Chapter 7

Discussion and Further Work

7.1 Characterization of Rab5a2

In total, 37 *rab* genes have been screened by knocking down the *rabs* using oligonucleotide MOs. Those *rabs* screened include members of the *rab1*, *rab6*, *rab7, rab8, rab11* and all the members of the *rab5* family, genes whose homologues in yeast are considered essential because of their lethality when knocked-down. *rab5a2* is the only *rab* gene in zebrafish which, when knocked-down, causes death before 24hpf.

In 1996, the Tubingen screen for new zebrafish mutants uncovered four zygotic mutants that affect epiboly. The most severe of these, *hab,* resulted in death before 24hpf (Kane et al., 1996; McFarland et al., 2005). Subsequently, it was shown that all these mutants resulted from mutations in E-cadherin (Kane et al., 2005). E-cadherin has been shown, in conjunction with Rab5c, to be responsible for Wnt11's function in cell cohesion during zebrafish gastrulation. The *hab* mutant is presently the only known zebrafish epiboly mutant. Although there is no evidence for E-cadherin involvement in the *rab5a2* MO injected phenotype, *wnt11* was seen to be decreased in the microarray data (Figure 6.2.1) for the *rab5a2* MO injected embryos. Disrupted cell adhesion may be responsible for the rougher appearance of the blasdoderm in *rab5a2* MO injected embryos. A loss of adhesion between the blastoderm cells and the yolk may also be responsible for the liquid observed building up between the yolk and the blastoderm in the *rab5a2* MO injected embryos (Figure 5.2.1). Interestingly, the homophilic cell adhesion gene *protocadherin8* was shown by the microarray data to be decreased in *rab5a2* MO injected embryos (Figure 6.2.1 and 6.2.3).

The reduction of Nodal signalling seen in the *rab5a2* MO injected embryos (Figure 5.1.2 and 5.2.2) implies a role for Rab5a2 in nodal signalling. This involvement of *rab5a2* with zebrafish signalling factors is not without precedent, as zebrafish *rab5* has been shown to play a role in the effective signalling range of Fgf8 (Scholpp and Brand, 2004). A reduction of Rab5, using the reportedly Rab5 specific GAP RN-tre, showed an increase in the range of Fgf8 signalling. In contrast to these results, the

microarray data suggests that *rab5a2* reduces Fgf8 signalling (Figure 6.2.1). The microarray shows that, at 30% epiboly, *fgf8* gene expression is severely reduced in *rab5a2* MO injected embryos.

The contrast between Scholpp's data and that reported by the microarray, may be due to the use of RN-tre, a Rab5 GTPase activation factor (GAP). Although it is reported to be a Rab5 specific GAP (Lanzetti et al., 2004), further research has shown that its Rab5 activity is weak and that RN-tre has a greater specific catalytic activity towards Rab41 (Haas et al., 2005). In addition, RN-tre is reported to act as a Rab5 effector, although this is also disputed in the later report. It is, therefore, unclear whether the increased Fgf8 signalling range is the result of a reduction in *rab5*. Scholpp (2004), however, addressed this issue by using antisense oligonucleotides to knock down *rab5a* function and showed that the result is the same as that seen for RN-tre.

The conflict beween those results seen in the microarray and Scholpp's data may, therefore, lie in the dose of MO used. The dose of MO used for the microarray was chosen to ensure the embryos showed a phenotype (Section 5.2.1). This is higher than that used for Scholpp's data, where the dose used would possibly have only been enough to reduce *rab5a* signalling by a small amount. Also, there is an additional *rab5a* gene and, although the clone used for overexpression analysis corresponds to *rab5a2*, the identity of the MO used is not stated. Scholpp implies that *rab5c* RNA is used in some of these experiments and on occasion refers to the mRNA as *rab5*. Therefore, it becomes unclear as to which RNA is being used, which could explain the conflict seen between the microarry data and Scholpp's data, since, although both *rab5a* and *rab5c* share high sequence homology, their functional homology in zebrafish has not been fully explored. However, overexpression of *rab5a* mRNA from the clone that corresponds to *rab5a2* does show reduction of Fgf8 signalling, allegedly corroborating the effect seen in the *rab5a* MO. The reduced range of Fgf8, seen in the *rab5a* overexpressing embryos, does not directly measure Fgf8 signalling range but uses expression of the Fgf8 target gene *spry4*. In addition, it measures this effect using exogenously supplied Fgf8. The *rab5a* MO used also looks at the target gene *spry4* but uses endogenous Fgf8. This thesis suggests that the conflict might be reconciled if a potential dual role is performed by *rab5a2* in early zebrafish development.

Overexpression of *rab5a2* results in expression of *gsc* and *ntl* in the animal pole of the embryo (Figure 5.3.2) in addition to the normal expression pattern of these genes seen at the margin. The *rab5a2* overexpression embryos also show either accumulation of cells in the animal pole or an enlarged organiser (Figure 5.3.1). These results, coupled with the lack of visible organizer and the reduction of *cyc, chd* and nodal responsive genes in *rab5a2* MO injected embryos, suggest a role for Rab5a2 in DV patterning and the establishment of the organizer. Interestingly, many of the genes that decreased in *rab5a2* MO injected embryos, when compared to controls, have been implicated in DV patterning (Fainsod et al., 1997; Fekany et al., 1999; Furthauer et al., 2004). At shield stage, this became more pronounced. In addition, some of the genes that increased in *rab5a2* MO injected embryos have also been implicated in DV patterning (Hild et al., 1999; Melby et al., 2000; Ramel et al., 2005). This is unsurprising, since DV patterning is a balancing act between genes which promote or antagonise ventral and dorsal signals. Those which promote ventral domains and inhibit dorsal include *bmp4, ved* and *vox*. While those which promote dorsal and inhibit ventral include *chd, fgf8* and *fgf24.*

