Chapter 1

Introduction

1.1 Zebrafish Development

Zebrafish (*Danio rerio*) are small, fresh water fish, which as adults are approximately two centimetres in length. They produce small, transparent eggs, which are fertilized shortly after laying. Once fertilized, the zebrafish embryos develop rapidly with the embryo, being recognisable as a fish by 24 hours post fertilization (hpf). By 48 hpf, the juvenile pigment has become apparent and they have started to hatch from their protective membrane, known as the chorion. Following three days of development the swim bladder has developed and after five days the eyes have become fully functional and the young are capable of independent feeding. Sexual maturity is reached between three and six months (Westerfield 2000).

1.1.1 From Fertilization to Gastrulation: An overview of morphological characteristics

Shortly after fertilization, yolk platelets separate from cytoplasm, which collects around the point of sperm entry at the animal pole (Kimmel et al., 1995). This animal pole cytoplasm cleaves approximately every 20 minutes to form a clump of cells, the blastoderm, situated on top of the large yolk cell (Kimmel et al., 1995). When the blastoderm reaches 512 cells the cell cycles lengthen and become asynchronous, and *de novo* transcription of the zygotic genome begins in a process called the mid-blastula transition (MBT) (Kane and Kimmel, 1993). At approximately four hpf the blastoderm cells move over the yolk cell and cover it, in a process called epiboly. When the cells have migrated to cover half the yolk - 50% epiboly – other gastrulation movements begin. Blastoderm cells internalise to form two cell layers; the outer epiblast and inner hypoblast (Kimmel et al., 1989). Concomitantly, a thickened marginal region, termed the germ ring, appears at the margin between the blastoderm and the yolk cell. This is followed by a local accumulation of cells in a structure called the embryonic shield which corresponds to the Spemann's organizer identified in amphibian development (Schier and Talbot, 2001; Spemann and Mangold, 1924). The appearance of the shield is the earliest visual marker of the

dorsal ventral axis in the embryo (Figure 1.1.1). During germ ring formation, epiboly temporarily arrests, but after shield formation, epiboly continues and the margin of the blastoderm eventually advances around the yolk cell to cover it completely (Houart et al., 1998; Kimmel et al., 1989)

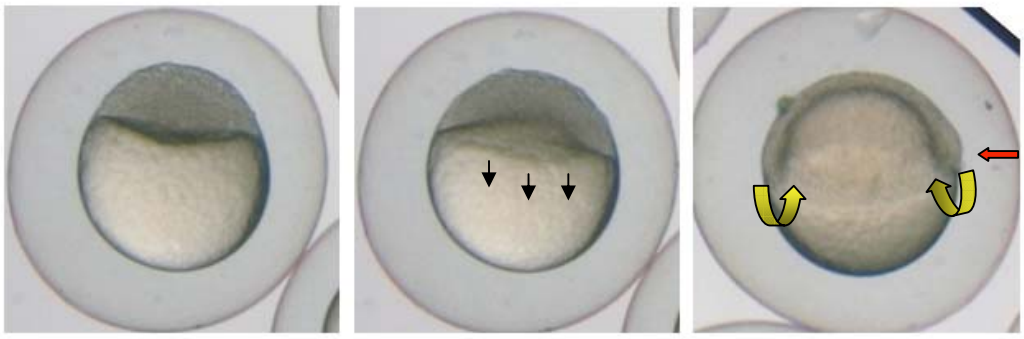

Figure 1.1.1: Figure showing movement of blastoderm cells during early zebrafish development.

Black arrows indicate the movement of cells during epiboly. Yellow arrows indicate the movement of cells during involution. Red arrow identifies the dorsal organizer (shield)

1.1.2 Dorsal Establishment.

Our understanding of the establishment of the dorsal axis remains incomplete. It has been shown however, that the yolk is an important early source of dorsal-ventral (DV) patterning signals (Piccolo et al., 1999). Dorsal identity in the embryo is established by a dorsal determinant, or determinants, that are initially located at the vegetal pole and translocated along microtubules to the future dorsal side before the first cleavage division occurs (Aanstad and Whitaker, 1999; Jesuthasan and Stahle, 1997). Experimental evidence for the presence of maternally inherited dorsal determinants has been shown by removal of the vegetal most part of the yolk shortly after fertilisation, which resulted in strongly ventralized embryos (Mizuno et al., 1996). This result suggests that the vegetal part of the yolk contains a dorsalizing factor, which is transferred to the future dorsal side early in development (Piccolo et al., 1999). Prior to shield formation β-Catenin, is seen to translocate to dorsal nuclei. It is thought that an unknown dorsalizing factor may bring about the stabilisation and nuclear translocation of β-Catenin and the activation of many dorsally associated

genes (Kelly et al., 2000; Kelly et al., 1995; Schneider et al., 1996). Cytoplasmic β-Catenin is targeted for degradation by a complex containing APC, axin, Glycogen synthase kinase-β (GSKβ) and other components which allow only low levels of β-Catenin to accumulate (Hinck et al., 1994; Papkoff et al., 1996). Activation of the canonical Wnt signalling pathway inhibits the β-Catenin degradation complex, stabilizing β-Catenin and allowing it to enter the nucleus where it then activates transcription of canonical Wnt target genes (Kelly et al., 1995; Papkoff et al., 1996; Schneider et al., 1996). β-Catenin accumulates specifically in nuclei of dorsal margin blastomeres as early as the 128-cell stage (Dougan et al., 2003; Schneider et al., 1996).

Many of the molecular details of the establishment of dorsal identity are derived from experiments done with the amphibian *Xenopus laevis*. Many of these results have been replicated in zebrafish embryos. In zebrafish when β-catenin is activated outside the dorsal region, a second dorsal axis is induced (Kelly et al., 1995) and, in *Xenopus laevis* if maternal *β-catenin* mRNA is inhibited using antisense oligonucleotides, then dorsal cell fates are inhibited (Tao et al., 2005). In zebrafish, the maternal effect loci *ichabod* and *tokkaebi* disrupt the nuclear localization of β-Catenin and lead to ventralized embryos (Kelly et al., 2000; Nojima et al., 2004). Indeed *ichabod* has been recently shown to encode *β-catenin-2* (Bellipanni et al., 2006). In *Xenopus laevis* one target of β-Catenin activation, is the transcription factor *siamois* (Wylie et al., 1996). In zebrafish, *dharma,* the gene mutated in *bozozok (boz)* mutants, is also a member of the hox family, as is *siamois* and although probably not performing exactly the same function *dharma* may also encode an important mediator of β-Catenin function (Nelson and Gumbiner, 1998). Mutant *boz* embryos often have no notochord or prechordal mesoderm and exhibit cyclopia and deficiencies in ventral regions of the CNS. Earlier in development, the shield does not form and the expression of several organizer genes is strongly reduced or eliminated at late blastula. The Dharma protein is thought to act downstream of β-catenin since injection of *β-catenin* into *boz* mutants does not rescue the mutant. Mutants develop some dorsal tissue, suggesting that Dharma is only essential for a portion of organizer functions (Kodjabachian et al., 1999). Both *siamois,* in frog, and *dharma,* in fish, have been shown to induce the formation of an ectopic body axis when overexpressed (Fekany et al., 1999; Nelson and Gumbiner, 1998).

In *Xenopus laevis*, β-Catenin has been shown to act on Vg1 (a transforming growth factor-β (TGF-β)) and VegT (a T-box transcription factor) (Nielsen et al., 2000; Tao et al., 2005; Zhang et al., 1998). Both Vg1 and VegT are localised to the vegetal pole. Vg1 is synthesized as an inactive precursor protein from maternally supplied mRNA and activated dorsally during cortical rotation (Melton, 1987; Yisraeli and Melton, 1988; Zhang and King, 1996). Absence of Vg1 leads to an absence of mesoderm (Melton, 1987; Zhang et al., 1998). VegT, on the other hand, can regulate generation of endoderm, in both a cell-autonomous manner and by inducing expression of the TGF-β family of secreted cytokines. In addition, the induction of TGF-βs means that VegT is also upstream of the induction of mesoderm (Clements et al., 1999). When maternal *vegT* mRNA is depleted in early embryos it results in the loss of the vegetal mesoderm-inducing signals and the loss of endodermal markers. Therefore, maternal *vegT* is essential for mesoderm and endoderm development (Clements and Woodland, 2003). The lack of mesoderm and endoderm, caused by Vg1 and VegT inhibition, can be rescued by injection of RNA encoding nodal proteins which are members of the TGF-β family which act downstream of β-Catenin, Vg1 and VegT (Melton, 1987; Zhang et al., 1998; Zhang and King, 1996). In zebrafish, the factors with functional homology to Vg1 and VegT are unknown as loss of function studies of the closest known orthologues do not produce similar phenotypes.

At MBT in zebrafish, the embryo switches from control by the inherited maternal products to control by the zygotic genome (Kane and Kimmel, 1993). Soon after this, other zygotic genes are activated by β-Catenin. Amongst these genes are *chordin*, *dickkopf1 (dkk1)*, *squint (sqt)* and *fibroblast growth factors* (*fgf*s). These genes act to inhibit the action of ventralizing factors or, in the case of *sqt*, to induce mesendodermal fates around the margin (Tao et al., 2005). The establishment of correct DV patterning is a balancing act between these dorsal associated genes antagonising ventral genes such as *wnt8* and *bone morphogenetic proteins (bmp*) (This is discussed in more detail in section 1.1.3.3).

Recent research has suggested that DV asymmetry in zebrafish can be identified experimentally as early as the two cell stage (Gore et al., 2005). Map kinase p38

antibody staining localises to the region of the embryo that will eventually become the dorsal side (Fujii et al., 2000). P38 has been shown not to specify dorsal fates since expression of dorsal genes still occurs in embryos expressing a dominant negative version of the gene (Fujii et al., 2000) It is thought, however, that p38 controls the rate of cell division in the dorsal blastomeres since, when dominant negative versions are overexpressed, there are larger, fewer blastomeres on the dorsal side (Fujii et al., 2000). Disruption of microtubules, which are thought to participate in the specification of dorsal, results in no activation of p38, suggesting that p38 may be regulated by the same factors that establish DV asymmetry (Fujii et al., 2000). Direct evidence for early asymmetry is provided in recent work that demonstrated that maternal transcripts of *sqt* are localized to two blastomeres of the four cell embryo (Gore et al., 2005). Removal of cells containing these transcripts from four-to-eight-cell embryos, or oocyte injection of antisense MO oligonucleotides (MOs) targeted to *sqt*, cause a loss of dorsal structures (Gore et al., 2005).

1.1.3 Dorsoventral Patterning and the Organizer.

Formation of the zebrafish embryonic shield is the first morphological indication of the DV axis although it is clear that the DV axis has been established prior to shield formation. The shield consists of two layers of cells - the epiblast and the hypoblast - and is covered by a tight epithelium the enveloping layer (EVL). Despite members of the *TGF-β* and *wnt* families having been implicated in the formation of the organizer (Feldman et al., 1998; Smith and Harland, 1991), no endogenous signalling molecule has been identified that results in the direct formation of the organizer. The function of the organizer during gastrulation is to inhibit ventral signals, differentiate axial structures and facilitate morphogenesis. Many of these organizer functions are mediated through the secretion of signalling factors (Feldman et al., 1998; Knecht et al., 1995). Amongst the first of the signalling factors to be secreted are members of the FGF, Wnt and TGF-β families (Feldman et al., 1998). The organizer is known to induce dorsal fates within mesoderm, anterior fates within endoderm and neural fates within ectoderm, achieved through opposing morphogenic activities, emanating from the ventrolateral regions of the embryo (Kodjabachian et al., 1999).

1.1.3.1 TGF-β signals.

TGF-β was identified in 1978 by de Larco *et al* due to its ability to induce phenotypic transformation of fibroblasts into diverse mouse cell types (de Larco and Todaro, 1978). Subsequently, many more TGF-β-related factors were identified resulting in a large family of TGF-βs, present in all metazoa. In humans, the TGF-β family contains at least 35 family members, including BMPs and Nodals (Derynck and Miyazono, 2006) which are also found in zebrafish. This section is concerned with the BMPs and their role in DV patterning, with the Nodals covered in greater detail in section 1.3.

