Chapter Eight

Discussion

8 Discussion

In recent years many families of genes have been identified to have vital roles in development. Many transcription factors have been identified as essential for proper development, acting in different cells to specify distinct fates. These transcription factors include the *Hox* gene family, which act in patterning both positional and temporal identity, the helix-loop-helix factors MyoD and Neurogenin, which act in specifying cells to a muscle or neural fate respectively and the t-box family, which includes the gene no-tail, which was identified as having an essential role in notochord development (Ma et al., 1996; Schulte-Merker et al., 1994; Weintraub et al., 1991; Weintraub et al., 1989). Further factors include secreted proteins, which act in signalling between developing cells and include the Wnt's, FGF's and the TGFβ family. More recently, a range of other factors have been identified as having essential roles in development. Work on the zebrafish notochord mutants generated as part of the 1996 ENU mutagenesis screen, has demonstrated a role for both the extracellular matrix and secretory maintenance in development. The mutants *gup, sly* and *bal* lack the laminin subunits β1, γ1 and α1 respectively and show defects in both neural and notochord development (Parsons et al., 2002b; Pollard, 2002). The mutants *sny, hap* and *dop*, which encode COPα, COPβ and COPβ' respectively, have demonstrated a role for secretory maintenance in development of both the notochord and the melanophores ((Coutinho et al., 2004) This thesis). Though much is now known concerning development, precisely how the range of developmentally essential factors interact to bring about the differentiation of cells and the generation of complete tissues is not well understood.

Much work, in a range of model organisms and developmental systems, is currently being performed to answer many of the un-resolved questions in developmental biology.

As part of this thesis, I have investigated the process involved in early vertebrate development, using the zebrafish notochord as a developmental system. Through the use of both characterised and un-characterised notochord mutants, I have aimed to further define what genes and systems are important in the process of notochord differentiation and to expand this understanding into the greater scheme of development. I have characterised the gene responsible for the notochord specific mutant *doc* and established that it encodes a novel and highly conserved 14 WD40 domain protein. I have also characterised the *hap* and *dop* loci, confirming that they encode the COPI subunits COPβ and COPβ' respectively. Continuing this, I have characterised the other COPI subunits and, through analysis of COPI expression and regulation, I have implicated the UPR as an essential regulatory system involved in development.

8.1 A Novel Gene with a Role in Zebrafish Notochord Development

8.1.1 Cloning the Zebrafish Notochord Mutant *doc*

By analysing the inheritance of polymorphic markers in *doc* mutant embryos with a systematic screen of SSLP markers, the mutant locus was defined to chromosome 18. The marker Z9484 demonstrated linkage to the *doc* locus and through analysis of known linked markers, the mutation was defined to a region

between the markers Z9484 and Z7417. Using publicly available genome sequence, these markers were placed on the genetic map, defining a region of ~12Mb between 5.5Mb and 18.2Mb. Through selection of identified INDEL's and BAC ends within this region, markers of ~200bp were designed and then examined for polymorphism. Polymorphic markers then assisted in fine mapping the *doc* locus to a region of ~0.5Mb between a BAC end marker at ~11.1Mb and an INDEL marker at ~11.5Mb. Examination of genomic sequence identified, three candidate genes, with homologies to Syntaxin-8, Cadherin-13 and a novel, conserved protein displaying multiple WD40 domains.

So, by examining the frequency of meiotic recombination, the *doc* locus was defined to a region of ~0.5Mb, where three candidate genes were identified. This approach was assisted by known and previously mapped SSLP markers, and further refined through the use of genome sequence to generate further markers. Genome sequence then allowed the identification of appropriate candidate genes, which could then be tested through antisense MO knock-down and whole mount *insitu* hybridisation.

