Chapter Five

Characterisation of *hap, dop* and the COPI Subunits

5 Characterisation of *hap, dop* and the COPI Subunits

In this this chapter I will provide a summary of the process of secretion, paying particular attention to the role and function of coatomer in the secretory network. The processes involved in coatomer vesicle coat formation will be discussed and the composition of the COPI complex will be reviewed. Additionally, current understanding of coatomer function in zebrafish will be briefly covered, leading into work performed as part of this thesis to characterise the *dop* and *hap* mutant loci and to characterise the remaining COPI subunits during zebrafish development.

5.1 Introduction

One of the key features of eukaryotic cells is the presence of an intricate network of endomembranes that function in a system involved with the exchange of macromolecules between cells and their environment. The secretory pathway constitutes a large part of this network, involving the endoplasmic reticulum (ER), Golgi and the trans-Golgi. The secretory network functions to deliver newly synthesised proteins, lipids and carbohydrates to the surface of the cell from the ER, with pre-secretory modification being made as the macromolecules move through the system. Much of the movement involved in this pathway is carried out by the transport of secretory cargo between distinct membrane bound organelles. However, the organelles themselves maintain their characteristic set of resident components. Coated vesicles function in all stages of secretion, acting in transport from the ER to

the Golgi, as in the case of COPII (Barlowe, 1998; Schekman and Orci, 1996), in vesicular trafficking within the endosomal membrane system between the plasma membrane and the trans-Golgi, as in the case of clathrin (Schmid, 1997), in anterograde and retrograde transport within the Golgi (Orci et al., 1997) and in the recycling of component proteins from the Golgi to the ER, as in the case of COPI (Letourneur et al., 1994). The action of these vesicles, specifically the COPI coated vesicles, contribute much to the maintenance of the complement of Golgi resident components.

The current model of the secretory network combines elements of the two classical models of secretion: the vesicular transport model and the cisternal maturation model. The vesicular transport model relies on the premise that the secretory network consists of stable, autonomous organelles and that secretory cargo is transported forward through the secretory network, by COPI and COPII coated vesicles, with 'resident' proteins recycled to their appropriate organelles via COPI mediated transport. The cisternal maturation model holds that the Golgi is a dynamic, steady state system, where secretory cargo is maintained within Golgi compartments and progresses through the Golgi stack as the Golgi compartments mature. In this model, maturation is balanced by a return flow of Golgi resident proteins by COPI vesicles. These two models have been combined to offer a third model, where aspects of both are incorporated. In this third model, large cargos of protein aggregates, for example procollagen, progress through the cisternal maturation model, with smaller cargo molecules being moved rapidly through the secretory system through forward transport in COPI vesicles. However, recent work has suggested that vesicular transport plays only a small part in anterograde transport of secretory cargo (Martinez-Menarguez et al., 2001; Mironov et al., 2001). Thus,

the true model of secretion may be closer to the cisternal maturation model than the vesicular transport model, where the cisternae move in a trans to cis direction as they mature. The transport and modification would thus occur as the cisternae mature, which itself occurs through the retrograde action of COPI vesicles in maintaining the complement of adaptory components at the proper position. This model can be compared to a conveyor belt, where cisternae move forwards, developing as they do so through changes in their compliment of adaptory proteins that proceed to modify the secretory cargo. COPI then functions on the underside of the conveyor belt, to maintain the appropriate type of compartment at the proper trans/cis location through the retrograde transport of resident components (see **Figure 5.1** for overview).

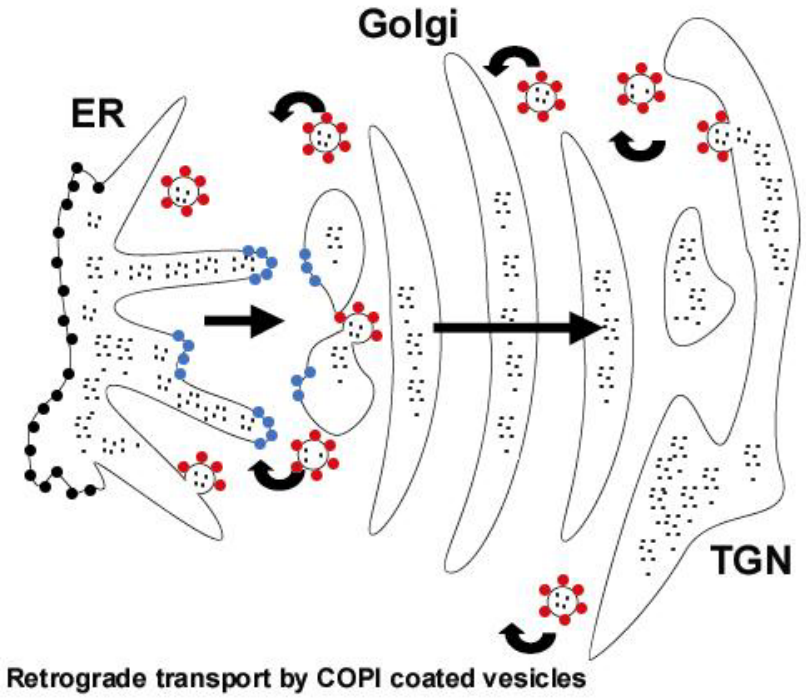

Figure 5.1 Overview of secretion.

Cargo is transported from the ER to the Golgi via COPII action (blue vesicles) and moves through the Golgi network within vesicles via vesicular maturation. Cargo eventually arrive at the trans-Golgi-network (TGN). COPI vesicles (in red) act to maintain Golgi functional identity at a specific trans/cis location through retrograde transport of components, both within the Golgi and from the Golgi to the ER.

COPI is a heptameric complex, made up of the coatomer subunits α, β, β', ε, γ, δ and ζ. Individual coatomer subunits were first identified through the analysis of vesicle coats formed in vitro from Golgi enriched membranes (Orci et al., 1986). In the case of the coatomer γ and ζ subunits, two novel isotypic variants have been identified in mammals (Wegmann et al., 2004) where only one isotype is known for the α, β, β', ε and δ subunits. The COPI subunits form two commonly isolated sub-complexes within the main complex, one consisting of the α, β' and ε subunits, which comprises the B-COPI sub-complex, and the other consisting of the γ, δ., ζ and β subunits, which comprises the F-COPI sub-complex (Fiedler et al., 1996) (**Figure 5.2**). In the case of the F-COPI complex, there is considerable homology to the subunits of the clathrin AP1 and AP2 subunits, where β-COP and γ-COP show homology to the large AP subunits and δ-COP and ζ-COP show homology to the medium and small AP subunits (Boehm and Bonifacino, 2001). More recent work has strengthened the proposed homology between subunits of clathrin and COPI. Structural studies on the γ-COP subunit have demonstrated significant similarity between a carboxyl terminal domain and the carboxyl terminal appendage domains of the α and β subunits of the AP2 unit of clathrin (Hoffman et al., 2003; Watson et al., 2004). This domain was demonstrated to interact with an ARF GTPase Adaptory Protein (ARFGAP) in both yeast and mammalian cells. These data reinforces the idea that both COPI and clathrin act through similar mechanisms in vesicle formation.

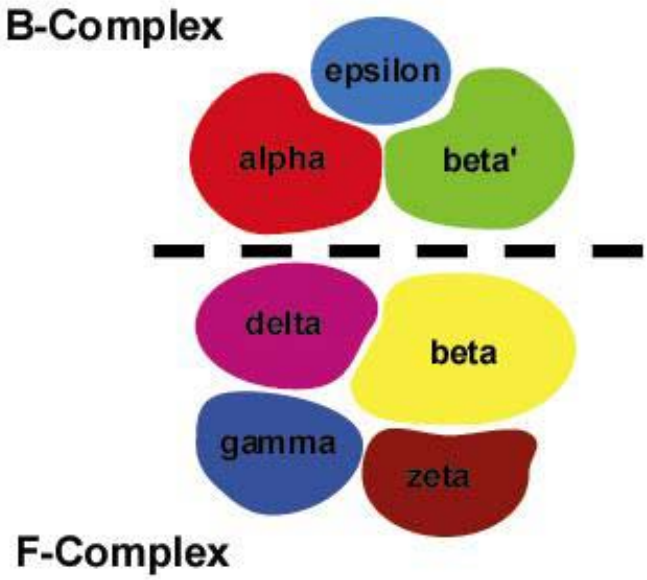

Figure 5.2 COPI complex subunit subcomplex composition.