The BMP family of growth factors were named for their ability to induce the formation of bone and cartilage. The BMPs induce ventral fates within the embryo and they accomplish this by binding to the extracellular domains of type I and type II BMP receptors (BMPRs) (Koenig et al., 1994; ten Dijke et al., 1994). These are transmembrane proteins, which contain intracellular serine/threonine kinase domains (Lin et al., 1992). Signal transduction through BMPRs results in mobilization of members of the Smad family of proteins, especially Smad1, Smad5 and Smad8 (Hild et al., 1999; Nakayama et al., 1998; Xu et al., 1998). These Smads are transcription factors, that are phosphorylated by ligand bound receptors, allowing them to translocate to the nucleus and, together with the non-receptor-regulated Smad protein Smad4, regulate target gene expression (Zhang et al., 1997). Secreted antagonists of this pathway include Chordin, Noggin and Follistatin, while the transmembrane protein Bambi functions as a decoy receptor (Fainsod et al., 1997; Onichtchouk et al., 1999). In addition to this, there are inhibitory Smads, Smad6/7, which are thought to interfere with Smad1/5/8 phosphorylation (Heldin et al., 1997; Nakao et al., 1997). The inhibitory Smads also recruit ubiquitin ligases, known as Smad ubiquitination regulatory factor 1 (Smurf1) and Smurf2, to the activated type I receptor, resulting in receptor ubiquitination and degradation, and termination of signalling (Ebisawa et al., 2001; Zhang et al., 2001)

In zebrafish, a number of BMP pathway components are essential for the formation of ventral cell types, including BMP2b and BMP7. These are widely expressed soon after MBT but become restricted to the ventral half of the embryo by the onset of

gastrulation (Dick et al., 2000; Schier and Talbot, 2005). Both of these genes have zebrafish mutants, (the *bmp2b* mutant *swirl* and the *bmp7* mutant *snailhouse*) that are strongly dorsalized. It is thought that the ventralizing signal *in vivo* is a BMP2b-BMP7 heterodimer (Schmid et al., 2000). Previous studies in *Drosophila, Xenopus laevis* and zebrafish have shown that BMP signalling is required for global DV patterning decisions during early gastrulation (Gelbart, 1989; Graff et al., 1994; Pyati et al., 2005). In DV patterning, the effect of the BMP pathway in zebrafish is a balancing act between many factors. The major modifiers in this pathway include Alk8, a type I BMP receptor required for the specification of ventral cell fates (Bauer et al., 2001; Connors et al., 1999; Mintzer et al., 2001), Smad5 (Hild et al., 1999), Chordin, Ogon/Sizzled and the protease Tolloid (Connors et al., 1999). *chordin* mutants have a ventralized phenotype (Schulte-Merker et al., 1997) whereas *bmp2b;chordin* double mutants are dorsalized, suggesting that *chordin* is not needed for dorsal development if BMPs are inactivated (Hammerschmidt et al., 1996b; Miller-Bertoglio et al., 1999; Piccolo et al., 1996). The sizzled gene is mutated in *ogon* mutants (Martyn and Schulte-Merker, 2003; Yabe et al., 2003) which have a ventralized phenotype similar to *chordin* mutants. This phenotype can be suppressed by overexpression of Chordin (Miller-Bertoglio et al., 1999; Wagner and Mullins, 2002). Tolloid promotes BMP signalling by cleaving and inactivating Chordin (Blader et al., 1997). The antidorsalizing morphogenic protein (ADMP) is a divergent member of the BMP family expressed on the dorsal side of the late blastula as well as in the axial mesoderm and anterior neuroectoderm during gastrulation (Lele et al., 2001; Lin et al., 1997). Overexpression of ADMP causes ventralization and a reduction of the organizer. Embryos injected with MOs (MO injected embryos) knocking down ADMP show moderate expansion of dorsal mesoderm. ADMP may be part of a negative feedback system which limits the size of the organizer region, possibly with the aid of *bmp2b* and *bmp7* (Lele et al., 2001; Willot et al., 2002).

1.1.3.2 Wnt Signalling

The Wnt pathway is essential for ventral and posterior fates within the embryo. Early in zebrafish development, maternal signalling divides nascent mesoderm into axial (dorsal) and non-axial (ventral) domains. Subsequently, subdivision of non-axial mesoderm into multiple DV fate domains involves zygotic Wnt8 and BMP signalling

as well as the Vent/Vox/Ved family of transcriptional repressors (Erter et al., 2001). Deletion or inhibition of both ORFs of the bicistronic (two non-overlapping open reading frames of *wnt8* produces a loss of ventroposterior structures and expansion of dorsal fates (Lekven et al., 2001). A reduction of Wnt3a and Wnt8 activity results in stronger expansion of dorsoanterior fates, suggesting that these genes have overlapping functions (Shimizu et al., 2005a); (Ramel et al., 2005).

The Wnt family of proteins signal through Frizzled-Lrp receptor complexes and employ a number of cytoplasmic proteins to stabilize β-Catenin (Cong et al., 2004; Sheldahl et al., 1999; Yang-Snyder et al., 1996) . These proteins enable β-Catenin to accumulate in the nucleus and activate target gene expression. Secreted antagonists of this pathway include secreted frizzled-related protein (SFRP), Cerberus and Wnt inhibitory factor (WIF), which act by binding to Wnt proteins. The Wnt antagonist, Dkk, acts by binding the LRP subunit of the receptor (Bafico et al., 2001; Brott and Sokol, 2002; Li et al., 2002) counteracting the ventralizing and posteriorizing effects of Wnt signalling (Hashimoto et al., 2000). *ddk* is an early target of maternal β-Catenin and is expressed early in the dorsal margin and dorsal yolk syncytial layer (YSL – see section 1.1.4.1) as well as later during gastrulation in the developing prechordal plate (Gonzalez-Sancho et al., 2005).

Genes that may be modified by Wnt signalling include *vox*, *vent* and *ved*. Inactivation of the redundant homeodomain transcriptional repressors Vox (also known as Vega1) and Vent (Vega2) leads to severe loss of ventroposterior structures (Kawahara et al., 2000a; Kawahara et al., 2000b). This loss of function phenotype in zebrafish is strain dependent, AB strain fish lacking *vox/vent* are essentially wild type (Imai et al., 2001) whereas inactivation of a third gene encoding a homeodomain transcriptional repressor *ved* strongly dorsalizes all strains of embryos, including the AB strain (Shimizu et al., 2002). This dorsalized phenotype is similar to that seen in zebrafish *wnt8* mutants and there is evidence that *wnt8* activates *vox* and *vent* expression repressing dorsal genes (Ramel et al., 2005). *chordin* is a key target of Vox and Vent and these proteins also repress other dorsal genes including *boz*, *goosecoid*, *floating head* and *dkk1* (Imai et al., 2001; Melby et al., 2000).

1.1.3.3 FGF signalling

The FGF family has been implicated in mesoderm formation, neural induction, DV patterning, and anterior-posterior (AP) patterning of the embryo (Furthauer et al., 2004; Kimelman and Kirschner, 1987; Lamb and Harland, 1995). In early zebrafish development, as soon as the zygotic genes become activcated, FGF has a role in restricting *bmp* RNAs to the ventral side of the embryo (Furthauer et al., 2004). The FGF signal is initiated on the dorsal side of the embryo by the expression of *fgf3*, *fgf8* (a mutation in which is responsible for the zebrafish mutant *acerebellar* (Reifers et al., 1998) and *fgf24,* and this expression then spreads progressively to lateral and ventral domains. The restriction of BMP signalling shows an important role for FGFs in DV patterning. The FGF pathway is first active at the dorsal blastoderm margin, along the entire margin and, finally, in the tail bud (Furthauer et al., 2004; Ulrich et al., 2003). FGFs bind and activate receptor tyrosine kinases, the dimerization of the receptor results in *trans*-phosphorylation, as well as the recruitment and activation of downstream effectors (Bellot et al., 1991; Kornbluth et al., 1988). Downstream targets of FGF include *pea3*, *erm*, and *sprouty4* which are induced over broad domains in neighbouring cells (Furthauer et al., 2004; Ulrich et al., 2003). These genes are activated at different thresholds of FGF. In zebrafish wild type embryos, it has been shown that *erm* is activated furthest from the source of FGF, followed by *pea3* and, finally, *sprouty4* is activated closest to the source (Scholpp and Brand, 2004).

In zebrafish, FGF signals may effect mesoderm formation via interactions with the Nodal signalling pathway (Furthauer et al., 2004), possibly relaying the action of TGF-β ligands over long distances. FGF is required downstream of Nodal signalling to induce the co-receptor One-Eyed Pinhead (Oep) in cells distant from the source of Nodal (Griffin and Kimelman, 2003; Mathieu et al., 2004). This mechanism contributes to the amplification and propagation of Nodal signals (Mathieu et al., 2004). Partial inhibition of FGF signalling, and blocking of Oep, disrupts posterior development and leads to the death of dorsal mesoderm cells by the end of gastrulation (Griffin and Kimelman, 2003; Mathieu et al., 2004). The FGF and BMP pathways have also recently been shown to have a repressive effect on endodermal precursors induced by Nodal signalling (Poulain et al., 2006).

1.1.3.4 Convergence Extension movements

The term convergent extension is used in the context of the developing zebrafish embryo to describe the movements that lead to the accumulation of cells at the dorsal side of the embryo followed by the anterior-posterior (AP) extension of the body axis (Warga and Kimmel, 1990) (Figure 1.1.2). Convergence and extension allows the thinning and spreading of the germ layers during epiboly. When Nodal signalling is lost both mesoderm formation and extension of the embryonic axis are inhibited. This loss does not, however, prevent the dorsal accumulation of cells suggesting that convergence and extension are not mutually exclusive (Feldman et al., 1998). It is thought that during convergence and extension, the cells move as a cohesive group as molecules that mediate cell polarity, sorting and adhesion have also been shown to be required for convergence and extension movements (Heisenberg et al., 2000; Ulrich et al., 2003; Wallingford et al., 2000). A mutation in zebrafish Wnt11 (*slb*) - which has been shown to control cell cohesion through E-cadherin - results in embryos with compromised convergence and extension movements during gastrulation (Heisenberg et al., 2000; Ulrich et al., 2005).

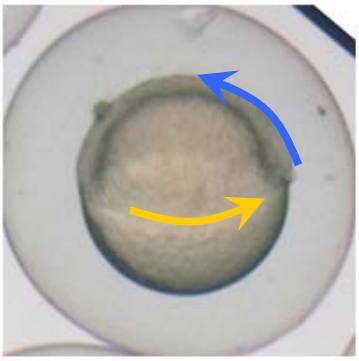

Figure 1.1.2: Photo of an embryo undergoing convergence and extession movements.

Yellow arrow indicates convergence movements as cells move towards the dorsal side of the embryo. Blue arrow indicates extension movements as cells extend along the AP axis.

1.1.4 The Yolk Cell

1.1.4.1 The Yolk Syncytial Layer (YSL) and its role in patterning

The YSL is an extra embryonic region, produced in zebrafish at the midblastula stage, when deep marginal blastoderm cells collapse and release their nuclei into the underlying yolk cell (Kimmel and Law, 1985). The YSL is a source of mesoderm and endoderm inducers (Chen and Kimelman, 2000; Hsu et al., 2006; Mizuno et al., 1996). Transplantation experiments have shown that when the YSL is transplanted into the animal-pole region of the host after MBT, the yolk cell and the YSL can induce mesoderm and organizer gene expression in the blastoderm (Mizuno et al., 1996). The YSL has been compared to the Nieuwkoop centre in *Xenopus*, a group of cells located in the dorsal-vegetal region of the blastula that are thought to induce the Spemann organizer in the overlying marginal zone in a non-cell-autonomous manner (Bischof and Driever, 2004; Gimlich and Gerhart, 1984). An important early step in the formation of the Nieuwkoop centre in *Xenopus* is the nuclear localization of β-Catenin in an area that will become dorsal (Guger and Gumbiner, 1995). In zebrafish, β-Catenin accumulates in the nuclei of dorsal blastomeres and the dorsal YSL, where, with the Tcf/Lef family proteins, it activates the expression of zygotic genes that mediate the formation of dorsal structures. One of the first of these zygotic genes activated in zebrafish is *boz,* which is expressed shortly before MBT within dorsal blastomeres and then at late blastula/early gastrula in the dorsal YSL (Solnica-Krezel and Driever, 2001). It has been suggested that *boz* is a direct target of β-Catenin-Tcf/Lef signalling (Geng et al., 2003; Leung et al., 2003).

1.1.4.2 Epiboly and the importance of the yolk cell

At the start of epiboly, the cells of the blastoderm sit on top of the yolk cell. The yolk itself is surrounded by a thin anuclear yolk cytoplasmic layer. Between the blastoderm and the yolk are the YSL and the blastoderm rim. The YSL is divided into two sections both of which contain yolk syncytial nuclei. These are the external YSL, a relatively thick belt of cytoplasm, and the internal YSL, a thinner layer (Betchaku and Trinkaus, 1986; Kimmel and Law, 1985). In the killifish, *Fundulus*

heteroclitus, the mechanism of epiboly has been more extensively researched (Trinkaus, 1963) but a similar process has been observed in zebrafish (Warga and Kimmel, 1990). Early work in *Fundulus* showed that epiboly begins with the contraction of the external YSL an autonomous process which can occur in the absence of the blastoderm. As epiboly progresses, the EVL, which tightly covers the blastoderm ensuring the correct physiological environment for the developing cells, moves down the yolk (Keller and Trinkaus, 1987). The underlying blastoderm cells link tightly with the YSL at the margin and are driven down the yolk by the YSL's epiboly movements, which are, in turn, driven by an intercrossing network of microtubules (Trinkaus, 1984). In zebrafish changes in configuration of the yolk microtubules are strictly correlated with epibolic movements, with disruption of microtubules blocking the vegetal movement of the YSL but only partially disrupting the epiboly of the EVL and deep cells (Solnica-Krezel and Driever, 1994). Recent evidence in zebrafish has shown that pregnenolone, a steroid in the yolk cell, is required to maintain an adequate level of polymerized microtubules and so normal epiboly (Hsu et al., 2006).

In addition to the movements of microtubules in the yolk, epiboly is driven by endocytosis (Betchaku and Trinkaus, 1986; Solnica-Krezel and Driever, 1994). In the external YSL, immediately beneath the EVL attachment region, endocytosis removes sections of the external YSL membrane reducing it and pulling the EVL vegetaly. The external YSL membrane is then fused with the internal YSL membrane to expand the internal YSL and drive further epibolic movement (Betchaku and Trinkaus, 1986). At approximately 50% epiboly an actin band forms in the external YSL, beneath the EVL margin - the region of active endocytosis (Cheng et al., 2004). This actin band is vital for epiboly, as shown by experiments in zebrafish inhibiting the formation of the actin cytoskeleton which result in a slowing of, and failure to complete, epiboly (Cheng et al., 2004; Zalik et al., 1999). *betty boop* the zebrafish homologue of MAPKAPK2 (a regulator of actin filament formation) displays premature constriction of the margin and is thought to regulate epiboly (Holloway, unpublished data, 2006; Wagner et al., 2004).