8.1.2 A Novel Multiple WD40 Domain Protein Encodes *doc*

Using MO antisense knock-down, the roles of the most likely candidate genes within the region defined for *doc* were examined. In this way, a novel protein containing multiple WD40 domains was determined to have an essential role in notochord development. Antisense MO knock-down of the mRNA encoding this protein results in a lack of AP extension, formation of 'U' shaped somites and a loss of proper notochord differentiation. Staged expression analysis of this mRNA

demonstrated up-regulation specifically within the notochord at 18 somite stage and a lack of notochord expression at 6 somite and 24 hpf. Hence, expression of *doc* is up-regulated in the notochord between ~11 hpf and 24 hpf and is thus expressed at stages before the notochord is fully differentiated and before early markers are extinguished. Expression of *ehh* in doc MO injected embryos has demonstrated that there is a lack of proper notochord differentiation in knock-down embryos. From this supporting evidence, it is suggested that the phenotype in *doc* mutants results from mutations in the gene encoding the novel, conserved WD40 domain protein. However, definitive proof that this gene does indeed comprise the *doc* locus remains to generated. Sequencing the precise mutation from both cDNA and genomic DNA would provide final confirmation that this gene is indeed the *doc* locus.

The precise function of this gene has yet to be determined and the novel nature of the gene has meant that no function can be derived from information on studied homologues. However, the multiple WD40 domains suggest that doc is interacting directly with other proteins in the process of notochord development. Though precisely what doc is interacting with remains to be established, it may be that it acts in establishing a link between the notochord BM sheath and the notochord itself. The multiple WD40 domains suggest that doc may be function via direct interections with other proteins. As such, doc may coordinate the cytoskeleton in response to basement membrane formation, or might stabalise a signal required for vacuolation that is received in response to BM formation. Additionally, the multiple WD40 domain may enable interactions with secretory components, for examble the COP subunits, which also demonstrate WD40 domains. So it may be that doc acts as a link between secretory maintanence and notochord vacuolation. A role for doc in signalling between notochord differentiaion and BM formation fits with observations

concerning general notochord differentiation, where a lack of sheath in *X. laevis* has been noted to result in a failure of notochord vacuolation and differentiation (Adams et al., 1990). Considering the nature of notochord development, where cells vacuolate and press against the surrounding sheath at high pressure to strengthen the notochord through turgor pressure, it seems reasonable to assume that a system of signalling to ensure proper sheath formation would be in place, since vacuolation prior to sheath formation would be fatal. It may well be that doc functions in just such a pathway, acting in some way to signal the completion of the BM sheath to the notochord cell. Expression analysis and MO knock-down analysis places just such a role firmly within the realm of doc action.

The fact that the doc remains highly conserved throughout evolution suggests that it has a vital function. A lack of functional information in other species has left many questions remaining concerning its function in zebrafish development, but also places the mutant *doc* in a position of considerable interest. Establishing the role of doc in the development of the zebrafish notochord would help define the role of this gene in other organisms and may well highlight a conserved mechanism of development throughout evolution. Thus, continued study of *doc* is of great interest.

8.2 The Role and Regulation of the Secretory Network in Development

8.2.1 A Role for COPI in Development

Work on the zebrafish notochord mutant *sny*, identified the gene COPα, a member of the seven subunit COPI coat complex, as essential for both notochord

development and pigmentation formation in melanophores (Coutinho, 2001). The phenotypic similarity between the mutants *sny, hap* and *dop* suggested that *hap* and *dop* may encode other COPI subunits, a suggestion that was reinforced by the ability of MOs against COPβ and COPβ' to phenocopy *hap* and *dop* respectively.

As part of this thesis, mutations were identified in COPβ and COPβ' in *hap* and *dop* embryos respectively. Sequencing of cDNA from *hap*^{tm285b} mutant embryos identified a substitution within COPβ that encodes a truncated protein of 499 amino acids. Sequencing of cDNA from *dop*^{m341} embryos identified a substitution within COPβ' that encodes a truncated protein of 761 amino acids. Thus, zygotic COPβ and COPβ' in *hap* and *dop* embryos is non functional and, since COPβ and COPβ' are both essential for the formation of the fully functional seven subunit COPI complex, there is a lack of zygotic COPI activity. Examination of COPβ and COPβ' mRNA expression during normal development demonstrated that both subunits are supplied maternally and expressed ubiquitously at early stages. These subunits then become specifically up-regulated in the chordamesoderm/notochord and neural structures from tailbud stage onwards. Not only did this expression match that previously observed for COPα, but it also matched that observed in the COPI subunits δ, ε, γ2 and ζ2.