In the *rab5a2* MO injected embryos *chd, fgf8 and fgf24* are all decreased. This would suggest that an increase in expression of ventralizing genes should be seen in the *rab5a2* MO injected embryos due to a reduction of pro-dorsal genes inhibiting them. However, this is not the case. The microarray data shows that genes which decrease in *rab5a2* MO injected embryos both promote and antagonise ventral and dorsal domains. Expression of *bmp4,* which is known to induce ventral fates within the embryo, was shown to be decreased (Figure 6.2.1 and 6.2.3) as was *wnt8a* another ventralizing gene. *wnt8a* has been shown to act through the transcriptional repressors *ved* and *vent* also shown to decrease in the microarray (Figure 6.2.1 and 6.2.3). *ved* and *vent* act to repress dorsalizing genes, their main target is *chd* but in addition, they repress *gsc* and *flh.* Therefore, a decrease in *vent* and *ved* expression should result in an increase in *gsc, chd* and *flh* expression. However, the expression of these genes is also decreased (Figure 5.1.2 and 5.2.2). In addition if *rab5a2* were responsible for the establishment of the organizer, the accumulation of cells in the animal pole of *rab5a2* overexpressing embryos might be a second organizer, while the enlarged organizer would show an expansion of the dorsal domain However,

there is no additional or expanded expression of *chd* in these embryos and a second axis is not apparent, which is what would be expected if the accumulation of cells in the animal pole were a second organizer. Instead, the embryos show mostly normal development, except for a severely reduced body axis (Figure 5.3.1). Interestingly, the microarray data shows that expression of the ventralizing gene *bmp2b* is increased in 30% epiboly stage *rab5a2* MO injected embryos. It was also increased in shield stage MO injected, but not to the extent seen at the 30% stage. While, the RT-PCR was inconclusive. This increase in *bmp2b* is unsurprising, as those factors which inhibit *bmp2b* are down regulated in *rab5a2* MO injected embryos. However, *bmp4*, which is also inhibited by these factors, is decresed (Table 6.2.1 and 6.2.3).

The transcriptional repressor genes *vent*, *ved* and *vox* prevent the transcription of *boz* and other dorsal genes, particularly *chd* but also *gsc* and *flh* (Imai et al., 2001) (Kawahara et al., 2000a; Kawahara et al., 2000b; Melby et al., 2000; Shimizu et al., 2002). All three genes *vox, ved* and *vent* are activated by *wnt8* (Ramel and Lekven, 2004), which has been suggested to regulate these genes in conjunction with *bmp2b*, since loss of function of *vox, vent* and *ved* phenocopies the *wnt8/swr (bmp2b)* double mutant phenotype (Ramel et al., 2005). Both *vent* and *ved* are decreased in *rab5a2* MO injected embryos, as is *wnt8* but to a lesser extent (Appendix 1 and 2). Interestingly, analysis of *vox* expression in control and *rab5a2* MO injected embryos, showed an increase in *vox* expression in the *rab5a2* MO injected embryos (Figure 7.1.1). The possible increase in *vox* expression may be due to the increase in *bmp2*, whilst the decrease in *ved* and *vox* may be the result of decreased *wnt8* expression. Therefore, it is possible that the reduction in *chd, flh* and *gsc* expression is the result of an increase in Bmp2b repression acting through *vox.* It has been suggested that *chd* is not needed if *bmps* are inactivated (Schulte-Merker et al., 1997), since *chordin* mutants have a ventralized phenotype (Chen and Schier, 2001), whereas *bmp2b;chordin* double mutants are dorsalized. This implies it is more likely that the increase in Bmp2b may be repressing *chd*, rather than decreased Chd effecting *bmp2b*.

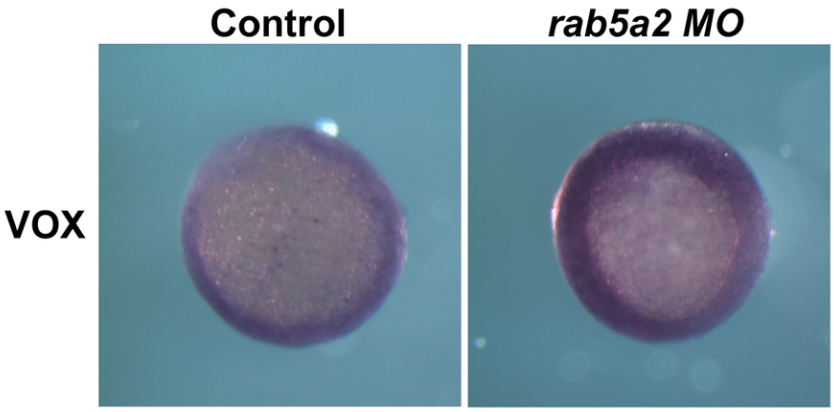

Figure 7.1.1 : Difference in expression pattern of the homeobox gene *vox* in *rab5a2* MO injected embryos when compared to controls.

bmp4 acts to specify the formation of ventral mesoderm and suppress neural fates, whilst *bmp2b* is described as necessary for mesoderm cell fate commitment (Graff et al., 1994; Hawley et al., 1995; Schneider et al., 1996; Suzuki et al., 1994). Both *bmp2b* and *bmp4* act to specify ventral identity and are negative regulators of endodermal cell fate specification. Therefore, it may be the difference between mesoderm cell fate commitment and mesoderm formation that results in the different expression profiles in the *rab5a2* MO injected embryos. Other genes down regulated in the *rab5a2* MO injected embryos also have roles in the mesoderm. *fgf8* and *fgf24* are coexpressed in mesoderm precursors during gastrulation (Draper et al., 2003). A defect in the gene *tbx16* is responsible for the mutant *spadetail*. *tbx6* and *ntl* are expressed in mesoderm and are known to regulate region-specific gene expression and developmental fate (Goering et al., 2003).