A further important factor in epiboly is the requirement for the intracellular adhesion molecule E-cadherin. Whilst a reduction in E-cadherin in zebrafish impairs epiboly, a

stronger loss-of-function interferes with early cells divisions and halts development completely (Babb and Marrs, 2004). Interestingly, the first zebrafish epiboly mutant identified, *half baked (hab)* (Kane et al., 1996), has a defect in the *E-cadherin* (*cdh1*) gene. In the *hab* mutant, the deep cells cease movement at mid-gastrulation, whilst epiboly of EVL and YSL proceeds further (Kane et al., 2005; Shimizu et al., 2005b).

1.1.5 Morphogen Gradients in the Developing Embryo

A morphogen can initially be described as a substance which governs the pattern of tissue development in an organism, where different concentrations of morphgen can induce different cell fates. In the early 20th century, biological morphogen gradients were first suggested by Child and Boveri from their work in *Hydra* and nematode respectively (Child, 1915; Child, 1941; Gilbert, 1997; Slack, 1994; Tsikolia, 2006), with the discovery of their importance in developmental biology being accredited to the work done in *Drosophila* by geneticist Thomas Hunt Morgan (Lawrence, 2004; Lawrence, 2001; Oppenheimer, 1983; Yucel and Small, 2006). These ideas were later refined in the 1960's by Lewis Wolpert with his 'French flag' model that described how morphogens could subdivide a tissue into discrete domains of different target gene expression (Figure 1.1.3) (Wolpert, 1996). Wolport's work in chick limb development demonstrated that grafting the region with morphogenic properties to successive positions along the antero-posterior axis could modify the positional information along the axis and so modify the pattern of digits obtained. He concluded that the interpretation of positional information can provide the basis for pattern formation in limb morphogenesis and that this pattern was consistent with a model based on diffusion of a labile morphogen (Tickle et al., 1975), similar to that proposed for the development of pattern in invertebrates (Wolpert, 1996).

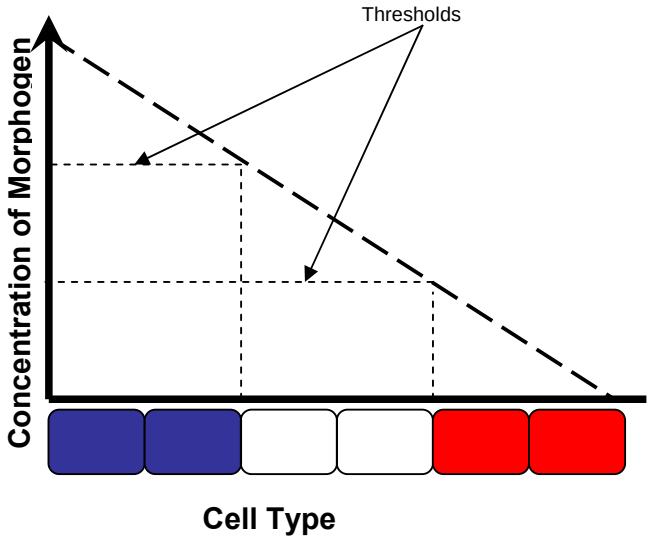

Figure 1.1.3: Diagram of Wolperts "French flag" model
Showing how morphogens could subdivide a tissue into discrete domains of different target gene expression (reproduced from (Wolpert, 1996).

In developmental biology, following the work of researchers such as Wolpert, morphogens can be described as signalling molecules which form spatial extracellular concentration gradients emanating from a restricted source of production. Cells receive a different concentration of morphogen depending on their distance from the source and this elicits a concentration-specific response. A more solid understanding of morphogens was obtained when Gurdon and collaborators used the animal cap assay in *Xenopus laevis* (Gurdon et al., 1985). This exogenous model system showed that the selection of genes expressed by animal cap cells is determined by the distance from a source of Activin - a peptide growth factor of the TGF-β family contained in vegetal cells and capable of inducing other cells to form mesoderm. Activin's long-range signal spreads over at least ten cell diameters in a few hours and does so by passive diffusion by-passing cells that do not respond to the signal or synthesize protein themselves. These results provided support for the operation of morphogen concentration gradients in vertebrate development as well as for a diffusion based model of these concentration gradients (Gurdon et al., 1994).

Following the general acceptance of morphogen gradients as a potential mechanism for patterning naïve tissue, four models have been proposed for the establishment and

maintenance of stable morphogen concentration gradients within tissues. The mechanisms underlying these models are trancytosis, cytonemes, argosomes and diffusion and will be discussed in the next sections.

1.1.5.1 Transcytosis

The transcytosis model suggests that a morphogen can be passed through a series of neighbouring cells in order to be transported over long distances (Strigini and Cohen, 1997; Bellaiche et al., 1998). Enchev *et al* investigated morphogenesis in the wing imaginal disk of *Drosophila.* Their research suggested that simple diffusion could not explain the morphogenic gradient of Dpp and that receptor-mediated endocytosis was essential for Dpp's long range movement (Entchev et al., 2000). Enchev *et al* proposed that Dpp was transported by transcytosis and suggested a role for Rabs, small GTPases of the Ras superfamily (Entchev et al., 2000). The transcytosis model will be expanded on in section 1.2 where the Rab family will be looked at in greater detail alongside the roles of Rabs in this method of morphogen movement.

1.1.5.2 Cytonemes

Cytonemes ('cell threads' neme = thread) is a term coined by Ramı´rez-Weber *et al* who discovered very thin projections from fluorescently labelled cells in the *Drosophila* imaginal disc emanating from the lateral flank towards the disc centre. These projections were shown to represent cytoplasmic extensions and were designated cytonemes. The cytonemes extend from disc cells toward the AP compartment border, but not from AP border cells outward. Ramı´rez-Weber *et al* (Ramirez-Weber and Kornberg, 1999) hypothesized that the cytoplasmic extensions grow in response to a chemoattractant. Different parts of the imaginal disc were placed next to anterior or posterior fragments to ascertain which parts of the disc could act as an attractant. The results showed that the anterior or posterior fragments only grew projections if placed next to the excised boundary region from between the anterior and posterior. Branchless – the drosophila homologue of FGF (dFGF) - was identified as a chemoattractant for these projections suggesting that activated FGF protein could be produced at the compartment border or that dFGF could function as a growth factor and stimulate non-polarized cell growth of cytonemes. Ramı´rez-

Weber *et al* (Ramirez-Weber and Kornberg, 1999) also showed that projections similar to cytonemes could be seen in cultures from mouse limb bud as well as those from chick. The authors suggested that cytonemes might play a role in disc cells defining their relationship with signalling centres so that cytonemes link outlying cells directly to signalling cells. This mechanism has been suggested as an efficient long distance transport for morphogens such as Hh and Dpp whose movement is said to be inconsistent with simple diffusion in the imaginal disc. The hypothesis is that Hh and Dpp are released at sites of cytoneme contact delivering the morphogens efficiently to target cells and limiting their spread (Ramirez-Weber and Kornberg, 1999) (Figure 1.1.4). Recently Hsiung *et al* has shown evidence for cytonemes involvment in Dpp signalling with the Dpp receptor, Thickveins, present in punctae that move along cytonemes (Hsiung et al., 2005).

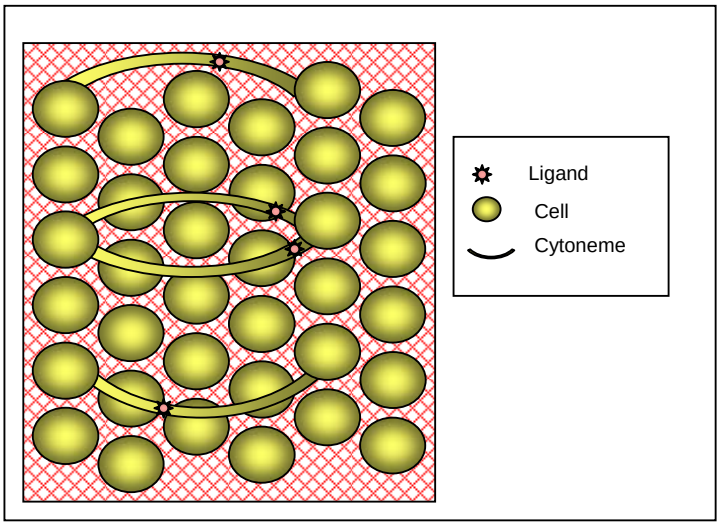

Figure 1.1.4: Diagramatic representation of cytonemes possible role in morphogen gradient formation.

Diagram shows cytonemes projectiong from distant to expressing cells (adapted from (Williams et al., 2004).

1.1.5.3 Argosomes

Many morphogens have been found to be tightly associated with membranes; the Wnt family bind heparin sulphate proteoglycans (HSPG) whilst hedgehog binds to membranes via covalently attached cholesterol and palmitate (Hashimoto et al.,

2000; Mann and Beachy, 2000; Nakato et al., 1995; Pepinsky et al., 1998). Greco *et al* observed that all hypotheses for the spread of morphogens assumed the release of the molecule from the membrane of the producing cells (Greco et al., 2001). Greco *et al* set out to investigate whether for the spread of morphogens might include their transport in vesicles at every stage of transport. This hypothesis was tested using fluorescent labelled markers (CFP-GPI) on the cytoplasmic and external faces of the membranes of the *Drosophila* imaginal disc. The expression of these markers was specifically driven in certain populations of cells within the wing disk but not others allowing the visualisation of vesicles derived from these populations in the non-expressing tissue. These 'exovesicles', found distant from the tissue expressing the membrane marker, were termed argosomes presumably from the mythical Greek ship, *Argo*, captained by Jason. Argosomes were shown to consist mainly of membrane with a fraction present in the early endocytic compartment. This led Greco *et al* (Greco et al., 2001) to surmise that argosomes are of endocytic origin. The endocytic compartments that contain the endosomes were shown to move rapidly with some compartments containing multiple argosomes (Greco et al., 2001). Two methods were suggested for argosome production. The first involves the production of vesicles within endosomes, when the endosome fuses with the plasma membrane the internal vesicles (exosomes) are released into the extracellular space (Figure 1.1.5). The second method suggests that part of the producing cell is internalised by its immediate neighbour forming a vesicle (derived from the producing cell) in the endosomes of the receiving cell (Figure 1.1.5). Greco *et al* suggested that Wingless can move through the disc epithelium on argosomes since Wingless in receiving cells colocalizes with argosomes derived from cells that synthesize wingless (Greco et al., 2001). Further, the rate of Wingless spread is consistent with the spread of argosomes. As removal of HSPG results in Wingless not associating with argosomes the authors propose that one function for the interaction of Wingless with HSPG might be to allow the incorporation of Wingless into argosomes (Greco et al., 2001).

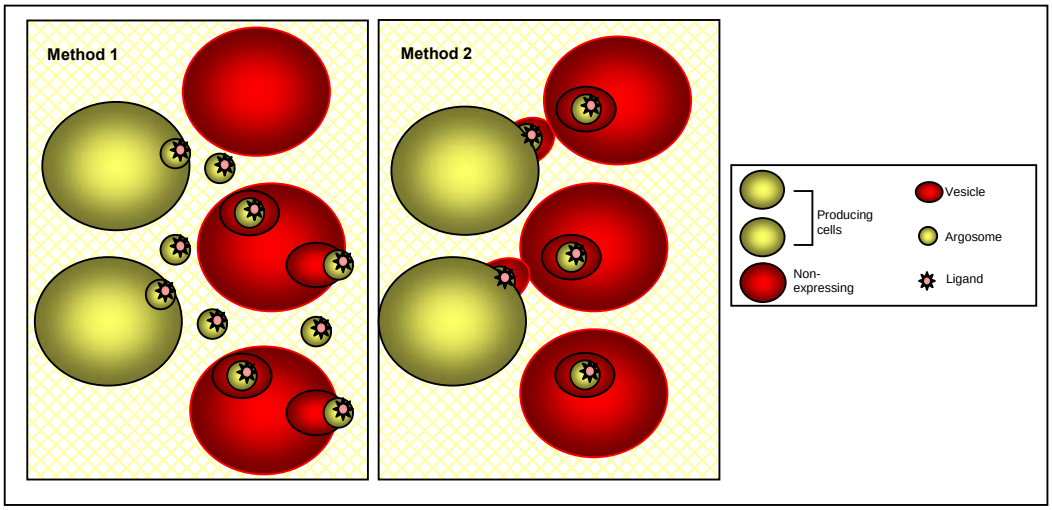

Figure 1.1.5: Diagramatic representation of argosomes possible role in morphogen gradient formation.

Diagram showing membranes from producing cells transporting ligands through reciving tissue in "exovesicles" (adapted from (Williams et al., 2004))

1.1.5.4 Diffusion

The original and simplest model for the movement of morphogens in a tissue has been by extracellular free diffusion. The diffusion model implies that intermediate cells do not play any role during the movement of the morphogen. Support for this is seen in *Xenopus laevis* animal caps, when endocytosis is blocked but TGF-β1 still spreads through the tissue (McDowell et al., 2001). Since morphogens signal over long distances doubt has been cast on the ability of free diffusion to form stable long-range concentration gradients with much of this stemming from the nature of the morphogen (Belenkaya et al., 2004; Entchev et al., 2000). The ideal morphogen to fit the diffusion model would need to be a small ligand which did not adhere to anything other than the receptor. Often this is not the case, both Wnt and Hh have been shown to adhere to proteoglycans in *Drosophila* and mouse cell culture, with Hh showing a high affinity for membranes (Blitzer and Nusse, 2006; Rietveld et al., 1999). This would result in their diffusion being hindered by extracellular binding proteins and proteoglycans (Kerszberg and Wolpert, 1998; Piek et al., 1999). TGF-β is a small ligand but its movement in *Xenopus laevis* is limited by the expression of the TGF-β type 2 receptor (McDowell et al., 2001), which in addition to its role in

transducing the signal, also targets the TGF-β ligand for degradation thus removing it from the extracellular environment and restricting its signalling.