Initial identification of *sny, hap* and *dop* as the COPI subunits α, β and β' respectively, was somewhat unexpected, since the COPI complex is an essential housekeeping complex, required to maintain the structure and composition of the secretory networks and considering that removal of COPI function in yeast is lethal. Thus, characterising these COPI subunits, and hence COPI function, as responsible for specific developmental defects was unexpected as removal of such a ubiquitously expressed gene complex would be thought to be lethal. However, in reconsiliation of

the essential nature of coatomer function, embryos do die by widespread necrosis by 48 hpf. By applying current understanding of notochord developmental processes, the specific phenotype observed can be readily understood. COPI function is provided maternally, since it is essential to cell survival and zygotic transcription does not initiate until the MBT. This maternal contribution of functional COPI is sufficient for the initial survival of the embryo, even when the zygotic contribution is absent. The half-life of the COPI complex in a mammalian cell lines has been measured to be 28 hours (Lowe and Kreis, 1996). However, the notochord, which is one of the first fully differentiated tissues in the zebrafish, experiences an increased demand for secretion. Notochord cells must assist in the formation and construction of the notochord basement membrane, of which laminins are a major component (Parsons et al., 2002b; Pollard, 2002), and must also undergo vacuolation. Both of these processes place increased demands on the secretory network. Thus, since COPI function is required for maintenance of the secretory pathway, a lack of COPI function will have a more dramatic effect on notochord development. As such, as the notochord differentiates, it out-strips the maternal complement of COPI due to its increased secretory requirements. The developing chordamesoderm/notochord must therefore support the maternal COPI supply through zygotic translation, which is supplied by the notochord specific expression of the COPI subunits between tailbud and 18 somite stages. However, in the mutants *sny, hap* and *dop*, the COPα, COPβ or COPβ' subunit is defective, respectively, and hence zygotic COPI function is compromised. This then results in the specific phenotype observed before general necrosis. This same reasoning can be applied to the observed defect in melanophore pigment development, since development of proper pigmentation requires the secretory network and thus places increased demands on COPI activity. Thus,

though COPI activity is required ubiquitously for cell survival, mutations in individual COPI subunits results in specific developmental abnormalities in specific cells and tissues that place an increased demand on the secretory network, early in embryogeneis, that exceeds the maternally contributed COPI activity. Later, loss of zygotic COPI activity results in wide spread cell death due to loss and dilution of the maternally provided, functional, COPI complex.

Previous analysis of *COPα* expression had demonstrated that mRNA is specifically up-regulated within the developing notochord from tailbud stage until approximately 18 somite stage, with the notochord specific expression becoming extinguished in all but the most posterior developing tip of the notochord by 24 hpf. Analysis of *COP*β and *COP*β' mRNA expression demonstrated a similar pattern of expression, which is befitting of the notochord defect in these COPI mutants. Perhaps most interesting though, was the observation that *COPα* expression was abnormally maintained in all three COPI mutants and in embryos treated with BFA, a general inhibitor of COPI function (Coutinho, 2001). This maintenance of subunit expression within the notochord of COPI mutants at 28 hpf was also demonstrated for *COP*β and *COP*β'. Though many early notochord markers, including *ehh*, are maintained in undifferentiated notochord, due specifically to the lack of differentiation, the COPI subunits are not. Where notochord expression of *ehh* is maintained in both *sly* mutants, which lack laminin α1, and *sny* mutants, which lack COPα, COPα is maintained only in the notochord of *sny* mutants at 28 hpf. Thus, it is a specific loss of zygotic COPI function that leads to the maintenance of subunit expression at 28 hpf. This observation, combined with the observed phenotype and expression of COPI subunit mRNA during normal development, suggested a

mechanism of regulation where COPI activity acts in some way to regulate expression of the COPI subunits.