An additional interesting twist is that *bonnie and clyde*, which is involved in endoderm formation, is down regulated in *rab5a2* MO injected embryos at 30% (Table 6.2.1) but increased slightly at shield (Appendix 2). Endodermal determination, initiated by the Nodal signalling pathway, has been shown to require a multitude of genes. Included in these are, as mentioned, *bonnie and clyde* (Trinh et al., 2003), as well as *eomesodermin* (*eomes*) (Bjornson et al., 2005), which is up regulated in the *rab5a2* MO injected embryos. *eomes*, a maternal T-box gene and transcriptional activator, has also been shown to determine dorsal identity. Initially

after fertilization *eomes* is expressed in a vegetal animal gradient in the embryo, with Eomesodermin protein (Eom) distributed cytoplasmically throughout the blastoderm. However, following midblastula transition, nuclear-localized Eomesodermin is only detected on the dorsal side of the embryo (Bruce et al., 2003). Overexpression of *eomes* results in Nodal-dependent and *nieuwkoid*/*dharma* (*nwk/dhm*) independent ectopic expression of the organizer markers *gsc*, *chd* and *flh*, and in the formation of a secondary axis (Bruce et al., 2003). Interestingly, the knocking down of *rab5a2* results in the increase of *eomes* (Figure 6.2.2 and 6.2.4) with the decrease of *gsc*, *chd* and *flh* (Figure 5.1.2 and 5.2.2) and pre-24hpf embryonic lethality (Figure 5.1.1).

Overexpression of *eomes* has been shown not to have any effect on *cyc*, *sqt, bmp2b, vega1/vox* or *nwk/dhm*, although *eomes'* effect on *gsc*, *chd* and *flh* is nodal dependent and it has been speculated that *eomes* possibly regulates *sqt* (Bruce et al., 2003). Therefore, this raises the possibility that *rab5a2* may affect nodal signalling by interfering with the pathway between *eomes* and *sqt*. *eomes* is suggested to activate, and possibly maintain, its own transcription (Bruce et al., 2003). If knock down of *rab5a2* does disrupt the pathway between *eomes* and *sqt* the *eomes* expression might be increased to compensate. However, *eomes* autoregulation is suggested to be Nodal-independent, since injection of *eomes* into MZoep embryos resulted in induction of *eomes* expression (Bjornson et al., 2005). This increased expression of *eomesodermin* in *rab5a2* MO injected embryos may be linked to the conflicting expression seen for *bmp2b* and *bmp4* although there is no direct evidence linking the *bmps* to *eomesodermin*

Another possibility is that *rab5a2* knock down results in mislocalization, or inhibition, of the early localization to individual cells of *sqt*, seen in the four cell embryo (Gore et al., 2005). Preliminarily data looking at the effect of *rab5a2* on early *sqt* expression shows no *sqt* localization to individual cells in the 8-16 cells stage of control or *rab5a2* MO injected embryos but does show localization in *rab5a2* overexpresing embryos (Figure 7.1.2). Since there is no localized expression of *sqt* in the controls, as reported by Gore *et al* (2005), the signal was increased by injecting 15pg in 1.4nl of *alexa-sqt* into the yolk of control MO injected, *rab5a2* MO injected and *rab5a2* overexpressing embryos. Gore reports that this localizes in the same manner as endogenous *sqt*. Localization to individual cells of the 8-16 cell

stage embryos was seen in control MO injected embryos, *rab5a2* MO injected embryos and *rab5a2* overexpressing embryos (Figure 7.1.2). However, the expression of *sqt* in the *rab5a2* overexpressing embryos developed before that of the control and *rab5a2* MO injected embryos. These data suggest that *rab5a2* may effect zebrafish development at a very early stage. This would be consistent with the misexpression of *β-catenin* expression seen in the *rab5a2* MOs, when compared to the gradient of *β-catenin* expression emanating from the dorsal side of control embryos (Figure 5.5.2). Such results imply a role for *rab5a2* early in development, although it is unclear how and what role the early maternal transcripts of *sqt* or *β-catenin* may play in this.