The COPI complex is formed from two subcomplex's, the B-complex, made up of COPα, COPβ' and COPε, and the F-complex, made up of COPγ, COPβ, COPδ and COPζ. The F-complex bears similarity to the clathrin AP1 and AP2 subunits.

COPI mediated retrograde transport, involved in carrying ER components from post-ER compartments back to the ER is mediated through direct physical interactions between the coat protein and the cargo protein. In the case of proteins such as p24 family members, a C-terminal dilysine (KKxx and KxKxx) motif allows the transport of proteins back to the ER (Cosson et al., 1996; Cosson and Letourneur, 1994; Fiedler et al., 1996). In this instance it is known that the γ subunit recognises these dilysine motifs, thus providing a mechanism for COPI transport of proteins containing these sequences (Harter et al., 1996; Harter and Wieland, 1998). Other proteins, such as BiP, contain a C-terminal Lys-Asp-Glu-Leu KDEL sequence that is recognised by the KDEL receptor and targets proteins for retrograde transport (Munro and Pelham, 1987; Orci et al., 1997). The recent characterisation of the Rer1p protein in yeast, a Golgi membrane protein required for the proper retrieval of a set of ER proteins, as able to interact in vitro with an α/γ coatomer complex and also as a protein that requires proper COPI activity for retrieval, since a lack of COPI activity causes mis-localisation to the vacuole, has demonstrated that other, possibly as yet uncharacterised, mechanisms of COPI cargo targeting may exist (Sato et al., 2001). COPI is also thought to act, somewhat indirectly, in localising proteins to the Golgi through retrieval of Golgi components from the vacuole to the Golgi. This has again been shown to be mediated by COPI binding to lysine rich, though not the conventional dilysine, C-terminal domains (Abe et al., 2004). Finally, some COPI coated vesicles have been observed carrying anterograde bound cargo, since a distinct population of vesicles was noted that carried cargo including proinsulin and VSV G protein (Orci et al., 1997). Thus, an as yet uncharacterised domain may act in directing cargo in an anterograde direction, in the manner that the KDEL sequence and dilysine motifs can direct cargo in a retrograde COPI pathway.

COPI dependent vesicular budding relies on the GTP dependent recruitment of GDP-bound ARF1 and COPI to Golgi membranes (Donaldson et al., 1992; Palmer et al., 1993; Rothman, 1994). The GTP loading of ARF1 is catalysed by GEFs, where the ARF1 interacting GEFs in yeast have been demonstrated to share a Sec7 domain (Peyroche et al., 1999). ARF1-GDP interacts specifically with the Golgi before nucleotide exchange (Gommel et al., 2001). In this context p23, a type I transmembrane protein, belonging to the p24 family, is known to play a key role in COPI coat assembly and has been identified as an ARF1-GDP receptor (Bremser et al., 1999; Gommel et al., 2001; Sohn et al., 1996). This p23 interacting ability has been localised to the c-terminal 22 residues of ARF1. However, ARF1 nucleotide exchange can take place in the presence of liposome's lacking any protein, and hence binding to p23 is not a requirement for nucleotide exchange (Beraud-Dufour et al., 1999). Though in a biological context p23 may act to direct ARF1 to desired sites and thus to its GEF. Interestingly, ARF hydrolysis of GTP is itself affected by p24 family members, so that ARF1 is likely undergoing continual cycles between the GDP and GTP bound states. Since only the GTP bound form of ARF1 interacts with the membrane, through the exposure of a myristoyl group (Goldberg, 1998), p23 may also function to maintain the concentration of ARF1-GDP at the Golgi membrane. Nucleotide exchange instigates release of ARF1-GTP from p23 (Gommel et al., 2001), generating two binding sites for COPI at the membrane; on ARF1-GTP (Zhao et al., 1997; Zhao et al., 1999), and on p23 (Dominguez et al., 1998; Sohn et al., 1996). However, the recruitment of COPI depends entirely on the proper activation and membrane localisation of ARF1 (Donaldson et al., 1992; Palmer et al., 1993). Following this recruitment, coatomer and ARF1-GTP polymerise to from a defined macromolecular structure that is likely to limit the size of forming vesicles (Bremser

et al., 1999; Reinhard et al., 2003). The rate of GTP hydrolysis and release of ARF1 from membranes has been suggested to act as a timer, triggering release of coat protein so that vesicles may fuse with target membranes. The rate of hydrolysis of GTP by ARF1 depends on the interaction with ARFGAP and COPI (Goldberg, 1999). However, recently the carboxy terminal FFxxRRxx sequence of the p24 protein hp24a, which binds to COPI, has been shown to reduce the ability of COPI to stimulate ARFGAP, though the FFxxKKxx sequence, in proteins that also bind COPI do not (Goldberg, 2000). This may represent 'preferred' cargo, which would fit with a model where COPI vesicles are continually forming on membranes and that only those that capture the 'preferred' cargo are stabilised, through inhibition of COPI stimulation ARFGAP, and are thus able to complete coat formation.

ARF GTPases are activated by GEFs of the Sec7 family, which promote the exchange of GDP for GTP (Peyroche et al., 1996). This oscillation between the GDP and GTP bound states switches ARF between the 'non membrane binding' and 'membrane binding' state (Amor et al., 1994; Goldberg, 1998). ARF1 activity is vital for coatomer coat formation and its activity can be inhibited through the activity of the fungal metabolite brefeldin A (BFA) (Donaldson et al., 1992; Helms and Rothman, 1992). Treatment of cell with BFA causes a redistribution of COPI components to the cytoplasm, a loss of COPI vesicle formation, a redistribution of Golgi components to the ER and a breakdown of Golgi structure (Donaldson et al., 1990; Lippincott-Schwartz et al., 1989; Orci et al., 1991). It has been demonstrated that BFA inhibits the action of GEFs for ARF1, blocking ARF1 activation and therefore the proper assembly of COPI vesicles (Donaldson et al., 1992; Helms and Rothman, 1992). More recent work has demonstrated that this inhibition occurs

through the stabilisation of the ARF1-GDP-Sec7 complex, rather that through the inhibition of interactions between Sec7 GEFs and ARF1 (Peyroche et al., 1999).

Loss of coatomer function in yeast, mammalian and zebrafish cells is lethal (Coutinho et al., 2004; Gerich et al., 1995; Guo et al., 1994). Thus, coatomer can be assumed to have a 'house-keeping' function, being necessary in all cells to maintain the secretory networks. However, it has been demonstrated specifically that loss of some coatomer subunits in zebrafish result in specific developmental phenotypes before widespread lethality is apparent ((Coutinho et al., 2004), this thesis). Moreover, COPα has been demonstrated to be expressed in a manner suggesting that demand for and activity of COPI acts in some way to regulate the expression of *COPα* and thus control the pool of available coatomer activity. In this section, I will characterise the coatomer subunits that are responsible for two zebrafish mutants, *dop* and *hap*. In addition, I will examine the expression profile of the remaining COPI subunits, both during normal development and under conditions where COPI activity is lost, to examine the possible regulation of COPI subunit expression by available coatomer function.

5.2 Identifying Mutations in *hap*^{tm285b} and *dop*^{m341}

5.2.1 Radiation Hybrid Mapping of COPβ

Using publicly available sequence EST and genomic sequence data for *COP*β, two primer sets were designed to RH map the loci. Using the LN54 zebrafish radiation hybrid panel (Hukriede et al., 1999), *COP*β was mapped to within 12.90

centi-Rays (cR) of the marker z20853 on LG7. Previous mapping efforts had successfully linked *hap* to the same LG.

