Zebrafish Rab5 proteins and a role for Rab5ab in nodal signalling

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A B S T R A C T

The RAB5 gene family is the best characterised of all human RAB families and is essential for in vitro homotypic fusion of early endosomes. In recent years, the disruption or activation of Rab5 family proteins has been used as a tool to understand growth factor signal transduction in whole animal systems such as Drosophila melanogaster and zebrafish. In this study we have examined the functions for four rab5 genes in zebrafish. Disruption of rab5ab expression by antisense morpholino oligonucleotide (MO) knockdown abolishes nodal signalling in early zebrafish embryos, whereas overexpression of rab5ab mRNA leads to ectopic expression of markers that are normally downstream of nodal signalling. By contrast MO disruption of other zebrafish rab5 genes shows little or no effect on expression of markers of dorsal organiser development. We conclude that rab5ab is essential for nodal signalling and organizer specification in the developing zebrafish embryo.

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Introduction

Cellular motility and cohesion are essential processes in vertebrate early embryonic development. Integral to the processes are intracellular trafficking events, which direct the signalling between cells and the movement and adhesion of cells. Intracellular signalling is, in turn, heavily dependent on vesicle transport events, under the control of RAB proteins, which localise to specific intracellular compartments and pilot vesicles to target membranes (Zerial and McBride, 2001).

The RAB family of small GTPase enzymes is the largest subfamily in the Ras super-family. RABS are found in all eukaryotes, with 64 (including 4 pseudogenes) RAB genes present in the human reference genome (Zerial and McBride, 2001; Collicelli, 2004; Seal et al., 2011). The current thinking is that five core RABs are required for the basic functions of a cell, RAB1, RAB5, RAB6, RAB7 and RAB11 (Chavrier et al., 1990; Bucci et al., 1992; Pereira-Leal and Seabra, 2001). The RAB5 family is perhaps the best characterised of all 43 human RAB families and its members have been shown to localise to clathrin-coated vesicles, early endosomes and the plasma membrane (Bucci et al., 1992). The proteins are essential for in vitro homotypic fusion of early endosomes and are able to increase the rate of endocytosis in vivo when overexpressed (Gruenberg and Howell, 1989; Gorvel et al., 1991; Li and Stahl, 1993).

For cell and developmental biology much interest in Rab5 activity has resulted from their use as a tool to alter endocytosis. For example, activation and disruption of Rab5 proteins have been used to understand cell movements during gastrulation and how signalling factors move though a developing embryo (Scholpp and Brand, 2004; Ulrich et al., 2005; Hagemann et al., 2009; Tay et al., 2010; Torres and Stupack, 2011). Similarly, Rab5 proteins have also been used to understand human diseases such as Alzheimer’s disease (Ginsberg et al., 2010) and the motility and invasiveness of tumour cells (Torres and Stupack, 2011).

There are three mammalian RAB5s: RAB5A, RAB5B and RAB5C, which have been studied in mammalian cell culture assays (Wilson and Wilson, 1992; Singer-Kruger et al., 1994). Normally, such studies do not distinguish individual RAB5 gene activities (Bernard et al., 2010; Hagiwara et al., 2011), though other cell-based studies have shown that each member of the family can differentially regulate trafficking (Baskys et al., 2007; Chen et al., 2009). For example, Rab5 proteins are differentially recognised by different kinases (Chiariello et al., 1999). RAB5A is efficiently phosphorylated by extracellular-regulated kinase 1 but not by extracellular-regulated kinase 2, whilecdc2 kinase preferentially phosphorylates Ser-123 of RAB5B. It was suggested that phosphorylation could be important to differentially regulate the function of the RAB5 isoforms (Chiariello et al., 1999).

In whole-animal studies of development, Rab5 family proteins have been used as a tool to understand trafficking of growth
Materials and methods

Probe synthesis and in situ hybridisation

Whole-mount in situ hybridisation was carried out essentially as described by Thisse and Thisse (2008). The rab5aa gene probe was transcribed directly from cDNA clone IMAGp998K098962Q (Source BioScience) and linearized with SalI (NEB) and transcribed with SP6 RNA polymerase (NEB). Embryos were manually dechorionated and fixed in 4% paraformaldehyde at 24 h post fertilisation (hpf).

S’ capped RNA synthesis

Capped rab5ab RNA was synthesised in vitro using 5 μg (5 μl) of linearized rab5ab DNA (IRAkp961M19104 sub-cloned into a pcS2 + vector or a GFP-pcS2 + vector) in a 50 μl reaction containing 10 μl of 5 × transcription buffer, 5 μl of 0.1 M DTT, 5 μl of 5 mM CAP (NEB), 5 μl of 1 mM GTP (NEB), 5 μl of 5 mM UTP (NEB), 5 μl of 5 mM ATP (NEB), 5 μl of 5 mM CTP (NEB), 2 μl of RNase inhibitor (NEB) and 3 μl of SP6 RNA polymerase (NEB). Embryos were manually dechorionated and fixed in 4% paraformaldehyde at 24 h post fertilisation (hpf).

MO injections

The following MOs (Genetools) were used in this study:

- rab5aa MO 5’-GACGATTGTAATCCACGGCCTCTTC,
- rab5ab MO 5’-TCGTTGCTCAATCCCTCCTGCGAT,
- rab5aa MO 5’-GACCCCAATCCCACGTCTTAC,
- rabbit MO 5’-GACCTGATCCATCCCCGCTTAC,
- rab5ab splice MO: 5’-ATGAAAGCTTGTCTCTCTCTTCTAT,
- rab5ab MO 5’-GCGGCTGCTCCACGCGGTTACATG,
- rab5c MO 5’-GCGGTTGCTCAATCCCTCCTGCGAT,
- Standard Control MO 5’-CTCTTACCTGATTAAGATTA,
- pCS2 MO: GGCCTGTCTTGGCAAATTG

Oligonucleotides were diluted in MO buffer (5 mg/ml phenol red (Sigma), 4 mM HEPEs pH 7.2 (Sigma), 160 mM KCl (Sigma)) and 1.44 ml of MO solution was mixed into the yolk of the 1–4 cell stage embryo. We used 10 ng of rab5aa MO, 3 ng or 5 ng of rab5ab MO, 8 ng for rab5b MO, 6 ng for rab5c MO and those quantities with an additional 2 ng for standard control MO injections.