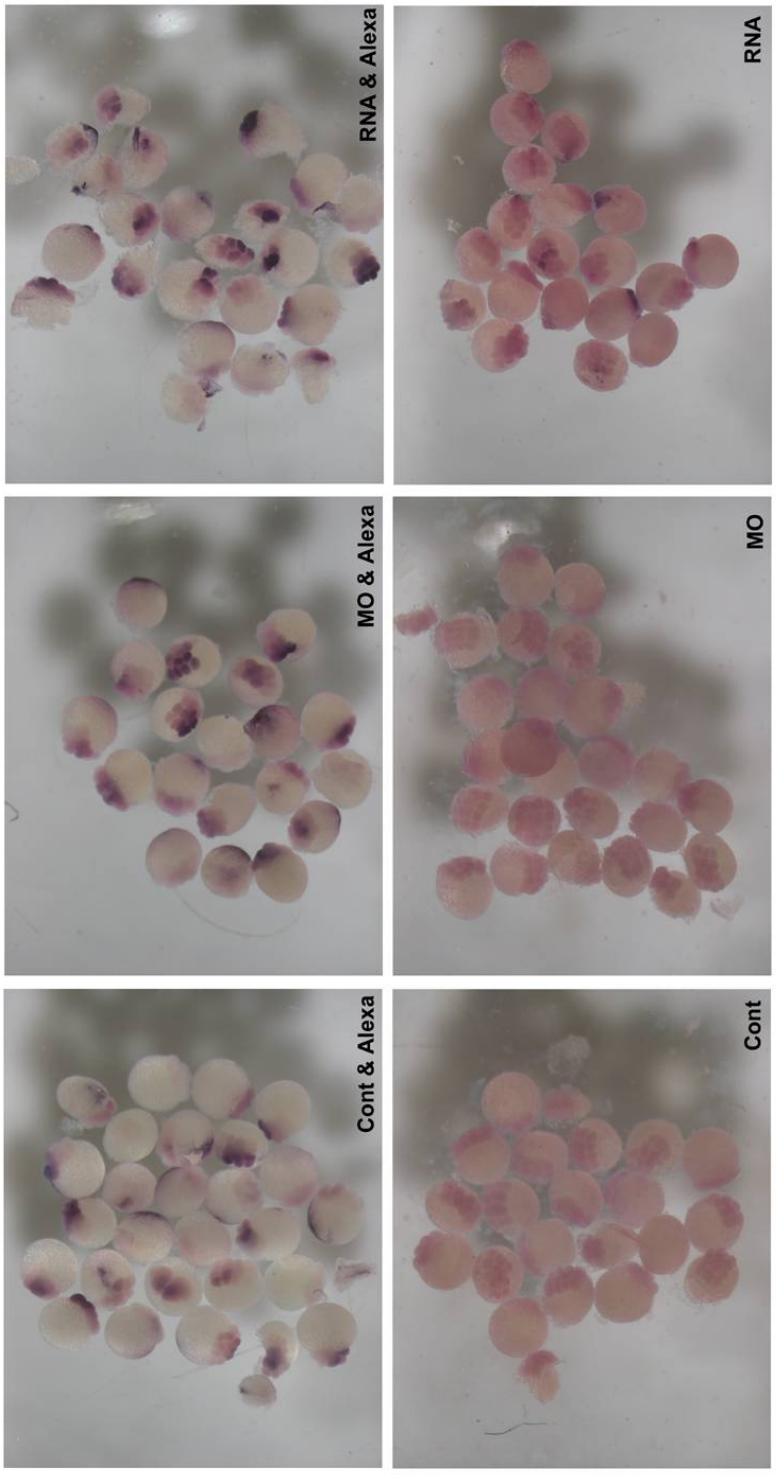

Figure 7.2.1: Identifying localized *sqt* expression in 8-16 cell embryos injected with control MO, 5ng *rab5a2* MO or *rab5a2* 1.5ng RNA. In addition to identifying localized *sqt* expression in 8-16 cell embryos injected with 15pg *alexa-sqt* and either control MO, 5ng *rab5a2* MO or 1.5ng of *rab5a2* RNA

The early phenotypes seen in *rab5a2* MO injected embryos, including the reduction of nodal target gene expression and *chd* expression, are possibly the result of the maternal *rab5a2* transcripts on early developmental factors. Knocking down only the

zygotic transcripts of *rab5a2* resulted in embryos with a visible organizer and normal speed of development, as well as expression of *bik, ntl, gsc* and *chd* being unaffected. However, the *rab5a2* MO injected embryos, deficient in only zygotic transcripts, died before 24hpf, suggesting an equally important role for zygotic *rab5a2* in later development.

To examine how Rab5a2 might directly affect nodal signalling, *sqt* or *cyc* was injected into one cell of a 128 stage embryo. Sqt has been proposed as a morphogen acting directly on target cells over a distance while Cyclops signals in a more localized manner (Chen and Schier, 2001).

In embryos overexpressing *rab5a2*, injection of either 5pg or 10pg of *sqt* resulted in similar *gsc* and *ntl* expression to that seen in controls (Figure 5.4.1 and 5.4.2). This conflicts with evidence from the signalling factor *fgf8*, where overexpression of *rab5a* resulted in the *fgf8* target gene *spty4* showing a reduction in range of expression. This reduction in range was suggested to result from increased clearance, by *rab5a,* of the Fgf8 ligand present in the extracellular space – a process termed "restrictive clearance". Injection of 5pg of *sqt* into *rab5a2* MO injected embryos resulted in a large proportion of embryos showing severe reduction of intensity of both *gsc* and *ntl* expression, with *gsc* showing a broader range of expression. However, many of the *rab5a2* MO injected embryos showed no expression of either *gsc* or *ntl.* When the dose of *sqt* was increased to 10pg, a greater proportion of the embryos showed no expression of *gsc* or *ntl.*

