Chapter 3

Screening the Zebrafish Rabs

3.1 Introduction

This study is a continuation of work by Dr. Isabel Campos, a former PhD student in the Stemple laboratory (Campos, 2004). With the sequencing of the zebrafish genome, more Rabs have been uncovered and there are now estimated to be over 80 *rab* genes (Clark, MD, pers. com.). cDNA clones for many of these genes have either been cloned by PCR and/or 5' and 3' RACE, or were obtained from outside sources (RZPD) and confirmed by sequencing. Design of the morpholinos (MO) for the first pass of this screen was primarily undertaken by Dr. Matthew Clark, with any additional MOs, for more in depth studies, designed by the author.

3.1.1 Loss of Function Screen

The MO for the initial pass of the loss of function screen are designed against 25 bases of the UTR region immediately 5' of the ATG start codon. There are three reasonsfor this: The first is that the UTR is considered to be more unique to the gene, a MO designed against this area is unlikely to bind any other gene, but to be sure each MO was checked by both Dr Clark and the author. Secondly, a MO in this area allows more efficient rescue by the injection of RNA encoding solely the open reading frame, as this does not contain sequence complementary to the MO. Lastly in our hands UTR MOs are usually more subtle than ATG MOs. An ATG MO, that would normally be lethal at a low dose, could show a distinct phenotype at higher does if a UTR MO were used.

In the initial pass of the Rab MO screen the embryos were injected with three different doses of MO: 8ng, 4ng or 2ng producing a dose response curve. The embryos were checked for a phenotype at gastrulation, 24 hours post fertilisation (hpf), and then each day until 5 days post fertilization (dpf). If all the embryos died then a lower dose was used, until the embryos no longer showed a phenotype. If the embryos show an abnormal phenotype, they were re-injected to check for reproducibility and photographed at shield stage if there was a gastrulation phenotype, 24hpf, 48hpf and, in some cases, later, up to 5 dpf. All *rab* MO injected

embryos were compared against embryos injected with a standard control MO supplied by Genetools at a dose 2ng higher than that of the highest does of *rab* MO.

This chapter concentrates on the Rabs screened solely by the author and shows the diverse range of phenotypes observed when *rab* expression is disrupted. These include heart phenotypes, pigmentation phenotypes, hatching phenotypes and some less specific phenotypes, such as greying of the brain indicative of cell death or tail abnormalities. Although, the Rabs do not typically exhibit a single phenotype but, rather, a host of malformations, this chapter has grouped the Rabs based on the common phenotypic features.

3.2 Pigmentation defects following knockdown of zebrafish rabs

This section focuses on those Rabs which following MO knockdown resulted in pigmentation defects. Stripe formation and colour patterning in zebrafish is an interesting system in which to study how spatial patterns form. It is also an interesting and visual way of examining at neural crest function. Most pigmented cells of vertebrates are derived from the neural crest, a transient population of cells that arises during neurulation along the dorsal neural tube and then migrates throughout the embryo (Kelsh et al., 1996). The neural crest also specifies many other cell types including neurons and glia of the peripheral and enteric nervous systems and cartilage of the head and neck (Eisen and Weston, 1993; Raible et al., 1992).

There are three types of pigment cells in zebrafish: the black melanin-containing melanophores; the yellow or orange pteridines and carotenoids containing xanthophores and the silvery guanine-rich reflecting platelets – iridophores (Parichy, 2003). Melanophores in zebrafish start to appear at 24hpf, increasing in number until around 60hpf (Yang et al., 2004), at which point, the embryo has approximately 400 melanophores. This number remains constant until approximately two weeks post fertilization, at which time the juvenile fish starts to develop its adult pigment (Milos et al., 1983; Parichy et al., 1999). A melanophore cell contains hundreds of melanin-

filled pigment vesicles, known as melanosomes. These melanosomes can be aggregated in the centre of the cell or dispersed through the cytoplasm. This pigment movement takes place in response to extracellular cues such as neurotransmitters (Levina and Gordon, 1983). The melanosome dispersion is induced by elevation of intracellular cAMP levels, while aggregation is triggered by depression of cAMP (Horowitz et al., 1980). Melanosomes are known to be associated with the microtubule based motors, dynein and kinesin (Lambert et al., 1999). Movement of the melanosome towards the centre of the cell is believed to involve the activation of the associated dynein motors (Skold et al., 2002). Movement towards the cell periphery is believed to involve the associated kinesin motors. Kinesin-2 is thought to mediate the long-range transport of melanosomes on microtubules, while myosin V, on actin filaments, is considered to be required for uniform distribution (Levi et al., 2006; Rogers and Gelfand, 1998; Wu et al., 1997). Interestingly two mammalian disorders casued by mutations in *rab27a* and *rab38* result in pigmentation defects (section 1.2.6.4 and 1.2.6.5).

3.2.1 Rab3c1

3.2.1.1 Initial Screening

Embryos injected with the *rab3c1* MO initially appeared normal and underwent successful gastrulation. By 24h hpf, about 80% of the 2ng *rab3c1* embryos had survived as the dose of *rab3c1* morpholino increased the number of embryos that survived decreased (2ng n = 31/38, 4ng n = 25/38, 8ng n = 22/43). While the 2ng embryos looked phenotypically normal many of the embryos that had been injected with either 8ng or 4ng of the *rab3c1* MO had small heads and brains (4ng n = 10/25, 8ng n = 22/22) (Figure 3.2.1 C), compared to control MO injected embryos (controls) (n = 40) (Figure 3.2.1 A). The MO injected embryos had poorly defined brain structures with the fore-, mid-, and hindbrain being disrupted (Figure 3.2.1 D). Some of these embryos also displayed heart oedema sometimes with no discernable heart tube (not shown).

At 48hpf, many of the embryos appeared normal, with the remainder possessing smaller brains, which appeared to be tinged yellow (2ng n= 2/31, 4ng n = 10/25, 8ng n = 22/22). In addition, some showed curved tails and malformed, oedematous hearts and had no circulation (2ng n= 2/31, 4ng n = 10/25, 8ng n = 12/22).

Figure 3.2.1: Lateral view of 24hpf embryos: control MO injected embryo (A) compared to 8ng *rab3c1* MO injected embryo (C). Dorsal anterior view of 24hpf embryos: control MO injected embryo (B) compared to 8ng *rab3c1* MO injected embryo (D).

By day five, the surviving embryos, were either normal or showed either defects in the distribution of melanophores, with thicker blotches of pigment being laid down on the head (2ng n = 7/31, 4ng n = 5/25, 8ng n = 10/22) (Figure 3.2.2 B and A) or a

shorter body, a smaller brain and pericardial oedema (2ng n = 2/31, 4ng n = 10/25, 8ng n = 12/22) (Figure 3.2.2 D and C). A closer look at the milder pigmentation phenotype revealed several aspects: Firstly, it showed a tight packing of melanophores on the head of the MO injected embryos (Figure 3.2.3 D and A). Secondly, the eyes of the MO injected embryos were smaller and the lens appears to be protruding (Figure 3.2.3 E and B). Finally, the swim bladder was either absent or not inflated (Figure 3.2.3 F and C). This interesting pigmentation phenotype is a characteristic of fish that have adapted themselves to darker surroundings (Logan et al., 2006). These observations led to the hypothes that the embryos were blind with constantly dark adapted pigment pattern, consistent with the defects observed in eye morphology.

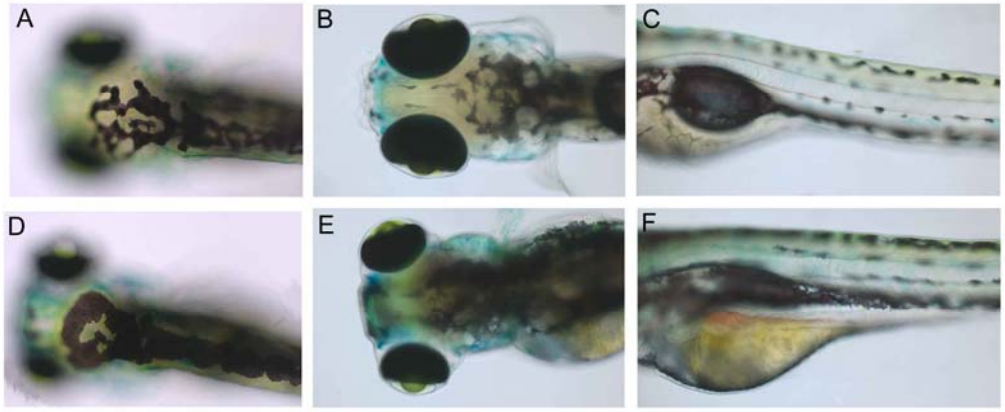

Figure 3.2.3: The dorsal view of pigment patterns in the head of five dpf control injected embryos (A) compared to five dpf *rab3c1* MO injected embryos (D). The dorsal view of eye morphology in 5 dpf control MO injected embryos (B) compared to *rab3c1* injected MO embryos (E). Side view of swim bladder in five dpf control MO injected embryos (C) compared to five dpf *rab3c1* MO injected embryos (F).