5.2.2 Radiation Hybrid mapping of COPβ'

As for *COP*β, publicly available sequence EST and genomic sequence data for *COP*β' was used to design two primer sets to RH map the loci. Using the LN54 zebrafish radiation hybrid panel (Hukriede et al., 1999), *COP*β' was mapped to within 7.47 cR of *elrA* on LG2. As with *hap*, previous mapping efforts had successfully linked *dop* to the same LG.

5.2.3 Cloning the Full Length COPβ Gene

Publicly available EST and genomic data was compared to the mouse COPβ to generate a scaffold of known sequence. This was then used to design primers to cover the gaps in available sequence from cDNA, 3' and 5' RACE products. Sequencing of these products allowed the reconstruction of a full length cDNA of 3,239 bases. The generated cDNA sequence predicted a protein of 953aa that demonstrated 93% identity to both the human and mouse *COP*β genes.

5.2.4 Identification of the Mutation in *hap*^{tm285b}

Using the full length cDNA sequence, primers were designed to generate overlapping COPβ cDNA fragments from *hap*^{tm285b} mutant embryos. Sequencing of these fragments identified a non-sense mutation in the codon encoding residue E_{499}, where a G to T substitution produces an early termination. Confirmation of this mutation was provided through re-sequencing of the loci containing the mutation from genomic DNA obtained from *hap*^{tm285b} mutant embryos. Final confirmation of the identified mutation in *hap*^{tm285b} mutants was provided through dCAPS analysis, whereby the identified mutation was shown to be present in all six mutant embryos but absent in one of the six wild type embryos, with the remaining five demonstrating heterozygosity.

5.2.5 Cloning the Full Length COPβ' Gene

Publicly available EST and genomic data was compared to the mouse COPβ' to generate a scaffold of known sequence. This was then used to design primers to cover the gaps in available sequence from cDNA, 3' and 5' RACE products. Sequencing of these products allowed the reconstruction of a full length cDNA of 3,170 bases. The generated cDNA sequence predicted a protein of 934aa that demonstrated 86% and 87% identity to the human and mouse *COP*β' genes respectively.

5.2.6 Identification of the Mutation in *dop*^{m341}

Using the full length cDNA sequence, primers were designed to generate overlapping COPβ' cDNA fragments from *dop*^{m341} mutant embryos. Sequencing of these fragments identified a non-sense mutation in the codon encoding residue Y_{761}, where a T to A substitution produces an early termination. Confirmation of this mutation was provided through re-sequencing of the loci containing the mutation from genomic DNA obtained from *dop*^{m341} mutant embryos. Final confirmation of the identified mutation in *dop*^{m341} mutants was provided through dCAPS analysis, whereby the identified mutation was shown to be present in all six mutant embryos tested but absent in two of the six wild type embryos, with the remaining four demonstrating heterozygosity.

5.3 Expression of COPβ and COPβ'

5.3.1 Expression of COPβ mRNA

Using the generated full length cDNA sequence, primers were designed to amplify a 997bp fragment from wild type cDNA. From this, an *insitu* riboprobe for *COP*β was synthesised. This probe was then used to examine the expression of *COP*β in staged wild type embryos; at approximately 32 cell stage, shield stage, tail-bud stage, 5 somite stage, 14 somite stage, 24 hpf and in the three coatomer mutants; *sny, hap* and *dop* at 28 hpf.

*COP*β is first observed at 32 cell stage, demonstrating that it is maternally expressed. The expression continues through to shield stage, when expression is

ubiquitous. By tail-bud stage, the general ubiquitous expression is lower, though still present and there is a noticeable up-regulation specifically within the chordamesoderm. This chordamesoderm/notochord localised up-regulation continues through to 14 somites. Expression above the background levels are also detected in the developing brain during somitogenesis. However, 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, where notochord cells are not yet fully differentiated (**Figure 5.3**).

The comparison of *COP*β expression in both mutant and wild type sibling *sny*, *hap* and *dop* mutants demonstrated that the chordamesoderm/notochord expression of *COP*β is maintained in 28 hpf *sny*, *hap* and *dop* embryos whereas in 28 hpf sibling wild type embryos, the chordamesoderm specific up-regulation is extinguished. The background levels of *COP*β remain unaffected in *sny*, *hap* and *dop* mutants (**Figure 5.4**).

5.3.2 Expression of *COP*β' mRNA

Using the generated full length cDNA sequence, primers were designed to amplify a 929bp fragment from wild type cDNA. From this, an *insitu* riboprobe for *COP*β' was synthesised. This probe was then used to examine the expression of *COP*β' in staged wild type embryos; at approximately 32 cell stage, shield stage, tail-bud stage, 5 somite stage, 14 somite stage, 24 hpf and in the three coatomer mutants; *sny*, *hap* and *dop* at 28 hpf.

As with *COP*β, *COP*β' is maternally expressed, with expression continuing through to shield stage at which point expression is ubiquitous. By tail-bud, the ubiquitous level of expression is lower, though still present and expression within the chordamesoderm is specifically up-regulated. This chordamesoderm/notochord localised up-regulation continues through to 14 somites. Elevated levels of expression are also detected in the developing brain 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 (**Figure 5.5**).

Comparison of *COP*β' expression in both mutant and wild type sibling *sny*, *hap* and *dop* mutants demonstrated that, as observed with *COP*β the chordamesoderm/notochord expression of *COP*β' is maintained in 28 hpf *sny*, *hap* and *dop* embryos whereas in 28 hpf sibling wild type embryos the chordamesoderm specific up-regulation is extinguished. Background levels of *COP*β' remain unaffected in *sny*, *hap* and *dop* mutants (**Figure 5.6**).

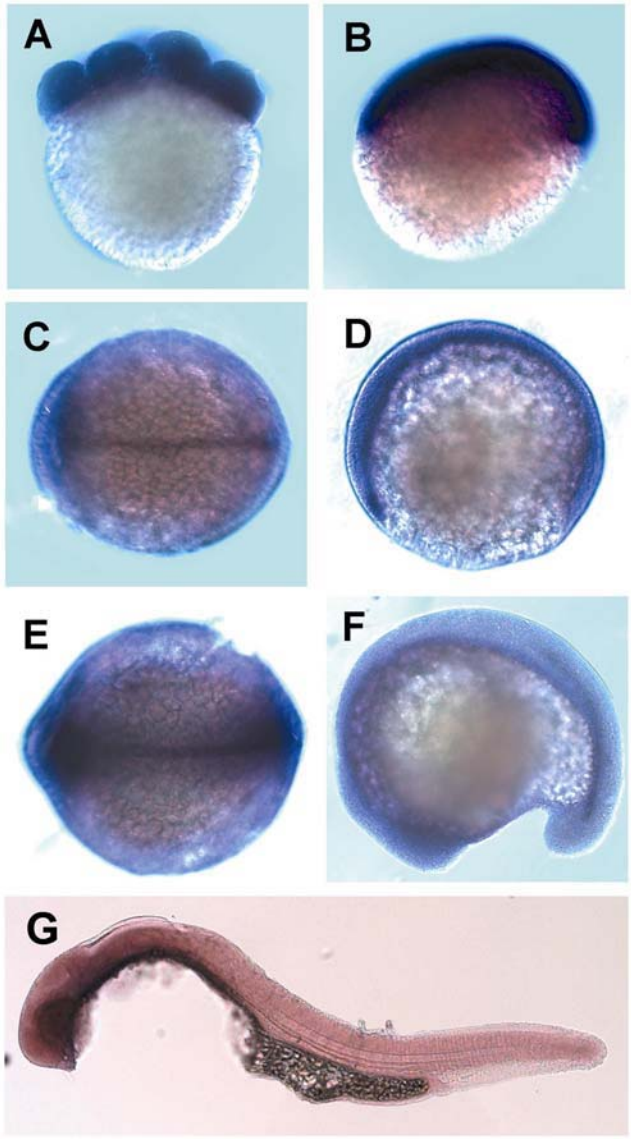

Figure 5.3 Staged expression profile of *COP*β.