This data is contradictory: the broader range of *gsc* expression would be consistent with the Scholpp's theory of "restrictive clearance", however, many embryos showed no expression, which is at odds with this (Scholpp and Brand, 2004). In addition, since *gsc* is induced at high doses of nodal and *ntl* induced at lower doses, the *ntl* expression would also be expected to be expanded. This contradictory data might be the result of the high background staining seen in *rab5a2* MO injected embryos, masking the already weak *gsc* and *ntl* expression. The weak signal, seen in the *rab5a2* MO injected embryos may result from the broader expression pattern. Therefore, if the embryo produced an even broader expression pattern this might be masked by the background staining. One theory is that extracelluar Sqt binds to its

receptor but the receptor cannot be internalized and, therefore, it cannot activate target genes. If the receptors cannot be internalized, then Sqt cannot be restrictively cleared form the extracellular space and extracellular Sqt can travel further. If there is not complete knock down of *rab5a2,* then some of the receptors will be internalized and will activate target genes. Since Sqt has not been cleared, the extracellular dose would be high, so *gsc* is induced. This theory assumes that percentage of receptor occupancy is responsible for the type of target gene expression, i.e. if greater than a certain percentage of receptors are occupied, one target gene is induced, whereas, if less than this percentage are occupied, a different target gene is induced.

The effect of Rab5a2 on Cyc signalling differed to that of Sqt. Injection of *cyc* into *rab5a2* MO injected embryos resulted in the majority of embryos showing a lack of *gsc* and *ntl* expression (Figure 5.4.3). Those which did show *gsc* expression failed to show the expanded expression pattern observed for the *sqt* injected embryos, while those that showed *ntl* derived from the exogenous *cyc* also showed endogenous *ntl* expression, suggesting that there was not complete knock down of *rab5a2*. Injection of *cyc* into *rab5a2* overexpressing embryos showed either absence or reduction of intensity of *gsc* expression in the majority of embryos, while *ntl* signalling was unaffected (Figure 5.4.3). These data seem contradictory but suggest a role for *rab5a2* in Cyc signalling. It is possible that *rab5a2* may have role in the secretion of the Cyc protein and with its internalization, once bound to a target cell receptor. If *rab5a2* had a role in secretion, reduction of this gene would cause impaired secretion of Cyc and no target gene expression. Overexpression, on the other hand, might result in normal secretion, since the production of Cyc would be unaffected, but would result in increased internalization. This increased internalization would result in Cyc being removed from the extracellular space, lowering its extracellular dose and resulting in *ntl* expression rather than *gsc.* Since the *rab5* genes have been well characterized as regulators of endocytosis (Bucci et al., 1992; Chavrier et al., 1990; Gorvel et al., 1991), it is probable that *rab5a2* wouldn't be directly responsible for secretion. The microarray data showed a reduction in the expression of *rab14* in *rab5a2* MO injected embryos (Figure 6.2.1 and 6.2.3). Rab14 has been shown to be involved in membrane trafficking between the golgi complex and endosomes. It is

possible that these two *rabs* work in co-operation to ensure normal Cyc signalling, however, the data is inconclusive.

The RT-PCR data, used to validate the microarray, is also inconclusive. Much of this is due to the large degree of variation seen in these samples. This variation is controlled in the microarray data by the increased number of replicates, as well as by normalization of the data. Although the RT-PCR data is inconclusive for some of the genes, particularly the supposed non-changing housekeeping genes, the trend observed does suggest that the microarray data is accurate. Stronger evidence corroborating this is found in the expression patterns of *chd, flh,* and *ntl* in chapter 4. The down regulation of *chd* seen in the microarray data for *rab5a2* MO injected embryos is validated by *ish* with the abolishment of *chd* expression seen in *rab5a2* MO injected embryos.

The microarray data showed hundreds of genes whose expression was changed when *rab5a2* was knocked down even when the base line for change was set as high as three fold. This data suggests an early effect for *rab5a2* and correspond with the early phenotype seen in these embryos. Even at the 30% stage, where there is no visual difference between the *rab5a2* embryos and the control embryos, hundreds of genes changed their expression. The shield stage data showed an increase in the genes which changed, when compared to 30% epiboly embryos. This is expected given the suggestion that *raba5a2* acts early in development and has an effect on signalling factors (Campos, 2004; Scholpp and Brand, 2004; Ulrich et al., 2005). The earlier in development *rab5a2* acts, the further up the signalling cascade it is likely to act and, in disrupting an upstream signalling factor, Rab5a2 affects all the downstream factors acted upon by that initial factor. Therefore, analysis of gene expression at later stages of development should be predicted to show an increased number of genes that change.

7.2 Further Work

7.2.1 Further analysis of Rab5a2

Due to the complex nature of *rab5a2* and the high degree of regulation of the protein it has been very difficult to fully explore its effect on Nodal signalling. It is possible that *rab5a2* knock down or overexpression could be indirectly interfering with the secretion or synthesis of the Sqt and Cyc proteins. Transplantation of *sqt* and *cyc* overexpression cells into *rab5a2* MO injected or overexpressing embryos would enable the role of *rab5a2* in cells producing or receiving the *sqt* or *cyc* to be separated. This results from the cells transcribing and secreting the Nodal related proteins having wild type *rab5a2* expression.

So far, much of the analysis has involved looking at Nodal responsive genes as an indicator of Nodal, rather than Nodal itself. To this end, *sqt-gfp* and *cyc-gfp* fusion proteins have been made by inserting a GFP region after the cleavage site, between the pro and mature domains of *sqt* and *cyc* (see Methods section 2.2.11). This ensures that when the proteins are cleaved, the GFP attaches to the mature domain.

