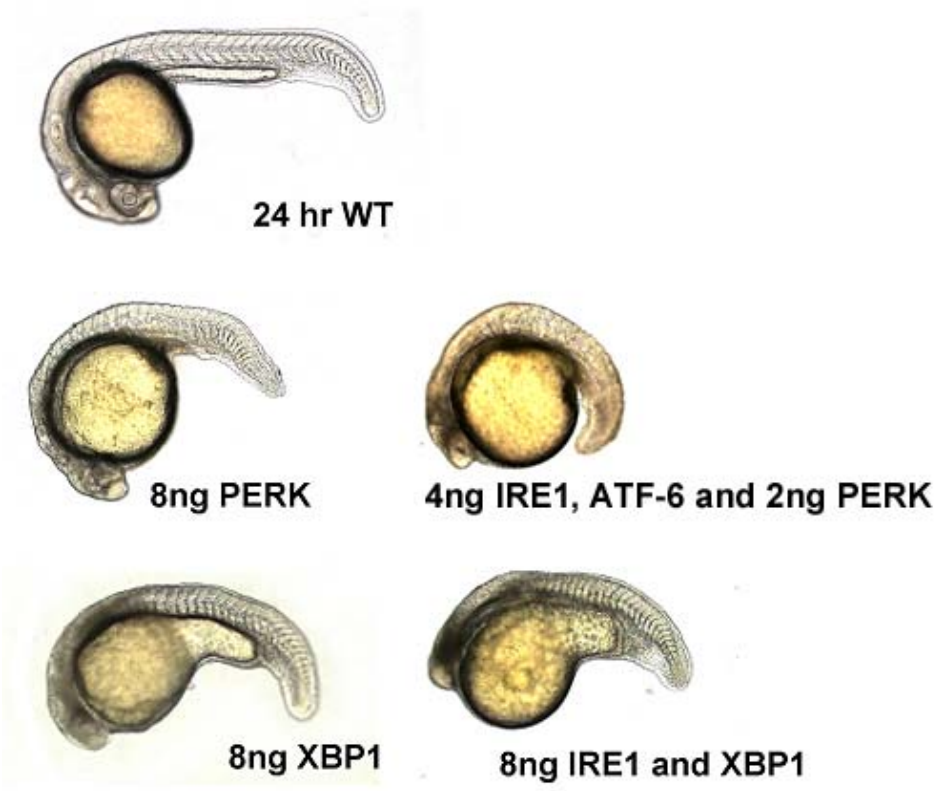

Figure 7.7 MO knockdown of PERK and XBP-1

Lateral view, anterior to the left, dorsal to the top, of live 24 hpf embryos.
Comparison of 24 hpf WT embryos (top) to PERK (middle left), PERK, IRE1 and ATF-6 (middle right), XBP-1 (bottom left) and IRE1 and XBP-1 (bottom right) MO injected embryos.
Loss of PERK alone or loss of PERK, IRE1 and ATF-6 combined, resulted in mild necrosis throughout the embryo at 24 hpf and a loss of proper neural and notochord development.
Loss of XBP-1 resulted in dramatic neural defects, obvious reductions in A-P axis extension and a complete lack of notochord development. Combined loss of IRE1 and XBP-1 is comparable to loss of XBP-1 alone, though moderately more severe.

7.5.2 the UPR 'Master Regulator' BiP

Injection of a MO targeted against the ATG start of translation of the zebrafish BiP resulted in both notochord and neural defects at 24 hpf. Injection of 2ng of MO against BiP resulted in a partially penetrant MO phenotype. A small percentage (~20%) of embryos demonstrated dramatic head defects, including defects in the developing eyes and stark neural abnormalities alongside a failure to differentiate notochord. In these embryos the posterior section of the tail loses any sense of structure and morphology and develops into a large growth of dying cells, though even in this, there can be observed some undifferentiated notochord cells. The less penetrant embryos show no obvious head abnormalities, developing eyes and showing no brain necrosis at 24 hpf. These embryos also demonstrate relatively normal extension along the A-P axis and a fairly well differentiated notochord, though there are minor defects in the posterior most limit of the developing tail where the tail curls and somites appear slightly compressed, suggesting a slight loss of A-P extension.