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 *COP*β 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 14 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.

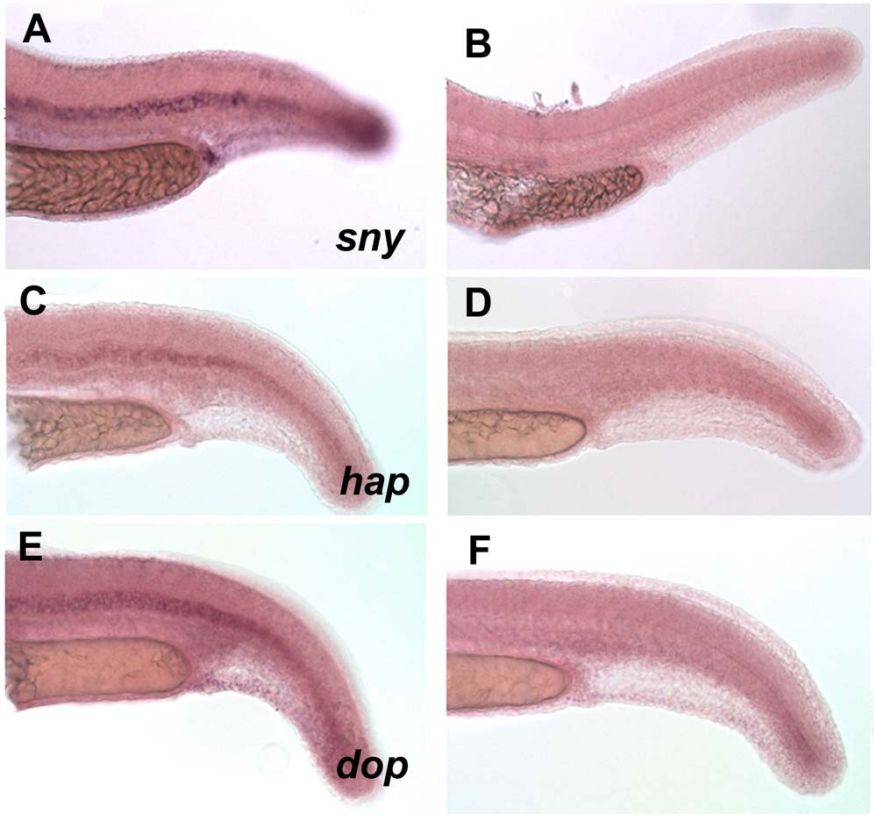

Figure 5.4 Expression profile of *COP*β in COPI mutants and wild type embryos at 28 hpf.

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 *COP*β is maintained within the undifferentiated notochord of the COPI mutants *sny* (**A**), *hap* (**C**) and *dop* (**E**). The chordamesoderm/notochord specific expression of *COP*β is shut down in the properly differentiated notochord wild type siblings of *sny* (**B**), *hap* (**D**) and *dop* (**F**).

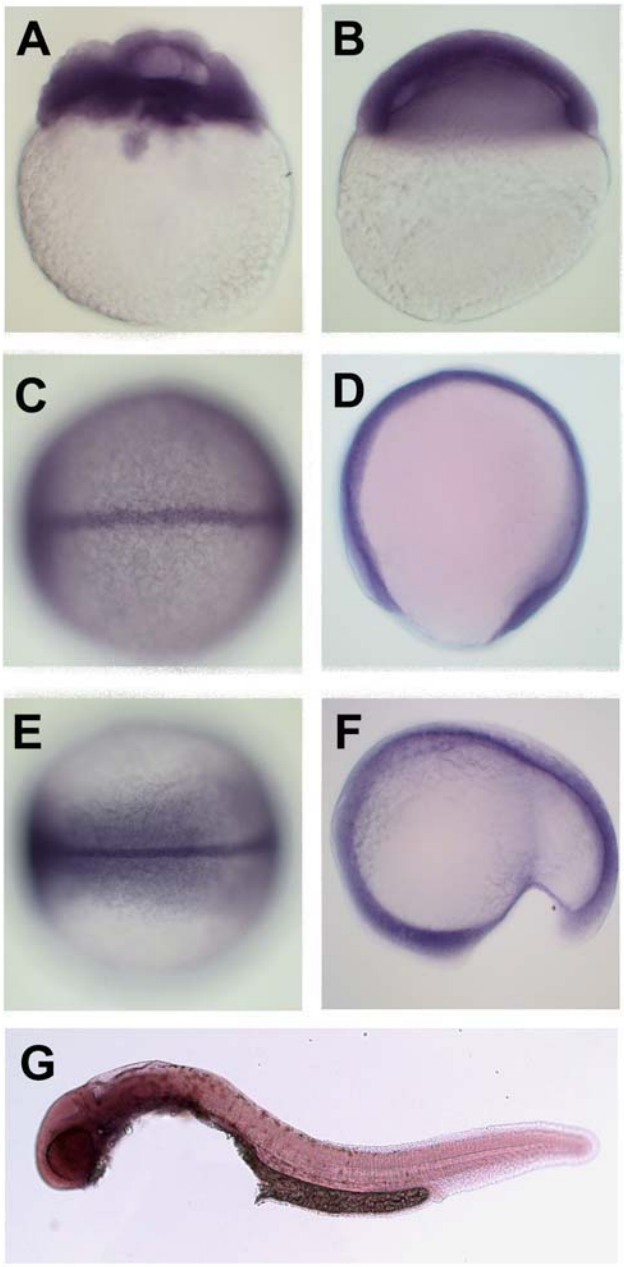

Figure 5.5 Staged expression profile of *COPβ*'.

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 *COP*β' 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 14 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.

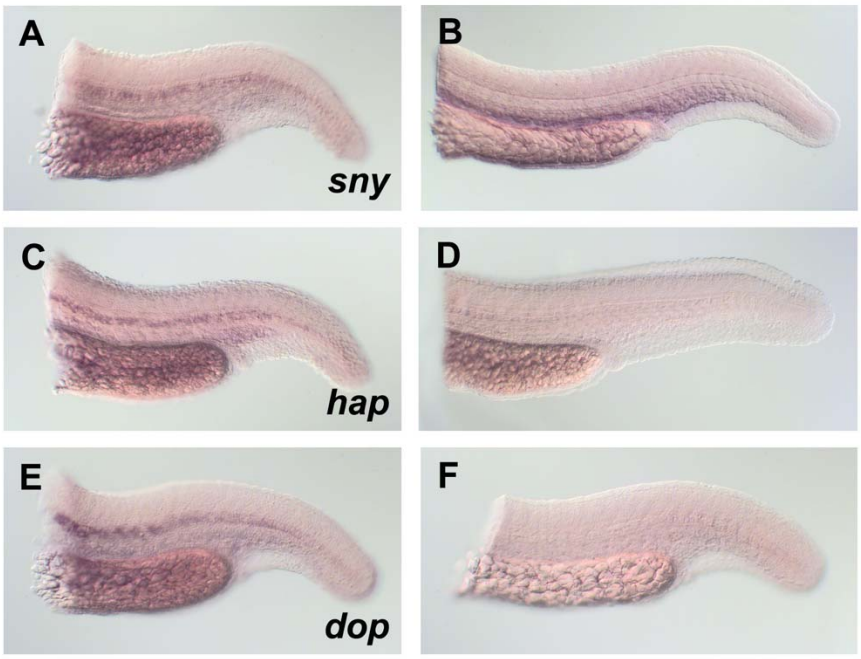

Figure 5.6 Expression profile of *COPβ'* in mutant and wild type embryos at 28 hpf.