To ascertain whether the *sqt-gfp* construct was producing the same effect as wild type *sqt*, 10pg of *sqt-gfp* RNA was injected into single cells of 128 cell stage embryos. These were then compared to embryos injected in the same manner with 10pg of wild type *sqt* RNA and those injected with 10pg of control RNA. Subsequently, each group of embryos was fixed at shield stage and examined for *ntl* expression. A similar procedure was completed for *cyc-gfp*, where it was compared against wild type *cyc* and control RNA. Expression of *ntl* was seen in the animal pole in both wild type *sqt* and *sqt-gfp* injected embryos, while the controls showed normal expression round the margin (Figure 7.2.1). Embryos injected with either *cyc* or *cyc-gfp* also showed expression of *ntl* in the animal pole (Figure 7.2.1). This suggested that the *sqt-gfp* and *cyc-gfp* was producing a similar affect to the wild type *sqt* and *cyc* RNA.

To further compare wild type *sqt* and *cyc* with *sqt-gfp* and *cyc-gfp,* 5pg or 10 pg of either *sqt, cyc, sqt-gfp* or *cyc-gfp* were injected into single cell embryos. These were left to develop until shield stage, when they were snap frozen and prepared for RT-PCR analysis (see Methods section 2.2.10), using taqman oligos for Nodal responsive markers. g*sc, flh, chd* and *lfy2* expression was increased compared to controls in embryos injected with either 5 or 10pg of *sqt,* 5 or 10pg of *cyc,* 5 or 10 pg of *sqt-gfp* or 10 pg of *cyc-gfp* (Figure 7.2.2). *ntl* and *lty1* expression was increased in embryos injected with either 5pg or 10pg of *sqt*, 5pg of *sqt-gfp* 5pg of 10pg of *cyc-gfp. lfy1* was also increase in embryos injected with 5 or 10pg of *cyc* (Figure 7.2.2)

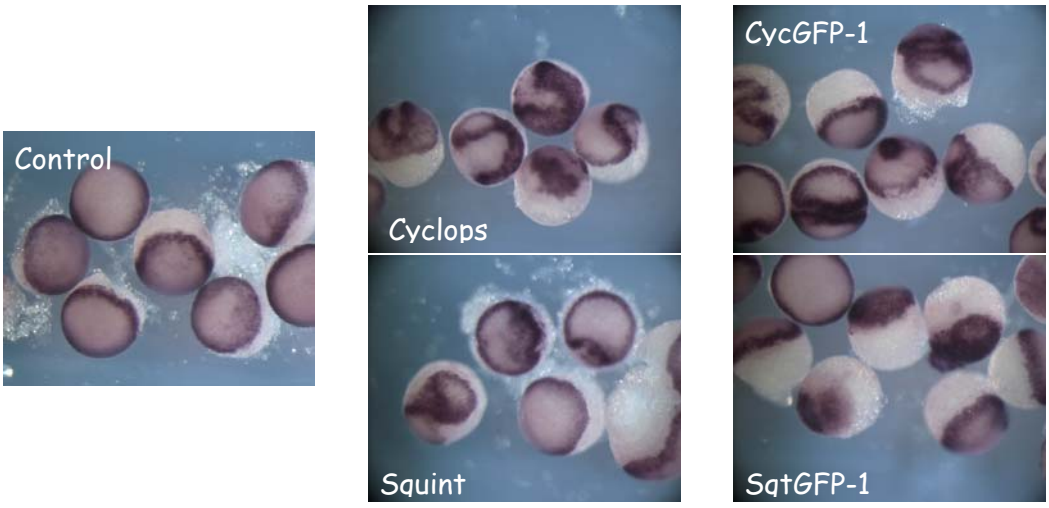

Figure 7.2.1: Comparison of *sqt* and *cyc* RNA to *sqt-GFP* and *cyc*-GFP fusion constructs.

Comparison of *ntl* expression in embryos injected with 10pg of *sqt* RNA compared to embryos injected with 10pg of *sqt-GFP* RNA and embryos injected with 10pg of *cyc* RNA compared to embryos injected with 10pg of *cyc –GFP* RNA. *ntl* expression in control injected embryos are also shown.

Finally, the *sqt-gfp* construct was checked for GFP activity. This was achieved by injecting a single cell in a 128 cell stage embryos with the lineage label rhodamine and 10pg of *sgt-gfp*. The embryos were then observed under a confocal microscope for GFP activity. GFP was seen accumulating round cells containing rhodamine, which suggested that the *sqt-gfp* was functional (Figure 7.2.3 supl mov 7.2.3).

Injection of these GFP-nodal fusion constucts would enable the Nodal ligand to be followed in the developing embryo. Therefore, it would be possible to observe the

range of the ligand directly, instead of using target gene expression. Injecting these constructs into one cell of 128 cell embryo, either overexpressing *rab5a2* or with *rab5a2* knocked-down, would potentially enable visualization of the ligand being secreted or produced, and enable investigation into whether *rab5a2* effects the secretion of the ligand. In addition, it would be interesting to transplant cells from embryos overexpressing these constructs into embryos, overexpressing *rab5a2* or knocked-down for *rab5a2*. This would enable the visualization of these Nodals through the developing embryo without the secretion, or production, of the Nodal-GFP ligands being altered by alterations in *rab5a2.*