At higher doses of 8ng, knock down of BiP results in a much more obvious phenotype. At 24 hpf a small, but substantial, number of the injected embryos have died (~10%). The remainder all share a similarly expressive phenotype. These embryos are comparable to the affected embryos at 2ng, although they appear more severe. These embryos have drastic head defects, showing abnormalities in eye development, neural development and have abnormally small heads. These embryos also show a obvious lack of A-P extension, with the most penetrant embryos showing no tail beyond the yolk extension. Embryos also show the amorphous cell growth at the posterior end of the tail in many, but not all, cases. These embryos

demonstrate observable chordamesoderm and there appears to be differentiation to notochord. Further, the majority of 8ng BiP MO injected embryos show early signs of cell death and by 30 hpf all injected embryos have died through systemic necrosis (**Figure 7.8**).

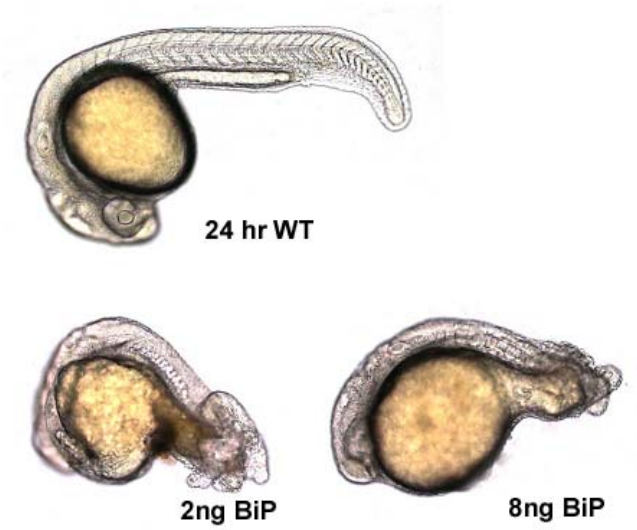

Figure 7.8 MO knock-down of BiP

Lateral view, anterior to the left, dorsal to the top, of live 24 hpf embryos.
Loss of BiP function results in deformations of the posterior tail tip, a lack of somite patterning and a loss of proper head development. Noticeably, both 8ng and 2ng injected embryos develop notochord and neural plate.

7.6 Role of the UPR in COPI expression

Using 8ng of XBP1 MO alone or 8ng of both XBP1 MO and ATF-6 MO in co-injected embryos, the expression of *BiP* and *COP*β' mRNA was examined under conditions of loss of UPR activation signal transduction, since the majority or UPR activation is transduced through IRE1 and ATF-6 signalling. To examine the role of the UPR in both normal expression and under conditions of COPI loss, MO injected and control embryos were raised to 28 hpf either in embryo water (as defined in Materials and Methods) or embryo water plus 1.8μM BFA.

Embryos injected with XBP1, co-injected with XBP1 and ATF-6 or with the control MO and then raised in blue water plus1.8μM BFA all demonstrated both a general up-regulation of BiP and COPβ' throughout the embryo as well as notochord specific maintenance at 28 hpf, demonstrating that loss of *Xbp1* or *Xbp1* and *ATF6* is insufficient to suppress activation of the UPR under conditions of COPI loss. Perhaps most interestingly though, was the observation that in untreated embryos, i.e. those raised in embryo water alone, XBP1 injected and XBP1 and ATF-6 co-injected embryos, but not control injected embryos, demonstrated a notochord specific maintenance of both BiP and COPβ'. Untreated control injected embryos demonstrated only normal expression of BiP and COPβ', with up-regulation limited to the most posterior limit of the tail (**Figure 7.9** and **Figure 7.10**).

This suggests that in MO injected embryos, which lack sufficient activation of the UPR, there is insufficient up-regulation of UPR response genes, including COPI subunits, specifically within the notochord during development. This results in a lack of ER and Golgi stress relief within the developing notochord. There is thus

continued UPR activation, since the increased secretory demand is not met, and the expression of *BiP* and *COP* subunits within the notochord is maintained.