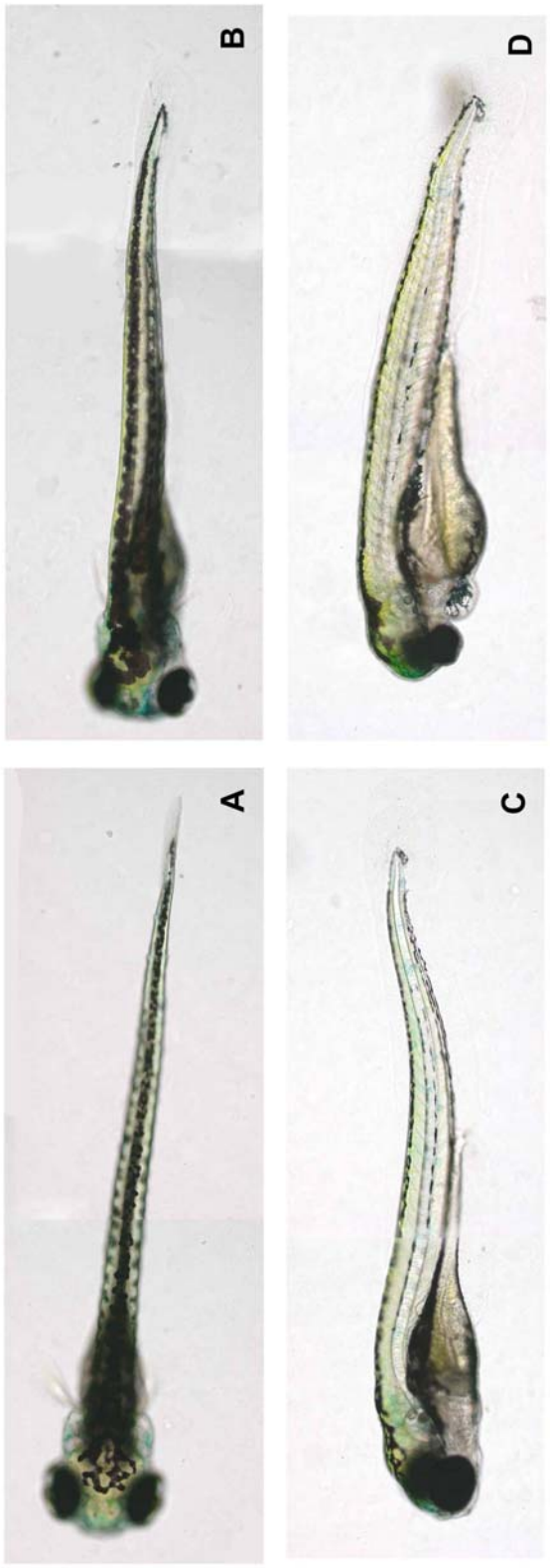

Figure 3.2.2: Dorsal view of 5dpf embryos: control MO injected embryo (A) compared to mild phenotype in *rab3c1* MO injected embryo (B) Lateral view of 5dpf embryos: control MO injected embryo (C) compared to severe phenotype in *rab3c1* MO injected embryo (D)

3.2.1.2 Assaying for blindness

To establish whether the embryos were blind, an optokinetic response apparatus was constructed (see Figure 2.5.1 Materials and Methods section 2.5.4). Each embryo was assayed individually so that movements from one embryo did not affect any other embryo. Both the control and the MO injected embryos were assayed for their ability to follow the clockwise and anti-clockwise motion of an alternating back and white spinning cylinder (see inset Figure 2.5.1 section 2.5.4). The control embryos followed the movement of the cylinder in both the anti-clockwise and clockwise directions (Figure 3.2.4 A and B see supplemental disk Mov 3.2.1A and Mov 3.2.1B). Conversely, the *rab3c1* MO injected embryos showed no response to the spinning cylinder, in either direction (Figure 3.2.4 C and D see supplemental disk Mov 3.2.1C and Mov 3.2.1D). The embryos were lightly touch-stimulated using a pair of forceps, resulting in eye movement in both control injected and *rab3c1* MO injected embryos.

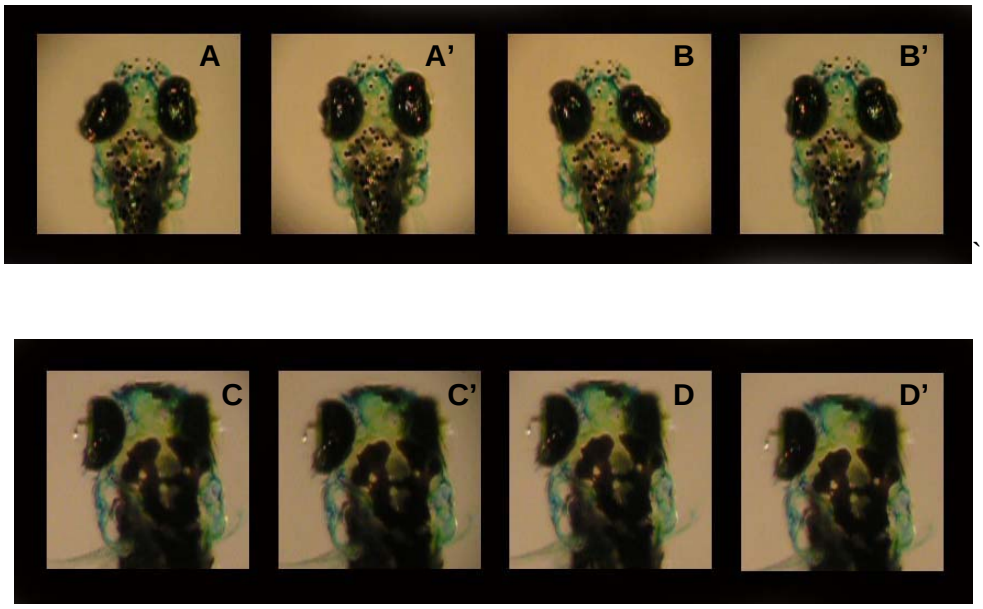

Figure 3.2.4: Still images taken from a short movie (see supplemental disk Mov 3.2.1). The stills at the top of the figure show the dorsal view of a five dpf control embryo following the spinning cylinder first in a clockwise direction (A), compared to no cylinder movement (A'), and then in an anticlockwise direction (B) compared to no cylinder movement (B'). The stills at the bottom of the figure show a five dpf *rab3c1* MO injected embryo not responding to cylinder movement in any of the directions (C, C' D and D').

3.2.1.3 Penetrance of phenotype

None of the three doses of the *rab3c1* MO produced a fully penetrant mild or severe phenotype. Therefore, the dose was increased until it became fully penetrant, or all the embryos died. The pigment phenotype increased in penetrance in a dose dependent manner (Figure 3.2.5) until 16ng. At 16ng, it was still not fully penetrant but at 19ng, all embryos displayed a phenotype far more severe. These embryos had very small brains and eyes, a short body axis and curved tail. These individuals were not assayed for blindness, since the eyes were so small that they appeared virtually absent.

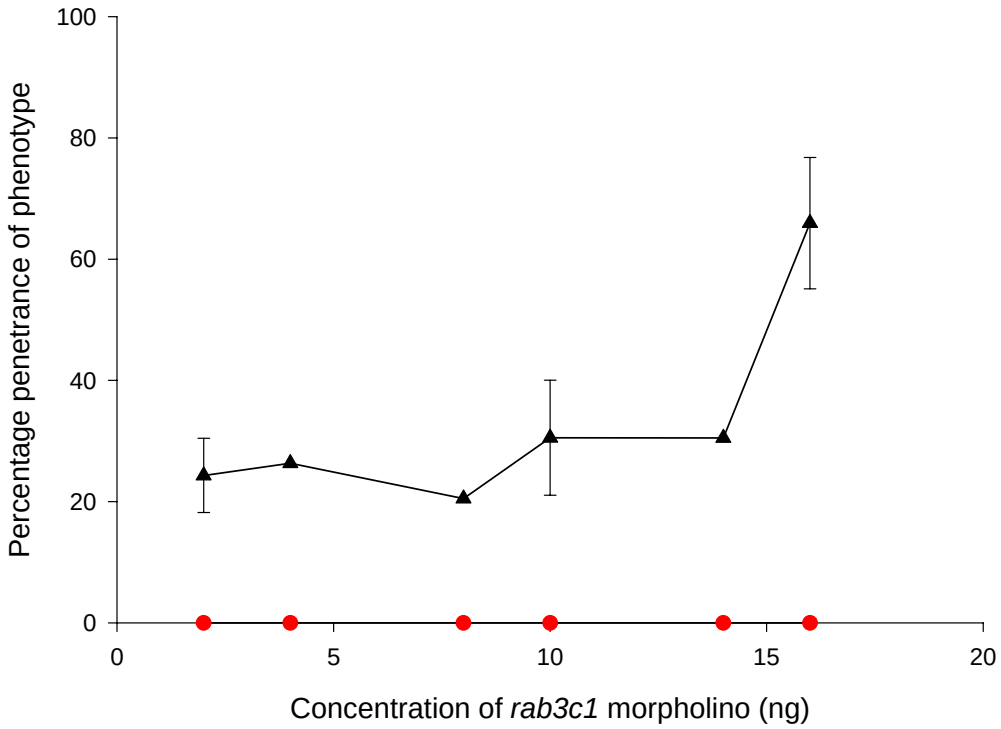

Figure 3.2.5: Graph showing the penetrance of *rab3c1* (black triangles) in a dose dependent manner (points with no error bars *n*=1 clutch, points with error bars *n*=2 or more clutches) compared to control embryos (red circles). The number of *rab3c1* injected embryos showing a phenotype at 2ng: n=7/23, 2/11 4ng: 5/19, 8ng: 10/39, 10ng: 4/19, 10/25, 14ng: 18/41 and 16ng: 35/48, 12/19, 35/39, 33/87. The number of control injected embryos showing a phenotype 2ng: 0/29, 0/15 4ng: 0/20 8ng: 0/44 10ng: 0/32, 0/30 14ng: 0/45 and 16ng: 0/45, 0/23, 0/41, 0/44.

3.2.1.4 Confirmation of *rab3c1* MO injected phenotype using alternative MOs.

To ascertain whether the phenotype observed due to the *rab3c1* MO, was a verifiable effect, two additional MOs were designed. The first was designed against the start codon of the open reading frame of the *rab3c1* gene and the second is a splice MO, designed to bind part of an intron and an exon. Either 16ng or 18ng of the splice MO was injected, as a comparison with the UTR MO. The 3 standard doses (2ng, 4ng, 8ng) of the ATG MO were injected for the screen.

At 24hpf, the 4ng ATG MO injected embryos showed smaller brains and eyes and a curved tail (Figure 3.2.6 D) (n = 35/35, 7 died), compared with controls (Figure 3.2.6 A). The 8ng ATG MO injected embryos showed a more severe phenotype, with very little brain, massively reduced eyes and reduced tail structures (n = 22/22, 17 died). The 16ng splice MO injected embryos showed a smaller brain and a mildly curved tail (n = 34/34, 5 died) (Figure 3.2.6 E), compared to controls (Figure 3.2.6 A). The 18ng splice MO injected embryos showed very small brain and head structures and significantly reduced tail structures (n = 30/30, 12 died). On closer inspection, both the splice MO injected (Figure 3.2.6 F) and the 4ng ATG MO injected (Figure 3.2.6 D) had less well defined brain structures, compared to controls (Figure 3.2.6 B).