Electron microscopy

Whole zebrafish embryos were dechorionated manually and fixed overnight with 2% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) (SCB). The following day, embryos were washed for 10 min in SCB and post-fixed for 1 h in 1% osmium tetroxide in SCB. They were washed again with SCB and stained en bloc with 1% aqueous uranyl acetate for 1 h. The samples were then dehydrated through a graded ethanol series, followed by two changes of propylene oxide over 20 min and embedded in Epon resin (Agar Scientific). We then cut 50 nm ultra-thin sections, mounted them on pioloform coated slot grids and stained with 1% aqueous uranyl acetate for 15 min, followed by Reynold’s lead citrate for 7 min. Sections were visualised in a Jeol 1200 EX electron microscope.

Epiboly movement assay

Embryos were dechorionated at dome stage, 30% epiboly or shield stage then placed in glass dishes containing 5 mg/ml of biotinylated-dextran (Molecular probes 10,000 mw lysine fixable) in 1 × Danieu solution for 20 min (Shih and Fraser, 1996). Embryos were then washed and fixed in 4% paraformaldehyde solution. Fixed embryos were dehydrated in methanol then rehydrated in PBT (10 mM phosphate buffered saline, 0.05% Tween-20, pH 7.4) and incubated for 30 min in 1:5000 horseradish peroxidase-labelled streptavidin in PBT. Embryos were washed three times with PBT and soaked for 30 min in DAB/PBT.
(0.4 mg/ml 3,3’-Diaminobenzidine in PBT). The solution was then changed for staining solution, DAB/PBT with 0.003% H2O2, and examined as reaction product developed over 30 min. Once stained the reaction was stopped by rinsing with PBT.

**RT-PCR**

RNA was isolated from control and rab5ab MO injected embryos using Trizol as per manufacturer’s protocol (Invitrogen).
We used 1.5 μg of RNA to produce cDNA by reverse transcription (Superscript III, Life Technologies). Quantitative PCR was performed using Applied Biosystems TaqMan Universal PCR Master Mix and TaqMan primers for nrd1, gsc, ntl, chd, tfr1b, wnt8a, bmp2b and bmp4 designed and made by Applied Biosystems on an ABI prism using 7000 system software. Each of the cDNAs, nrd1, ntl, gsc, chd, tfr1b, wnt8a, bmp2b and bmp4 were analysed separately. Preliminary inspection supported normalisation of the cycle threshold (Ct) data transformation by 1/log2(Ct) to stabilise variances and allow intuitive visualisation using box-whisker plots. Such a transformation allows intuitive interpretation of changes in gene expression (a difference of one equates to a two fold change in expression), with further reciprocal transformation stabilising the mean-variance relationship (Eastwood et al., 2008; Bull et al., 2012). Expression of tfr1b was shown not to change between control and MO injected embryos (L.R. = 0.097, p = 0.76) but did change between stage (L.R. = 13.4, p < 0.001, 30% v shield stage) therefore all other genes were normalised to tfr1b within stage. The transformed data were analysed using mixed effect models (Pinheiro and Bates, 2000) with MO-injected versus control embryos as a categorical fixed effect and the experimental design captured as hierarchical random effects, with technical replication (RNA preparations) nested within biological replication (individual embryos). Hypotheses on the effects of MO injections were tested using likelihood ratios, with the test statistic assumed to be Chi-squared distributed (Pinheiro and Bates, 2000). R software was used for calculations and graph generation (R Core Development Team, 2008).

Phalloidin labelling

At 48 h post fertilisation (hpf) zebrafish embryos were fixed in 4% PFA overnight. Embryos were washed in PBS-triton and incubated in the dark with gentle agitation at room temperature overnight in 2.5 μg/ml Rhodamine phalloidin (Molecular Probes R415). Embryos were repeatedly washed in PBS-tween and imaged on an agarose plate using a Nikon SMZ800 fluorescent stereomicroscope and camera.

Results

Identification of zebrafish homologues of Human RAB5A, RAB5B and RAB5C

The three RAB5 genes in humans were used as query sequence to identify zebrafish Ensembl predictions (Ensembl Zebrafish 19.3.2) (Flicek et al., 2011), which was based on the zebrafish genome assembly (2v3) (Howe et al., 2013), mRNAs and ESTs using WU-BLAST followed by MSPrunch filtering (Sonhammer and Durbin, 1994). We used a threshold of 60% identity as a human to zebrafish match. Zebrafish EST sequences were retrieved along with capillary sequence traces (http://www.ncbi.nlm.nih.gov/Traces/), if available, which were quality clipped and vector masked with PreGap (Bonfield and Staden, 1996). These were then used to assemble cDNAs and ESTs in Gap4 (Bonfield et al., 1998) giving a total of four zebrafish rab5 genes with recent genome assembly’s not revealing any further orthologs. These four rabs are currently known as rab5aa (ENSDARG0000018602, ZDB-GENE-030131-139), rab5ab (ENSDARG0000007257, ZDB-GENE-040122-3), rab5b (ENSDARG0000016059, ZDB-GENE-040426-2593) and rab5c (ENSDARG00000026712, ZDB-GENE-031118-30). The rab5ab gene generates at least two coding variants while rab5aa, rab5b and rab5c have only one annotated coding variant each. Using Genomics version 61.01 (Muffato et al., 2010) and confirmed by Ensembl release 62 we find that the rab5a duplication is likely to have resulted from the teleost specific whole-genome duplication, as similarly duplicated rab5a genes are also present in the Tetraodon, Medaka and Stickleback genomes (Fig. 1).