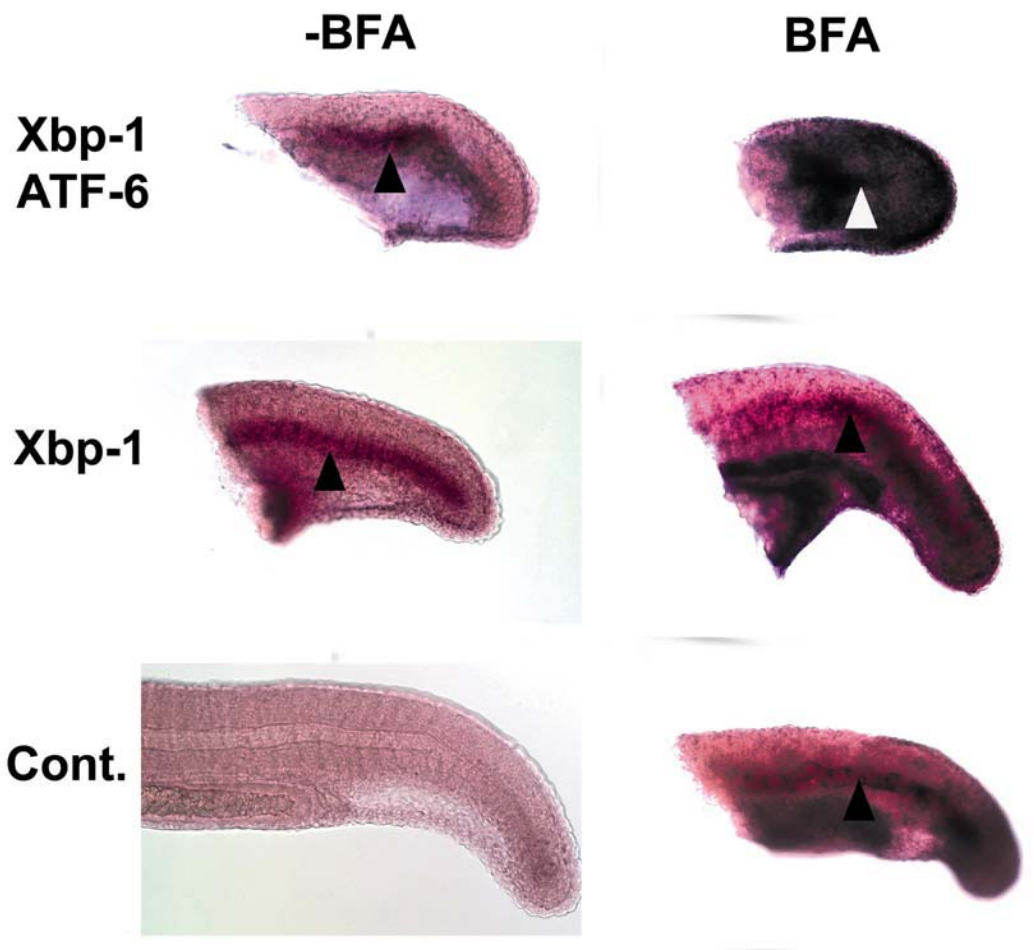

Figure 7.9 Expression of *COP*β' in normal and UPR deficient embryos.

Lateral view of fixed, 24hpf tails, anterior to the left, dorsal to the top.
XBP-1 and ATF-6 (top row) and XBP-1 (middle row) MO injected embryos demonstrate embryos lacking full UPR activation. Under conditions of COPI loss of function (+BFA) there is maintained *COP*β' expression in UPR deficient and control embryos. Under normal conditions (-BFA), there is a lack of *COP*β' expression in control embryos. Under these condition, UPR deficient embryos show maintained expression of *COP*β' specifically within the notochord.
Arrows mark maintained expression of *COP*β' within the notochord.