By 5dpf, the 16ng *rab3c1* splice MO injected embryos had smaller eyes and the concentrated melanophores, seen in the UTR MO injected embryos. They also showed a severely curved body axis (n = 17/34) (Figure 2.3.7 B) when compared to controls (Figure 2.3.7 A). When assayed, some of the *rab3c1* splice MO injected embryos did not respond to the spinning cylinder of the optokinetic response apparatus (n = 7/34). As with the UTR MO injected embryos, this phenotype is not fully penetrant and many of the injected embryos appear normal (n = 17/34) and respond to the optokinetic response assay. By 5dpf, the 18ng splice MO injected embryos have all died. The 2ng *rab3c1* ATG MO injected embryos appear normal but have a mildly curved body axis (n = 25/40) (Figure 2.3.7 D). When assayed, most of the 2ng *rab3c1* ATG MO injected embryos responded to the spinning cyclinder of the optokinetic response apparatus (n = 36/40). At 4ng, they present a more severe phenotype, with the head becoming smaller (n = 35/35) but, as with the

2ng ATG MO injected embryos, these embryos were assayed and not considered blind as they responded to the spinning cyclinder of the optokinetic response apparatus (n = 34/35). The embryos injected with 8ng of ATG MO had striking defects: the body axis was greatly reduced; tail structures were reduced; and there was pericardial oedema. In the head, not only was the brain reduced but the eyes were drastically reduced in size or absent (n = 18/18) (Figure 3.2.7 F and G).

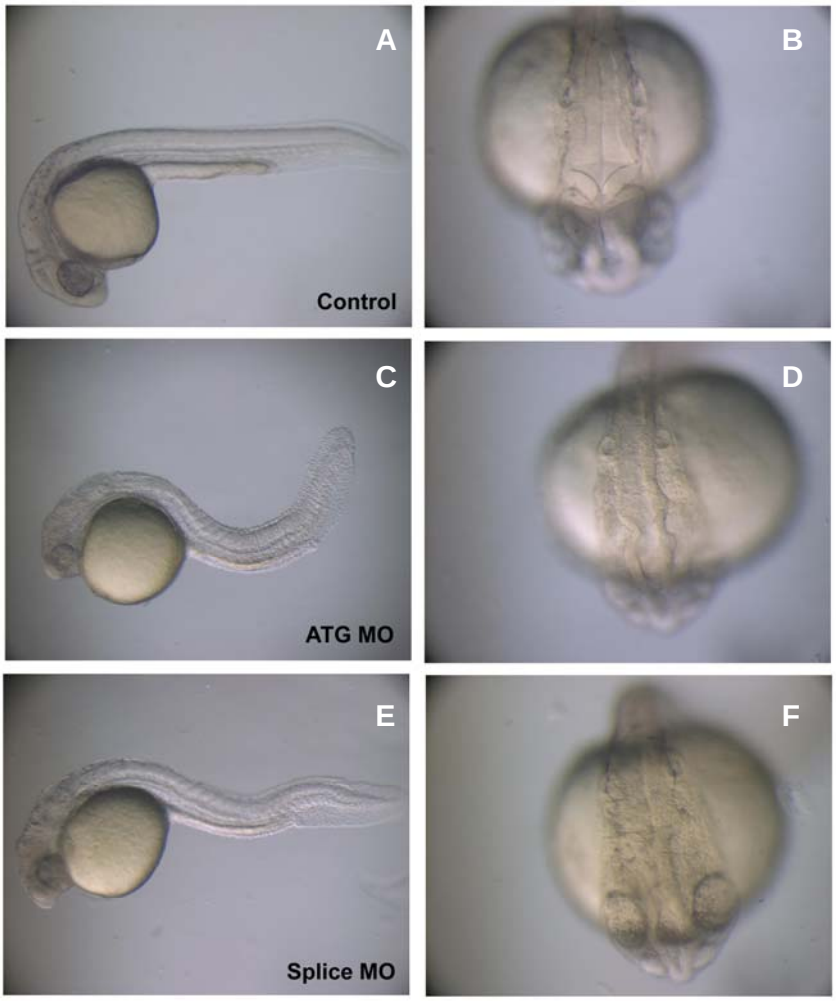

Figure 3.2.6: Lateral view of 24hpf embryos comparing control MO injected embryos (A) with 4ng injected *rab3c1* ATG MO injected embryos (C) and 16ng injected *rab3c1* splice MO injected embryos (E). A close up view comparing the brain structures of control MO injected embryos (B) with 4ng injected *rab3c1* MO injected embryos (D) and 16ng injected *rab3c1* splice MO injected embryos (F).

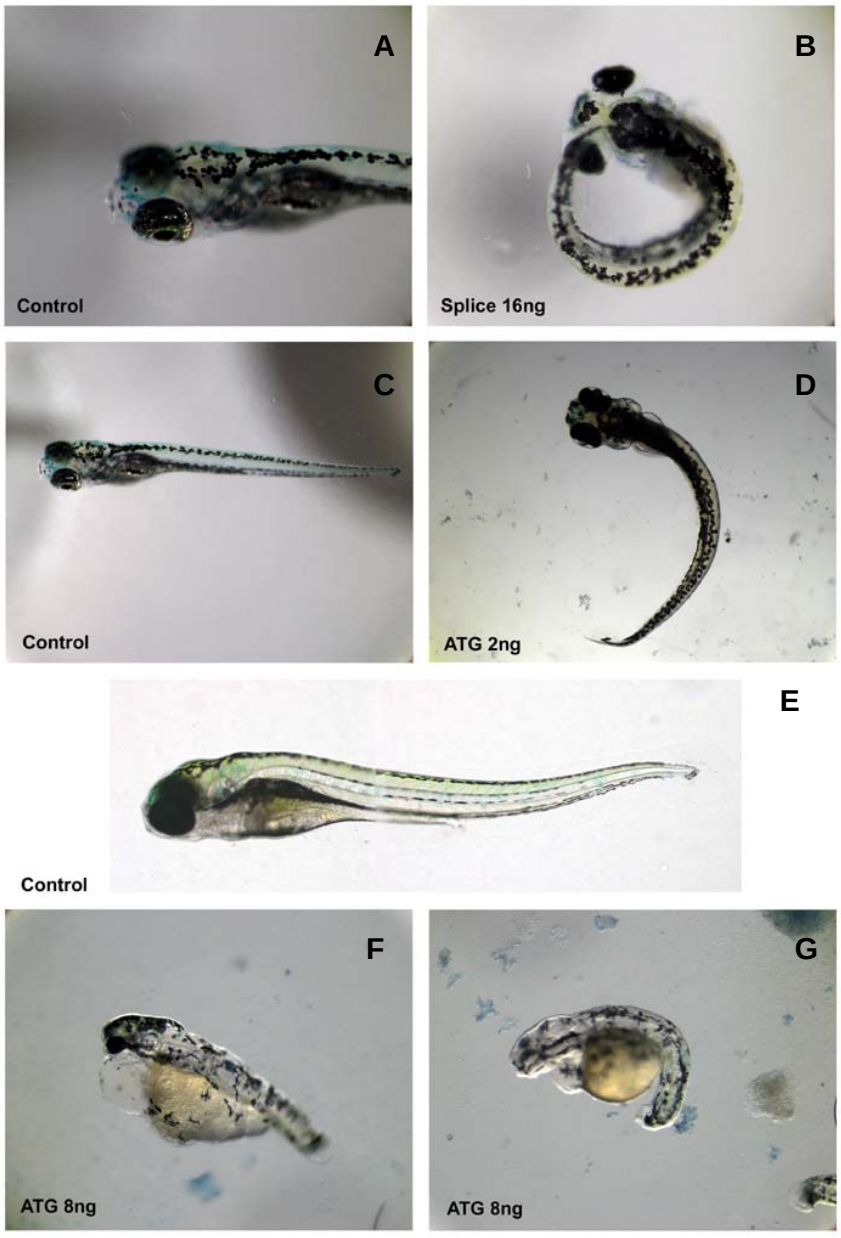

Figure 3.2.7: Dorsal view of five dpf control injected embryos (A) compared to five dpf 16ng splice MO injected embryos (B) and five dpf 2ng ATG MO injected embryos (D) compared to control (C). Lateral view of five dpf control injected embryos (E) compared to 8ng ATG MO injected embryos (F and G).

3.2.2 Rab1a3

The *rab1a3* embryos all gastrulated normally and were similar to the controls. By 24hpf, the 2ng and 4ng MO injected embryos appeared normal (2ng n = 42/42, 4ng n = 40/43). However a small proportion of the 4ng MO injected embryos had slightly reduced head size, compared to control MO injected embryos (n = 3/43). At 8ng, c. 60% of the MO injected embryos had died (n = 28/49), with 25% of the embryos exhibiting a normal phenotype. The last 15% of embryos showed a slightly reduced head size (n = 7/49).

At 48hpf, the 2ng injected embryos still appeared normal, as did the majority of the 4ng injected embryos. The remaining 4ng *rab1a3* MO injected embryos and 15% of the 8ng injected embryos still displayed slightly reduced brain size and reduced melanophore density (4ng n = 3/43 , 8ng n = 7/49) (Figure 3.2.8 D) when compared to controls (Figure 3.2.8 A). Upon closer inspection, the head (Figure 3.2.8 E) and yolk sac (Figure 3.2.8 F) had a reduced density of melanophores, in the 4ng and 8ng MO injected embryos, when compared with controls (Figure 3.2.8 B and C).

Since this phenotype was not fully penetrant the dose was increased to 10ng. Many of the embryos died prior to 24hpf, as would be expected (n = 28/46). Surviving embryos, however, all exhibited a common phenotype (n = 18/18). By 48hpf, the 10ng injected *rab1a3* MO injected embryos displayed a slightly reduced head size and a curved body axis, as well a reduced density of melanophores (Figure 3.2.9 B), compared to controls (Figure 3.2.9 A). Since the curved body axis was a new phenotype, compared with the last pass of injections, further study was warranted. The MO injected embryos possessed U-shaped somites and, interestingly, a shortened body axis, kinks in the notochord (Figure 3.2.9 D and C) and intermittent failure of vacuolation in some of the notochord cells (Figure 3.2.9 F and E).