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 *COP*β' is maintained within the undifferentiated notochord of the COPI mutants *sny* (**A**), *hap* (**C**) and *dop* (**E**). The chordamesoderm/notochord specific expression of *COP*β' is shut down in the properly differentiated notochord wild type siblings of *sny* (**B**), *hap* (**D**) and *dop* (**F**).

5.4 Identification of the zebrafish COPI subunits

Publicly available zebrafish EST and genomic data was compared to the mouse COPε, COPδ, COPγ, COPγ2, COPζ and COPζ2 genes to generate scaffolds of known sequence. Compiled EST data predicted cDNAs of 1258, 1818, 3005, 1034 and 915 bases for COPε, COPδ, COPγ2, COPζ1 and COPζ2 respectively. A homologue for zebrafish COPγ1 has yet to be found and exhaustive EST searches have returned no sequence, however, the incomplete nature of the genome and the presence of two COPγ genes in many other higher organisms suggests that a secondary COPγ homologue has yet to be identified. The generated cDNA sequences for COPε, COPδ, COPγ2, COPζ1 and COPζ2 predicted proteins of 481, 509, 873, 198 and 189 amino acids respectively.

5.5 Expression of COPI subunit mRNA

Using the generated sequence for *COPε*, *COPδ*, *COPγ2*, *COPζ* and *COPζ2* primers were designed to amplify a 722bp, 932bp, 790bp, 864bp, and 742bp fragments respectively, from wild type cDNA. From these fragments, *insitu* riboprobes for the *COPI* subunits were synthesised. These probes were then used to examine the expression of the *COPI* subunits in staged wild type embryos; at approximately 32 cell stage, shield stage, tail-bud stage, 5 somite stage, 14 somite stage, 24 hpf and in the three coatomer mutants; *sny*, *hap* and *dop* at 28 hpf.

5.5.1 Expression of *COPε*, *COPδ*, *COPγ2* and *COPζ2* mRNA

Similar to the expression of *COP*β and *COP*β',the COPI subunits ε, δ, γ2 and ζ2 are all maternally expressed. Ubiquitous expression continues through to shield stage and by tail-bud stage, the ubiquitous level of expression is lower, though still present. At this stage, expression within chordamesoderm cells is specifically up-regulated, as observed with *COP*β and *COP*β'. This specific chordamesoderm/notochord up-regulation continues through to 14 somites. Expression of all subunits is elevated within the developing brain during somitogenesis. As with *COP*β and *COP*β', expression of these COPI subunits is returned to low levels of ubiquitous expression throughout the embryo, with the exception of the most posterior tip of the developing notochord, by 28 hpf (**Figure 5.7 through to 5.10**).

Expression of *COPε*, *COPδ*, *COPγ*2 and *COPζ*2 in both mutant and wild type sibling *sny*, *hap* and *dop* mutants demonstrated that, as observed with *COP*β and *COP*β', the chordamesoderm/notochord expression of these COPI subunits is maintained in 28 hpf *sny*, *hap* and *dop* embryos whereas in 28 hpf sibling wild type embryos the chordamesoderm specific up-regulation is extinguished. Background levels of these COPI subunits remain unaffected in *sny*, *hap* and *dop* mutants (**Figure 5.11 through to 5.14**).

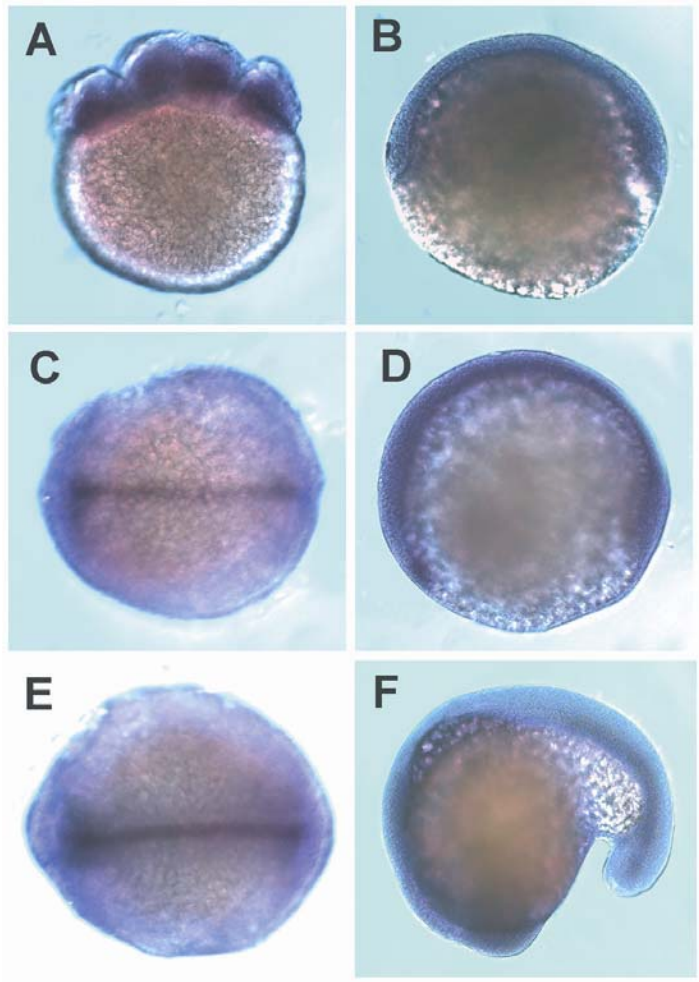

Figure 5.7 Staged expression profile of *COPε*.

At (**A**) 32-cell, (**B**) shield, (**C**) tailbud dorsal view, (**D**) tailbud lateral view, (**E**) 5-somite dorsal view and (**F**) 14 somite lateral view, anterior to the left. (**A**) High level of expression at 32 cell stage demonstrates that *COPε* 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 14 somite stages (**F**), with noticeably high expression in the developing brain at these stages.

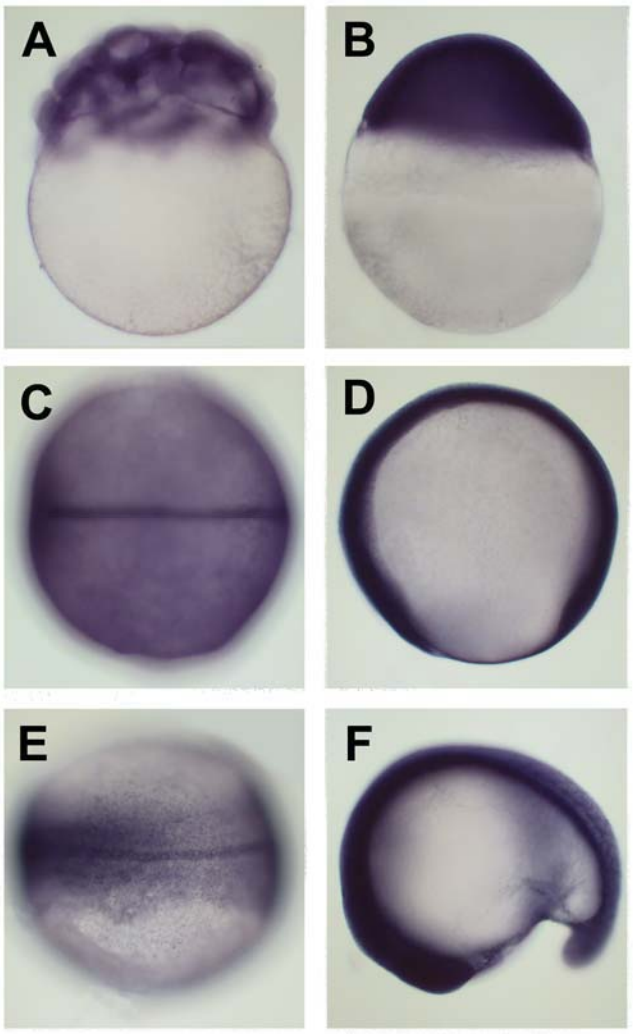

Figure 5.8 Staged expression profile of *COP*δ

At (**A**) 32-cell, (**B**) shield, (**C**) tailbud dorsal view, (**D**) tailbud lateral view, (**E**) 5-somite dorsal view and (**F**) 14 somite lateral view, anterior to the left. (**A**) High level of expression at 32 cell stage demonstrates that *COPδ* 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 14 somite stages (**F**), with noticeably high expression in the developing brain at these stages.