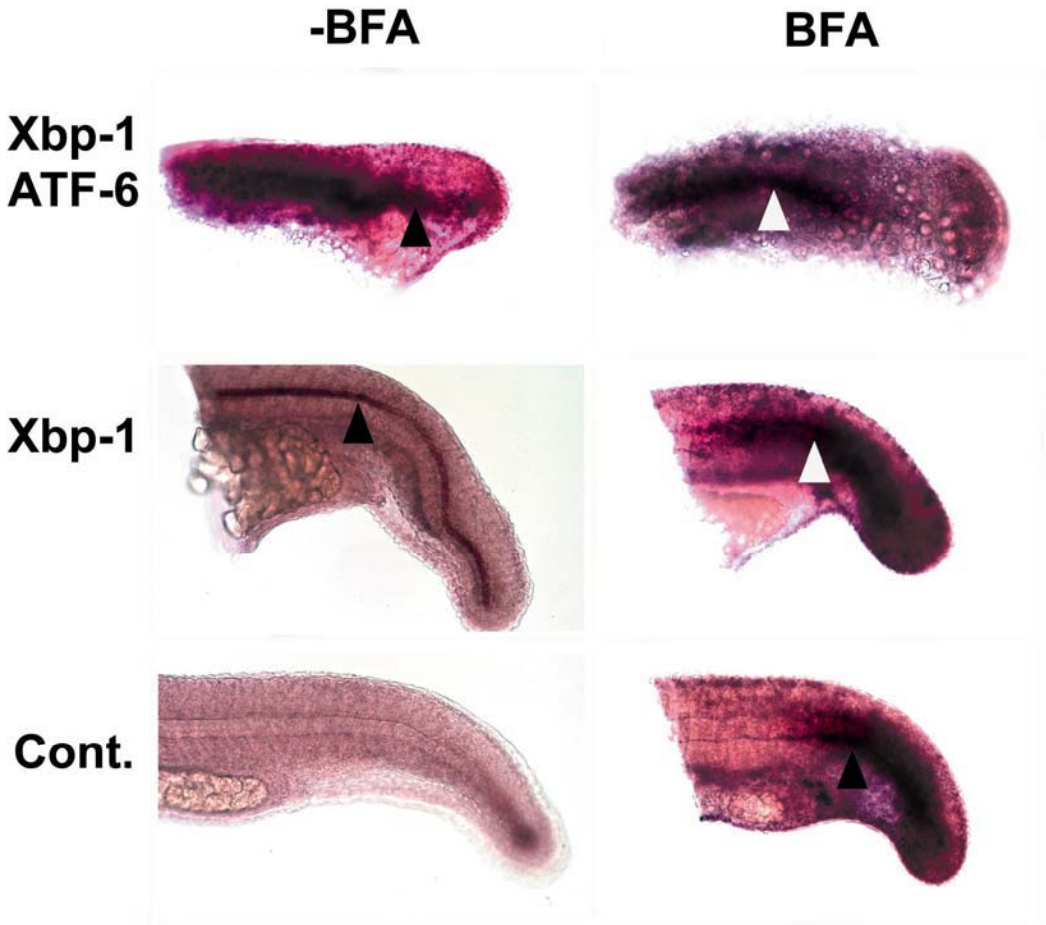

Figure 7.10 Expression of *BiP* in normal and UPR deficient embryos.

Lateral view of fixed, 24hpf tails, anterior to the left, dorsal to the top.
XBP-1 and ATF-6 (top row) and XBP-1 (middle row) MO injected embryos demonstrate embryos lacking full UPR activation. Under conditions of COPI loss of function (+BFA) there is maintained *BiP* expression in UPR deficient and control embryos. Under normal conditions (-BFA), there is a lack of *BiP* expression in control embryos. Under these condition, UPR deficient embryos show maintained expression of *BiP* specifically within the notochord.
Arrows mark maintained expression of *BiP* within the notochord.

7.7 Discussion

The expression of *ehh* within the notochord of *sny* demonstrates that there is a lack of notochord differentiation in these mutants, as observed in the *sly* mutant, which lack the zebrafish laminin γ1 gene. However, since *COP*β' is only maintained within the undifferentiated notochord of *sny* mutants at 28 hpf and is not present in the notochord of *sly* mutant embryos at 28 hpf, it can be argued that the maintenance of *COP*β', and by association the other COPI subunits that are observed within the chordamesoderm during development, is not due simply to a lack of notochord differentiation. Rather, it is the specific loss of COPI as occurs in the mutants *sny, hap* and *dop* that results in the maintenance of COPI subunit expression at 28 hpf. Reinforcing the ideal that COPI is involved in a system of auto-regulation, where conditions in which available COPI function is exceeded by demand for COPI activity lead to an up-regulation of a complete set of COPI subunits.