Investigations into the four proposed methods of morphogen movement were carried out in *Xenopus laevis* using the *Xenopus* nodal-related 2 protein (Xnr2) (Williams et al., 2004). Cytonemes were ruled out as a possible explanation since even though *Xenopus* cells did extend protrusions these were no longer than one cell diameter. A variation on the animal cap assay was used to examine whether argosomes could be responsible for the movement of Xnr2 (Williams et al., 2004). This was considered not to be the case when eGFP-Xnr2 was not observed in association with CFP-GPI positive vesicles in receiving cells. Transcytosis was also ruled out, since affecting endocytosis using constitutively active or dominantly negative Rab5 had no effect on the expression domain or level of activation of endogenous *Xbra* a target gene of Xnr2. *Williams et al* showed that eGFP-Xnr2 in the receiving cells was extracellular and confined to interstices between cells. They therefore, proposed that eGFP-Xnr2 exerts its effects by diffusion. Time lapse corroborates this notion and shows that Xnr2 can travel ten cells within two hours on the exogenous animal cap assay. The authors admit the limitations of the animal cap assay and suggest that different morphogens in different developmental contexts use different means of transport (Williams et al., 2004).

1.2 Cell Trafficking and Transport

Embryonic development relies on many different cell biological processes including cell signalling and cell adhesion. These would not be possible, however, without the cells ability to traffic and transport substances into, around and out of the cell. Eukaryotic cells routinely traffic substances, not only between organelles but also across the outer cell membrane, using vesicles - microscopic fluid filled sacs surrounded by a lipid membrane. Vesicles are involved in secretion, endocytosis and various recycling processes within the cell (Armstrong, 2000). There are four main stages to vesicle trafficking: formation - the vesicle must be formed and packed with its cargo; targeting – the vesicle is released from its membrane and directed towards the target membrane; tethering/docking – the vesicle aligns and attaches with the target membrane; and finally fusion - allowing the vesicle to release the cargo at its destination (Armstrong, 2000; Zerial and McBride, 2001). Each stage of the vesicle trafficking process requires specialized protein machinery, many of which belong to the Ras superfamily of guanosine triphosphatases (GTPases).

1.2.1 Introduction to Rabs

In 1983, the accidental discovery of Ypt1p, in *Saccharomyces cerevisiae* by Gallwitz et al (Gallwitz et al., 1983) showed that vesicular transport is governed by conserved monomeric GTPases. Subsequently, Salminen and Novick (Salminen and Novick, 1987) provided the first evidence that a Ras-like GTPase (Sec4) was directly involved in vesicular transport whilst Tavitian and colleagues (1987) cloned the first mammalian homologs of SEC4/YPT termed *rab* (*ras*-like in rat brain) genes (Touchot et al., 1987). Ypt1p was shown to be necessary for the docking process of vesicles with the Golgi. Yeast cells lacking functional Ypt1p showed accumulation of endoplasmic reticulum (ER) membranes, vesicles and ER core glycosylated proteins before they died (Schmitt et al., 1988). The *ypt1p* gene showed 70% sequence homology to the *rab1* gene found in mammalian cells and, furthermore, *rab1* could replace the essential function of *ypt1p* in yeast, suggesting that these genes are orthologous (Touchot et al., 1987). Continuing studies in yeast and

mammalian cells showed that different Ypt/Rab-GTPases were performing the same function, however, each localized to different parts of the secretory and endocytic pathways (Lazar et al., 1997). The localization of these proteins and their effectors, aided by SNARE proteins, help determine the specificity of the vesicle, ensuring they are transported, docked and fused with the correct target (Hammer and Wu, 2002; Ossig et al., 1995; Price et al., 2000a). The complete sequencing of the *Saccharomyces cerevisiae* genome has revealed the total number of *ytp* genes to be 11, some of which are phenotypically redundant (Lazar et al., 1997). Only those *ytp* genes involved in the biosynthetic pathway are essential for cell viability. Loss of the Ytp proteins Ypt1p, Ypt51p , Yptp6, Ypt7p, Sec4p and Ypt31p/Ypt32p (Benli et al., 1996; Haubruck et al., 1989; Schmitt et al., 1986; Singer-Kruger et al., 1994; Wichmann et al., 1992), proved fatal for the cell and demonstrated their essential nature. These essential Ytp proteins correspond to Rab1, Rab5, Rab6, Rab7 Rab8 and Rab11 in mammalian cells and are now considered 'core' Rabs (Lazar et al., 1997; Singer-Kruger et al., 1995; Ullrich et al., 1996).

The Rabs are now known to be the largest sub family in the Ras superfamily. The Ras-superfamily comprises of over 150 human members (with evolutionarily conserved orthologs found in *Drosophila*, *Caenorhabditis elegans*, *S. cerevisiae*, *Saccharomyces pombe* and *Dictyostelium* as well as in plants (Bush et al., 1993; Garrett et al., 1993; Haubruck et al., 1990; Nonet et al., 1997). The Ras superfamily is divided into five major branches on the basis of sequence and functional similarities: Ras, Rho/Rac, Rab, Ran and Arf. (Colicelli, 2004; Pereira-Leal and Seabra, 2000; Wennerberg et al., 2005). The Rab family is found in all eukaryotes, with over 60 *rab* genes uncovered in the human genome (Colicelli, 2004; Zerial and McBride, 2001) and over 80 in zebrafish (Clark, MD, pers. com.). Rab proteins localize to specific intracellular compartments, consistent with their function in distinct vesicular transport processes (Zerial and McBride, 2001). This localization is dependent on prenylation (addition of hydrophobic molecules to facilitate protein attachment to the cell membrane) and specificity is dictated by divergent C-terminal sequences (Khosravi-Far et al., 1991). For example, Rab1 is found in the intermediate compartment of the *cis*-Golgi network and is involved in ER to Golgi transport (Ayala et al., 1989), while Rab5 is located in early endosomes (vesicles

involved in endocytosis) and regulates clathrin-mediated vesicle transport from the plasma membrane to early endosomes (Gorvel et al., 1991).

Although Rab proteins are localized within individual cells, at the level of the whole organism many of the *rab* transcripts identified to date have ubiquitous distribution, although their level of expression may vary from one cell type to another. This is surprising given the high degree of regulation at the protein level (see section 1.2.3). However, there are exceptions with some Rabs being cell type- or tissue-specific. For example, in mammals Rab3A is only expressed in neurons and neuro-endocrine cells (Fischer von Mollard et al., 1990; Johnston et al., 1991) while Rab17 has only been detected in epithelial cells (Lutcke et al., 1993).

1.2.2 Rab Homology and Classification.

The similarities between the members of the Ras-superfamily make them difficult to classify. All of the Ras family contain the conserved regions referred to as G1–G3 and PM1–PM3. These regions are involved in binding guanine nucleotide and phosphate/$Mg2^{+}$ respectively (Valencia et al., 1991). There are two regions that undergo a significant conformational change upon GTP binding and hydrolysis: the switch I (Ras residues 30-38) domain and the switch II (59-67) domain (Figure 1.2.1). Although the GTP-bound and GDP-bound state of the Rabs (as with rest of the Ras family) have similar conformations they also have pronounced differences. Critically the GTP-bound conformation has higher affinity for effector targets (Bishop and Hall, 2000; Milburn et al., 1990). This difference corresponds to the changes in the switch I and switch II regions. It is mainly through the conformational changes in these two switch regions that regulatory proteins and effectors distinguish the nucleotide status of the GTPases (Dumas et al., 1999). Although the GTP-bound form is the active form for all Ras superfamily GTPases it is the cycling between the two states, in which distinct functions are associated with each nucleotide-bound form, that is also critical for the activities of Rabs (Geyer et al., 1996; Pereira-Leal and Seabra, 2000).

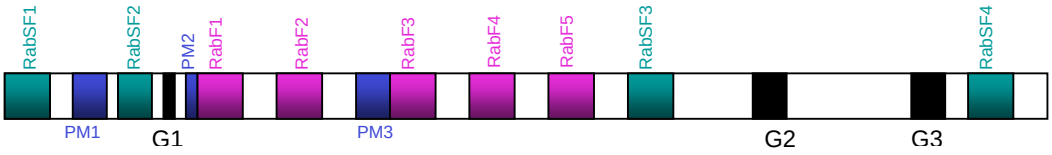

Figure 1.2.1: Diagram representing the structure of *rab* GTPases

G1-3 (black) are regions involved in guanine nucleotide binding, PM1-3 (blue) are regions involved in phosphate/$Mg2^{+}$ binding, RabF1-5 (pink) are Rab family motifs and RabSF1-4 (green) are subfamily-specific sequences. Adapted from (Bucci et al., 1995).

Studies undertaken by Pereira-Leal and Seabra (2000) facilitated the classification of Rabs. They showed the existence of five short conserved stretches of residues which appear to be diagnostic for the Rab family (Pereira-Leal and Seabra, 2000). These are termed Rab family motifs (RabF), with the section termed RabF1 localising to the effector domain. Most, though not all Rabs, also have a double-cysteine motif in the C-terminus which is regarded as a good diagnostic of a Rab protein. Rab8 and Rab13 are the exceptions as they contain only a single cysteine residue. Therefore the double-cysteine motif may confirm that a given small GTPase is a Rab, but it's absence should not be used to prove it is not (Pereira-Leal and Seabra, 2000).

The *rab* genes can also be placed into sub groups (such as Rab1a and Rab1b) in which the genes share over 70% amino acid identity to one another. It could be assumed that these genes are functionally related and interact with the same type of effector but this is not necessarily the case. For example, the Rab3 subgroup has at least 4 members in humans showing 77-85% amino acide identity with the greatest variance evident within the N- and C-terminal regions. Although they have high sequence homology their subcelluar targets and functional roles are distinct (Pavlos et al., 2001). Rab3a is expressed in neurons and neuroendocrine cells and together with Rab3c regulates neurotransmitter release, Rab3b is specifically expressed in polarized epithelial cells, while Rab3d is mainly expressed in adipocytes and is possibly involved in glucose transporter trafficking in response to insulin stimulation (Bucci et al., 1995). While working on Rab3a Ostermeier and Brunger (1999) identified three regions that contribute to an effector region pocket that mediates binding to the effector. These regions were named Rab complementary-determining regions (RabCDR's) with the individual regions called RabCDRI-III. These regions

were shown to correspond to subfamily-specific sequences RabSF3 and RabSF4 found by Moore (Moore et al., 1995; Pereira-Leal and Seabra, 2000). Taken together these bioinformatic analyses suggests that a GTPase can be identified as a Rab if it has the five Rab specific domains and usually, but not necessarily, a conserved PM/G (GTPase binding region) motif and a double-cystine C-terminal motif. Sub-families can be classified as such if they have 70% identity and conservation at the RabSF and RabF motifs (Pereira-Leal and Seabra, 2000).

Lastly, alternative splicing of a gene can result in different forms or a protein. *rab6a* is the only know *rab* to do this. The gene has a duplicated exon, and incorporation of either of the two exons by alternative splicing is shown to generate the isoforms named Rab6a and Rab6a' in human, which differ in only three amino acid residues (Echard et al., 2000). Interestingly this is conserved in zebrafish where two isoforms of Rab6a have also been found (Clark, MD, pers. com.).

1.2.3 Rab Regulators

Rabs are described as molecular switches, able to cycle between GTP bound (active) and GDP bound (inactive) forms and thus, recruiting a diverse group of "effector proteins" to the cytoplasmic leaflet of vesicular membranes (Zerial and McBride, 2001). When a Rab switches conformation to the inactive form, the effectors dissociate, the Rab is removed from the membrane and recycled back to a donor compartment. Rab function is promoted by the following factors: 1) guanine-exchange factors (GEFs) which accelerate guanine-nucleotide exchange and promote formation of the active, GTP-bound form on targeting to a donor membrane (Camus et al., 1995; Schmidt and Hall, 2002). 2) GTPase activation factors (GAPs) which stimulate guanine-nucleotide hydrolysis to promote formation of the inactive GDP-bound form (Settleman et al., 1992) and 3) GDP-dissociation inhibitors (GDIs) which prevent nucleotide dissociation, extracting the Rab from the target membranes and recycling it back to the donor membranes by binding it only in its GDP rather than GTP state (Fischer von Mollard et al., 1994; Sasaki et al., 1991; Seabra et al., 2002). GDI's transport the Rab back to the donor compartment for recycling without the Rab becoming active (Figure 1.2.2).

All members of the Ras superfamily of proteins undergo protein prenylation, a post-translational lipid modification that involves the attachment of a farnesyl (15-carbon) or geranylgeranyl (20-carbon) group to cysteine or serine residues at or near the C-terminus. These modifications are essential for the GTPases to associate tightly with cell membranes. Without this lipid modification, the proteins remain in the cytosol and are unable to associate with membranes or localise subcellularly, processes which are critical for their biological activities (Wennerberg et al., 2005, Casey and Seabra, 1996). Three different protein prenyltransferases have been identified which perform this role, protein farnesyl transferase (PFT or FTase), protein geranylgeranyl transferase type-I (PGGT or GGTase-I) and Rab geranylgeranyl transferase (RGGT or GGTase-II). These proteins have a heterodimeric structure and consist of distinct α- and β-subunits (Leung et al., 2006). FTase and GGTase-I recognise only substrates containing a CAAX motif at the C-terminus and these are found in the Ras sub familys of Rac and Rho. GGTase-II recognises only Rab substrates of which there are six different C-terminal motifs -CC, -CXC, -CCX, -CCXX, -CCXXX and –CXXX (Pereira-Leal and Seabra, 2000).