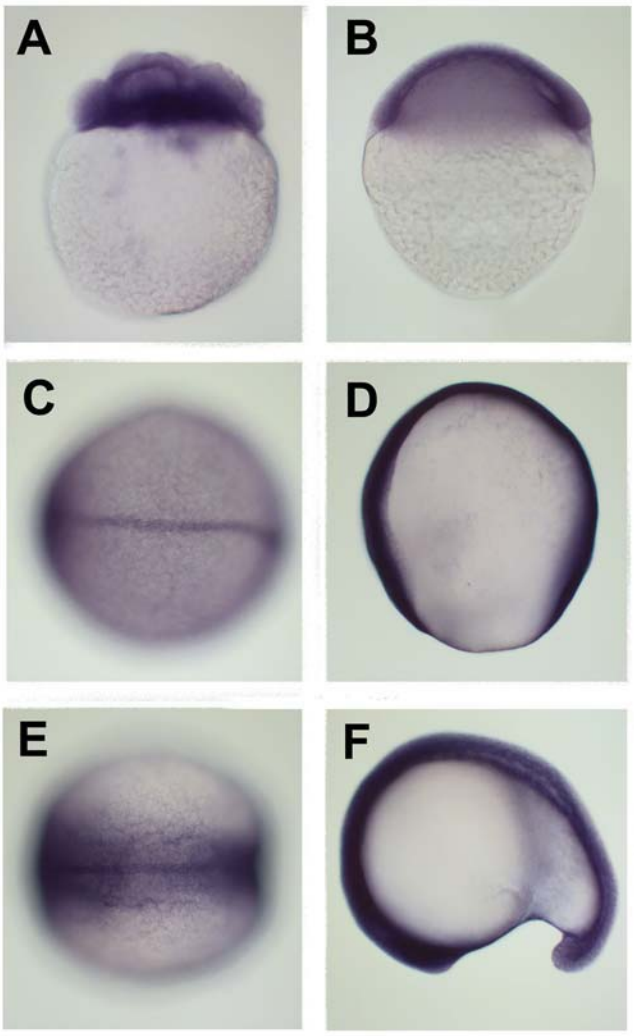

Figure 5.9 Staged expression profile of *COPγ2*

At (**A**) 32-cell, (**B**) shield, (**C**) tailbud dorsal view, (**D**) tailbud lateral view, (**E**) 5-somite dorsal view and (**F**) 14 somite lateral view, anterior to the left. (**A**) High level of expression at 32 cell stage demonstrates that *COPγ2* 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 14 somite stages (**F**), with noticeably high expression in the developing brain at these stages.

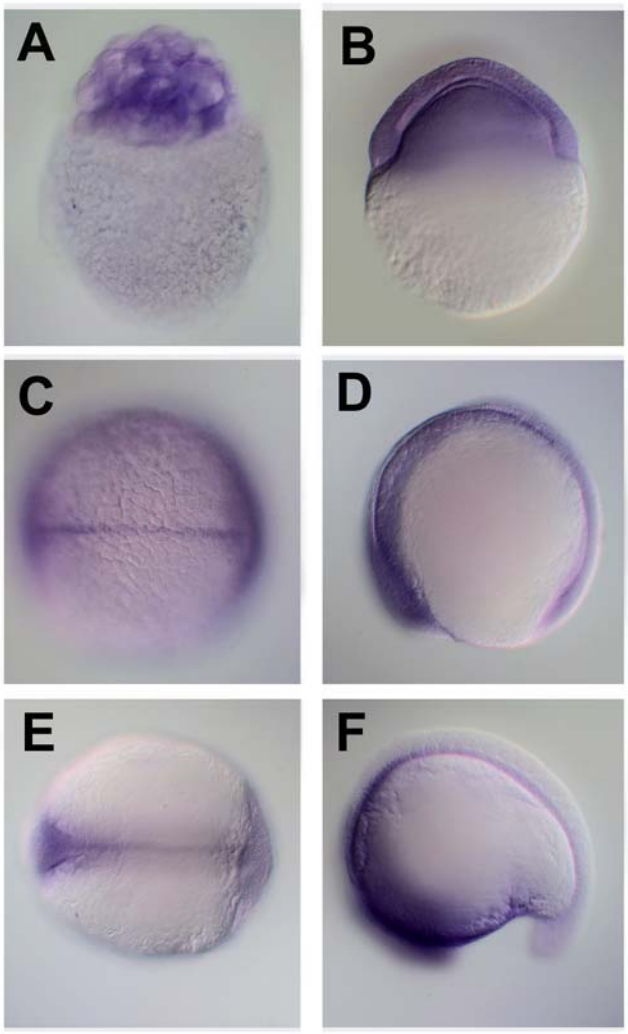

Figure 5.10 Staged expression profile of *COPζ2*

At (**A**) 32-cell, (**B**) shield, (**C**) tailbud dorsal view, (**D**) tailbud lateral view, (**E**) 5-somite dorsal view and (**F**) 14 somite lateral view, anterior to the left. (**A**) High level of expression at 32 cell stage demonstrates that *COPζ2* 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 14 somite stages (**F**), with noticeably high expression in the developing brain at these stages.

Figure 5.11 Expression profile of *COP*ε 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 *COP*ε is maintained within the undifferentiated notochord of the COPI mutants *sny* (**A**), *hap* (**C**) and *dop* (**E**). The chordamesoderm/notochord specific expression of *COP*ε is shut down in the properly differentiated notochord wild type siblings of *sny* (**B**), *hap* (**D**) and *dop* (**F**).

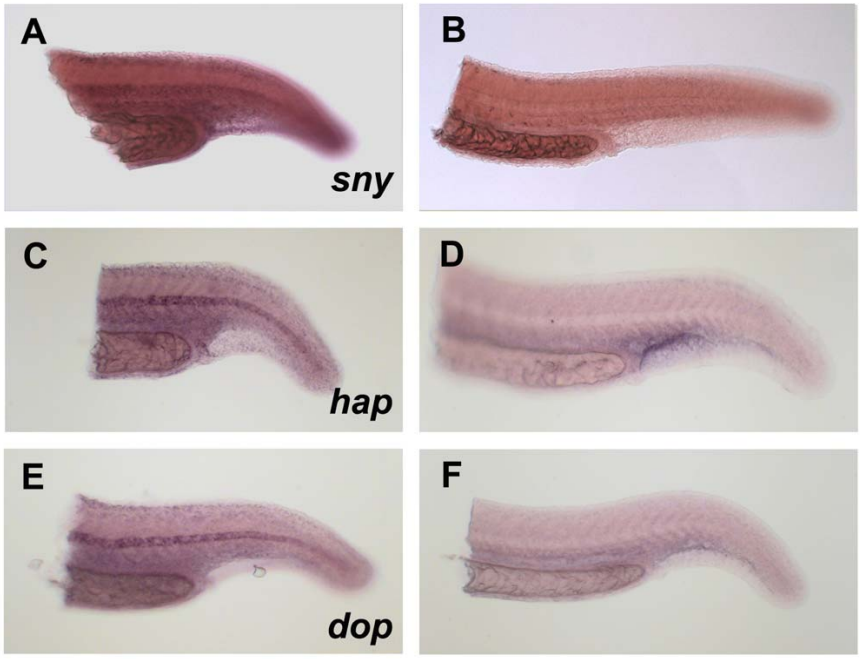

Figure 5.12 Expression profile of *COP*δ in mutant and wild type embryos at 28 hpf.

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 *COP*δ is maintained within the undifferentiated notochord of the COPI mutants *sny* (**A**), *hap* (**C**) and *dop* (**E**). The chordamesoderm/notochord specific expression of *COP*δ is shut down in the properly differentiated notochord wild type siblings of *sny* (**B**), *hap* (**D**) and *dop* (**F**).