Loss of COPI function leads to a loss of secretory network homeostasis, since there is no retrograde transport to allow movement of secretory machinary, involved in processes such as glocosylation and protein folding, back to their correct location within the secretory network. As such, the composition of the Golgi and ER is compromised, leading to defects in post translational modification and a loss of proper protein folding, which in turn leads to a build of secretory cargo. Hence, we thought that the UPR could be engaged when COPI function is compromised and could provide a mechanism for the regulation of coatomer transcription. The expression of *BiP*, which is up-regulated under conditions of UPR activation and is commonly used as a marker for the UPR, closely resembles the expression profiles of the coatomer subunits α, β, β', ε, δ, γ2 and ζ2. Further to this *BiP* is maintained

beyond its normal temporal expression domain in the COPI mutants *sly, hap* and *dop* and in embryos treated with BFA, indicating that a loss of COPI activity leads to activation of the UPR beyond it's normal developmental expression profile. Thus, *BiP* is expressed in the same manner as the COPI notochord specific subunits, indicating that the UPR is active in the same physical and temporal domains as the notochord specific COPI subunits under the same conditions. Such observations suggest that loss of COPI function activates the UPR.

Though the UPR is active at the same time and in the same domains as the COPI subunits, it may well be that conditions where COPI activity is required and where COPI subunits are up-regulated are permissive to the activation of the UPR. Using MO knockdown techniques to either shut down the UPR response, through the removal of the signalling components ATF-6, PERK, IRE1 and Xbp1 alone and in combination, or hyperactivate the UPR, through the knockdown of BiP, the role of the UPR in development was examined through associated phenotypes.

The observed phenotype for IRE1 was considerably less severe than that noted in Xbp1 injected embryos, though they are involved in the same signalling pathway, with IRE1 acting in the intron removal dependant activation of Xbp1. The apparent difference in phenotype can be explained by the identification of only one IRE1 gene in zebrafish, where two isoforms are known in both humans and mouse but only one in *C. elegans*. Thus, the MO designed against the identified IRE1 may only knockdown one isoform, whereas knockdown of Xbp1 blocks all signalling through the IRE1 pathway. Knockdown of XBP1 resulted in defects in both the developing brain and within the notochord, with the trunk of injected embryos resembling the trunk defects observed in the COPI mutants. Embryos co-injected with Xbp1 and ATF-6 were more severe than Xbp1 alone, however they also demonstrated a lack of

proper notochord differentiation with the trunk again bearing similarities to the COPI mutants. One defining characteristic of the COPI mutants is the loss of pigment in the melanophores. In embryos co-injected with both IRE1 and ATF-6, there is an almost complete loss of pigmentation at 48 hpf. Injection of PERK MO also resulted in notochord and neural defects. Thus, removal of some elements of UPR activation, which causes a lack of UPR response, results in neural defects, a failure to differentiate notochord and a loss of pigmentation. Demonstrating a role for the UPR in, development of the brain, differentiation of the notochord and proper development of the melanophores. The latter two of these three defects are also observed in the COPI mutants, suggesting a link between the requirement for COPI and the UPR in certain developmental processes.

Knockdown of BiP results in a continual activation of the UPR system, since BiP protein acts to inhibit the activation of IRE1, ATF-6 and PERK. BiP also acts to chaperone proteins during proper protein folding within the ER. Embryos lacking BiP demonstrate amorphous and necrotic posterior trunks as well as minor neural defects when compared to UPR suppression. There is, however, some notochord differentiation in the most anterior trunk sections. BiP MO injected embryos thus demonstrate the most severe defects in the tissues that demonstrate BiP expression. This in turn suggests that BiP knockdown renders embryos more sensitive to activation of the UPR, leading to earlier activation of the apoptotic components of the UPR. Both UPR modified embryos, i.e. Xbp1, IRE1, ATF-6 and PERK injected embryos, and UPR activated embryos, i.e. BiP injected embryos, show initial establishment of dorsal-ventral and anterior-posterior axis and both demonstrate specification of chordamesoderm, though this remains undifferentiated in the UPR suppressed embryos. It therefore appears that the UPR functions after establishment

of the shield and mesendoderm, in the development of neural structures and differentiation of notochord. This is supported by the developmental profile of BiP expression, which demonstrates activation of the UPR in specific tissues initially at tailbud stage, in the chordamesoderm, and then later in both the developing brain and the differentiating notochord.