Such a system would fit with observations of the COPI mutants. Under normal conditions, in wild type embryos, the maternal contribution of COPI activity is sufficient to ensure cell and embryo survival until zygotic transcription can begin. It is also sufficient for survival in the majority of cells and tissues in the developing embryos until approximately 48 hpf, when embryos lacking zygotic COPI function undergo widespread cell death. However, in the notochord and melanophores, where there is an early increased demand for secretory network activity, to act in producing the basement membrane sheath and pigmentation respectively, the maternal contribution is insufficient. In these cells, the requirement for COPI exceeds the level of available activity. Under such conditions, the loss of sufficient COPI activity acts to up-regulate expression of COPI subunits, causing the observed notochord specific expression of COPI subunits at tailbud to 18 somite stage. The loss of zygotic COPI subunits in the mutants *sny, hap* and *dop* results in an inability to supplement the maternal COPI activity and thus, the tissues in which the maternal compliment of COPI is insufficient are affected before widespread cell death is apparent. However, since these embryos are unable to provide additional zygotic COPI activity, the increased demand on the secretory network is maintained as is the requirement for COPI activity, thus, the expression of the COPI subunits within the notochord is maintained beyond its normal temporal profile.

In such a system, available COPI can be viewed as a pool of activity, initially supplied maternally. This maternal pool is sufficient for cell maintenance throughout the early embryo, with the exception of the notochord and the melanophores, where demand for secretion exceeds the activity available in the maternal COPI pool.

Under these conditions of loss of available activity, where all COPI is recruited to the highly active secretory network, the free activity available in the pool is insufficient. This signalling results in the the up-regulation of COPI subunits to increase the pool of zygotic COPI activity. This restores the free activity of COPI to a level that is sufficient so that additional COPI is no longer required and subunit expression is extinguished. So, in wild type embryos, by tailbud stage, secretory network activity is sufficiently high to exceed the pool of maternal COPI and cause the up-regulation of COPI subunits, but by 24 hpf, zygotic COPI transcription has re-established the pool of free COPI activity and therefore COPI subunit expression is extinguished in the notochord. However, in the COPI mutants, the zygotic COPI is non-functional and hence subunit expression within the notochord is not extinguished, as the pool of free COPI activity cannot be supplemented. Thus, COPI is acting in an auto-regulatory feedback loop to maintain sufficient activity during development.

Examination of the remaining *six* coatomer subunits demonstrated that the subunit ε, δ, γ2 and ζ2 are also up-regulated specifically within the notochord and maintained in the notochords of COPI deficient embryos, thus demonstrating that, like *COPα*, *COP*β and *COP*β', these subunits are up-regulated under conditions of depleted free COPI activity. However, the subunit COPζ is not. So, under conditions where the pool of available COPI activity is depleted, a complete set of seven COPI subunits is up-regulated, via an auto-regulatory feedback mechanism, to restore the level of COPI activity.

8.2.2 The Effect of Loss of COPI Function

Visualisation of Golgi and ER structure in living cells, using fluorescent tagged proteins enabled the effect of COPI loss of function to be examined through knock-down of the COPα subunit with an ATG targeted MO or general inhibition with BFA. Loss of COPI function most prominently results in a complete loss of vacuolation within notochord cells. Wild type notochord cells demonstrate clear and distinct Golgi and ER structure, though this is confined to a small region of the cell as a result of vacuolation. In contrast, the notochord cells of COPα MO injected embryos have a complete lack of vacuolation, as demonstrated by their vastly diminished size and the observation that both the Golgi and the ER are spread throughout the cell. This dispersal of the ER and Golgi was also observed in the muscle cells of COPα MO injected embryos. In both morphant notochord and muscle cells, there is a breakdown and dispersal of the ER and a fusion and dispersal of the Golgi, due to the loss of retrograde transport. In wild type notochord and muscle cells, the ER and Golgi are distinctly localised around the nucleus and organised into discrete organelles. Thus, COPI function is vital to maintain the proper structure of both the Golgi and the ER, by acting to transport "lost" components back to their proper organelle position.