Morphological loss of function screen of rab5a family

tfr1b

rab5aa

MO-injected embryos (n=63/63) were morphologically indistinguishable from control-injected embryos (n=54/54). Due to a lack of obvious phenotype, we studied the expression pattern of rab5aa in detail. Before 24 hpf, rab5aa mRNA expression was found to be low and uniform throughout all tissues (data not shown). At 24 hpf, however, rab5aa mRNA was expressed in a subset of cells in the brain especially in the ventral anterior part of the neural tube, forebrain and midbrain region (Fig. 2(A)). Additionally, we found rab5aa mRNA expression in a bilateral patch of telencephalic cells (Fig. 2(B)). The hindbrain showed expression in the central region of each rhombomere (Fig. 2(C)), in cells in the outer layer of the neural tube at the midbrain/hindbrain boundary (arrow in Fig. 2(D)) and at the posterior end of the hindbrain.
(arrows in Fig. 2(E)), which may correspond to expression in the anterior and posterior lateral line, respectively. Expression could also be seen in trunk region (Fig. 2(E)) showing positive cells scattered in the dorsal half of the embryo.

**rab5b**

At 24 hpf, rab5b MO-injected embryos showed thin and bar-shaped somites, as well as brain abnormalities. Specifically we found reduced forebrain and general brain necrosis (Fig. 2(G)), when compared to control-injected embryos (Fig. 2(F)). Double-injected rab5b MO / p53 MO embryos showed the same curved axis and bar-shaped somites (Fig. 2(K)) as rab5b MO single-injected embryos (Fig. 2(G)) but with slightly fewer brain defects and less necrosis at 24 hpf. By 48 hpf rab5b MO-injected embryos showed a reduced and curved axis with curved notochord (Fig. 2(I)), more pronounced bar-shaped somites (Fig. 2(O)) and disrupted muscle fibres (Fig. 2(Q)) when compared to control-injected embryos (Fig. 2(H), (N) and (P)). When control and rab5b MO-injected embryos were co-injected with the p53 MO, both control and p53 MO injected embryos (Fig. 2(F)) appeared similar to control embryos (Fig. 2(G)). By 48 hpf, rab5b MO/ p53 MO double-injected embryos injected embryos showed similar reduced and curved axis with curved notochord (Fig. 2(M)) and pronounced bar-shaped somites (Fig. 2(S)) as rab5b MO-injected embryos (Fig. 2(I), (O) and (Q)). Additionally, rab5b MO / p53 MO double-injected embryos showed reduced head size (Fig. 2(M)) and disrupted muscle fibres (Fig. 2(U)).

**rab5c**

Similar to rab5b MO-injected embryos, rab5c MO-injected embryos showed U-shaped somites, shortened axis, forebrain defects and brain necrosis, with the head region appearing poorly developed at 24 hpf (Fig. 2(W)) when compared to control-injected embryos (Fig. 2(V)). On the second day of development, rab5c MO-injected embryos continued to develop poorly, with reduced head size, shorter axis and curved tail (Fig. 2(Y)), when compared to control-injected embryos (Fig. 2(X)). Additionally, in rab5c MO-injected embryos muscle fibres failed to align as smoothly (Fig. 2(A)) and notochord cells failed to form proper vacuoles (Fig 2AE) when compared to controls (Fig. 2(AD) and (AF)). When the control and rab5c MO-injected embryos were co-injected with the p53 MO the control and p53 MO injected embryos (Fig. 2(Z)) looked similar to control embryos (Fig. 2(Y)). The rab5c MO and p53 MO injected embryos showed the same curved axis, U-shaped somites, forebrain defects and brain necrosis (Fig. 2(AA)) as rab5c MO-injected embryos (Fig. 2(W)) at 24 hpf. By 48 hpf rab5c MO and p53 MO injected embryos were more adversely affected with a more pronounced curved axis and poorly developed head region (Fig. 2(AC)) than rab5b MO-injected embryos (Fig. 2(Y)). Double-injected rab5c MO / p53 MO embryos showed similar U-shaped somites (Fig. 2(AI)) and more disorganised muscle fibres (Fig. 2(AK)) than embryos injected with rab5c MO alone (Fig. 2(AE) and (AG))