To examine the role of the UPR in regulating the expression of the COPI subunits, UPR suppressed embryos, that is, embryos injected with Xbp1 and both Xbp1 and ATF-6, were incubated with 1.8μM BFA at 18 somite stage to precipitate conditions of COPI loss of function (Coutinho et al., 2004). The expression of both *BiP* and *COP*β' in these embryos was then compared to untreated embryos at 28 hpf. In control, Xbp1 and Xbp1 and ATF-6 injected embryos, treatment with BFA resulted in the maintenance of both *COP*β' and *BiP* expression within the undifferentiated notochord and un specific up-regulation through out the embryo. Hence, removal of Xbp1 alone or Xbp1 and ATF-6 together is not sufficient to prevent activation of the UPR in response to COPI loss of function, as demonstrated by the up-regulation of *BiP* throughout the embryo and maintained within the notochord. However, this answers little about the role of the UPR in the regulation of COPI subunit expression. The observation that untreated embryos, injected with either Xbp1 or Xbp1 and ATF-6 show specific maintenance of both *COP*β' and *BiP* in the undifferentiated notochord at 28 hpf. suggests that, the suppressed UPR response in these embryos, which lack either the IRE1 signalling pathway, or the ATF-6 and IRE-1 signalling pathway, is insufficient for proper notochord differentiation. However, a lack of notochord differentiation alone does not result in the maintenance of COPI subunit or BiP expression within the notochord. Thus it can be argued that insufficient activation of the UPR during development, through

knockdown of the UPR signalling components, causes a loss of notochord differentiation and results in a lack of sufficient COPI activity. This in turn results in the expression of COPI beyond it normal temporal domain, due to a loss of COPI activity. It can therefore be argued that the UPR is not only an ER stress response, but also an essential system involved in maintaining the protein adaptory and secretory network. In this way, the UPR functions during development in tissues and cells that experience increased translatory and secretory loads as part of their normal development. In this way, the UPR is therefore suggested as a regulatory mechanism that functions in the notochord, and other tissues, during development to up-regulate many genes, including the COPI components, required to meet an increased secretory load. The UPR also acts in COPI compromised individuals, initially to maintain expression of these genes, including the COPI subunits, in an attempt to remedy abnormalities in secretion and then later in activating apoptosis in response to continued UPR activation.

The evidence presented in this chapter provides strong argument that the UPR is acting as a vital regulatory mechanism during development to maintain the ER and Golgi in cells encountering increased secretory and translatory demands. However, the evidence that the UPR is acting to regulate COPI subunit expression is less than certain. Further work, to provide more definitive evidence, therefore remains. By examining the expression of COPI subunits and *BiP* under conditions of BiP overexpression, it should be possible to more accurately defeine the role of the UPR in the expression of COPI subunits. Examination of gemonic sequence upstream of the COPI subunits may reveal common regulatory elements. Such elements could then be compared to characterised UPRE sequences and then examined through the attachment of marker genes to such regulatory elements. Despite the work still to

perform, the demonstration that the UPR is active when and where COPI subunits are upregulated and that a lack of UPR activation results in similar developmental defects to those observed in COPI mutants strongly links the UPR to COPI subunit expression. Combined with the demonstration that a lack of proper UPR activation leads to both maintained UPR activation and COPI subunit expression, the evidence provides strong, though incomplete, support for a model where the UPR is functioning during the development, including within the notochord, to up-regulate genes, including the COPI subunits, involved in secretion and post-translational modification.

7.8 Summary

- Maintenance of COPI subunits in COPI mutants is not due to a lack of notochord differentiation, but a specific loss of COPI function.
- *BiP* mRNA has the same expression profile as the notochord specific COPI subunits during development and in the COPI mutants.
- Knockdown of the UPR results in comparable notochord and melanophore phenotypes to *sny, hap* and *dop*.
- Over-activation of the UPR results in apoptosis in the trunk and head.
- Knockdown of XBP1 or XBP1 and ATF-6 is insufficient to prevent COPI maintenance and prolonged UPR activation in response to COPI loss of function.
- Knockdown of XBP1 or XBP1 and ATF-6 results in COPI maintenance and prolonged UPR activation in wild type 28 hpf embryos.

- The UPR may be involved in both the normal developmental expression profile of COPI subunits and abnormal maintenance in COPI mutants.
- The UPR plays an essential role in development.