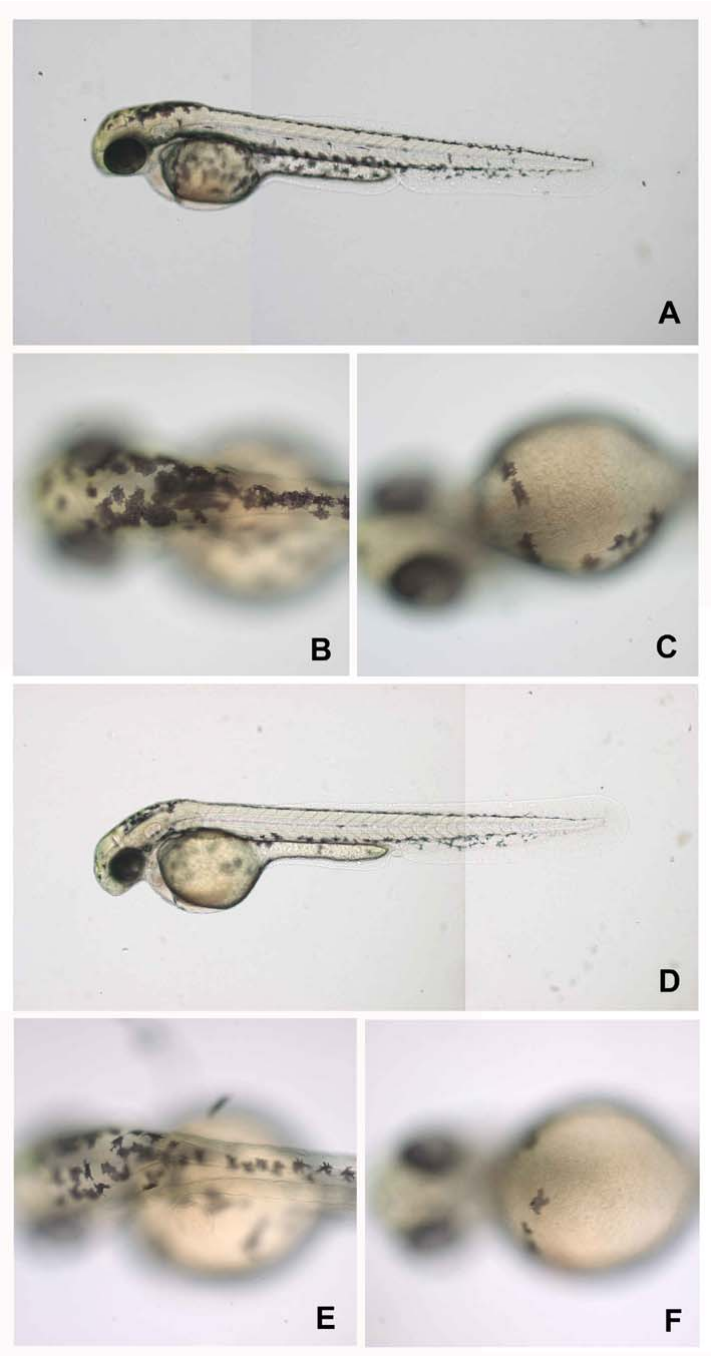

Figure 3.2.8: Lateral view of a 48hpf control MO injected embryo (A) compared to an embryo injected with 8ng *rab1a3* MO (D). Dorsal magnified view of head pigmentation of a control injected embryo (B) compared to an embryo injected with 8ng *rab1a3* MO (E). Ventral magnified view of the yolk pigmentation of a control MO injected embryo (C) compared to an embryo injected with 8ng *rab1a3* MO (F).

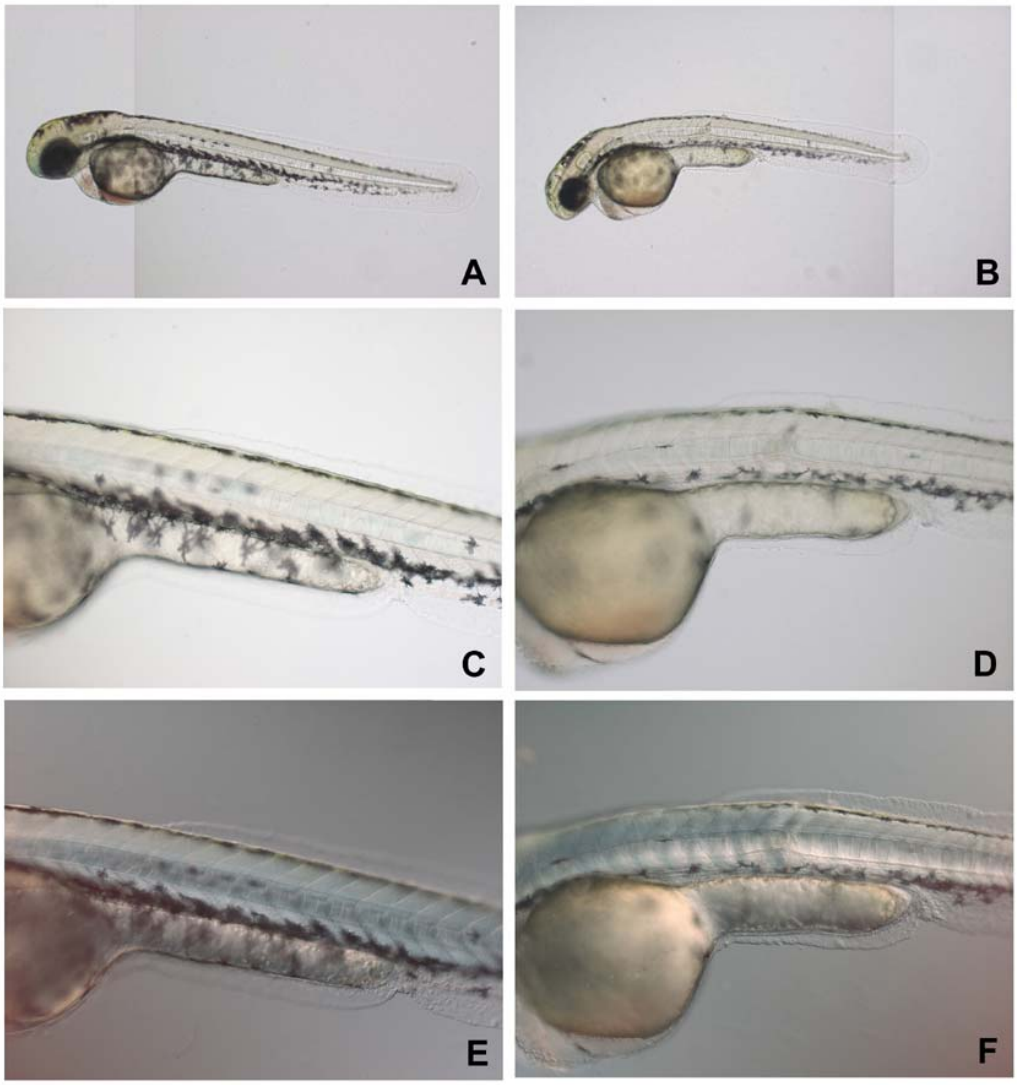

Figure 3.2.9: Lateral view of 48hpf control MO injected embryo (A) compared to 10ng *rab1a3* MO injected embryo (B).

10x magnification of the lateral view of the mid section of control MO injected embryo (C) compared to 10ng *rab1a3* MO injected embryo (D).

10x magnification of the lateral view of the mid section of a control MO injected embryo (E) compared to 10ng *rab1a3* MO injected embryo (F) under reflected light enabling a clearer view of embryo somites and notochord.

3.3 Slowed Development

This section focuses on those Rabs which following MO knockdown resulted in developmental delay. In all cases this delay was seen as early as shield stage with many MO injected embryos reaching shield stage hours after their control MO injected siblings.

3.3.1 Rab 11a1

Injection of 2ng, 4ng or 8ng's of *rab11a1* MO showed an early phenotype. When the control injected embryos had reached shield stage (n = 45) (Figure 3.3.1 A), the *rab11a1* MO injected embryos were still beginning epiboly (2ng n = 46/48, 4ng n = 38/38, 8ng n = 48/50) (Figure 3.3.1 B) and took two hours more than control injected embryos to reach shield stage (Figure 3.3.1 C). At shield stage an accumulation of cells became apparent at the animal pole (2ng n = 43/45, 4ng n = 38/38, 8ng n = 46/50) (Figure 3.3.1 C and A).

By 24hpf, the majority of embryos died, leaving approximately 10% of the 2ng *rab11a1* MO injected embryos (n = 5/48) and approximately 8% of the 4ng MO injected embryos (n = 3/38). All of the embryos injected with the highest dose of *rab11a1* MO died. The surviving *rab11a1* MO injected embryos (2ng and 4ng) had all either arrested at late gastrulation or had shortened tails with U-shaped somites and small heads (2ng = 5/5, 4ng = 3/3) (Figure 3.3.1 E and D).

When the embryos reached 48hpf, the embryos injected with *rab11a1* MO exhibited reduced distance between the eyes and smaller brains (Figure 3.3.1 G and F) and possessed a bent tail, a malformed heart and no evident circulation (Figure 3.3.1 G) (2ng n = 5/5, 4ng n = 3/3)

By 5dpf, the embryos injected with 4ng *rab11a1* MO had died while many of the remaining 2ng injected embryos had failed to hatch. (n = 5). Survivors showed some circulation, however, it was absent from the intersomitic region and blood cells appeared to adhere to the yolk.