**rab5ab**

In contrast to rab5aa, knockdown of rab5ab produced a striking morphological phenotype during gastrulation. Specifically MO-injected embryos did not develop a dorsal organizer and died before the completion of epiboly (Fig. 3(B)). Development was dramatically slowed (Fig. 3(B)) in comparison with controls (Fig. 3(A)). Injection of 5 ng of rab5ab MO resulted in the embryos dying at between 30% and 50% epiboly. At 30% epiboly fluid had accumulated between blastoderm cells and the yolk cell (Fig. 3(B)). In these embryos, the cells of the blastoderm appeared substantially grainier in texture and less cohesive (Fig. 3(B)), when compared with the smooth blastoderm of the control-injected embryos (Fig. 3(A)) and stage matched control embryos (Fig. 3(C)). Further decreasing the dose of rab5ab MO to 3 ng (Fig. 3(E), (G), (I), (K)) resulted in rab5ab MO-injected embryos surviving to 80–90% epiboly (Fig. 3(K)). Embryos injected with rab5ab MO underwent a slowed epiboly (Fig. 3(E), (G), (I), (K)). Control embryos, however, underwent epiboly at a constant rate over approximately 5 h (Fig. 3(D), (F), (H), (J)). By the time control embryos reached 80% epiboly (Fig. 3(H)), MO-injected embryos had only progressed to 50% epiboly (Fig. 3(I)) and when MO-injected embryos eventually reached 80% epiboly (Fig. 3(K)), at 9 hpf, control embryos were at the 7-somite stage (Fig. 3(J)). Although many MO-injected embryos did not reach 80% epiboly, in cases where they did, the blastoderm margin contracted and pinched off the yolk, causing its contents to leak leading to the death of blastoderm cells. To confirm this phenotype we injected embryos with a second translation blocking MO to rab5ab (rab5ab MO2) and compared them with a 5 base-pair mismatch control MO (rab5ab MM MO2) and with uninjected control embryos. Once again the rab5ab MO injected embryos underwent a slowed epiboly, did not develop a dorsal organizer and died before the completion of epiboly (Fig S1H) when compared with both the rab5ab MM MO2 (Fig S1G) and the uninjected control (Fig S1F). The proportion of embryos that survive to 30% epiboly is significantly (p = 0.002) reduced in embryos injected with rab5ab MO2 compared to those injected with rab5ab MM MO2 (Fig S1L).

The lack of visible dorsal organiser led us to investigate the mRNA components of the nodal signalling pathway. By in situ hybridisation we found that rab5ab MO-injected embryos showed no gsc expression (Fig. 3(M)), fhh (Fig. 3(O)) or bhik (Fig. 3(Q)), expression compared to control MO-injected embryos (Fig. 3(L), (N), (P)) respectively. There was some marginal expression of nth (Fig. 3(S)) in rab5ab MO-injected embryos and reduced expression of ndr1 (Fig. 3(U)) and ndr2 (Fig. 3(W)) compared with controls (Fig. 3(R), (T), (V)) respectively. Embryos injected with the second rab5ab MO also showed disruption of gsc expression (Fig S1K) while the 5-bp mismatch MO injected embryos (Fig S1J) and the uninjected control embryos showed normal expression (Fig S1L). To quantify and validate the in situ results we performed qRT-PCR on control and rab5ab MO injected embryos at 30% epiboly and shield stage. Empirical distributions of expression are shown in Fig. 4. For gsc, chd, nth and ndr1, average expression was lower in MO-injected embryos, compared to control-injected embryos (nth: Likelihood Ratio = 12.4, p < 0.001; gsc: L.R. = 16.7, p < 0.001; chd: L.R. = 4.38, p = 0.037), although this was only the case at 30% epiboly stage for ndr1 expression (morpholino × stage interaction: L.R. = 15.6, p < 0.001). For these measurements transferrin receptor 1b (tfr1b) was used as a control and showed no significant difference in expression between MO-injected and control-injected embryos (L.R. = 0.097, p = 0.76).

**A role for rab5ab in nodal signalling**

To test whether the lack of nodal—responsive gene expression is specific to the rab5ab MO we compared expression of gsc in embryos co-injected with GFP-rab5ab RNA/rab5ab MO with those injected with a control MO or those injected with rab5ab MO alone. In embryos injected with GFP-rab5ab RNA/rab5ab MO (Fig S1C) and those injected with control MO (Fig S1A) we saw normal gsc expression. In those embryos injected with rab5ab MO alone (Fig S1B) we failed to see gsc expression. To ensure the morpholino was specific we repeated the experiment using a second morpholino (rab5ab MO2) that binds to the UTR of rab5ab. Here we saw that the proportion of embryos that survived to 30% epiboly was significantly increased (p = 0.004) in embryos co-injected with rab5ab RNA/rab5ab MO2 when compared with those injected with rab5ab MO2 alone (Fig S1L). gsc expression was also normal in embryos co-injection with rab5ab RNA/rab5ab MO2 (Fig S1Q) when compared with
Fig. 3. Loss of function of *rab5ab*. (A) Control embryo at 70% epiboly compared to (B) a 5 ng *rab5ab* MO-injected embryo at the same time point showing apparent accumulation of extracellular fluid between the yolk and the cells. (C) Control embryo at shield stage. (D) Animal view and (F) side view of a control-injected embryo at shield stage compared to (E) animal view and (G) side view of a 3 ng *rab5ab* MO-injected embryo at the same time point. (H) Control-injected embryos at 90% epiboly compared to (I) the same time point in the 3 ng *rab5ab* MO-injected embryos. (J) 8 somite stage control embryo compared to (K) 3 ng *rab5ab* MO-injected embryo at the same time point. Expression pattern of *gsc* in (L) control MO-injected embryos (n = 40/40) compared to (M) 3 ng *rab5ab* MO-injected embryos (n = 41/41). Expression pattern of *flh* in (N) control MO-injected embryos (n = 20/20) compared to (O) 3 ng *rab5ab* MO-injected embryos (n = 21/21). Expression pattern of *bhik* in (P) control MO-injected embryos (n = 29/29) compared to (Q) 3 ng *rab5ab* MO-injected embryos (n = 31/31). Expression pattern of *ntl* in (R) control MO-injected embryos (n = 40/40) compared to (S) 3 ng *rab5ab* MO-injected embryos (n = 39/39). Lateral view of expression pattern of *ndr1* in (T) control MO-injected embryos (n = 30/30) compared to (U) *rab5ab* MO-injected embryos (n = 30/30). Lateral view of expression pattern of *ndr2* in (V) control MO-injected embryos (n = 30/30) compared to (W) *rab5ab* MO-injected embryos (n = 29/29).
As injection of *rab5ab* RNA resulted in an unexpected pattern of expression of nodal downstream genes *ntl* and *gsc* while injection of *rab5ab* MO resulted in abolishment of these genes and qRT-PCR showed a reduction in *cht* and *ndr1* expression, we investigated the role of *rab5ab* in the expression of ventral markers *wnt8a, bmp4, vox* and *bmp2b*. Injection of *rab5ab* MO resulted in widespread expression of *wnt8a* around the whole margin (*Fig. 6(B*)) compared to control embryos where expression was excluded from the dorsal margin (*Fig. 6(A*)). Measurement of mRNA expression using qRT-PCR showed the level of *wnt8a* to be lower (L.R. = 32.0, *p* < 0.001) in *rab5ab* MO injected embryos compared to controls (*Fig. 4*). Injection of *rab5ab* RNA resulted in the expression of *wnt8a* being restricted to the ventral most half of the embryo (*Fig. 6(C*)).