By examining the localisation of the fluorescent marker proteins over time, the nature of Golgi and ER breakdown was observed and, additionally, the time scale of this dispersal was identified. At 28 hpf, morphant cells closely resemble those observed in wild type embryos. By 31 hpf however, the ER has become completely dissembled and its contents are dispersed throughout the cells, with no distinct localisation. This breakdown and dispersal can be observed to occur gradually between 28 hpf and 31.hpf, demonstrating that the lack of COPI function becomes critical to ER structure at this stage. Golgi structure is also grossly amorphous by 31

hpf, showing a fusion of individual organelles and a dispersal of Golgi contents throughout the cell. Thus, it appears that the loss of Golgi and ER structure is not apparent at 28 hpf, but develops from this time causing the breakdown of the ER, fusion of Golgi organelles and the dispersal of secretory network contents throughout the cytoplasm.

Beginning at 28 hpf, the Golgi and ER loose normal structure and disperse throughout the cell rapidly, becoming grossly malformed over a period of two hours. This loss of Golgi and ER structure occurs in cells throughout the developing embryos. Defects in notochord differentiation are obvious by 32 hpf, COPα morphant notochord cells are considerably smaller than their wild type counter parts. Morphant notochord cells show a complete lack of vacuolation and the ER and Golgi are dispersed throughout the cytosol.

8.2.3 The UPR as an Essential Regulatory Mechanism in Developmental

The UPR is an important and only partially understood response mechanism to ER stress. Under conditions of increased secretory or protein folding demand, the UPR is active and is responsible for up-regulating many genes involved in post-translational modification, protein folding and protein transport, which then act in the ER and Golgi to alleviate stress. Much of the current understanding of UPR activation and activity stems from work in yeast and recent analysis in *S. cerevisiae* has demonstrated that amongst the many thousands of genes up-regulated in response to UPR activation, are several COPI subunits (Travers et al., 2000). This observation, combined with the nature of COPI and the observed auto-regulatory

expression of the COPI subunits, suggested that the UPR may be responsible for regulating coatomer expression during development.

The expression profile of *BiP*, a major regulator of the UPR and a commonly used marker of UPR activation, is highly similar to that observed for all seven of the notochord specific COPI subunits. Thus, it appears that the UPR is active at the same time and in the same tissues that there is specific up-regulation of COPI subunits. This observation, along with the observation in yeast that activation of the UPR result in up-regulation of COPI subunits, reinforced the suggestion that the UPR is acting to regulate the expression of COPI. Given the nature of notochord differentiation, this makes considerable sense. During notochord differentiation there is a requirement for basement membrane sheath formation, which can be provided by both surrounding tissues and by the notochord itself (Coutinho et al., 2004; Parsons et al., 2002b). The formation of this basement membrane requires the secretion of proteins, such as laminin, from notochord cells. The demand for these proteins places an increased demand on the ER's protein folding capacity and on the ER's and Golgi's ability to properly secrete proteins. This increased demand would, in cells with only the basic, maternal complement of protein folding and secretory compounds, result in a build of unfolded proteins within the ER. Thus, the UPR would be activated and the necessary components to relieve this unfolded protein backlog would be up-regulated. It therefore seems, considering the observation that UPR is active within the notochord during development, maintained within the notochord of COPI deficient embryos and that the UPR results in expression of COPI components in yeast, that the UPR is acting during development as an essential regulatory mechanism to meet specific demands in cells and tissues, in response to

increased secretory and protein folding demands, to maintain the structure and composition of both the Golgi and ER.

To further define the role of the UPR during development, MO's were targeted against the ATG start of translation sites of the key signalling components in the UPR: IRE1/XBP1, ATF-6, PERK, and the key regulator of UPR activation, BiP. By knocking down the signalling components of the UPR, the ability of the UPR to activate in response to unfolded protein build up is compromised. As such, the level of UPR response is severely reduced. In such embryos, there is a distinct lack of notochord differentiation. The observed defect in notochord differentiation in embryos deficient in UPR activation closely resembles the notochord differentiation defect observed in the COPI mutants *sny, hap* and *dop*. Additionally, embryos raised to 48 hpf demonstrate an obvious similarity to the COPI mutants, not only in terms of lack of notochord differentiation and associated defects, but also in the distinct lack of proper melanophore development.