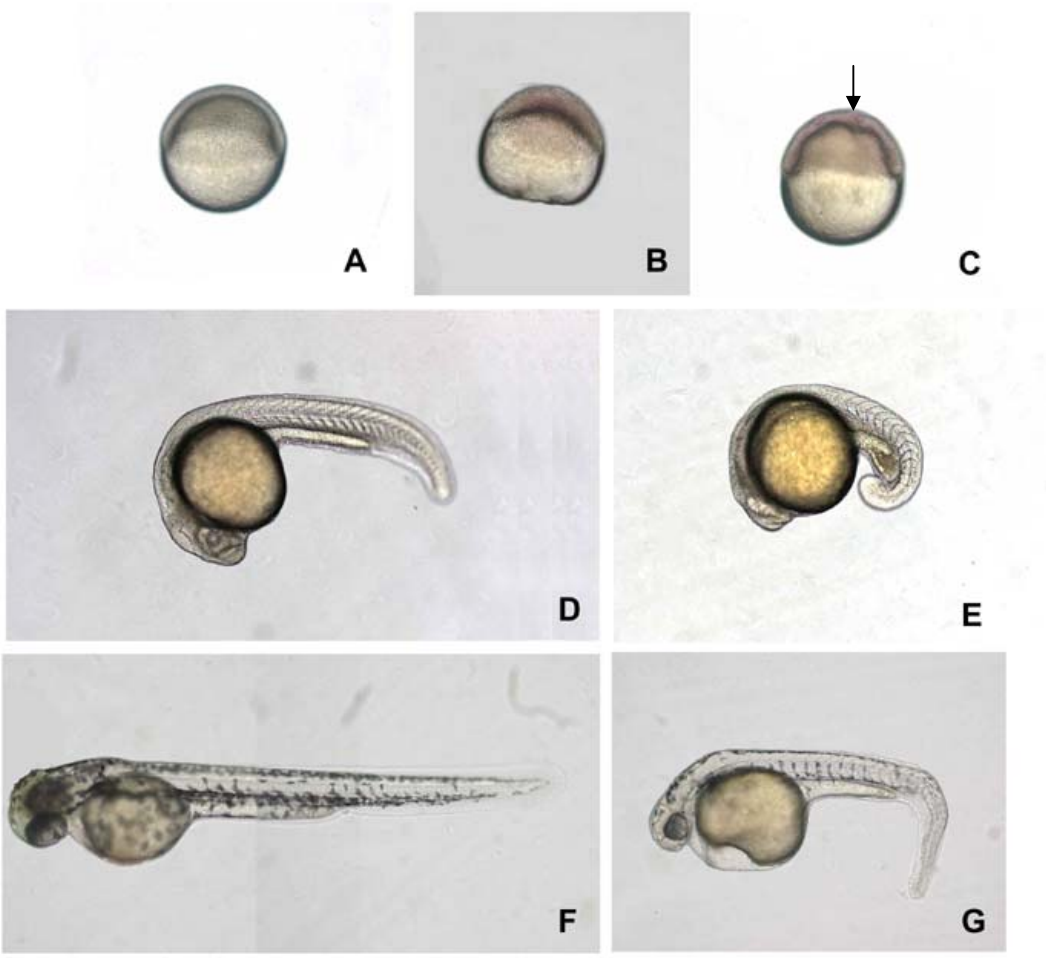

Figure 3.3.1: Shield stage embryos injected with control MO (A) compared to the same time point in embryos injected with *rab11a1* MO (B) and shield stage in embryos injected with *rab11a1* MO, arrow indicates accumulation of cells (C). 24hpf embryos injected with control MO (D) compared to 24hpf embryos injected with *rab11a1* MO (E). 48hpf embryos injected with control MO compared to (F) to 48hpf embryos injected with *rab11a1* MO (G).

3.3.2 Rab1a4

At shield stage, the embryos injected with 2ng of *rab1a4* MO (n = 45), along with the majority of the 4ng *rab1a4* MO injected embryos (n = 40/46), were comparable to control MO injected embryos (n = 47). A small proportion of the 4ng *rab1a4* MO injected embryos (6/46) and all the 8ng *rab1a4* MO injected embryos (n = 41) exhibited delayed gastrulation, this resulted in the *rab1a4* MO injected embryos only reaching approximately 20% epiboly (Figure 3.3.2 B) by the time the control embryos reached shield stage (Figure 3.3.2 A). When the 8ng *rab1a4* MO injected embryos did reach shield stage, approximately two hours later than controls, they possessed an enlarged shield (n = 40/41) (Figure 3.3.2 C and A).

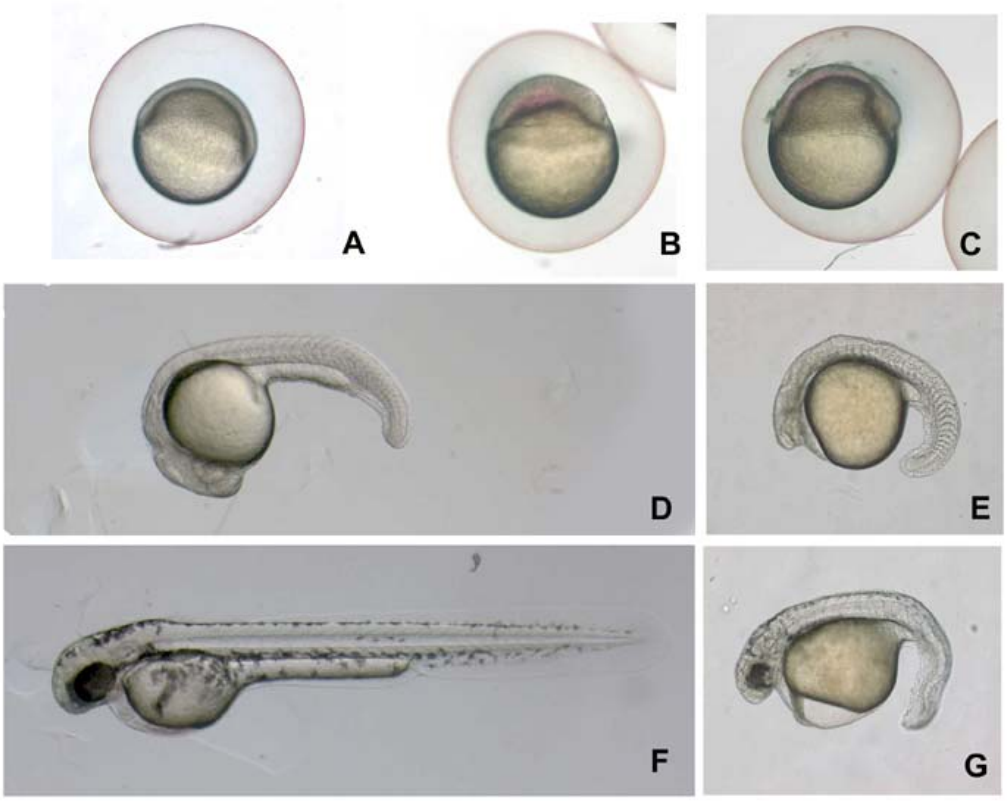

Figure 3.3.2: Shield stage control MO injected embryo (A) compared to the same time point embryo injected with 8ng of *rab1a4* MO (B) and shield stage embryo injected with 8ng of *rab1a4* MO (C). Lateral view of 24hpf control MO injected embryo (D) compared to 24hpf embryo injected with 8ng of *rab1a4* MO (E). Lateral view of 48hpf control MO injected embryo (F) compared to 48hpf embryo injected with 8ng of *rab1a4* MO (G).

By 24hpf, the majority of the 2ng and 4ng *rab1a4* MO injected embryos appeared normal (2ng n = 45, 4ng n = 40/45) although a small proportion displayed smaller brains and kinked tails (2ng n = 0, 4ng n = 6/45). When the 8ng *rab1a4* MO injected embryos reached 24hpf, about. 35% of the embryos were dead (n = 14/41). Those that survived had reduced head structures and a curved tail (n = 27/27) (Figure 3.3.2 E and D).

At 48hpf many of the 2ng and 4ng *rab1a4* MO injected embryos appeared normal; however, a small proportion still possessed small brains and shortened curved tails (2ng n = 0, 4ng n = 6/45). In addition the 4ng *rab1a4* MO injected embryos also showed some pericardial oedema (n = 6/45) and did not hatch (n = 22/45). The 8ng *rab1a4* MO injected embryos did not hatch and displayed pericardial oedema, similar to that seen in the 4ng *rab1a4* MO injected embryos. These embryos also displayed yellowing of the brain, smaller eyes, shortened tails with U-shaped somites and a curving notochord (n = 24/27) (Figure 3.3.2 G and F).

At 3dpf, all the 2ng *rab1a4* MO injected embryos hatched (n = 45) although some had an inward curving body axis and swam in circles (n = 4/45). Although fewer embryos hatched this phenotype was exaggerated in the 4ng *rab1a4* MO injected embryos where the embryos exhibited a curved body axis and swam in a large circular motion on their sides (n = 24/46). Those 4ng *rab1a4* MO injected embryos that failed to hatch exhibited abruptly curved, or ‘kinked’, tails and pericardial oedema (n = 22/46). At 8ng, only about 10% of embryos hatched (n = 3/27) and these, in common with the 4ng *rab1a4* MO injected embryos, had difficulty swimming normally swimming on their back and sides. Those that did not hatch showed the same phenotype as the 4ng *rab1a4* MO injected embryos (n = 24/47).

3.3.3 Rab18(2)

Injection of 2ngs, 4ngs or 8ng's of *rab18(2)* MO resulted in an early phenotype, with gastrulation in these embryos delayed by approximately two hours (2ng n = 42, 4ng n = 45, 8ng n = 37/39). When the embryos reached shield stage, they showed an elongated and pinched yolk (2ng n = 42, 4ng n = 45, 8ng n = 37/39) (Figure 3.3.3 B and A).

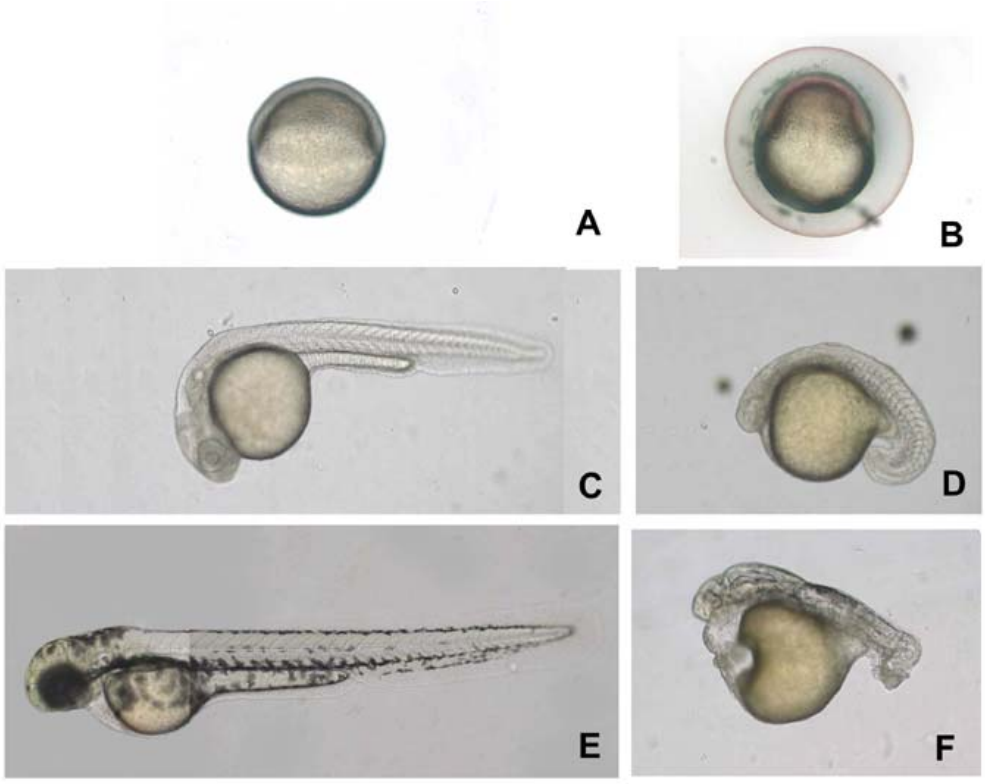

Figure 3.3.3: Shield stage embryos: control MO injected embryo (A) compared to embryo injected with 4ng of *rab18(2)* MO (B). Lateral view of 24hpf embryos: control MO injected embryo (C) compared to embryo injected with 4ng of *rab18* MO (D). Lateral view of 48hpf embryos: control MO injected embryo (E) compared to embryo injected with 4ng of *rab18(2)* MO (F).