*In situ* hybridisation showed that *bmp4* expression in embryos injected with *rab5ab* MO was primarily in the margin (*Fig. 6(E*) compared to control embryos where *bmp4* expression could be observed over the ventral half of the embryos (*Fig. 6(D*)). Similarly, qRT-PCR showed the level of *bmp4* to be lower (L.R. = 23.4, *p* < 0.001) in *rab5ab* MO injected embryos compared to controls.
Injection of rab5ab RNA resulted in the expression of bmp4 being further restricted to the ventral most part of the embryo when compared with controls (Fig. 6(F)). Expression of vox in embryos injected with rab5ab MO was observed over the whole animal pole of the embryo (Fig. 6(H)) compared to controls where the expression was excluded from the dorsal most part of the embryo (Fig. 6(G)). Expression of bmp2b in embryos injected with rab5ab MO was observed predominantly in the margin of the embryo (Fig. 6(K)) compared to controls where the expression was excluded from the dorsal most part of the embryo only (Fig. 6(J)). Measurement of bmp2b levels by qRT-PCR showed no significant difference
In embryos injected with rab5ab MO, bmp2b expression was excluded from the majority of the dorsal half of the embryo (Fig. 6(L)).

Control of epiboly movements by Rab5ab

In rab5ab MO-injected embryos the movement of all tissues layers was significantly delayed. Embryos injected with rab5ab MO
underwent a slowed epiboly from the outset and slowed further as epiboly progressed, whereas controls underwent epiboly at a constant rate over approximately 5 h (Fig. 7(D)). This delay was initially synchronous but in embryos that survived through later stages of epiboly, we found that the delayed movement of distinct layers was out of sync (Fig. 7(A), (B) and (C)). Since epiboly in zebrafish involves endocytic removal of the yolk cell membrane in the cells (Betchaku and Trinkaus, 1978; Solnica-Krezel and Driever, 1994; Lepage et al., 2014) we investigated endocytosis in the rab5ab MO-injected embryos. To measure the endocytosis activity directly we incubated rab5ab MO-injected embryos and control-MO injected embryos in a physiological solution containing biotinylated dextran, then fixed the embryos at three stages.

Control embryos, at dome stage and 30% epiboly, all showed a ring of staining for biotinylated dextran around the leading edge of the blastoderm (Fig. 7(E) and (G)). At shield stage, this staining formed a gradient from the dorsal to ventral side of the embryo (Fig. 7(I)). In contrast, rab5ab MO-injected embryos showed very little staining at dome stage, less staining at 30%, and no staining at shield stage (Fig. 7(F), (H) and (J)).

Despite the defects in endocytosis in the rab5ab MO-injected embryos, epiboly did proceed but at a slower pace and did not finish. This suggested that the microtubules in the yolk were unaffected and were responsible for epiboly proceeding as far as it did. As cold depolymerizes microtubules (Jesuthasan and Stahle, 1997), we held 5 ng rab5ab MO-injected and control embryos at 20 °C and monitored for 18 h (Mov S1). Control embryos developed normally (Fig. 7(K), (M), (O) and (Q)) albeit with some developmental delay, whereas rab5ab MO-injected embryos arrested and started to die at 13 hpf (10 h in to monitoring) at sphere to early epiboly stages (Fig. 7(L), (N), (P) and (R)).

Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.ydbio.2014.11.007.

Activity of rab5ab in endocytosis

The mammalian version of RAB5A has been shown to function as a regulatory factor in the early endocytosis pathway by stimulating membrane fusion during endocytosis (Gorvel et al., 1991; Bucci et al., 1992; Stenmark et al., 1994). We investigated the cell morphology of rab5ab MO-injected cells at the ultrastructural level (Fig. 8) and found that rab5ab MO-injected cells showed enlarged, smooth membrane profiles with highly irregular shapes (Fig. 8(B) and (D)), which were not observed in the cells of control-injected embryos (Fig. 8(A) and (C)). Additionally, the cells of rab5ab MO-injected embryos had an increased number of what appear to be large secondary lysosomes (Fig. 8(B) and (D) white arrows).

Discussion

The RAB5 family has been one of the most extensively studied of the Rabs (Li and Stahl, 1993; Singer-Kruger et al., 1994; Zerial and...
Their role in vesicle trafficking and endocytosis within the cell is well characterised (Gorvel et al., 1991; Bucci et al., 1992) and, for this reason, the Rab5 family has been used to understand signalling in the developing embryo (Scholpp and Brand, 2004; Hagemann et al., 2009). The transport of signalling factors in and out of a cell is integral to the patterning of the embryo, with the Rab5 family being used to understand the endocytosis that control this signalling. However, comparative studies between the genes within the Rab5 family have not been undertaken in whole animal systems previously and the possibility that the various Rab5 genes perform different developmental roles is hitherto unexplored. We sought to distinguish the possibility that Rab5 genes are functionally redundant with overlapping activities, from the possibility that Rab5 genes have divergent functions during development.