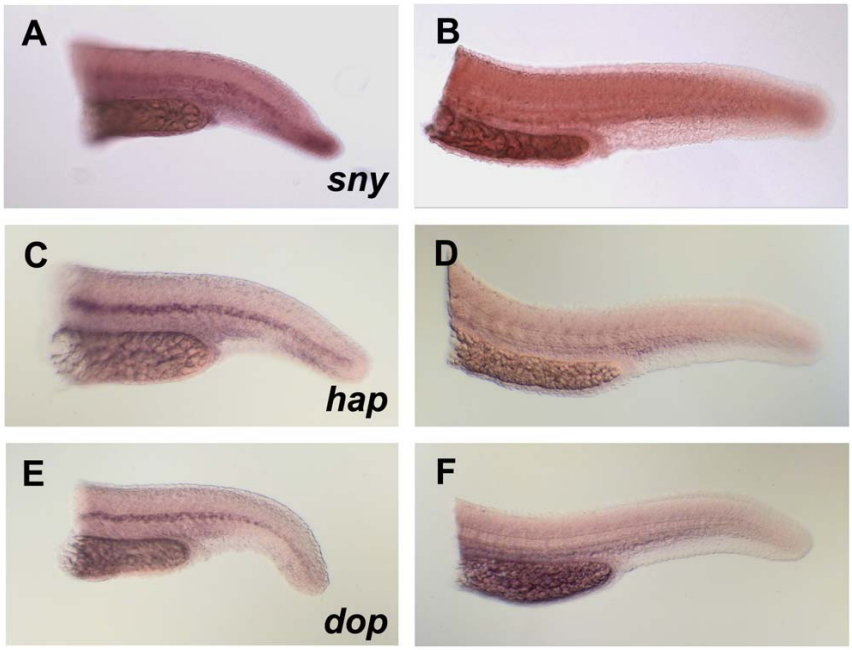

Figure 5.13 Expression profile of *COP*γ2 in mutant and wild type embryos at 28 hpf.

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 *COP*γ2 is maintained within the undifferentiated notochord of the COPI mutants *sny* (**A**), *hap* (**C**) and *dop* (**E**). The chordamesoderm/notochord specific expression of *COP*γ2 is shut down in the properly differentiated notochord wild type siblings of *sny* (**B**), *hap* (**D**) and *dop* (**F**).

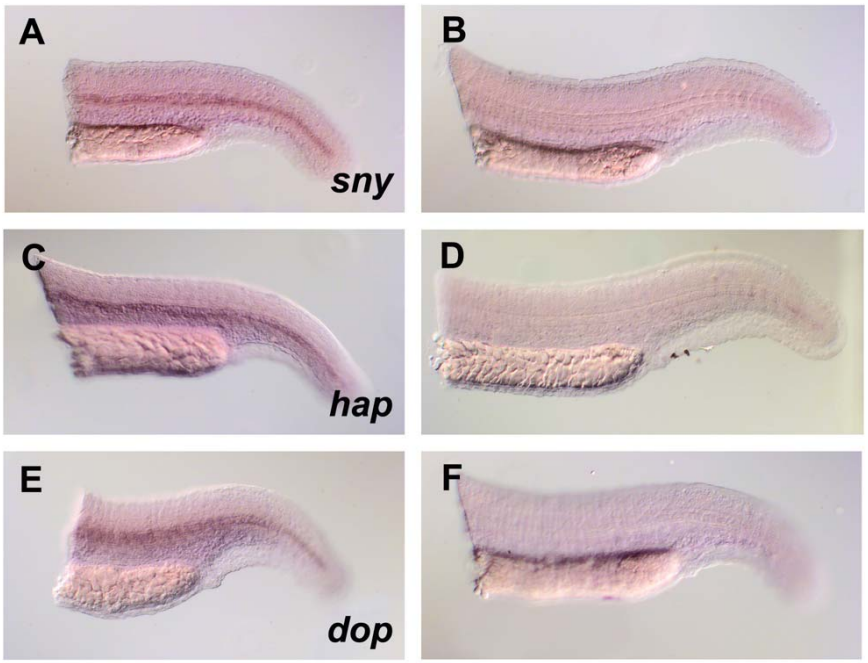

Figure 5.14 Expression profile of *COP*ζ2 in mutant and wild type embryos at 28 hpf.

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 *COP*ζ2 is maintained within the undifferentiated notochord of the COPI mutants *sny* (**A**), *hap* (**C**) and *dop* (**E**). The chordamesoderm/notochord specific expression of *COP*ζ2 is shut down in the properly differentiated notochord wild type siblings of *sny* (**B**), *hap* (**D**) and *dop* (**F**).

5.5.2 Expression of *COPζ* mRNA

Expression of the *COPζ* subunit differs from that of the other coatomer subunits. *COPζ* are first observed at the 32 cell stage, indicating maternal expression. By shield stage, the expression of *COPζ* is considerably lower than that observed for other subunits, though there are low levels of expression throughout the embryo. This low level ubiquitous expression continues through tail bud stage and throughout somitogenesis, with no up-regulation in the chordamesoderm/notochord. However, *COPζ* does show high levels of expression in the developing brain (**Figure 5.15**).

The comparison of expression of *COPζ* in both mutant and wild type sibling *sny*, *hap* and *dop* mutants demonstrated that there is no notochord expression of either subunit in either mutant embryos or their wild type siblings at 28 hpf (**Figure 5.16**).

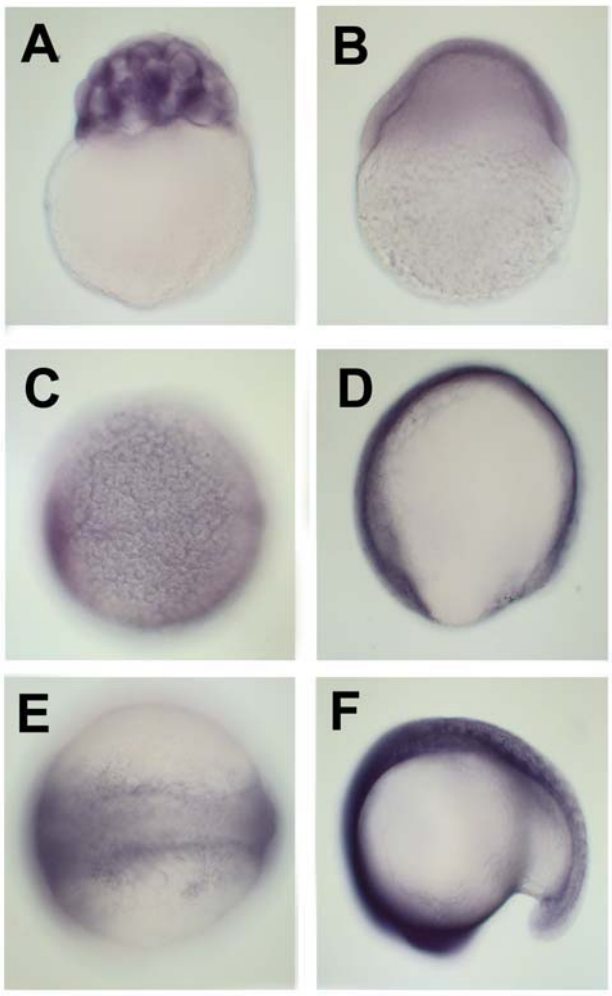

Figure 5.15 Staged expression profile of *COPζ*

At (**A**) 32-cell, (**B**) shield, (**C**) tailbud dorsal view, (**D**) tailbud lateral view, (**E**) 5-somite dorsal view and (**F**) 14 somite lateral view, anterior to the left. (**A**) High level of expression at 32 cell stage demonstrates that *COPζ* 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 and there is a lack of the specific up-regulation within the chordamesoderm observed with other COPI subunits. This is continued through 5 somite (**E**) and 14 somite stages (**F**), with noticeably high expression in the developing brain at these stages.