Thus, a lack of UPR activation appears to phenocopy the COPI mutants in terms of the lack of notochord differentiation and loss of pigmentation, supporting a role for the UPR in the requirement for COPI in both notochord and melanophore development. The UPR is thus acting in response to increased secretory and post-translational adaptory demands by up-regulating the proteins involved in such processes, including the COPI subunits. Interestingly, knockdown of UPR activation also results in obvious neural defects. This fits with the observed expression profile of BiP, where expression is noted not only in the developing notochord, but also in the developing brain. Interestingly, though COPI subunits are also expressed with the developing brain, the COPI mutants show no significant defects in neural development. However, it may be that the UPR is acting in both the notochord and

the developing brain in response to differing pressures on the ER as a result of differentiation and that this activation results in the up-regulation of all UPR target genes, including the COPI subunits. However, only a subset of these genes are necessary to alleviate the specific stress of each developing tissue. Thus, in notochord, the UPR up-regulates all the UPR genes, where only a subset of genes, including COPI, are required for proper notochord differentiation, whereas in the brain, a different subset, not including COPI, is necessary for proper development. Hence the notochord specific defect in COPI mutant embryos and the combined notochord and neural defects in UPR inactivated embryos.

Further evidence for the UPR's role in notochord development has been resolved from expression analysis in UPR inactivated morphant embryos. In such embryos it was clearly demonstrated that UPR inactivated morphant's do not have a completely inactivate UPR, despite the obvious notochord, melanophore and neural defects, since BiP and COPβ' were both up-regulated in response to BFA treatment. Indicating that a partially active UPR is insufficient to maintain proper notochord development. However, more informatively, the partially UPR inactivated embryos demonstrated a distinct maintenance of *COP*β' and *BiP* at 28 hpf when raised under normal conditions, similar to the COPI mutants. In such embryos, the partially inactivated UPR is insufficient for the required gene up-regulation necessary to meet the increased demands for Golgi and ER activity, resulting in the observed defects in development. This also results in maintained activation of the UPR, since the stress on the ER and Golgi is not alleviated by zygotic transcription. This maintained expression of *COP*β' and *BiP* at 28 hpf closely resembles the maintained expression of *COP*β' and *BiP* in COPI mutants. In both the COPI mutants and UPR activation morphant's, there is a lack of components necessary to alleviate the increased

demands on the Golgi and ER, due to either a specific lack of functional COPI or the lack of many important genes, including the COPI subunits, respectively, and as such, both result in maintained activation of the UPR and therefore the maintained expression of *COP*β' and *BiP* at 28 hpf.

It thus appears that the UPR is active during development in tissues and cells that encounter increased translational and secretory demands, to help maintain the structure, composition and function of the ER and Golgi. The UPR is active in both the notochord and brain during development, and a loss of proper activation causes defects in the development of neural structures, notochord and melanophores as well as resulting in widespread necrosis by 48 hpf. However, the specific stresses of these tissues vary, such that loss of COPI function, which is up-regulated by the UPR, results only in defects in notochord and melanophore development. So, the UPR responds to general ER stress during development, to up-regulate the range of gene regulated by UPR activation, though only a subset are required for alleviating the specific stresses within the developing tissue or cell. Further work to demonstrate decisively that the UPR does indeed act to regulate COPI expression during development remains, and is a source of continued effort. However, the evidence put forward in this thesis supports the idea that the UPR is required for proper development and that this requirement may result in the upregulation of UPR target genes involved in membrane trafficking, secretion, ER and Golgi maintenance, translation and post-translational modification, which would include the COPI subunits and numerous other genes involved in vesicular transport.

Such a role for the UPR is further supported by recent work in plasma cell differentiation (Brewer and Hendershot, 2005; Iwakoshi et al., 2003a). In such work, it has been demonstrated that XBP1 is essential for differentiation of plasma cells

(Iwakoshi et al., 2003b). This work demonstrated that IRE1 spliced XBP1 was able to restore immunoglobulin production production in $XBP1^{-/-}$ cells, where unspliced XBP1 was unable to do so and that XBP1 splicing occurs normally during terminal B cell differentiation, linking the UPR to differentiation of plasma cells. This, coupled with the observation that XBP1 splicing is dependent on the production of immunoglobulin, since the prevention of protein synthesis by cycloheximide in B cells results in a loss of XBP1 splicing (Iwakoshi et al., 2003b), links the UPR with the differentiation of plasma cells via its role in ER stress and in maintaining the secretory and translatory potential of a cell. Indeed, when viewing plasma cells as antibody factories', which are responsible for secreting massive quantities of soluble protein, a role for the UPR in their development can be compared directly to the role proposed for the UPR in notochord, as put forward in this thesis.