There are two conditions in which prenylation can occur. In the first, a newly synthesized Rab must form a stable complex with Rab escort protein (REP) to enable its recognition by GGTase-II. In the second, a newly synthesized Rab can associate with a preformed REP:GGTase-II complex (Mruk et al., 2005). The first scenario suggests association of an unprenylated Rab protein with REP where the interaction relies mostly on ionic bonds and does not involve the two C-terminal cysteine residues (Anant et al., 1998). This complex is then presented to GGTase-II, which adds two geranylgeranyl moieties to the Rab protein without prior dissociation of REP (Thoma et al., 2001b; Thoma et al., 2001c). After the transfer of the isoprenoids onto C-terminal cysteines, the complex remains associated until the binding of a new geranylgeranyl diphosphate (GGpp) molecule, which stimulates the release of the Rab-GG:REP complex. The REP then delivers the Rab to the target membrane (Leung et al., 2006). The second scenario implies that REP-1 and RGGT can form a tight complex in the presence of GGpp. This complex can associate with a Rab protein, but this occurs ten times more slowly than in the first scenario (Thoma et al., 2001a). This model depends on the *in vivo* concentrations of the proteins involved.

At high concentrations of all the components the second scenario maybe preferred whereas at low concentrations, the first scenario may be preferred.

Upon completion of its function at the target membrane the Rab/REP complex must then be returned to its membrane of origin. This is mediated by the cytosolic protein Rab GDP dissociation inhibitor (GDI). Rab-GDI interacts promiscuously with the GDP-bound Rab extracting it from membranes to the cytosolic reservoir for re-use (Wu et al., 1996). GDI/Rab forms a soluble complex in the cytoplasm which then delivers the Rab to the donor membrane. Once there, the GDI is released to the cytosol and the Rab remains membrane-associated. As GDI has a strong binding affinity for GDP bound Rabs the less well-characterized GDI-dissociation factor (GDF) catalyses the dissociation of Rab GDI complexes in order to enable transfer of Rabs from GDI onto membranes. Work in mammalian cells has shown that the integral membrane protein Yip3 acts catalytically to dissociate complexes of endosomal Rabs bound to GDI and to deliver them onto the membranes (Sivars et al., 2003). Rab-GDI's share homology with REP, but are thought to bind only prenylated Ypt/Rab proteins, preferentially in their GDP-bound form, inhibiting GDP dissociation and masking the prenyl modification (Seabra and Wasmeier, 2004). Once at the membrane the Rab is activated to the GTP-bound form by GEF and is ready to transport vesicles from the donor membrane to the target membrane. At this point the Rab is hydrolysed back to its GDP bound state with the help of GAP. The Rab is then ready to be recycled back to the donor compartment by GDI and the whole process starts again. All of the Ras superfamily of small GTPases exhibit high-affinity binding for GDP and GTP, and possess low intrinsic GTP hydrolysis and GDP/GTP exchange activities. GTPases within a family branch use shared GAPs and GEFs whereas GTPases in different branches exhibit structurally distinct but mechanistically similar GAPs and GEFs (Bernards and Settleman, 2004; Pan and Wessling-Resnick, 1998).

1.2.4 Rab Effectors and SNARES

Rab effectors are a soluble and diverse collection of proteins that transduce the Rab signal. A Rab effector preferentially binds to the GTP-bound conformation of Rab and competes for interaction with GAP proteins (Nakafuku et al., 1993; Schimmoller et al., 1998). They can be described as a protein - or protein complex - which binds the GTPase directly in a GTP-dependent manner and is required for the Rab-specific downstream function (Grosshans et al., 2006; Nakafuku et al., 1993). These Rab effectors perform diverse functions including vesicle budding, tethering and docking, vesicle transport by way of the cytoskeleton and vesicle motility. The Rab effectors show structural heterogeneity with some effectors sharing structural features. For example, p115/Uso1p, Rabaptin-5 and early endosome antigen 1 (EEA1), all contain predicted coiled-coil regions, Rab3-interacting molecule (Rim1), EEA1 and Rabenosyn-5 contain FYVE zinc-fingers (named after four proteins Fab1, YOTB/ZK632.12, Vac1, and EEA1) (Christoforidis et al., 1999a; McBride et al., 1999; Stenmark et al., 1996). The best characterized Rab effectors have been shown to mediate tethering and docking of a Rab-bearing vesicle with the target membrane to which it will fuse (McBride et al., 1999; Zerial and McBride, 2001). Other well characterised effectors are involved in vesicle motility while in recent work interesting insights into cell adhesion and cell signalling have been identified (Eggenschwiler et al., 2001; Eggenschwiler et al., 2006; Gibbs et al., 2004; Lock and Stow, 2005; Pellinen et al., 2006; Scholpp and Brand, 2004).

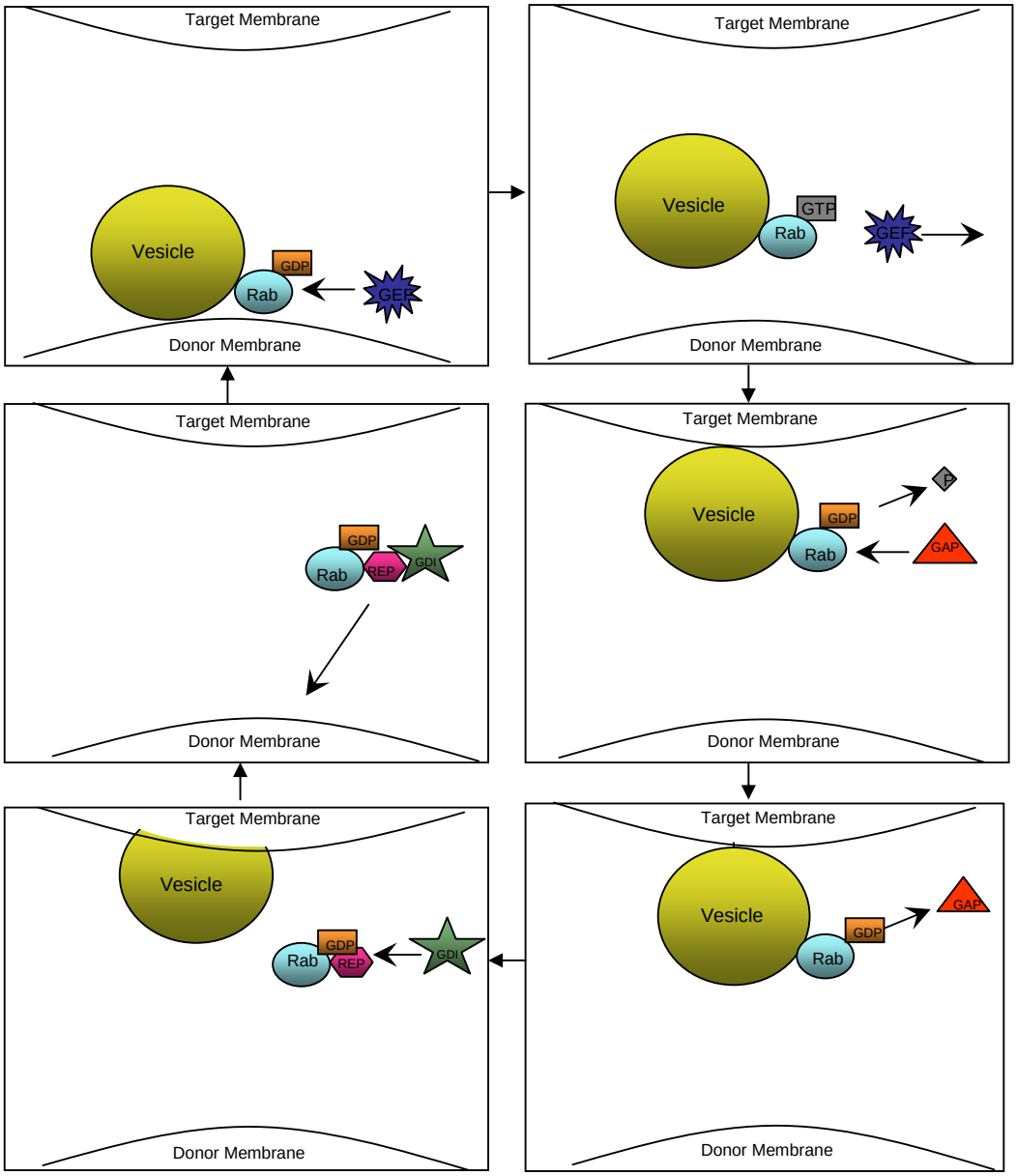

Figure 1.2.2: Diagram showing the regulation of Rab GTPases during vesicle trafficking.

1.2.4.2 Tethering Effectors

The best characterized Rab effectors have been shown to mediate tethering and docking of a Rab-bearing vesicle with the target membrane to which it will fuse (McBride et al., 1999; Zerial and McBride, 2001). Membrane tethering is a conserved mechanism that depends on Rab effectors. In yeast, tethering of ER-derived vesicles to the Golgi complex depends on the membrane recruitment of Uso1p by Ypt1p which is exclusively required on target Golgi membranes (Cao and Barlowe, 2000). In mammals the homologue of Uso1p, p115 binds directly to Rab1 (Beard et al, 2005). Rab1 recruits p115 onto coat protein 2 (COPII) vesicles at the budding step committing the vesicles to targeting and fusion (Allan et al., 2000). In yeast a multi-protein complex called TRAPP (transport protein particle) also targets ER-derived vesicles to the Golgi apparatus by accelerating nucleotide exchange on Ypt1p in the Golgi (Sacher et al., 1998; Wang et al., 2000). The delivery of post-Golgi vesicles to the plasma membrane in yeast depends on Sec4p and the tethering factor exocyst. The exocyst is a complex of eight proteins that are specifically required for exocytosis with an equivalent complex exists in mammals (Kee et al., 1997; TerBush et al., 1996). The exocyst complex in yeast contains eight different proteins namely Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p Exo70p and Exo84p. Most of these units were discovered when mutations resulted in an accumulation of vesicles destined for the plasma membrane (Wiederkehr et al., 2004). The complex localizes to sites of exocytosis on the plasma membrane in a process which is determined by the subunit Sec3p (Finger et al., 1998). The exocyst mediates vesicle targeting and, through another subunit (Sec15p) interacts specifically and directly with Sec4p in a GTP-dependent manner, a process that is considered to control the assembly of the whole complex (Guo et al., 1999). The mammalian exocyst comprises homologues of the eight subunits found in yeast. In mammals the subunit responsible for association with Rabs is Sec5, rather than Sec15 in yeast. Another difference between the yeast and mammalian exocyst is that the mammalian complex associates with a protein called RaIA instead of a Rab (Brymora et al., 2001).

In early endocytosis, mammalian Rab5 regulates both clathrin-coated vesicle-mediated transport from the plasma membrane to the early endosomes and homotypic early endosome fusion (Bucci et al., 1992; Gorvel et al., 1991). Early

endosomal autoantigen 1 (EEA1) is the Rab5 effector that mediates tethering/docking of early endosomes (Christoforidis et al., 1999a). This will be discussed in further detail in Chapter 5.

1.2.4.1 SNAREs

Vesicle tethering and docking requires another group of proteins for completion. Once the vesicle reaches its target, the effector triggers the interaction of vesicular (v-SNARE) and target (t-SNARE) proteins. This brings the vesicle and the target membranes into close apposition and mediates fusion (Kierszenbaum, 2000).

SNARE (Soluble NSF Attachment Protein REceptor) proteins have been proposed to mediate all intracellular fusion events (Sollner et al., 1993). The first of these to be discovered were the synaptic proteins; SNAP-25, syntaxin (STX1), and VAMP (Vesicle –Associated Membrane Protein or synaptobrevin) (Bennett et al., 1992; Oyler et al., 1989; Trimble et al., 1988). STX1 and VAMP are anchored to the membrane by a carboxyl-terminal transmembrane domain while SNAP-25 is peripherally attached to the membrane by the covalent attachment of 16-carbon saturated fatty acids (palmitate) to four cysteine residues in the central region of the protein (Chen and Scheller, 2001). It was thought that each type of transport vesicle had a distinct v-SNARE, which paired with an unique complementary t-SNARE at the appropriate target membrane (Rothman and Warren, 1994; Sollner et al., 1993). This interaction enabled the vesicle to dock at the appropriate membrane, leading to the subsequent dissociation of the SNARE complex, with the ATPase activity of NSF (*N*-ethylmaleimide Sensitive Fusion protein) driving the fusion. This view has since been modified and it is now thought that NSF acts as a chaperone to reactivate SNAREs after fusion instead of directly driving fusion (Morgan and Burgoyne, 1995). The specificity of targeting is brought about by the Rab protein family, with assembly of the SNARE complex driving the lipid fusion (Chen and Scheller, 2001).

Rabs and SNAREs interact via intermediate proteins the nature and number of which can be quite diverse between different steps of membrane traffic (Armstrong, 2000; Tall et al., 1999). The active form of the Rab protein generally nucleates the assembly of a protein complex that can ultimately bind to or tether the target

membrane and then a SNARE complex can form (Armstrong, 2000). Certain organelles are enriched in SNAREs which helps them to identify the correct target and to limit non-specific fusion events. During vesicle transport a given SNARE will spread throughout many cellular compartments such that organelles will contain SNARE complexes which must remain inactive until they return to their place of specific function. This is where the Rabs provide their additional layer of regulation (Zerial and McBride, 2001).

1.2.4.3 Budding effectors

It is thought that Rab proteins influence vesicle budding, in a transport event-specific manner (Allan et al., 2000; Bucci et al., 1992; Riederer et al., 1994). *in vivo* studies in mammals have indicated a possible role for Rab1 in budding of vesicles from the ER (Allan et al., 2000; Nuoffer et al., 1994). While Rab9 has been implicated in budding from endosomes directed to the *trans*-Golgi network (TGN) (Riederer et al., 1994). In yeast a component (Vam2p/Vps41p) of the Ypt7p (Rab7)-tethering complex HOPS has been implicated in the budding of vesicles from the Golgi (Price et al., 2000a). HOPS stands for homotypic fusion and vacuole protein sorting and is also referred to as Class C Vps protein complex (Rieder and Emr, 1997). Components of this complex include as mentioned the Vam2p/Vps41p proteins as well as Vam6p/Vps39p proteins (Price et al., 2000a; Seals et al., 2000). It should be noted that Ytp7p has not been shown to be directly implicated in Golgi budding (Price et al., 2000b). *in vivo* mammalian Rab5 has been shown to modulate the half-life of clathrin-coated pits on the plasma membrane during vesicle formation (Bucci et al., 1992). *in vitro* mammalian Rab5 is required for vesicle formation with the suggested Rab5 GAP, RN-Tre, downregulating Rab5 and inhibiting receptor internalization (Lanzetti et al., 2000; McLauchlan et al., 1998).