It was important first to account for all of the Rab5 gene family members in zebrafish and we found that there are four Rab5 family members: Rab5aa, Rab5ab, Rab5b and Rab5c, which compares with the three Rab5 genes in human. The duplicated Rab5a gene is particularly interesting, since the knockdown of each of these two genes resulted in very different phenotypes in our study, whereas Rab5b and Rab5c showed similar phenotypes. Specifically, Rab5aa MO-injected embryos were phenotypically indistinguishable from controls, while Rab5ab MO-injected embryos showed an early lethal phenotype.

The lack of an abnormal phenotype associated with Rab5aa knockdown suggests that it is redundant for early development, although it may be required for post-embryonic development. Indeed, expression data suggests a subtle role for Rab5aa in later brain development (Fig. 2) with other animal models showing a role for Rab5a in the brain (Allende and Weinberg, 1994; de Hoop et al., 1994; Sadler et al., 2007; Rosenegger et al., 2010). In rats, Rab5a has been detected in axons and dendrites, with Rab5a co-localised with synaptophysin-containing vesicles, suggesting a role for Rab5a in axonal and dendritic endocytosis (De Hoop et al., 1994). Our results show expression of rab5aa in zebrafish is restricted to discrete parts of the brain and spinal cord.

We found that knockdown of Rab5b and Rab5c lead to similar abnormal phenotypes. Specifically, MO-injected embryos showed no obviously abnormal phenotype through gastrulation but by 24 hpf had thin and bar-shaped somites, forebrain defects and cell death, suggesting a later role for these Rab5 genes (Fig. 2). In situ hybridisation shows rab5b expression from 1–13 somites in the YSL and pronephric mesoderm, then after 20 somites in the YSL, pronephric ducts and dorsal telencephalon (Thisse and Thisse, 2004). Taken together, these data imply a role for Rab5b in nervous system development. In rat hippocampal cultures, Rab5b has been shown to be upregulated by the neuroprotective agent DHPG (Blaabjerg et al., 2003). Neuroprotection by DHPG against NMDA-mediated injury may involve facilitation of NMDA receptor endocytosis stimulated by a DHPG-induced increase in Rab5b synthesis and may therefore play a role in synaptic plasticity (Arnett et al., 2004; Baskys et al., 2007).

Knockdown of Rab5c, although phenotypically similar to knockdown of Rab5b, suggests that Rab5c and Rab5b may have different roles in development. In situ hybridisation data for Rab5c showed expression from the 20 somites to the Prim-15 stage in the intermediate cell mass of mesoderm, the site of primitive haemopoiesis (Detrich et al., 1995; Thisse and Thisse, 2004). At later stages of development, Rab5c expression is seen in axial vasculature and blood (Thisse and Thisse, 2004). A previous study of

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**Fig. 8. Activity of rab5ab in endocytosis.** Transverse sections of cells of the leading edge of the enveloping layer from a 3 ng control-injected embryo 80% epiboly (A) and (C), a 3 ng rab5ab MO injected embryo (fixed at 40% epiboly but when control embryos were at 80% (B) and (D). White arrows show large secondary lysosomes with membraneous contents. (A) and (B) are at 10,000 × ; and (C) and (D) are at 18,750 × .
knock down of rab5c reported cell death over the whole embryo resulting in reduced yolk extension and expanded hindbrain at 28 hpf and at 56 hpf there was significant cell death, resulting in small head and eyes and bending of the body axis (Kalen et al., 2009). This result corresponds well with what we have observed, which included shortened axis and brain cell death. Taken together these observations suggest a distinct function for rab5c in mesoderm development at the late gastrula stage as it has been observed that Wnt11 functions in gastrulation by controlling cell cohesion through Rab5c and E-cadherin (Ullrich et al., 2005).

Depletion of rab5ab led to loss of the dorsal organiser and embryonic lethality by 90% epiboly stage. We therefore examined the expression of the nodal genes ndr1 and ndr2 and their downstream genes gsc, flh and ntl and found all but ntl to be abolished in rab5ab MO-injected embryos. Further this expression for gsc could be reversed by injection of synthetic rab5ab or taram-a mRNA. Indeed injection of large amounts of rab5ab RNA resulted in ectopic expression of downstream genes gsc and ntl but not the dorsal marker gene chd. It also resulted in embryos with larger organizers and shorter body axes. Interestingly ntl showed no ectopic expression at shied stage but instead showed expansion of the margin into the animal pole. This and the presence of ntl in rab5ab MO injected embryos could be explained by the fact that the expression of ntl is not entirely nodal-related, but is also regulated by Wnt and BMP signalling (Harvey et al., 2010). We therefore investigated whether bmp and wnt signalling might be affected in embryos overexpressing rab5ab or in those injected with a rab5ab morpholino. Although wnt8a was present around the whole margin of the embryo total wnt8a expression was lower in embryos injected with rab5ab MO. In embryos injected with rab5ab RNA expression of wnt8a was restricted to the ventral half of the embryo. Expression of bmp family members was more complex and while bmp4 expression was decreased in rab5ab MO injected embryos, there was no significant change in bmp2b expression in these embryos. Expression of bmp4, bmp2b and vox showed abnormal distribution both in embryos overexpressing rab5ab and those injected with rab5ab MO. It is possible that bmp2b may be driving ntl expression in those embryos lacking rab5ab. All together this shows an important role for rab5ab in nodal signalling and dorsal-ventral patterning.