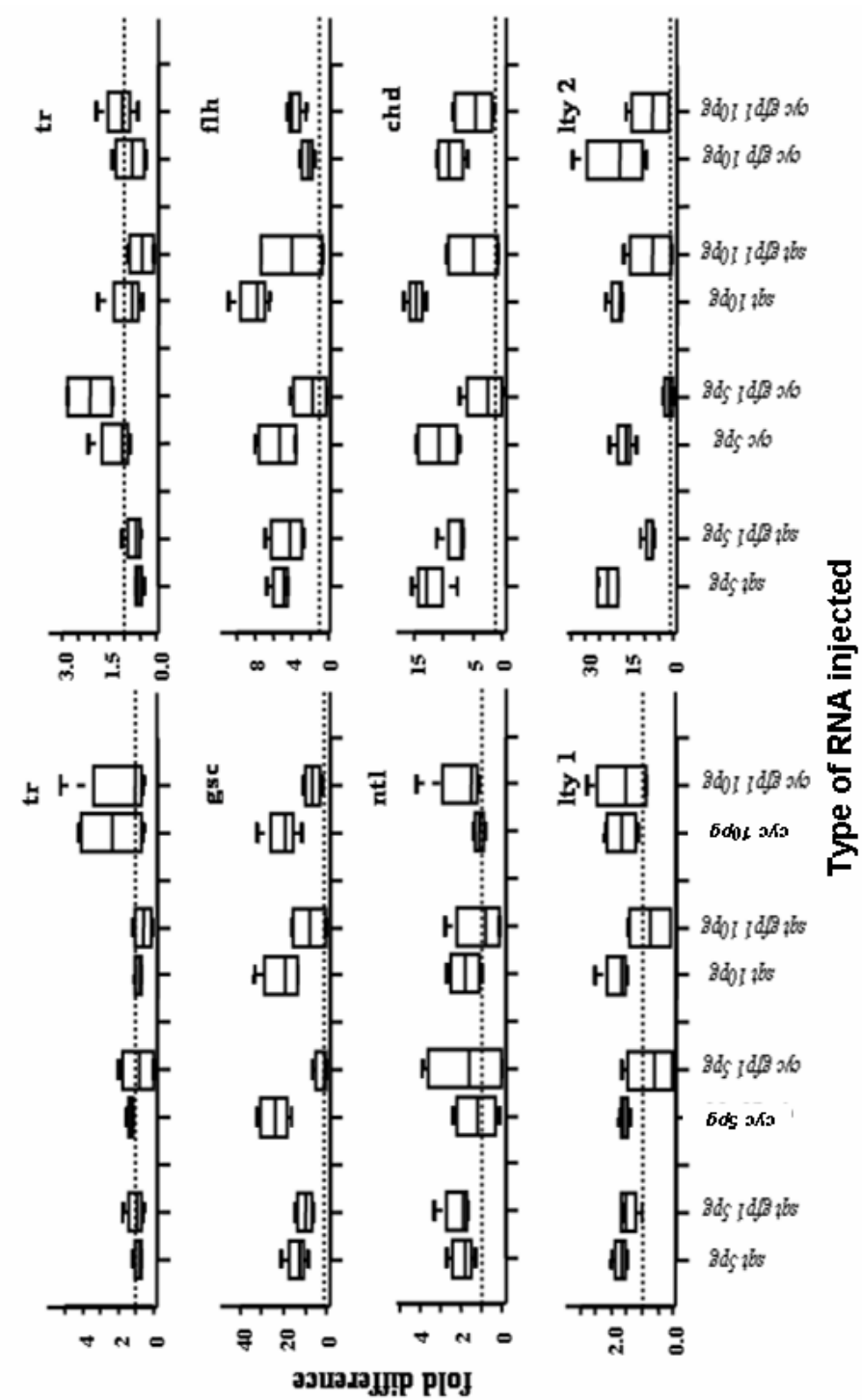

Figure 7.2.2: Box and whisker plots showing the fold change (compared to wild type embryos) of *gsc*, *ntl*, *flh*, *tr*, *lty1*, *lty2* and *chd* expression in embryos injected with 5pg of *sqt*, 5pg of *sqt-gfp*, 10pg of *sqt*, 10pg of *sqt-gfp*, 5pg of *cyc*, 5pg of *cyc-gfp*, 10pg of *cyc* or 10pg of *cyc-gfp*.