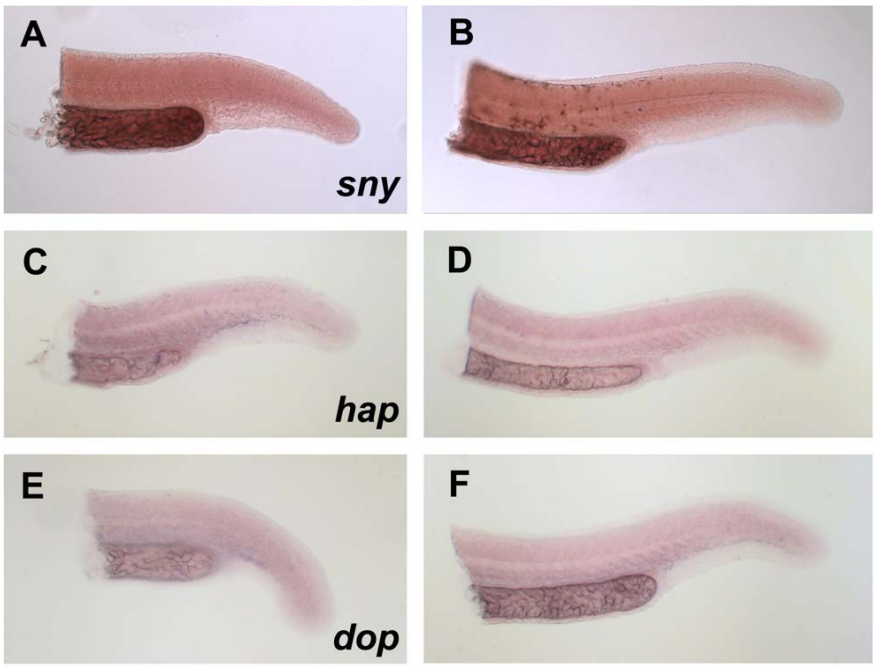

Figure 5.16 Expression profile of *COP*ζ in mutant and wild type embryos at 28 hpf.

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 *COP*ζ is not maintained within the undifferentiated notochord of the COPI mutants *sny* (**A**), *hap* (**C**) and *dop* (**E**). The chordamesoderm/notochord specific expression of *COP*ζ is shut down in the properly differentiated notochord wild type siblings of *sny* (**B**), *hap* (**D**) and *dop* (**F**).

5.6 Discussion

The *hap* and *dop* loci were suggested to encode subunits of the COPI complex based on the identification of *COPα* as the gene responsible for *sny* (Coutinho, 2001). This work suggested that the *hap* and *dop* loci encoded *COP*β and *COP*β', demonstrating that injection of antisense MO's against *COP*β and *COP*β' could indeed phenocopy *hap* and *dop*.

As part of this thesis, I cloned the full length cDNA for both *COP*β and *COP*β'. The full length cDNAs are 3239 bases and 3170 bases long, encoding proteins of 953aa and 934aa respectively. The zebrafish COPβ shows 93% identity to both the human and mouse COPβ, whereas zebrafish COPβ' shows 86% and 87% identity to the human and mouse homologues of COPβ' respectively. Using these full length cDNA sequence, *COP*β and *COP*β' cDNA was sequenced from *hap* and *dop* mutant embryos, identifying a G to T substitution at residue E_{499} in *hap*^{tm285b} embryos and a T to A substitution in *dop*^{m341} embryos at residue Y_{761}. Both mutations result in premature termination of the protein. Hence, the mutants *hap* and *dop* lack a functional COPI complex and the three notochord mutants *sny, hap* and *dop*, which all present almost indistinguishable phenotypes, all lack vital subunits of the essential coatomer complex. These mutants are all therefore deficient in COPI function.

Expression profiling of *COP*β and *COP*β' demonstrated that, like *COPα*, mRNA is provided maternally. During development, expression is ubiquitous with high levels of specific up-regulation occurring within the developing chordamesoderm and notochord. Once the notochord has differentiated this specific

expression is shut down, similar to other early notochord markers, such as *ehh*. Examination of the expression of *COP*β and *COP*β' within mutants and wild type siblings at 28 hpf demonstrated a maintenance of COPβ and COPβ' mRNA within the undifferentiated notochord of mutant embryos. This is, again, similar to the observation that other early notochord markers are maintained beyond their normal temporal expression domain in mutants demonstrating a failure of notochord differentiation.

In an effort to fully characterise the coatomer complex, I cloned the remaining subunits; *COPε*, *COPδ*, *COPγ2*, *COPζ* and *COPζ2* and used the scaffolds to generate *insitu* probes to examine their expression profile. Such an examination revealed that expression of *COPε*, *COPδ*, *COPγ2* and *COPζ2* is, like *COPα*, *COP*β and *COP*β', maternal and ubiquitous throughout early development, with a specific up-regulation within the chordamesoderm/notochord during development that is shut down by 24 hpf. These subunits were also demonstrated to be maintained within the notochord of the COPI mutants *sny, hap* and *dop* at 28 hpf though not in the differentiated notochord of wild type siblings. Hence, a complete set of seven COPI subunits, which are thus capable of forming a functional coatomer complex, are up-regulated specifically within the developing notochord and maintained beyond their normal temporal expression domain in the mutants *sny, hap* and *dop*, which fail to form a fully differentiated notochord. This maintenance of COPI subunit expression within mutants lacking COPI function suggests the possibility of a regulatory feedback mechanism where COPI functions both in the secretory network and in repressing expression of the coatomer subunits α, β, β', ε, δ, γ2 and ζ2. Though it remains a possibility that COPI subunits are maintained within the notochord of the COPI mutants merely due to a lack of notochord differentiation, as is observed with

notochord markers such as *ehh* (Parsons et al., 2002b; Stemple et al., 1996), rather than the specific loss of coatomer function.

Finally, the expression of *COPζ* differed from the observed expression of the other coatomer subunits. This subunit shows maternal expression, and is expressed ubiquitously at low levels at later stages and within the developing brain during somitogenesis, as observed for the other COPI subunits. However, *COPζ* is not up-regulated within the chordamesoderm and notochord during development and is also not present with the notochord of the COPI mutants at 28 hpf. Hence, only those subunits that are up-regulated with the chordamesoderm during early notochord development are maintained within the undifferentiated notochord of COPI mutants at 28 hpf.

Interestingly, insertional mutagenesis screening in zebrafish has generated a mutant for COPζ1 (Golling et al., 2002). These mutants were reported to show degeneration of the eyed after 4 days development, with no reports of notochord or melanophore defects, which befits its specific expression within the developing head. It may be that other COPI subunits, which are also up-regulated within the head, are also required for proper eye development. However, loss of COPα, COPβ or COPβ' in *sny, hap* or *dop* embryos results in loss of COPI function throught the embryo where loss of COPζ1 does not, since COPζ2 can function in its place, and thus eye defects are not observed in these mutants due to early lethality. The specific expression profiles of *COPζ1* and *COPζ2* combined with the specific eye defect in COPζ1 mutants (Golling et al., 2002) suggests that these two subunits may function in distinct developmental processes.

5.7 summary

- The zebrafish mutant *hap*^{tm285b} has a G→T substitution at residue E_{499}, which results in early termination.
- The zebrafish mutant *dop*^{m341} has a T→A substitution at residue Y_{761}, which results in early termination.
- The coatomer subunits β, β', ε, δ, γ2 and ζ2 are up-regulated within the developing notochord at early stages and shut down by 24 hpf.
- *COP*β, *COP*β', *COPε*, *COPδ*, *COPγ2* and *COPζ2* are all maintained within the undifferentiated notochord of COPI mutant embryos at 28 hpf.
- The COPI subunit *COPζ* is not up-regulated within the notochord at early stages and is not present within the notochord of COPI mutants at 28 hpf.