1.2.4.4 Motility Effectors

Rabs have been shown to determine the distribution of cellular compartments by regulating the movement of vesicles and organelles along cytoskeletal filaments (Cheney and Rodriguez, 2001). Probably the best charachterized of these is the Rab motility effector myosin Va. In collaboration with Rab27a and melanophilin (another Rab effector), myosin Va has been shown to be involved in correct pigment

patterning in both mice and humans as defects in any of these genes is responsible for the human disorder Griscelli's syndrome (see section 1.2.6.4) (Bahadoran et al., 2001; Matesic et al., 2001; Menasche et al., 2000). In yeast, interactions have been found between Sec4p and the myosin heavy chain Myo2p (Pruyne et al., 1998) suggesting that vesicles are propelled by motor proteins along polarized actin cables towards the site of exocytosis (Pruyne et al., 1998; Schott et al., 1999). In a screen looking for mammalian Rab effectors it was found that Rab6 interacts with a kinesin-like protein (Echard et al., 1998), Rabkinesin-6, which has since been shown to be important for cytokinesis (Echard et al., 1998; Hill et al., 2000). Rab5 has been shown to regulate the attachment of early endosomes to microtubules and their motility along those microtubules (Nielsen et al., 1999).

1.2.4.5 Cell Adhesion Effectors

Recent papers have shown a role for Rabs in cell adhesion (Kohler and Zahraoui, 2005; Ulrich et al., 2005). Small GTPases, such as Rab3b, Rab8 and Rab13 have been shown to localize to tight junctions between cells (Lutcke et al., 1993). These tight junctions are multifunctional complexes involved in various signalling events controlling cell–cell adhesion, differentiation and polarity. Kohler et al (2004) demonstrated that mammalian Rab13 directly interacts with PKA and inhibits PKA-dependent phosphorylation of the actin-remodelling protein VASP resulting in the recruitment of VASP, ZO-1 and claudin1 to cell–cell junctions to be inhibited (Kohler et al., 2004). Kohler also suggested a link between Rab13 and protein kinase A signalling during tight junction assembly in epithelial cells (Kohler et al., 2004).

Pellinen has shown an association, in both yeast and mammalian cells, of Rab21 and Rab5 with integrins (Pellinen et al., 2006). Continual trafficking of integrin cell adhesion molecules to and from the cell surface is vital to cell migration. Rab21 and Rab5 associate with the cytoplasmic domains of α-integrin chains, and their expression influences the endo/exocytic traffic of the integrins (Pellinen et al., 2006). Knock down of Rab21 impairs integrin-mediated cell adhesion and motility, whereas its overexpression stimulates cell migration and cancer cell adhesion to collagen and human bone (Pellinen et al., 2006). If there is a point mutation in the integrin in the region where the integrin associates with Rab21, when Rab21 is overexpressed, it

fails to induce cell adhesion (Pellinen et al., 2006). It is thought that these Rabs target the integrins to intracellular compartments and this regulates cell adhesion. (Pellinen et al., 2006).

1.2.5 Disorders caused by Mutations in Rabs

Although there are now over 60 Rabs uncovered in the human genome only a small number of mammalian Rabs have been show to be directly linked with specific disorders: These Rabs are Rab3a, Rab7, Rab23, Rab27a and Rab38 (Eggenschwiler et al., 2001; Kapfhamer et al., 2002; Loftus et al., 2002; Menasche et al., 2000; Verhoeven et al., 2003).

1.2.5.1 Rab3a and the earlybird mouse.

In a screen for mouse mutants with abnormal rest–activity and sleep patterns, a "semidominant" mutation called earlybird - which shortens the circadian period of locomotor activity and homeostatic response to sleep loss was - identified (Kapfhamer et al., 2002). Sequence analysis of *rab3a* identified a point mutation in a conserved amino acid (Asp77Gly) within the GTP-binding domain resulting in significantly reduced levels of Rab3a protein suggesting that the Asp77Gly change may affect the overall stability of the protein. Phenotypic assessment of earlybird mice and mice with a null allele of *rab3a* revealed anomalies in circadian period and sleep homeostasis, providing evidence that Rab3a-mediated synaptic transmission is involved in these behaviours (Kapfhamer et al., 2002). Rab3s are known to be the most abundant Rab proteins in the brain and have a regulatory role in synaptic vesicle trafficking. Mice with a targeted loss-of-function mutation in *rab3a* have defects in Ca2+-dependent synaptic transmission which lead to an increased number of vesicles released in response to an action potential in the mutant mice (Kapfhamer et al., 2002).

1.2.5.2 Rab7 in Charcot-Marie-Tooth type 2B Neuropathy

Charcot-Marie-Tooth type 2B (CMT2B) neuropathy is a clinical disorder characterized by distal muscle weakness and wasting and a high frequency of foot ulcers, which results in the need for amputations of the toes due to recurrent infections. The gene responsible has been mapped to chromosome 3q13-q22 where two mis-sense mutations (Leu129Phe and Val162Met) were found in Rab7. The alignment of Rab7 orthologues shows that both missense mutations target highly conserved amino acid residues. Rab7 is a late endosomal protein with expression found in sensory and motor neurons (Verhoeven et al., 2003). Later work showed a further mutation a heterozygous A to C mutation, changing asparagine to threonine at codon 161 whose phenotype lacked the motor features of the other mutations. This mutation is situated adjacent to one of the previous mutations suggesting hotspot for mutations in the highly conserved C terminus of Rab7 (Houlden et al., 2004).

1.2.5.3 Rab23 and the openbrain mouse

Sonic hedgehog (Shh) is essential for many aspects of mammalian embryogenesis including the patterning of the neural tube and limbs (Chandrasekhar et al., 1998; Couve-Privat et al., 2004; Tsukazaki et al., 1998). Hedgehog acts as an extracellular ligand which binds the trans-membrane receptor Patched (Ptc). In the absence of Hedgehog, Patched represses the activity of a second transmembrane protein, Smoothened (Smo), thereby blocking the downstream signalling pathway (Murone et al., 1999).

The mouse *open brain (opb*) gene product has been shown to have an opposing role to Shh (Gunther et al., 1994). In neural patterning *opb* is required for dorsal cell types and Shh is required for ventral cell types in the spinal cord (Eggenschwiler and Anderson, 2000). Mutations in *opb* causes the embryos to die during the second half of gestation with an open neural tube in the head and spinal cord, abnormal somites, polydactyly and poorly developed eyes (Gunther et al., 1994). *opb* mutants lack dorsal cell types specifically in the caudal spinal cord while Shh mutants lack ventral cell types throughout the spinal cord (Eggenschwiler and Anderson, 2000). The *opb* mutant phenotypes resemble those produced by partial loss of function of Ptc which acts as a negative regulator of the Shh pathway (Eggenschwiler et al., 2001). The *opb* mutant phenotype could be the result of partial activation of the Shh signalling

pathway in dorsal and lateral neural cells. When *opb* was cloned it was found to encode for Rab23 (Eggenschwiler et al., 2001). Dorsalizing signals are thought to activate transcription of *rab23* in order to silence the Shh pathway in dorsal neural cells. (Eggenschwiler et al., 2001). More recent research using Rab23-GFP showed that Rab23 co-localizes in endosomes with Ptc but it was thought that it may act more distally in regulating Shh (Evans et al., 2003).

Additional proteins which influence Shh signalling and whose cellular trafficking could be regulated by Rab23 have been uncovered (May et al., 2005). A forward genetic approach in mice identified a role for intraflagellar transport (IFT) genes in Shh signal transduction, downstream of Ptc and Rab23 (May et al., 2005). IFT proteins are essential for cilia assembly and have recently been associated with a number of developmental processes, such as left-right axis specification and limb and neural tube patterning (Haycraft et al., 2005; May et al., 2005) (Wang et al., 2006).

Work this year (Eggenschwiler et al., 2006) has shown that, contrary to initial speculations suggesting that Ptc and Smo are the targets of Rab23 action, Rab23 mutants do not appear to affect the localization and dynamics of either protein. Genetic analyses have now shown that Rab23 actually functions downstream of Smo and affects the function of the Shh-regulated Gli family of transcription factors (Eggenschwiler et al., 2006). Double mutant analysis has shown that the primary target of Rab23 is the Gli2 activator and that Rab23 and Gli3 repressor have additive effects on patterning (Eggenschwiler et al., 2006). Analysis of Gli3 protein suggests that Rab23 also has a role in promoting the production of Gli3 repressor (Eggenschwiler et al., 2006). Antibodies generated against Rab23 showed that the protein is highly enriched in the adult rodent brain and present in low levels in multiple tissues of the adult rodent. This suggested a role for Rab23 not only in the embryo but also in the adult (Guo et al., 2006).

1.2.5.4 Rab27a in Griscelli syndrome and Ashen mice

Griscelli's syndrome (GS) is a rare autosomal recessive disease characterized by an immune deficiency. Patients have uncontrolled T-lymphocyte and macrophage

activation (haemophagocytic syndrome, HS) leading to death in the absence of bone-marrow transplantation. In addition, patients show partial albinism with striking silvery-metallic hair sheen and mild cutaneous depigmentation (Griscelli et al., 1978). The disease has been attributed to an abnormal melanosome distribution (Griscelli et al., 1978). Melanosomes are melanin-containing vesicles in the epidermis the uniform distribution of which leads to normal pigmentation.

Originally the mutation responsible for this syndrome was mapped to 15q21 and the myosin-V gene (Pastural et al., 1997). However a mutation in the *rab27a* gene can also cause GS (Pastural et al., 2000). Studies have shown that in normal melanocytes Rab27a colocalizes with melanosomes but in melanocytes isolated from a patient with GS, there is abnormal melanosome distribution and a lack of *rab27a* expression. Re-expression of *rab27a* in GS melanocytes restores melanosome transport to dendrite tips, leading to a phenotypic reversion of the diseased cells implicating Rab27a as a key component of vesicle transport machinery in melanocytes (Bahadoran et al., 2001). Unlike the GS patients with the *myosin-V* gene mutation GS patients with the *Rab27a* mutation exhibited reduced T cell cytotoxicity and cytolytic granule exocytosis (Menasche et al., 2000).

The pigmentation defect mutants *dilute* (*d*), *leaden* (*ln*), and *ashen* (*ash*) in mice show mutations in *myosin Va*, *melanophillin* and *rab27a* respectively, with defects in *myosin V* and *rab27a* and more recently melanophilin corresponding to genes shown to be defective in GS (Matesic et al., 2001; Menasche et al., 2003; Mercer et al., 1991; Wilson et al., 2000). All three mouse mutations produce a lightened coat colour due to defects in pigment vesicle transport (Wilson et al., 2000) in addition *ash* mice show platelet defects resulting in increased bleeding times and a reduction in the number of platelet dense granules.

Melanosomes are transported by microtubules to the dendrite tips and then are retained there by MyoVa-mediated interaction with the cortical actin cytoskeleton (Wu et al., 1998). It is thought that Rab27a, melanophilin, and myosin Va form a ternary complex in the human melanocyte cells where melanophilin has a role in bridging Rab27a on melanosomes and myosin Va on actin filaments during

melanosome transport. (Costa et al., 1999; Karcher et al., 2001; Nagashima et al., 2002).

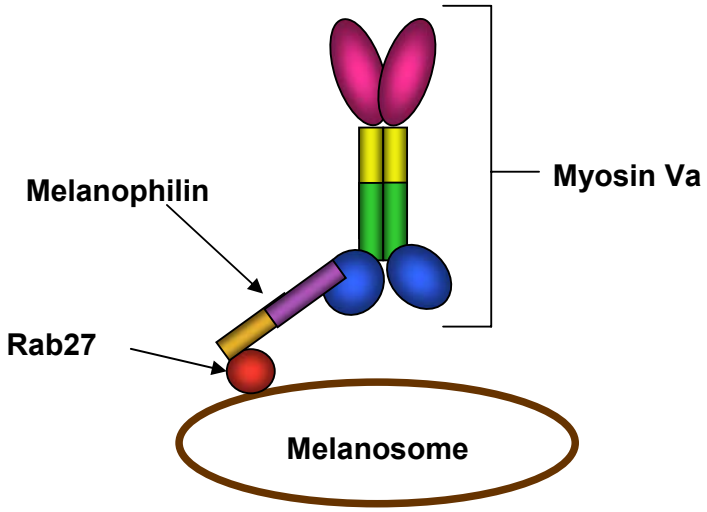

Figure 1.2.3: Diagram of myosin Va attachment to melanosomes via melanophilin.