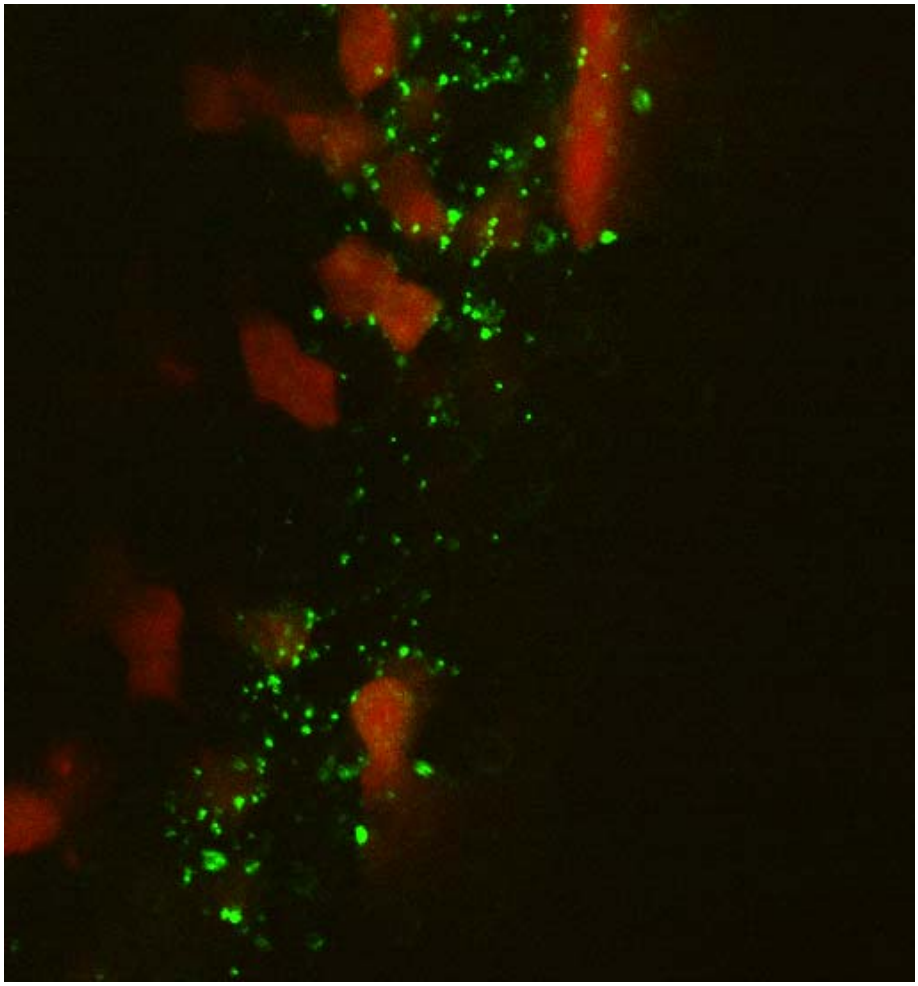

Figure 7.2.3: Assessing the viability of the Squint-GFP fusion protein.

Confocal image of 10pg *squint*-GFP RNA injected embryos. The green colour identifies the Squint-GFP protein and the red (rhodamine) identifies the lineage of the cells injected with the *squint*-GFP RNA.

7.2.2 Investigations into the other members of the rab5 family.

In zebrafish, there are four members of the Rab5 family. Rab5c and Rab5b show similar phenotypes, which include brain cell death and tail defects, while Rab5a1 shows no phenotype. This thesis has shown that, out of all four Rabs, Rab5a2 is the most essential for early embryo development. Therefore, it is now important to

establish whether these remaining Rab5s are even partially redundant and whether they have a role in cell signalling, in particular Nodal signalling.

Initial studies looking at the expression of dorsal and Nodal responsive gene have shown that *rab5a1* appears to be redundant in regard to Nodal signalling (Figure 7.2.4). However, *rab5c* and *rab5b* show a degree of down regulation. Rab5b particularly showed downregulation of all the genes investigated, while Rab5c showed down regulation of *bhik*. Therefore, it would be interesting to conduct microarray analysis on each of these genes to further quantifly to what extent these *rabs* effect cell signalling and which of these pathways, if any, overlap with other members of the Rab5 family.

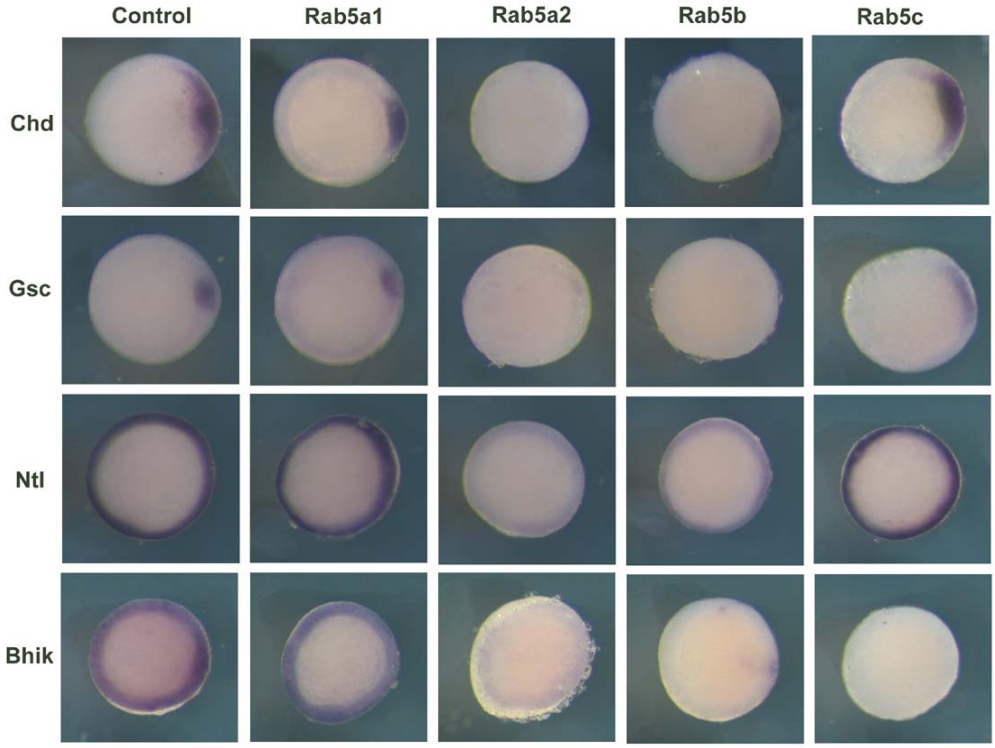

Figure 7.2.4: Animal views of shield stage embryos: *gsc, ntl, chd* and *bik* expression in control MO injected embryos on the left compared to embryos injected with 12ng of *rab5a1* MO, 8ng of *rab5a2* MO and 8ng *rab5c* MO.