The demonstration that the UPR, which is considered a stress response mechanism, acting to protect and restore the function of the ER under conditions unfolded protein stress, acts during development to meet the specific secretory and translatory demands of developing cells demonstrates that cell stress protection mechanisms can and do function specifically during development. Such an observation opens up the possibility that other "stress" response mechanism might also have vital roles during development to maintain cells under the strenuous conditions they encounter as they differentiate.

8.3 Future Work

The notochord has proven to be a useful developmental system in zebrafish. Work on the ENU generated zebrafish notochord mutants has demonstrated essential

and specific roles in early development for both Laminin and Coatomer, despite their roles throughout the embryo. Such roles would have been difficult to define using mouse, where loss of laminin can lead to implantation defects and where loss of coatomer function would lead to early lethality. This thesis has concentrated on two related projects; the positional cloning and identification of the last zebrafish notochord mutant, *doc* and the characterisation of COPI expression and regulation during development. Though many questions still remain, especially concerning the nature of doc's role in notochord differentiation and its possible signalling function downstream of sheath formation, much has been learnt about the process of both notochord development and the general mechanisms of development.

Obvious immediate aims are the further characterisation of the role of doc in notochord development. One of the most beneficial examinations would be to determine what other proteins interact with doc, since doc may be acting directly in signalling the notochord cell to differentiate in response to sheath formation and thus, may be interacting with either the basement membrane sheath directly or with transmembrane proteins that are themselves interacting with the sheath. Expression of a tagged protein would enable isolation of doc from embryos, and associated proteins could rapidly be examined through mass spectroscopy techniques. Associated binding partners could also be easily identified through the use of yeast two hybrid screens, of which there are several commercially available for the zebrafish. It should therefore be relatively simple to identify proteins that are able to interact with doc and from this information, it should hopefully be possible to place doc into a system or signalling mechanism that befits its role in notochord differentiation. It may be that such information will enable the linking of doc to the differentiation and vacuolation of notochord cells and to the formation of the

basement membrane sheath, or it may be that doc is involved in other processes required for differentiation. The role of any identified interactors could be relatively easily tested using a MO knockdown approach and by identifying proteins that interact with doc it should hopefully be possible to place this novel gene into a system involving known and characterised factors, thus helping to reveal the mechanism by which doc functions in notochord differentiation. Further to this, information about how this protein functions should also be revealed by its structure and so another obvious avenue for investigation is the crystallisation of the protein and examination of its three-dimensional structure. By comparing this information to the known structures of well characterised proteins, it should be possible to hypothesise how doc functions.

The identification of *doc* represents the cloning of the last of the seven "dwarf" mutants in zebrafish. These mutants have revealed specific roles for the laminin chains α1, β1 and γ1, as well the coatomer coat protein, in early development. Both of these families of mutants represent well-characterised proteins that can be attributed clear roles in the development of the notochord, where laminin forms a vital part of the notochords basement membrane sheath and where COPI acts in both secreting the components of the sheath and in vacuolation. The identification of *doc* and the further characterisation of how it is acting during development to maintain notochord differentiation should hopefully provide a link between the formation of the sheath, vacuolation and proper differentiation, offering the opportunity to identify further processes and components that act during the development of the notochord.