(Adapted from Cheney and Rodriguez, 2001; Costa et al., 1999; Karcher et al., 2001; Maciver, 2006)

1.2.5.5 Rab 38 and the chocolate mouse.

Disorders with reduced pigmentation can be placed into two groups according to whether they affect melanocyte differentiation or melanosome function. Those that affect melanocyte differentiation are characterized by a localized absence of melanocytes resulting in ‘‘white patch’’ patterns. Disorders with affected melanosome function include Oculocutaneous albinism (OCA) I-IV and Griscelli syndrome (GS). Microarray analysis has revealed that *rab38* demonstrates a similar expression profile to melanocytic genes and further comparative genomic analysis has linked human *rab38* to the mouse chocolate (*cht*) locus (Loftus et al., 2002). Mutant mice exhibit a brown coat similar in colour to mice with a mutation in tyrosinase-related protein 1 (*Tyrp1*). This forms a mouse model for OCA (Loftus et al., 2002; Suzuki et al., 2003). In the melanocytes of these mice the targeting of TYRP1 protein to the melanosome is impaired, suggesting that Rab38 plays a role in the sorting of TYRP1 (Loftus et al., 2002). More recent work has also shown that *rab38* mRNA and native protein are expressed in a tissue-specific manner in the lung, skin, stomach, liver, and kidney. Cellular analysis has shown Rab38 mainly

colocalized with endoplasmic reticulum-resident proteins and also partly with intermittent vesicles between the endoplasmic reticulum and the Golgi complex. This implies that Rab38 abnormality may cause multiple organ diseases as well as OCA (Osanai et al., 2005).

1.2.6 Morphogen Signalling and Vesicular Trafficking.

1.2.6.1 Endocytosis

Endocytosis has been shown to be essential for cell motility, cell signalling and cell adhesion and can be broadly placed into two categories; clathrin mediated (CME) and non-clathrin mediated (Le Roy and Wrana, 2005). Much of the literature is concerned with CME which is of particular interest in development, as a possible route by which TGF-β receptors, such as those for the Nodals, are internalized (Anders et al., 1998). Clathrin independent endocytosis is used to describe any endocytic process that does not involve clathrin (Le Roy and Wrana, 2005). One form which is suggested to also play a role in development relies on cholesterol-rich membrane domains such as lipid rafts and caveolae (raft/caveolae endocytosis)(Polo and Di Fiore, 2006). In this form of endocytosis receptors are internalized and thought to be delivered for degradation (Le and Nabi, 2003; Pelkmans et al., 2005). Investigations using epiderimal growth factor receptors have suggested that during signalling a choice is made between CME and raft/caveolae endocytosis (Polo and Di Fiore, 2006). If low doses of receptor are internalized then endocytosis progresses via CME. High doses of receptor internalization have been shown to correlate with the monoubiquitination of EGFR and so are probably destined for degradation via raft/caveolae endocytosis. It has therefore been suggested that CME is preferred at low doses for prolonged endosomal signalling but some of the receptors are routed through the raft/caveolae endocytosis pathway for degradation at higher does. Such routing avoids excess stimulation with signalling considered as the ratio of CME to raft/caveolae endocytosis (Polo and Di Fiore, 2006). This view, however, has been recently challenged by the suggestion that clathrin coated vesicles may play a part in the degradative endocytic pathway (Lakadamyali et al., 2006).

The correct sorting of endocytic ligands and receptors is essential for proper cell function. Early endosomes are considered to be the initial sorting station, where cargos for degradation are separated from those for recycling (Lakadamyali et al., 2006). Live mammalian cell imaging used to monitor individual endosomes and ligands has shown that a sorting mechanism takes place prior to early endosome entry and that endosomes are comprised of two distinct populations. The first of these endosomes are highly mobile on microtubules and mature rapidly toward late endosomes. The second are static endosomes that mature much more slowly. Those cargos destined for degredation are targeted to the dynamic endosomes whereas those destined for recycling are enriched in the larger, static population (Lakadamyali et al., 2006). This pre-early endosome sorting process is thought to be dependent on microtubule motility, involve endocytic adaptors and interestingly begin at clathrin-coated vesicles (Lakadamyali et al., 2006).

In metazoans, CME requires dynamin, a member of a family of self assembling GTPases. During endocytosis, dynamin forms an oligomeric 'collar', in a GTP-dependent manner, which is thought to induce fission of vesicles from the plasma membrane (Takei et al., 1995), in addition to recruiting effectors such as actin-binding proteins (Elde et al., 2005). The actin cytoskeleton is important in endocytosis as demonastrated by interference with actin turnover in mammalian cells by pharmacological agents which inhibits endocytic uptake and the formation of coated vesicles (Lamaze et al., 1997). However, this block in endocytosis in mammalian cells seems to be partial (Fujimoto et al., 2000; Moskowitz et al., 2003).

1.2.6.2 Evidence for vesicle trafficking in morphogen movement

The trancytosis model of morphogen movement proposes that morphogens use vesicular trafficking through the cells to reach their target cells. Much of the evidence for this comes from work in *Drosophila* other investigations, however, suggest a role for this mechanism in vertebrates.

The *Drosophila* Dpp protein, the orthologue of Bmp2/4, has been shown to act as a morphogen, patterning the early embryo including well characterised roles in specifying cell fates along the AP axis of the wing (Eldar et al., 2002; Lecuit et al.,

1996; Weigmann and Cohen, 1999). Functional GFP-Dpp fusions have enabled visualisation of Dpp showing that it forms a long-range dose gradient across 30 cells (Entchev et al., 2000; Teleman and Cohen, 2000) travelling at a rate of more than four cells per hour in all directions. This is not unusual for signalling factors and is consistent with diffusion, however Dpp cannot form a stable gradient by diffusion (Entchev et al., 2000; Teleman and Cohen, 2000). It is possible this is due to the mature Dpp peptide binding to the extracellular matrix components and so restricting its extracellular movement. The binding of Dpp to the receptor may also decrease the range of Dpp. Additionally Dpp signalling range may be reduced by internalized Dpp being degraded in receiving cells upon targeting to the late endosome/lysosome. Labelled GFP-Dpp disappears within 3h and is considered to be actively degraded (Entchev and Gonzalez-Gaitan, 2002).

Dpp has been show to be targeted towards degradation by a sorting mechanism which depends on the small GTPase Rab7 (Entchev et al., 2000). Dpp's signalling range has been shown to be determined by Rab7-dependent rates of degradation (Entchev et al., 2000). Entchev *et al* have shown that Dpp is internalized by the receiving cells where it localizes in endosomes and is then excised with the help of dynamin in clathrin-coated pits to form endocytic vesicles (Entchev et al., 2000). In the dynamin mutant (*shibire*) Dpp is not internalized and can only be observed extracellularly adjacent to its source (Entchev et al., 2000). In endocytic and endosomal-defective tissue Dpp has a range which, Entchev *et al* propose is consistent with their estimated range - less than five cells - of Dpp in the absence of transcytosis (Entchev et al., 2000). They proposed that receiving cells are required to perform transcytosis in order to form a long-range Dpp gradient (Figure 1.2.4). Their transcytosis model implies that Dpp is internalized in the receiving cell, traffics through the endocytic pathway and is re-secreted allowing it to signal in adjacent cells. Consistent with this is the observation that Rab5 activity can alter the signalling range of Dpp (Entchev et al., 2000). Rab5 controls endocytic trafficking by directing the budding of endocytic vesicles from the plasma membrane (McLauchlan et al., 1998) to their fusion with the early endosome (Bucci et al., 1992). Cells expressing a dominant negative Rab5 showed reduced range of expression of the Dpp target gene *spalt,* whilst cells overexpressing Rab5 showed increased expression range of *spalt* (Entchev et al., 2000). Enchev *et al* suggested

that once Dpp enters the endocytic pathway, it can be sorted towards degradation or recycling (Entchev et al., 2000). Recycled Dpp is re-secreted and moves forward into the target tissue. Dpp transcytosis is considered to be controlled by endocytic Rab proteins which include Rab5 and Rab7.

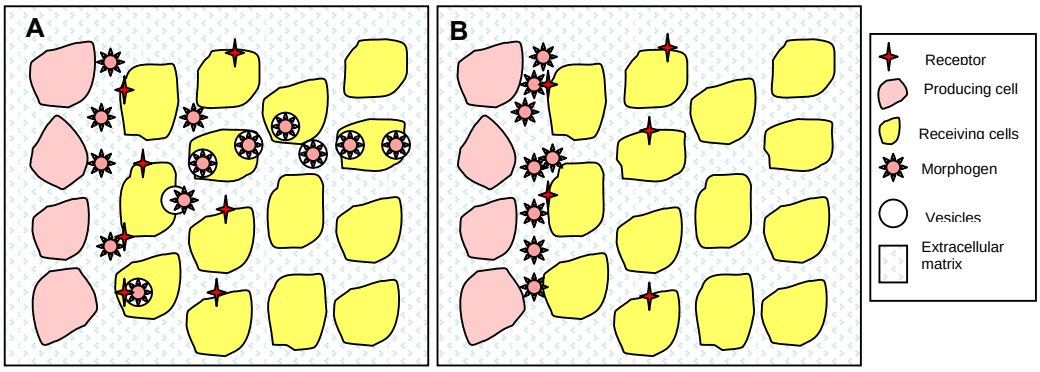

Figure 1.2.4: Diagramatic representation of planar transcytosis possible role in morphogen gradient formation.

Diagram A shows morphogens moving through receiving cells in vesicles. Diagram B shows planar transcytosis impeded by a reduction in endocytosis (i.e.*rab5* deficient cells).

Teleman and Cohen (2000) have an alternative explanation to transcytosis. They agree that Dpp-GFP forms an unstable extracelluar gradient which can travel over long distances. However, they show with the slow spread of target gene induction that this extracellular gradient differs from the activity gradient. They propose that the activity gradient is shaped at the level of receptor activation possibly by the need to downregulate a Dpp repressor (Teleman and Cohen, 2000). A subsequent paper by Belenkaya et al (2004) contradicted earlier studies and showed that Dpp is mainly extracellular with its extracellular gradient coinciding with its activity gradient. They demonstrated that blocking endocytosis using *shibire* does not block Dpp movement but inhibits Dpp signal transduction suggesting endocytosis is needed but not essential for transport. They suggest a model where Dpp moves along the cell surface by restriced extracellular diffusion involving glypicans Dally and Dally-like (Belenkaya et al., 2004).

The *Drosophila* Wnt homolog Wingless (Wg), has been found in vesicular structures at the target tissue (Gonzalez et al., 1991). It has therefore been suggested that it

spreads throughout the target tissue by trafficking through the receiving cells during patterning of the epidermis in the Drosophila embryo (Bejsovec and Wieschaus, 1995). Wg is thought to be internalized by Dynamin-dependent endocytosis, since in dynamin mutant embryos Wg is only able to elicit signalling in the cells neighbouring the source (Bejsovec and Wieschaus, 1995). This indicates that long-range Wg activity requires endocytosis. The finding that a response is elicited in neighbouring cells implies that Wg signal transduction in these neighbouring receiving cells could occur in the absence of Wg internalization (Bejsovec and Wieschaus, 1995). Alternatively this suggests that dynamin is required for secretion (Strigini and Cohen, 2000). In the *Drosophila* wing, impaired dynamin function reduces Wg transcription but when Wg production is unaffected, extracellular Wg levels are increased by impaired dynamin (Seto and Bellen, 2006). Despite this, target gene expression is reduced, suggesting that internalization at the target is also required for efficient Wg signalling *in vivo*. Rab5 deficient cultured cells showed a reduction of the activity of the Wg reporter Super8XTOPflash suggesting that internalization and endosomal transport facilitate Wg signalling (Seto and Bellen, 2006). When endosomal transport is impaired, Wg signalling is reduced however, when transport to endosomes is increased expression of Wg targets is enhanced. This increased signalling correlates with greater colocalization of Wg, Arrow, and Dishevelled on endosomes (Seto and Bellen, 2006). Regulation of endocytosis is the mechanism through which Wg signalling levels are determined (Seto and Bellen, 2006).

Hedgehog (Hh) has been shown to restrict the propagation of Wg signalling suggesting that this signalling pathway sets a barrier for the spreading of Wg (Sanson et al., 1999). It has been suggested that it does this by vesicular trafficking (Dubois et al., 2001). Wg degradation is specifically enhanced posteriorly by a mechanism thought to be initiated by Hedgehog activating EGF receptor signalling (Scott, 2001). Sequentially EGF receptor signalling is thought to activate the transcription of an unknown factor in posterior cells and this in turn enhances Wingless degradation (Dubois et al., 2001). In the absence of the Hh ligand, Patched (Ptc) antagonizes Hh signalling by binding Smoothened and blocking its signalling activity and possibly destabilizing it (Chen and Struhl, 1996; Denef et al., 2000). When Hh is present it binds to Ptc triggering its internalization, followed by phosphorylation of

Smoothened which becomes stabilized and accumulates at the surface and signals (Denef et al., 2000). There is the possibility that Hh elicits signalling by diverting Ptc into a distinct trafficking route releasing Smoothened from its repression (Chen and Struhl, 1996; Denef et al., 2000) and implying a role for Hh in vesicular trafficking. Vesicle trafficking may have an additional role in the movement of Nodal and Lefty. In chick embryos Nodal-GFP and Lefty-GFP fusion proteins are localized in the endosome (Sakuma et al., 2002) suggesting the possibility that Nodal and Lefty dispersal occurs by intracellular trafficking. Vesicular trafficking has been suggested to play one of two roles depending on the morphogen (Entchev and Gonzalez-Gaitan, 2002). Intracellular trafficking could push forward the ligands when the extracellular space restricts its movement as seems to be the case for Dpp in the wing. Alternatively, internalization and degradation of the morphogens could restrict the range of the morphogen if the extracellular matrix permits its long-range diffusion as appears to apply to Wg in the Drosophila embryo (Entchev and Gonzalez-Gaitan, 2002).