By 24hpf, the embryos injected with 2ngs of *rab18(2)* MO possessed a smaller head, while, about. 50% of the 4ng *rab18(2)* MO injected embryos were dead (n = 24/45). The remaining 4ng *rab18(2)* MO injected embryos displayed brain cell death and small eyes (n = 21/21) (Figure 3.3.3 D and C). In addition the 4ng *rab18(2)* MO

injected embryos had tails that were either very short and curved with U-shaped somites, or absent (n = 21/21) (Figure 3.3.3 D). When the dose of the *rab18(2)* MO was increased to 8ng, approximately 87% of the MO injected embryos were dead before 24hpf (n = 34/39). Those that remained were an accumulation of dying cells on the yolk (n = 5/5).

By 48hpf, the 8ng *rab18(2)* MO injected embryos were dead. The 2ng *rab18(2)* MO injected embryos failed to hatched, showed kinked tails and pericardial oedema (n = 37/37). The 4ng *rab18(2)* MO injected embryos showed a similar, if more severe, phenotype, with a small proportion of embryos exhibiting no tail structures and eyes that were substantially reduced, or absent (n = 16/16)(Figure 3.3.3 F). In addition the head structures were greatly reduced (Figure 3.3.3 F and E).

At 3dpf, the 2ng *rab18(2)* MO injected embryos had not hatched and their heads started to develop a yellow/green colour and a thick layer of melanophores (n = 37/37). Consistent with the pericardial oedema the heart was beating slowly and there was no movement of blood. The 4ng *rab18(2)* MO injected embryos did not hatch and exhibited a similar, if slightly more severe, phenotype than the 2ng *rab18(2)* MO injected embryos (n = 16/16).

3.3.4 Rab1a1

Embryos injected with *rab1a1* MO exhibited a developmental delay (2ng = 45, 4ng = 38, 8ng n = 42). When the control embryos had reached shield stage, all of the *rab1a1* MO injected embryos, regardless of the dose of *rab1a1* MO injected, were at least two hours delayed.

By 24hpf, c. 50% of the 2ng *rab1a1* MO injected embryos were dead (n = 23/45). Approximately 10% of the embryos appeared normal (n = 4/45), whereas, the remainder had massive brain cell death and curved tails (n = 18/45) (Figure 3.3.4 B and A). In addition, a small proportion of the phenotypic *rab1a1* MO injected embryos displayed grey cells in the tail indicative of cell death (n = 3/18) (Figure 3.3.4 B). The majority of the 4ng *rab1a1* MO injected embryos had died by 24hpf (n = 35/38). Approximately, 10% survived and these had severe defects, missing head

structure, some tail structure and massive widespread cell death over the whole embryo (n = 3/38). The 8ng *rab1a1* MO injected embryos were all dead.

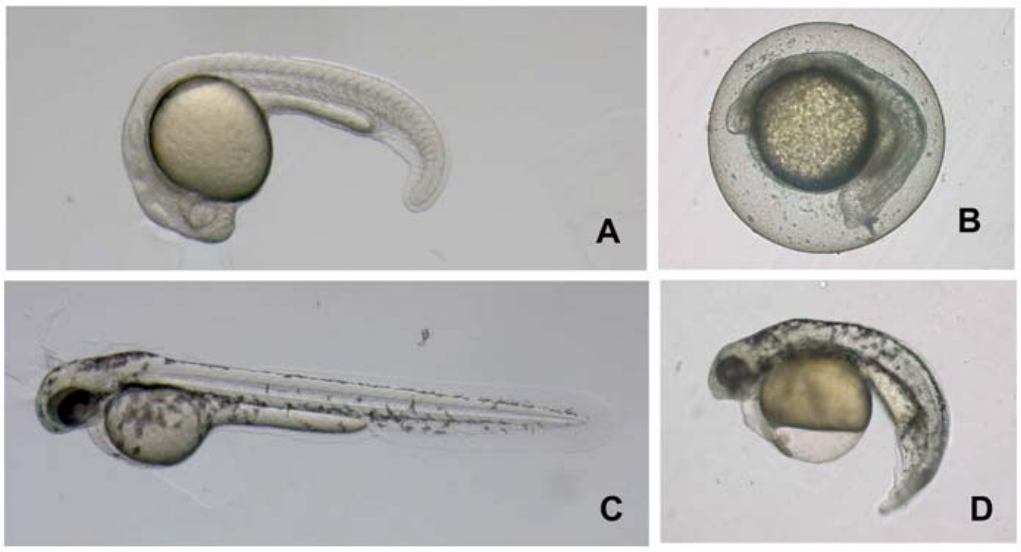

Figure 3.3.4: Lateral view of 24hpf embryos: control MO injected embryos (A) compared to 2ng *rab1a1* MO injected embryos (B) Lateral view of 48hpf embryos: control MO injected embryos (C) compared to 2ng *rab1a1* MO injected embryos (D)

By 48hpf only about 25% of the original clutch of 2ng *rab1a1* MO injected embryos survived (n = 12/45) a third of those remaining appeared normal (n = 4/12). Those *rab1a1* MO injected embryos with a phenotype exhibited reduced head structures with brain cell death, some being so disrupted that they comprised only a pair of eyes on an elongated yolk (n = 3/12). Other *rab1a1* MO injected embryos had pericardial oedema, in addition to short, bent tails with indistinguishable somites (n = 5/12) (Figure 3.3.4 D and C). By this time point, the 4ng *rab1a1* MO injected embryos were all dead.

At 3dpf, remaining *rab1a1* MO injected embryos were either normal (n = 4/9) or displayed a similar phenotype to those at 48hpf, with kinked tails, indistinguishable somites and reduced head structure when compared to controls (n = 5/9).

3.4 Hatching Defects

This section describes those *rabs* that, when knocked-down led to a failure to hatch. Hatching results from the combined effort of the hatching enzyme secreted from the hatching gland, along with osmotic and mechanical mechanisms (Denuce, 1985; Yamagani, 1988). The hatching gland is derived from the prechordal plate which, in turn, is derived from the embryonic shield in the area of the dorsal margin. The prechordal plate cells differentiate to form various different types of cells. Those cells that will become the hatching gland migrate from the dorsal margin of the embryo along the dorsal midline to anterior of the forebrain, where they accumulate to form the polster, upon completion of gastrulation. By 24hpf, the cells of the hatching gland are located on the pericardial membrane. They can be visualised as a semi-circle around the yolk and are prominent due to the presence of brightly refractive cytoplasmic granules within the hatching gland cells (Houart et al., 1998).

3.4.1 Rab11b1

Figure 3.4.1: Lateral view of 24hpf embryos: control MO injected embryo (A) compared to embryo injected with 4ng of *rab11b1* MO (B). Dorsal anterior view of 24hpf embryos: control MO injected embryo (C) compared to embryo injected with 4ng of *rab11b1* MO (D).

All of the embryos gastrulated successfully with the majority of the 2ng *rab11b1* MO injected embryos appearing normal by 24hpf (n = 36/42). However a few 2ng *rab11b1* MO injected embryos exhibited a grey brain, indicative of cell death and a reduction in the size of the eyes (n = 6/42). Increasing the dose of *rab11b1* MO to 4ng resulted in all embryos exhibiting this abnormal phenotype, in addition they exhibited a shortened tail with U-shaped somites (n = 46/46) (Figure 3.4.1 B and A). Injection of 8ng of the *rab11b1* MO resulted in a more severe phenotype with the entire embryo turning grey, indicative of cell death. The 8ng *rab11b1* MO injected embryos exhibited massively reduced brain and head size as well as tail defects (n = 39/39). Closer inspection of 4ng *rab11b1* MO injected embryos revealed small, malformed eyes and badly defined brain structures, resembling an unstructured mass of cells than clearly defined regions (Figure 3.4.1 D and C).

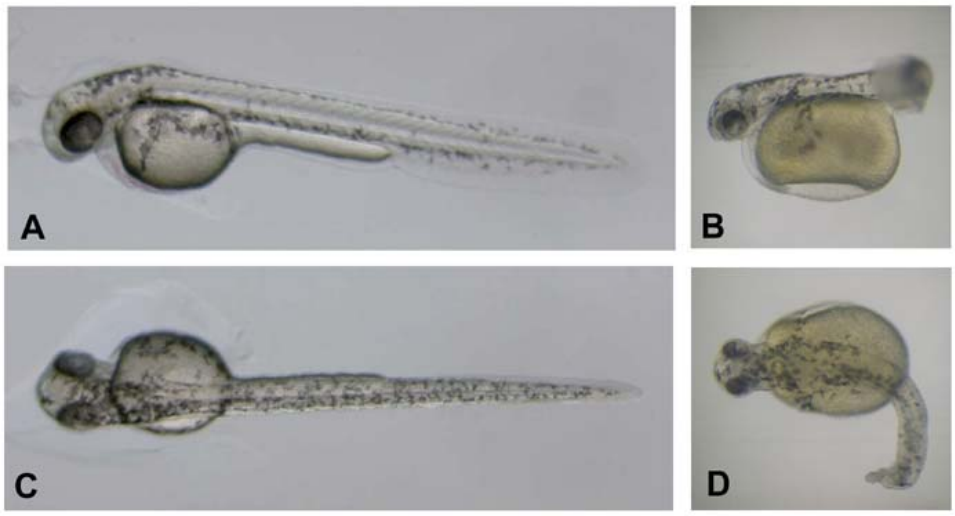

Figure 3.4.2: Lateral view of 48hpf embryos: control MO injected embryo (A) compared to embryos injected with 4ng of *rab11b1* MO (B). Dorsal view of 48hpf embryos: Control MO injected embryo (C) compared to embryos injected with 4ng of *rab11b1* MO (D).

When the phenotypic *rab11b1* MO injected embryos reached 48hpf, they possessed smaller brains and eyes (2ng n = 36/42, 4ng n = 46/46, 8ng n = 39/39) (Figure 3.4.2 B and A) and exhibited bent tails and a curved body axis (Figure 3.4.2 D), compared to controls (Figure 3.4.2 C).