In addition to its role in Nodal signalling we find rab5ab plays a role in cell movement within the developing embryo. In rab5ab MO-injected embryos, epiboly is slowed. This is understandable, as RAB5 family members are known for their role in endocytosis (Bucci et al., 1995). Epiboly is thought to be the result of two processes, endocytosis at the margin, which moves cells over the yolk, and the contraction of the actin cytoskeleton within the yolk cell proper (Solnica-Krezel and Driever, 1994). Therefore cell movements within the developing embryo are disrupted when endocytosis associated with epiboly is diminished (Fig. 6) (Lepage et al., 2014) but when we further disrupt the microtubule cytoskeleton in the yolk, epiboly is not rescued. Moreover, it appears that certain events such as closing of the actin ring at the end of epiboly are independent of the epiboly process as this occurs whether epiboly completes or not. It is also possible that the start of gastrulation may be independent of the stage of epiboly, as marginal expression of bmp4 and bmp2b is seen in embryos injected with rab5ab MO at 30% epiboly where as in control embryos this is not be observed until the embryos enter gastrulation at 50% epiboly.

Non-embryonic nodal transcripts in the YSL can mediate inter- action between the embryonic and non-embryonic tissues that maintain nodal related gene expression in the margin (Fan et al., 2007). Additionally ndr1 function is required in the YSL to induce the morphological shield, and the YSL is a source of Nodal signals that is independent of the population in the overlying blastomeres. Both Nodal ligands Ndr1 and Ndr2 are expressed by the YSL and induce nrd1 mRNA expression in the overlying blastomeres. It has been suggested that the three non-embryonic sources of Nodal ligands, maternal ndr1 and non-embryonic ndr1 and ndr2, account for the complete spectrum of early nodal signalling and, therefore, organizer specification and induction of mesoderm and endoderm (Hong et al., 2011). A recent paper (Kumari et al., 2013) has shown that maternal control of Nodal signalling is via the conserved Y box-binding protein 1 (ybx1) and that maternal-effect mutations in zebrash ybx1 lead to deregulated Nodal signalling, gastrulation failure, and embryonic lethality. The paper suggests that Ybx1 prevents ectopic Nodal activity.

Our data and the published literature lead us to propose that Nodal signals emanating from the YSL are taken up by blastomeres via endocytic vesicles under the control of Rab5ab. This model could explain the abnormal accumulation of fluid we observe between the blastoderm and the yolk in rab5ab MO-injected embryos (Fig. 3(B)). In addition, rab5ab over–expressing embryos showed ectopic expression, as well as normal expression of the markers of Nodal signalling gsc and ntl. This would be consistent with the model if early expression of these genes was controlled by maternal and/or non-embryonic sources of Nodal ligands but later expression was due to embryonic sources. An alternative scenario is that that maternal rab5ab is somehow involved in the ybx1 maternal control of Nodal signalling leading to deregulation of nodal signalling, its downstream genes and deregulation of DV patterning.

It was recently shown that zebrash dynamin, a GTPase required for receptor-mediated endocytosis, plays a fundamental role within the blastoderm during epiboly. Dynamin is required for completion of epiboly and maintains epithelial integrity and the transmission of tension across the EVL (Lepage et al., 2014). Embryos lacking dynamin show a similar phenotype to those we have shown lacking rab5ab. In Drosophila, remodelling of the apical surface during epithelial morphogenesis has been shown to be regulated by dynamin and the Rab5-effecto Rabankyrin-5 (Fabrowski et al., 2013) while research in the sea urchin embryo suggests that dynamin-mediated endocytosis acts as a sink to limit the range of Nodal signalling (Ertl et al., 2011). In sea urchins, inhibition of dynamin, resulted in embryos that became radialised and phenocopied embryos that overexpress nodal. Although this does not correspond with what we see with knock down of rab5ab it does suggest a possible relationship between rab5ab, dynamin and nodal which is worthy of further study.

In conclusion, the key finding of this study is the crucial role for Rab5ab in early nodal signalling and organizer specification in the developing zebrash embryo. It should also be noted that various members of the Rab5 family are associated with different roles in early embryonic development. Corroborative evidence from whole organism phenotypic analysis in zebrash is more consistent with functional divergence, than redundancy, between rab5 genes.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.11.007.
References

Allende, M.L., Weinberg, E.S., 1994. The expression pattern of two zebrafish achaete-scute homolog (ash) genes is altered in the embryonic brain of the cyclops mutant. Dev. Biol. 166, 509–530.

Aoki, T.O., Mattheij, J., Saint-Etienne, L., Rebagliatti, M.R., Peyriera, N., Rosa, F.M., 2002. Regulation of nodal signalling and mesendoderm formation by TARM-L1, a TGFbeta-related type I receptor. Dev. Biol. 241, 273–288.

Aquín-Beck, A., Ilagan, K., Liu, Q., Liang, J.O. 2007. Nodal signalling is required for closure of the anterior neural tube in zebrafish. BMC Dev. Biol. 7, 122.

Arnett, A.L., Bayraktar, L., Blaabjerg, M., Fang, L., Zimmer, J., Baksy, A. 2004. Antisense oligonucleotide against GTPase Rab5b inhibits metabolic agonist DHG-induced neuroprotection. Brain Res. 1028, 59–65.

Baksy, A., Bayraktar, I., Zhu, E., Fang, L., Wang, R. 2007. Rab-mediated endocytosis: linking neurodegeneration, neuroprotection, and synaptic Plasticity? Annu. N. Y. Acad. Sci. 1122, 313–329.

Bernard, E., Solignat, M., Gay, B., Chazal, N., Higgs, S., Devaux, C., Briant, L. 2010. A microarray analysis of transcript profiles of marginal cells of the enveloping layer and of the yolk syncytial and yolk cytoplasmic layers of furthlds before and during and epidermis. J. Exp. Zool. 206, 381–426.

Blaabjerg, M., Baskys, A., Zimmer, J., Vawter, M.P., 2003. Changes in hippocampal signalling in Xenopus. Development 136, 2803–2813.