1.3 Nodal signalling

Zygotic Nodal signalling is essential for mesoderm and endoderm formation in the developing embryo (Feldman et al., 1998) with evidence in chick suggesting a possible role for endocytosis in its movement (Sakuma et al., 2002). Recently maternal transcripts of the zebrafish nodal Squint have been shown to be localized to cells that may form the dorsal side of the embryo (Gore et al., 2005) suggesting a role for maternal transcripts of Nodal protein in DV patterning. Nodal proteins are therefore considered to be of great importance in the study of zebrafish development

1.3.1 Introduction to Nodal Signalling

Nodal is a gene found to be essential for the establishment of the primitive streak, in mice, from which mesoderm and endoderm are derived. *nodal* encodes a TGF-β superfamily ligand (Zhou et al., 1993) identified by an insertional mutagenesis screen in mouse. Mutant embryos, lacking a functional *nodal* gene fail to gastrulate and the expression of mesoderm markers such as *brachyury* is severely inhibited (Rebagliatia et al., 1998). Zebrafish have 3 homologues of this gene *squint* (*sqt*), *cyclops* (*cyc*) and *southpaw*. Squint and Cyclops are expressed at the blastoderm margin and are required for prechordal plate and notochord formation. In their absence there is a lack of mesoderm and endoderm formation (Dougan et al., 2003; Feldman et al., 1998) (Figure 1.3.1). An additional role for Nodal signalling has been observed in patterning axial mesoderm precursors in the organizer along the anterioposterior axis (Gritsman et al., 2000). This patterning occurs by differential activation of the Nodal signalling pathway (Gritsman et al., 2000). Embryos mutant for both *squint* and *cyclops* do not develop a shield and, in addition markers for dorsal mesoderm such as *goosecoid* and *floating head* are not expressed at the onset of gastrulation (Feldman et al., 1998). The nodal related gene *southpaw (spaw)* has been shown to regulate left-right asymmetry in the visceral organs and brain of the developing embryo. *spaw* is expressed bilaterally in paraxial mesoderm precursors then subsequently within the left lateral plate mesoderm (Long et al., 2003) but is not detected prior to

somitogenesis. Since much of this thesis is concerned with events preceding somitogenesis, the actions of *spaw* will not be fully discussed.

The *nodal* related genes encode a pro and mature region that once transcribed are cleaved to produce a mature ligand which is a covalently disulfide-linked dimer. All TGF-β related ligands show a conserved cysteine this is essential for the secretion and biological function of the protein (Sampath et al., 1998). A mutation of this cysteine is thought to disrupt the formation of disulphide bonds and possibly ligand dimerization.

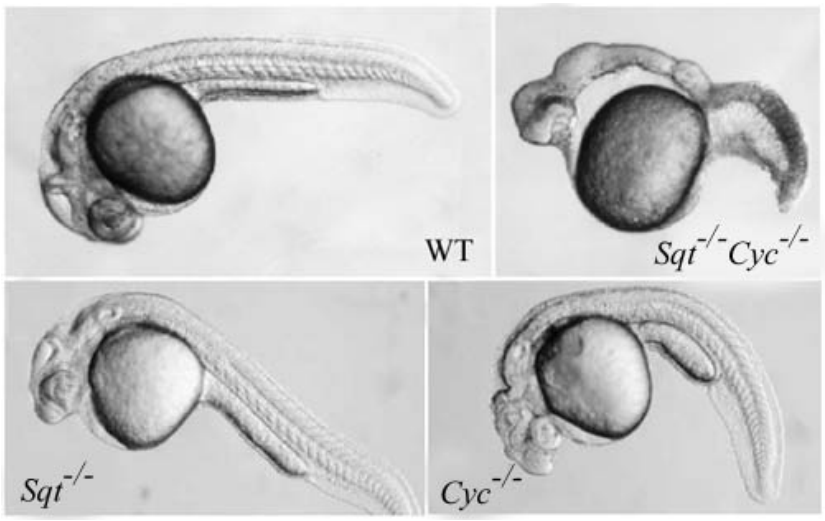

Figure 1.3.1: The zebrafish nodal mutants.

Comparing wild type embryos to ***squint*** homozygous mutants, ***cyclops*** homozygous mutants and both ***sqt*** and ***cyc*** homozygous mutants (Feldman et al., 1998).

1.3.2 Cyclops

cyclops muatants have a variety of alleles of differing types and strengths most derived from ENU mutagenesis (Table 13.1) (www.zfin.org) The *cyclops (cyc)* gene product is required for the formation of ventrally located cells in the neuroectoderm, both in the forebrain and more posteriorly in the floor plate (Solnica-Krezel et al., 1996). *cyclops* mutants have been shown to have a failure in specification of cells

that come to occupy the ventral midline of the neural tube (Hatta et al., 1991) giving a cyclopean phenotype (Figure 1.3.1).

Allele	Mut. Type	Brief Description
b16	Deficiency	Strong cyclopia, interrupted floor plate.
m101	Unknown	Cyclopia; CNS and floor plate defects.
m122	Unknown	Cyclopia; CNS and floor plate defects.
m294	Point	Cyclopia; CNS and floor plate defects; null allele.
sg1	Point	Temperature-sensitive allele. Incompletely penetrant at 22 deg. C, completely penetrant at 28.5 deg C.
st5	Deficiency	
te262c	Unknown	Patchy floor plate; weak allele.
tf219	Unknown	Cyclopia, no FP, abnormal RT projection; allele strength = *b16*.
tu43x	Unknown	
B299	Deletion	
B213	Translocation	

Table 1.3.1: Different alleles of *cyc*, their mutation type and a brief description
Adapted from the Zfin website (*www.zfin.org*)

cyclops transcripts are not detectable in the fertilized egg, but are activated after the MBT, reaching maximal expression level at shield stage, after which there is a decrease in expression (Rebagliatia et al., 1998). Expression begins in early gastrulation (30% epiboly) in cells encircling the blastoderm margin and is progressively restricted to the dorsal organizer. Mid-gastrulation expression extends along the midline mesendoderm and by the end of gastrulation *cyclops* is expressed in the prechordal mesendoderm and posterior tail bud mesoderm but disappears from both regions by the 2-3 somite stage. In *cyclops* mutants the expression is initially indistinguishable from WT but declines thereafter and is absent in the prechordal plate. *cyclops* expression is initially normal in the mutant *oep* (see Chapter 3.4), but decreases during gastrulation contributing to the cyclopia and floorplate defects of *oep* mutants. This suggests that *cyclops* and *oep* are necessary for maintenance rather than activation of *cyclops* expression (Rebagliati et al., 1998). Subsequently it was found that *oep* is a co-receptor required for nodal signalling (Gritsman et al., 1999). In *bozozok (boz)* mutants which show a loss of chordomesoderm (Fekany et al., 1999) dorsal *cyclops* expression is greatly reduced or absent throughout gastrulation

identifying *boz* as an early-acting upstream regulator of *cyclops* (Sampath et al., 1998).

1.3.3 Squint

There are two known mutant alleles of the *squint* both of which are insertion mutations (Table 1.3.2) (www.zfin.org). Expression of *sqt* is both maternal and zygotic with *sqt* RNA found to be uniformly expressed in oocytes at all stages of oogenesis (Gore and Sampath, 2002). Five minutes after egg activation *sqt* RNA aggregates in clusters throughout the yolk and is detected in the emerging blastoderm. At the one cell stage RNA is restricted to the blastoderm and excluded from the yolk cell (Gore and Sampath, 2002). This movement from yolk to blastoderm is microtubule dependent (Gore and Sampath, 2002). At the four cell stage *sqt* localizes to two blastomeres that may become the dorsal region of the embryo. Removal of these blastomeres can result in loss of dorsal structures. This localization requires a highly conserved sequence of the 3' untranslated region (UTR) (Gore et al., 2005).

Allele	Mut. Type	Brief Description
Cz35 (z1)	Insertion	Cyclopia; prechordal plate, ventral nervous system defects; null allele.
hi975	Insertion	Mild eye cyclopia, u-shaped somites.

Table 1.3.2: Different alleles of *squint*, their mutation type and a brief description.
Adapted from Zfin (*www.zfin.org*)

Subsequently in the developing embryo the *sqt* gene has been shown to be expressed in the dorsal region of the blastula including the embryonic YSL which has been implicated as a source of signals that induce organizer development and mesendoderm formation. Mis-expression of *sqt* RNA within the embryo or specifically in the YSL induces ectopic or expanded dorsal mesoderm (Feldman et al., 1998). The expression of Sqt is shown to be at peak abundance around sphere stage and declines sharply after shield stage. Loss of *sqt* results in a phenotype that

resembles that of *cyc,* but is usually less severe. The range of phenotypes varies from mild phenotypes such as eyes being to close together (hence the name) to more severe phenotypes including complete cyclopia (Figure 1.3.1). This is, again, due a failure to specify prechordal mesendoderm and subsequent failure to specify the midline of the anterior neural plate.

Although very similar and partially functionally redundant, Sqt has been shown to differ from Cyc in a number of important ways. While Cyc only has short range activity, Sqt can act as morphogen (Chen and Schier, 2001) capable of acting in a dose dependent manner directly at a distance. Sqt producing cells can induce expression of *gsc* - a nodal target gene - at the source and in its immediate neighbours where there are high levels of nodal signalling. Further away Sqt induces first *flh* and then at a greater distance *ntl* and *bik* where the levels of nodal signalling are lower. At reduced levels of Sqt, *gsc* is not induced and *ntl* is only induced close to the source (Chen and Schier, 2001). This long range signalling was not seen in cells producing Cyclops (Chen and Schier, 2001).

1.3.4 Receptors, Co-receptors, Extracellular Inhibitors

The TGF-β superfamily ligands signal by binding to transmembrane serine-threonine kinase receptors. Two sets of receptors have been identified, which are assembled into a receptor complex (Dougan et al., 2003). Type I receptors are predominantly involved in the activation of downstream transducers while type II receptors phosphorylate and activate type I receptors in response to ligand binding (Whitman, 2001). In addition to these receptors there is a class of factors specifically required for signalling by Nodal related ligands. These are EGF-CFC factors; a family of membrane attached extracellular glycoproteins that include OEP in zebrafish and Cripto in mice. Maternal and zygotic loss of *oep* function phenocopies the loss of both Sqt and Cyc. Loss of *oep* function renders cells unable to respond to ectopic *cyc* or *sqt* but does not alter responsiveness to activin (Whitman, 2001) another TGF-β family member that signals through the same receptors as the Nodals (Reissmann et al., 2001; Sun et al., 2006). This suggests that the requirement for EGF-CFC factors is specific to the Nodal superfamily of TGF-β ligands (Gritsman et al., 1999).

The best characterized Nodal antagonists in zebrafish are the Lefties (Goering et al., 2003; Parsons et al., 2002a; Schmid et al., 2000). There are at least two zebrafish lefties, *lefty 1* and *lefty 2*. Overexpression of these in zebrafish induces phenotypes strongly resembling *cyclops;squint* double mutants or maternal-zygotic *one-eyed pinhead* mutants (Sakuma et al., 2002). Loss of Lefty function leads to enhanced Nodal signalling during mesoderm induction with expansion of mesendoderm and loss of ectoderm in addition to left-right pattern defects (Parsons et al., 2002a; Sakuma et al., 2002). Individually, loss of Lefty1 causes aberration during somitogeneseis stages including left-right pattern defects, while Lefty2 depletion has no obvious consequences. The gastrulation defects of embryos depleted of Lefty1 and Lefty2 have been shown to result from the deregulation of Sqt signalling (Parsons et al., 2002a). In contrast, de-regulation of *cyclops* does not affect morphology or the transcription of Nodal target genes during gastrulation. Cyclops is thought to be specifically required for the maintenance of *lefty 1* and *lefty 2* transcription. Severe gastrulation defects do not arise in zebrafish unless two Nodals or two Leftys are removed (Parsons et al., 2002a). This suggests that the activity of Leftys is controlled at the level of transcription with Lefty expression being dependent on Nodal signalling in most tissues.

1.4 Thesis aims

With the publication of the zebrafish genome, Dr Matthew Clark has undertaken to identify all the Rabs in the genome and classify them according to sequence homology to their mammalian counterparts. To date, there are estimated to be over 80 zebrafish rabs (Clark, MD, pers. com.), compared to over 60 in mammals (Colicelli, 2004). This thesis aims to characterise the function of specific members of the extensive *rab* family. To achieve this anti-sense MO oligonucleotides will be used to knock-down individual *rabs* with further analysis including overexpression assays and microarrays

This thesis aims to uncover new roles for the *rab* family in zebrafish development with the ultimate aim of identifying the function of all the zebrafish *rabs*. This thesis presents the results obtained when 13 *rab* genes were knocked-down, bringing the total number of genes screened to 37 with a small number of these genes exhibiting specific and fascinating defects when knocked-down. In mammalian development a small minority of *rab* genes have been implicated in disorders such as Griscelli's syndrome or the *openbrain* mutant in mice. Investigations into *rabs* in zebrafish have shown that sequence homology does not necessarily translate to functional homology (Campos, 2004).

In addition to screening the zebrafish *rabs*, this thesis further characterizes the function of *rab5a2*: the only *rab* gene screened to date which resulted in mortality before the completion of epiboly. The essential nature of this *rab* is not surprising, since *rab5* has been identified as one of the core *rab* genes. What is surprising is that, although many of the other core *rabs* have also been screened, none of them have shown such a dramatic early phenotype. In addition, there are four *rab5* genes in zebrafish, however, none of the other *rab5s* display such early morbidity. Recent experiments in zebrafish have shown the zebrafish *rab5* family to be important in cell signalling and cell migration during early development. This research, coupled with results from Dr Campos showing a lack of organizer and *nodal* responsive

markers in *rab5a2* MO injected embryos, has suggested a role for *rab5a2* in Nodal signalling.

It is therefore the aim of this thesis to further characterise *rab5a2* and in particular its effect on Nodal signalling by using antisense morpholino oligonucleotides and overexpression of RNA analysis. This thesis also looks at the contribution of maternal and zygotic transcripts of *rab5a2* in nodal signalling in addition to analysing the effect of *rab5a2* on exogenously supplied *nodal* RNA. Finally this thesis uses microarray analysis to identify other genes and possible pathways affected by the knocking down of *rab5a2.* The results show a complex and vital role for *rab5a2* in cell signalling and zebrafish development and that its loss of function impacts on many hundreds of genes.