At 4dpf, none of the phenotypic *rab11b1* MO injected embryos had hatched (2ng n = 36/42, 4ng n = 46/46, 8ng n = 39/39) and many displayed pericardial oedema (2ng n = 6/42, 4ng n = 46/46, 8ng n = 39/39).

3.4.2 Rab11b2

The *rab11b2* MO injected embryos all gastrulated comparably with the control embryos (2ng n = 40/40, 4ng n = 42/42, 8ng n = 45/45). At 24hpf, all embryos, regardless of which dose was injected, showed the same phenotype (2ng n = 40/40, 4ng n = 42/42, 8ng n = 45/45). The MO injected embryos all had reduced sized, greying brains, indicative of cell death. Furthermore, they had U-shaped somites, shortened tails and pericardial oedema (Figure 3.4.3 B and A).

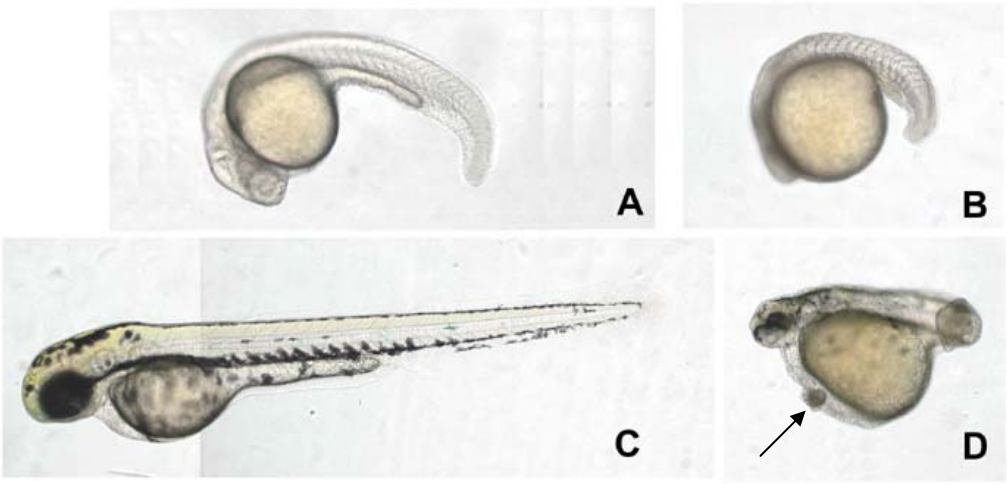

Figure 3.4.3: Lateral view of 24hpf embryos: control MO injected embryo (A) compared to an embryo injected with 4ng of *rab11b2* MO (B). Lateral view of 48hpf embryos: control MO injected embryo (C) compared to an embryo injected with 4ng of *rab11b2* MO (D arrow represents hatching gland cells).

When the embryos reached 48hpf, the *rab11b2* MO injected embryos showed a disjointed 'patchy' hatching gland, with the cells accumulating in peaks in individual areas around the yolk (shown by arrow in Figure 3.4.3 D). The brains of these embryos remained small and had a yellowish tinge (Figure 3.4.3 D), compared to controls (Figure 3.4.3 C). In addition *rab11b2* MO injected embryos had curved and shortened tails, the eyes were small and closer together and they had pericardial oedema (Figure 3.4.3 D) (2ng n = 40/40, 4ng n = 42/42, 8ng n = 45/45).

By 72hpf, none of the *rab11b2* MO injected embryos hatched, the brain remained yellow, the embryos were shortened and pericardial oedema persisted (2ng n = 40/40, 4ng n = 42/42, 8ng n = 45/45). By 5dpf, all the *rab11b2* MO injected embryos were dead.

3.4.3 Rab6a

When the embryos reached shield stage, the *rab6a* MO injected embryos were visually normal. When they reached 24hpf, the 2ng *rab6a* MO injected embryos had small heads, with the distance between the eyes severely reduced, compared to controls. In addition the 2ng *rab6a* MO injected embryos possessed short bent tails with straight somites (n = 43/43) (Figure 3.4.4 B), compared to the V-shaped somites of the controls (n = 45/45) (Figure 3.4.4 A). The majority of the 4ng (n = 42/43), and all the 8ng *rab6a* MO injected embryos (n = 47/47) died. Those of the 4ng *rab6a* MO injected embryos that survived possessed no head structures and greatly reduced tail structures (1/1).

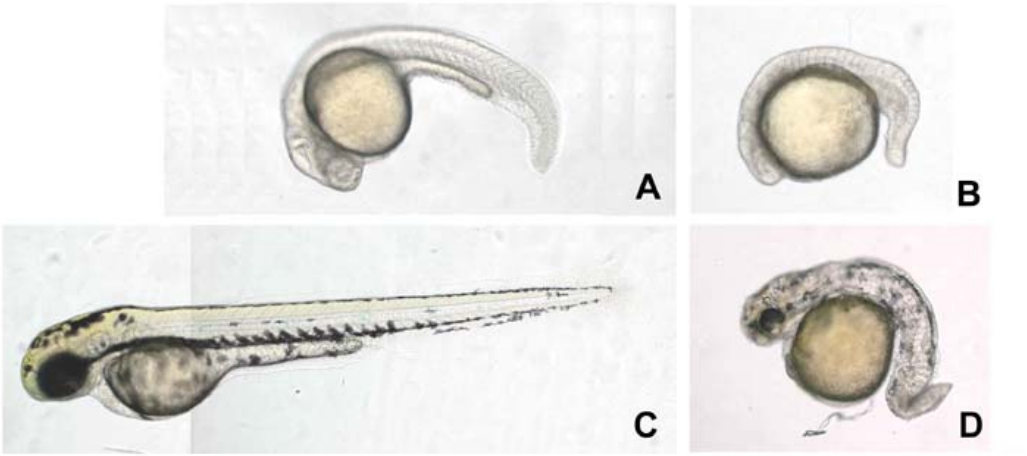

Figure 3.4.4: Lateral view of 24hpf embryos: control MO injected embryo (A) compared to embryo injected with 2ng of *rab6a* MO (Figure B). Lateral view of 48hpf embryos: control MO injected embryo (Figure C) compared embryo injected with 2ng of *rab6a* MO (Figure D).

By 48hpf, all of the 4ng *rab6a* MO injected embryos were dead (n = 1/1), the 2ng *rab6a* MO injected embryos had very small brains and small closely spaced eyes. The tail was shortened and bent posteriorly (n = 43/43) (Figure 3.4.4 D and C).

By 4dpf, most of the *rab6a* MO injected embryos had not hatched and the hatching gland appeared disjointed and patchy with possible pericardial oedema (n = 39/43).

3.4.4 Rab 11a2

All embryos appeared phenotypically normal at gastrulation. At 24hpf, the embryos injected with 2ng of *rab11a2* MO displayed a slightly reduced head size (Figure 3.4.5 B and A) and shortened tails, with U-shaped somites (n = 44). When the dose of *rab11a2* MO was increased to 4ng or 8ng the phenotype remained consistent, but a greater proportion of embryos died before 24hpf (4ng n = 38/41, 8ng n = 43/46 died).

By 48hpf, the 2ng *rab11a2* MO injected embryos had kinked or, in some cases, absent tails, and some pericardial oedema (n = 44). The 4ng *rab11a2* MO injected embryos displayed a yellow tinged head with thick pigment (n = 3/3). At the higher dose of 8ng, the majority of the *rab11a2* MO injected embryos were dead (n = 2/3) while survivors were phenotypically similar to the 4ng *rab11a2* MO injected embryos.

Figure 3.4.5: Lateral view of 24hpf embryos: control injected embryo (A) compared to embryo injected with 2ng of *rab11a2* mopholino (B).

When the embryos reached 4dpf, a large proportion of the 2ng *rab11a2* MO injected embryos remained unhatched and had pericardial oedema (n = 39/44). Most of the 4ng *rab11a2* MO injected embryos were dead and the remainder appeared normal, except for the thick pigment on the dorsal side of the embryo (n =1/1). At 8ngs, all of the *rab11a2* MO injected embryos were dead by 4dpf.

3.5 Swimming Defects

3.5.1 Rab28

During gastrulation the *rab28* MO injected embryos are phenotypically normal. By 24hpf all *rab28* MO injected embryos exhibited a reduced head size (Figure 3.5.1 C and A) regardless of the dose of *rab28* MO injected (2ng n = 43/43, 4ng n = 42/42, 8ng n = 39/39). The 8ng *rab28* MO injected embryos exhibiting greying of the brain, indicative of cell death, and, in a small percentage of cases (n = 5/39) the tail curved upwards.

Figure 3.5.1: Lateral view of 24hpf embryos: control MO injected embryo (A) compared to embro injected with 4ng of *rab28* MO (Figure C). Lateral view of 48hpf embryos: control MO injected embryo (Figure B) compared to embryo injected with 4ng of *rab28* MO (Figure D).

By 48hpf, the 2ng *rab28* MO injected embryos appeared normal (n = 43/43), whereas the 4ng *rab28* MO injected embryos had a curved and shortened body axis, in addition to a smaller head and pericardial odeama (n = 42/42) (Figure 3.5.1 D and B). These 4ng *rab28* MO injected embryos showed modified swimming behaviour, with the embryos twitching and swimming in circles. When the dose of *rab28* MO was increased to 8ng embryos showed a similar phenotype to the 4ng *rab28* MO

injected embryos and in addition the 8ng MO injected embryos possessed kinked tails (n = 39/39).

By 4dpf, 2ng *rab28* MO injected embryos appeared phenotypically normal (n = 43/43). The 4ng *rab28* MO injected embryos continued to show pericardial oedema, a shortened body axis and a high proportion of them had bent tails. In addition, the 4ng *rab28* MO injected embryos still displayed abnormal swimming behaviour (n = 42/42). The 8ng *rab28* MO injected embryos again showed a similar phenotype to those injected with 4ng of *rab28* MO (n = 39/39).

3.6 Non-Specific Defects

This section focuses on those Rabs that, when knocked-down, exhibited no specific phenotypes. Each of these Rabs showed brain defects and defects in the tail.

3.6.1 Rab20

The *rab20* MO injected embryos gastrulated normally, regardless of the dose of MO injected. By 24hpf, approximately 20% of the 2ng and 4ng *rab20* MO injected embryos died (2ng n = 9/46, 4ng n= 13/49). The majority of those that survived were phenotypically normal (2ng n = 34/37, 4ng n = 35/36) however, some exhibited reduced head and brain size and slightly shortened tails (2ng n = 3/37, 4ng n = 1/36). When the dose of *rab20* was increased to 8ng a larger percentage of embryos died (n = 34/56) while all the surviving embryos showed reduced head and brain size and slightly shortened tails. In addition, they displayed a slow heart beat and no blood flow (n = 22/22) (Figure 3.6.1 B and A).