Chen, P.I., Kong, C., Su, X., Stahl, P.D., 2009. Rab5 isoforms differentially regulate the endocytic pathway. Cell 70, 715–726.

Detrich 3rd, H.W., Kieran, M.W., Chan, F.Y., Barone, L.M., Yee, K., Rundstadler, J.A., de Hoop, M.J., Huber, L.A., Stenmark, H., Williamson, E., Zerial, M., Parton, R.G., 2004. Microarray analysis of hippocampal CA1 neurons implicates early endosomal dysfunction during Alzheimer’s disease progression. Biol. Psychiatry 68, 885–896.

Dotti, C.G., 1994. The involvement of the small GTP-binding protein Rab5a in cell signalling. Curr. Protein Pept. Sci. 12, 43–50.

Hagiwara, M., Shinomiya, H., Kashihara, M., Kobayashi, K., Tadokoro, T., Yamamoto, Y., Nishida, E., 2011. Interaction of activated Rab5 with actin-bundling proteins, L- and T-plastin in endocytosis: insights from in vitro analysis. J. Biol. Chem. 286, 21278–21289.

Humphray, S., McLaren, K., Matthews, L., et al., 2013. The zebrafish genome sequence and its relationship to the human genome. Nature 496, 529–537.

Howe, K., Clark, M.D., Torroja, C.F., Torrance, J., Berthelot, C., Muffato, M., Collins, J.E., Humphrey, S., McLaren, K., Matthews, L., et al., 2013. The zebrafish reference genome sequence and its relationship to the human genome. Nature 496, 521–530.

Henger, S., Nishida, E., 2003. Dynamic microtubules and specification of the zebrafish embryonic axis. Curr. Biol. 7, 31–42.

Kalen, M., Wallgard, E., Asker, N., Nasevicius, A., Athley, E., Billgren, Larson, J.D., Wadman, S.A., Norsborg, E., Clark, K.J., et al., 2009. Combination of reverse and chemical genetic screens reveals angio genesis inhibitors and targets. Chem. Biol. 16, 432–441.

Kumari, P., Gilligan, P.C., Lim, S., Tran, L.D., Winkler, S., Philip, R., Sampath, K. 2013. An essential role for maternal control of Nodal signalling. elife 2, e00683.

Lepaje, S.E., Tada, M., Bruce, A.E., 2014. Zebra fish D8001 is required for maintenance of enveloping layer integrity and the progression of endoderm. Dev. Biol. 385, 52–66.

Li, G., Stahl, P.D., 1993. Structure–function relationship of the small GTPase Rab5. J. Biol. Chem. 268, 24475–24480.

Muffato, M., Louis, A., Poinsel, C.E., Roest Crollius, H., 2010. Genomicus: a database and a browser to study gene syngen in modern and ancestral genomes. Bioinformatics 26, 1119–1121.

Nasevicius, A., Elker, S.C. 2001. A targeted gene knockout in zebra fish. Nat. Genet. 26, 216–220.

Pereira-Leal, J.B., Seabra, M.C., 2001. Evolution of the Rab family of small GTP- binding proteins. J. Mol. Biol. 313, 898–901.

Pinheiro, J.C., Bates, D.M. 2000. Mixed-Effects Models in S and S-PLUS. Springer-Verlag, New York, NY.

R Core Development Team, 2008. R: A language and environment for statistical computing [ed. R. R. F. S. Computing]. Vienna, Austria.

Rosenegger, D., Wright, C., Gross, M., 2010. A quantitative proteomic analysis of long-term memory. Mol. Brain 3, 564–570.

Sadler, K.C., Krahn, K.N., Gaur, N.A., Ukonomic, C., 2007. Liver growth in the embryo and during liver regeneration in zebrafish requires the cell cycle regulator, uhrf1. Proc. Natl. Acad. Sci. USA 104, 1570–1575.

Scholpp, S., Brand, M. 2004. Endocytosis controls spreading and effective signalling range of Fgf8 protein. Curr. Biol. 14, 1843–1844.

Seal, R.L., Gordon, S.M., Lush, M.J., Wright, M.W., Bradford, E.A., 2011. genenames.org: the HGNC resources in 2011. Nucl. Acids Res. 39, D544–D549.

Shih, J., Fraser, S.E., 1996. Characterizing the zebrafish organizer: microsurgical analysis at the early-stage development. Development 122, 1313–1322.

Severs, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Soding, J., et al., 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol. Syst. Biol. 7, 539.

Singer-Kruger, B., Stenmark, H., Dusterholt, A., Philippson, P., Yoo, J.S., Gallwitz, D., Zerial, M. 1994. Role of three rab5-like GTPases, Ypt51p, Ypt52p, and Ypt53p, in the endocytic and vacuolar protein sorting pathways of yeast. J. Cell Biol. 123, 283–298.

Solnica-Krezel, L., Driever, W., 1994. Microtubule arrays of the zebrafish yolk cell: organization and function during development. Development 120, 2443–2455.

Somahlemmer, E.L., Durbin, R. 1994. A workbench for large-scale genome homology analysis. Comput. Appl. Biosci. 10, 301–307.

Stenmark, H., Rutten, M.G., Lindberg, E., Fich, P., Paule, B., 2006. Determination of α-tubulin gene expression during vertebrate development. Proc. Natl. Acad. Sci. USA 92, 10713–10717.

Steen, J., Christian, J., Boekhorst, J., 2004. The ChIPaligner: fast mapping of ChIP-Seq data to a reference genome. Bioinformatics 20, 901–907.

Thisse, C., Thisse, B., 2008. High-resolution in situ hybridization to whole-mount zebrafish embryos. In: ZFIN Direct Data Submission. Thisse, C., Thisse, B., 2004. Fast release clones: a high throughput expression analysis. In: ZFIN Direct Data Submission.