The mutant *sly* was demonstrated to encode a non-functioning COPα protein (Coutinho, 2001) and MO knockdown of the subunits *COP*β and *COP*β' was

demonstrated to phenocopy the mutant *hap* and *dop*. Work as part of this thesis demonstrated that the mutants *hap* and *dop* encode non functioning COPβ and COPβ' subunits respectively. Analysis of the COPI mutants has revealed that the majority of COPI subunits are up-regulated within the notochord during development and maintained in the notochords of COPI mutants. Examination of the mechanism of this regulation demonstrated that the UPR stress response mechanism acts as an essential regulatory mechanism during development to maintain the function and composition of the ER and Golgi. The UPR is active in the notochord during development and acts to up-regulate genes essential for differentiation, including the COPI subunits. However, this work also revealed the essential nature of the UPR for proper neural development, where a lack COPI function does not appear to result in obvious neural defects. Thus, examination of which UPR regulated genes are required for proper neural development is an obvious extention to current work. It may be that the UPR is functioning in several developmental process, including later processes that have not been revealed through MO knockdown and that UPR regulated genes can be divided into different classes depending on which developmental process/processes they are required for. For example, though the majority of the COPI subunits were up-regulated in all tissues in response to UPR activation, one subunit demonstrated discrete expression patterns where mRNA levels were raised only in specific regions of UPR activation. Thus, it may be that the UPR has several specific responses, comprising varying sets of response genes, as well as a unanimous gene set, up-regulated under any conditions of UPR activation. Examination of what genes are up-regulated in either neural or notochord tissue during development, through, for examble, micro-array analysis, would help to define if the UPR is able to function in such a manner and if so, then what genes are

regulated under what conditions. On a more general note, identifying what genes respond to UPR activation would also be of interest. Since many UPR responsive genes have been classified in yeast, it should be possible to identify homologues for many of these in zebrafish from the genomic sequence and then characterise if they respond to the UPR in zebrafish as they do in yeast. It would then be relatively simple to identify upstream genomic sequences for these genes, which could then be used to perform comparative searches for conserved regulatory elements. Such searches have demonstrated success in yeast (Patil et al., 2004). These regulatory elements could then be used to search for further UPR regulated genes.

Much of the emphasis of developmental biology has been on the events involved in altering both the genetic and physiological make-up of cells during development of a properly differentiated and patterned adult. However, the mechanisms involved with maintaining the basic functions of the cell during these complex events have been little considered. Here it has been shown that the UPR, which is involved with maintaining the function and composition of the ER and Golgi under conditions of increased secretory and translatory load, acts during development to maintain the development of cells that exceed the natural complement of Golgi and ER functional proteins. Moving beyond the specific scope of the UPR, the demonstration that such a protective mechanism is required for proper development opens up the possibility that other such mechanisms could also be functioning in such a manner. Work on the heat shock response in *Arabidopsis* has demonstrated a chaperone function for heat shock proteins under normal conditions, allowing aberrant proteins to fold correctly and acting as a "buffer" for mutations (Queitsch et al., 2002). Thus, it may be that the heat shock response functions in a similar way during zebrafish development, allowing proteins to fold

correctly under the stressful conditions encountered during development. In a broader view, it is possible to see how such stress response mechanisms might evolve in simple organism to allow survival in non-optimal conditions but, as they evolve into complex muticellular organisms, such mechanisms become vital for cell survival during the strenuous conditions encountered during differentiation. Such mechanisms would provide protection against potentially lethal conditions that would arise as the processes of development evolved and may well be essential to provide room for the 'evolutionary experimentation' that occurred during the transition from single celled eukaryotes to a complex, highly patterned, muticellular organisms.

Analysis of the UPR and other "stress" response mechanisms may yield much information about how a developing embryo is protected and buffered against the stresses encountered during normal development and may provide much information about the regulation of the essential maintenance processes involved in the complex events that occur during the development from a simple unspecified collection of cells to a fully functioning adult.

8.4 Summary

- *doc* encodes a novel and conserved 14 WD40 domain protein.
- The mutants *hap* and *dop* encode COPβ and COPβ' respectively.
- All of the COPI subunits, with the exception of COPζ, are expressed in the notochord during development
- The same subunits are maintained in COPI deficient embryos.
- Loss of COPI results in a breakdown of the ER and Golgi.

- The UPR is vital for proper notochord, melanophore and neural development.
- The UPR may be responsible for up-regulting COPI subunits under conditions of ER stress to maintain Golgi and ER function.