By 48hpf, the 8ng *rab20* MO injected embryos exhibited curved tails (Figure 3.6.1 D and C), a sigmoidal body axis (n = 22/22) (Figures 3.6.1 F and E) and reduced head structures.

At 5dpf, the *rab20* MO injected embryos displayed reduced hatching success (n = 15/22) and a shortened curved tail (not shown) when compared to controls. In excess of one third of the *rab20* MO injected embryos failing to hatch exhibited pericardial oedema and had no circulation (n = 5/22).

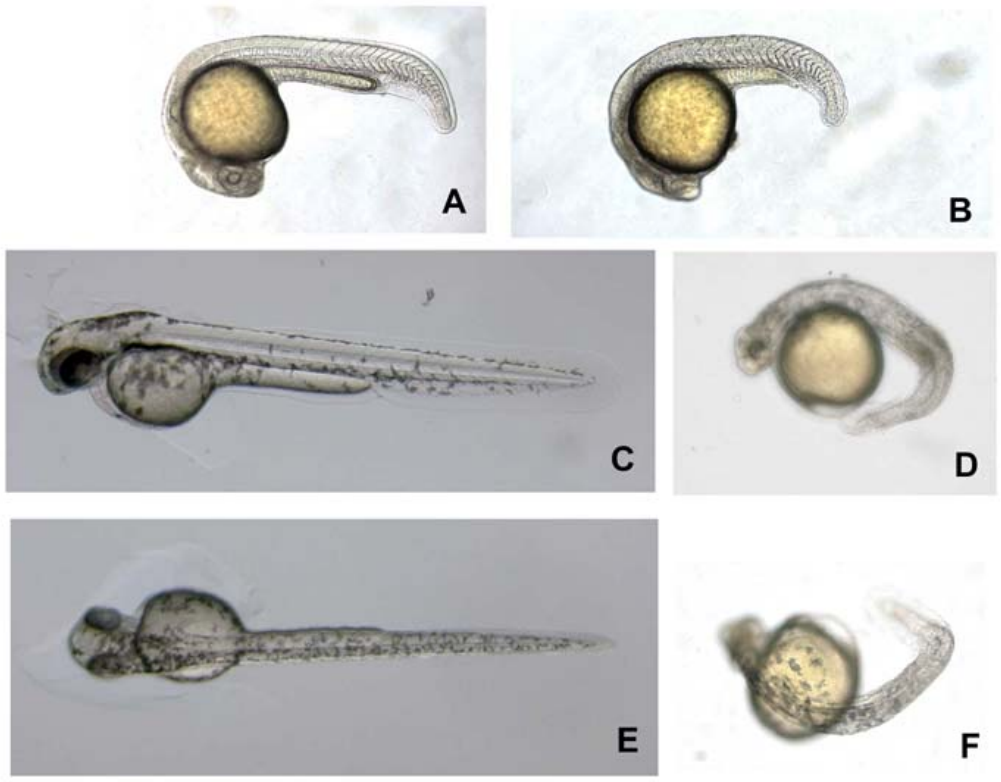

Figure 3.6.1: Lateral view of 24hpf embryos: control MO injected embryo (A) compared to embryo injected with 8ng of *rab20* MO (B). Lateral view of 48hpf embryos: control injected embryo (C) compared to embryo injected with 8ng of *rab20* MO (D). Dorsal aspect of 48hpf embryos: control MO injected embryo (E) compared to embryo injected with 8ng of *rab20* MO (F).

3.6.2 Rab1a2

At shield stage, *rab1a2* MO injected embryos were comparable with the control MO injected embryos, exhibiting no delayed gastrulation and no morphological differences between the organizers, at any of the doses injected. By 24hpf, the majority of the 2ng *rab1a2* injected embryos appeared normal (n = 42/42). The 4ng *rab1a2* MO injected embryos exhibited reduced head size and brain cell death (n = 40/40), when compared to controls (n = 48/48). Increasing the *rab1a2* MO dose to 8ng resulted in the embryos exhibiting a more severe reduction in head size and severe brain cell death (n = 33/33) (Figure 3.6.2 D), when compared with controls (n = 48/48) (Figure 3.6.2 C). In addition to this the 8ng *rab1a2* MO injected embryos

showed severe reduction in tail structures (Figure 3.6.2 B) when compared with controls (Figure 3.6.2 A)

Figure 3.6.2: Lateral view of 24hpf embryos: control MO injected embryo (A) compared to embryo injected with 8ng of *rab1a2* MO (B). 10x magnification of anterior dorsal view of 24hpf embryos: control MO injected embryo (C) compared to embryo injected with 8ng of *rab1a2* MO (D).

At 48hpf, the 2ng *rab1a2* MO injected embryos had smaller heads and smaller eyes (n = 42/42), when compared to controls (n = 48/48). In addition, the 2ng *rab1a2* MO injected embryos had curved tails. The 4ng *rab1a2* MO injected embryos displayed reduced head size, eye size and brain cell death. They also exhibited shortened axis and curved tails (n = 40/40). The 8ng *rab1a2* MO injected embryos had severely reduced head and eye size and brain cell death (Figure 3.6.3 D and C). In addition the 8ng *rab1a2* MO injected embryos showed an oedematous area around the yolk, severely reduced tail structures, with the remainder of the tail being kinked posteriorly (n = 33/33) (Figure 3.6.3 B and A). The notochord in the 8ng *rab1a2* MO

injected embryos was either, curved and misshapen (Figure 3.6.3 F and E) or was not visible (Figure 3.6.3 G and E).

At 4dpf, the 2ng and 4ng *rab1a2* MO injected embryos had smaller heads and eyes than controls and curved tails. The 8ng *rab1a2* MO injected embryos showed severely reduced head and eyes, when compared to controls. In addition they exhibited shortened, kinked tails and an oedematous area round the yolk. Only about 20% of the 8ng *rab1a2* MO injected embryos hatched (n = 7/33).

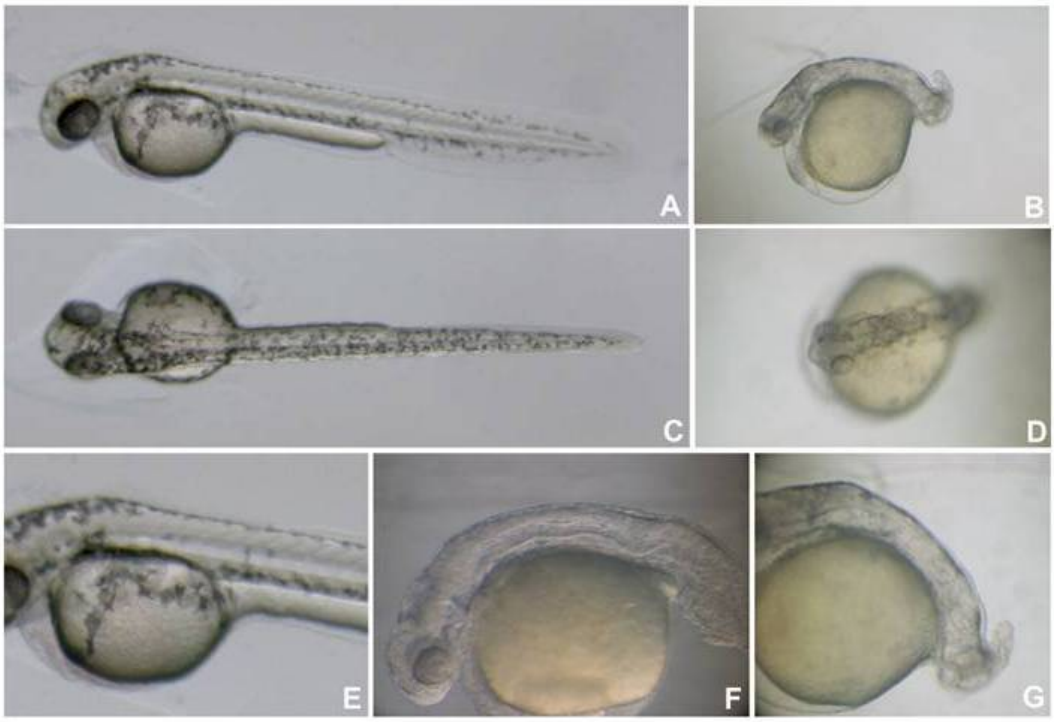

Figure 3.6.3: Lateral view of 48hpf embryos: control MO injected embryo (A) compared to embryo injected with 8ng of *rab1a2* MO (B). Dorsal view of 48npf embryo: control MO injected embryo (C) compared to embryo injected with 8ng of *rab1a2* MO (D). 10x magnified lateral view of rear of head and midtrunk region of 48hpf embryos: control MO injected embryo (E) compared to embryo injected with 8ng of *rab1a2* MO (F). 10x magnified posterior lateral view of 48hpf embryo injected with 8ng of *rab1a2* MO (G).

3.6 Summary of phenotypes.

Gene	Summary of phenotypes in *rab* morpholino injected embryos
Rab1a1	Developmental delay, brain cell death, tail defects, pericardial oedema
Rab1a2	Brain cell death, tail defects inc notochord, pericardial oedema
Rab1a3	Reduced head size, reduced pigment, kinks in notochord
Rab1a4	Developmental delay, enlarged organizer, reduced head size and tail defects, pericardial oedema, reduced hatching success
Rab3c1	Reduced head size, thicker bands of pigment, smaller eyes, protruding lens, swim bladder defect, blind
Rab6a	Reduced head size, tail defects, fail to hatch
Rab11a1	Developmental delay, shield - accumulation of cells on animal pole, tail defects, reduced head size, malformed heart, limited circulation
Rab11a2	Reduced head size, tail defects, pericardial oedema, fail to hatch
Rab11b1	Brain cell death, tail defects, pericardial oedema, fail to hatch
Rab11b2	Brain cell death, tail defects, pericardial oedema, patchy hatching gland, fail to hatch
Rab18(2)	Developmental delay, shield - elongated embryo, reduced head size, tail defects, pericardial oedema, reduced blood flow, fail to hatch
Rab20	Reduced head size, tail defects, pericardial oedema, limited circulation
Rab28	Brain cell death, curved axis, abnormal swimming behaviour, pericardial oedema

Table 3.6.1: Showing the range of phenotypes seen in embryos injected with morpholinos to different rabs.