Progressive loss of RacGAP1/ogre activity has sequential effects on cytokinesis and zebrafish development

Rachel M. Warga*, April Wicklund, Sarah E. Webster, Donald A. Kane

Department of Biological Sciences, Western Michigan University, Kalamazoo, MI 49068, USA

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A B S T R A C T

RacGAP1 is one of the two components of the centralspindlin complex essential for orchestrating cytokinesis in all animal cells. We report here that the early arrest mutant ogre is a maternal and zygotic loss of function mutation in the zebrafish homolog of racgap1. Like the other model organisms in which racgap1 is mutated, cells in the mutant stop dividing. In vivo cell recordings reveal that gradual loss of wild-type RacGAP1 leads progressively from a failure of abscission, then to cleavage furrow ingression, and finally complete absence of furrow formation. Despite the lack of cytokinesis, gross patterning occurs overtly normally in ogre mutants and cells continue to cycle slowly, some even attaining four or eight nuclei. Many multinucleate cells differentiate and survive, but the majority of cells enter apoptosis that we demonstrate is due to cumulative rounds of defective cytokinesis. Investigation of the cells that differentiate in the mutant indicate that RacGAP1 is also needed for long-term survival of motoneurons and the cytoskeletal organization of sensory axons. We conclude that while RacGAP1 function is crucial for cytokinesis and its activity at different levels controls different aspects of cytokinesis, these defects have occluded other critical roles of this interesting protein.

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1. Introduction

Cytokinesis is the final step of mitosis, cleaving the cytoplasm of the dividing cell into two separate daughter cells. In animal cells, cytokinesis begins with assembly of the central spindle, a structure composed of bundled antiparallel microtubules, and a complex of proteins known as centralspindlin. Once assembled at the midzone, the central spindle sets up a signaling center at the equator of the cell for positioning, assembly, and contraction of an actomyosin ring that drives cleavage furrow ingression. By the end of furrow ingression the central spindle has compacted into a structure known as the midbody: here it plays both structural and regulatory roles in coordinating abscission and ending cytoplasmic continuity between daughter cells. In some animals and during very early development this may not occur until well into the next cell cycle (reviewed in: Agromayor and Martin-Serrano (2013), Douglas and Mishima (2010), Glotzer (2013), Green et al. (2012), Schiel and Prekeris (2013), White and Glotzer (2012)). Mechanistically it seems centralspindlin controls the myriad functions of the central spindle including its own assembly (Pavicic-Kaltenbrunner et al., 2007). Comprised of two proteins, the mitotic kinesin-like protein MKLP1 and the Rho family GTPase-activating protein (GAP) RacGAP1, centralspindlin is only active as a complex, not as its individual components (Mishima et al., 2002; Pavicic-Kaltenbrunner et al., 2007). Moreover, the complexes must also self-associate into higher order clusters before concentrating on the spindle midzone microtubules (Basant et al., 2015; Hutterer et al., 2009). In this paper we focus on the RacGAP1 subunit of centralspindlin (known also as mgcRacGAP in mammals, Cyl4 in nematodes, and RacGAP50C in fruit flies).

Much of our knowledge concerning the role of RacGAP1 during cytokinesis has been gleaned from studies in early cleavage stage Caenorhabditis elegans embryos or cultured Drosophila and human cells. Because loss of function mutations in nematodes and fruit flies are embryonic lethals (Jantsch-Plunger et al., 2000; Jones and Bejsovec, 2005; Unhavaithaya et al., 2013; Zavortink et al., 2005), less is known about other roles RacGAP1 may have. Nevertheless, there is evidence that during polarization of the arcade cell epithelium in C. elegans, myotube extension and neurite outgrowth in Drosophila as well as neural migration in mammals, RacGAP1 (together with MKLP1) may have organizing properties of the cytoskeletal machinery (del Castillo et al., 2015; Falnikar et al., 2013; Guerin and Kramer, 2009; Lores et al., 2014; Portereiko et al., 2004). Furthermore, there is mounting evidence that GAPs in general are critical players in neuronal development and survival making them underlying factors in many neurodegenerative disorders (reviewed in: Bai et al. (2015), Stankiewicz and Linseman (2014)).
Composed of multiple domains, RacGAP1 is a Swiss Army knife of proteins. First, RacGAP1 contains a conventional RhoGAP. This domain inactivates the small GTPase Rac1 at the cell equator, necessary for successful furrow ingress (Bastos et al., 2012; Canman et al., 2008; D’Avino et al., 2004; Touré et al., 1998). Second, RacGAP1 provides specific binding sites for a number of key effectors of cytokinesis and abscission. Notably, ECT2 (Burkard et al., 2009; Kim et al., 2014; Somers and Saint, 2003; Wolfe et al., 2009; Zavortink et al., 2005), the guanine nucleotide exchange factor (GEF) needed to activate the small GTPase RhoA (Prokopenko et al., 1999; Su et al., 2011; Yüce et al., 2005), which controls formation and contraction of the actomyosin ring (Matsumura, 2005; Otomo et al., 2005; Piekný and Glotzer, 2008). Finally, RacGAP1 possesses an evolutionary conserved atypical C1 domain promoting protein-lipid interactions (Colon-Gonzalez and Kazanietz, 2006), a domain essential for linking the central spindle to the plasma membrane and successful abscission (Lekomtsev et al., 2012).

Here we describe ogre, a loss of function racgap1 mutation in the zebrafish. Like the other model organisms in which racgap1 is mutated, we find that cells in the ogre mutant become binucleate. However, this does not occur until gastrulation, long after onset of zygotic transcription (Kane et al., 1996b), consistent with the idea that maternal stores of cell cycle gene products regulate cytokinesis during the first 15 or so cell cycles of zebrafish (Kane and Kimmel, 1993). Sequencing of the mutant allele revealed a nonsense mutation in the C1 domain, just upstream of the GAP domain. By monitoring cytokinesis in vivo we show that abscission rather than cleavage furrow ingress is most sensitive to levels of RacGAP1 activity. However, once maternal racgap1 transcripts become depleted, cleavage furrow ingress is compromised. Notably, we also find a weak dominant maternal effect. Cells lacking zygotic RacGAP1 continue to re-enter the cell cycle after becoming binucleate albeit more slowly than wild type, however if cells are prevented from becoming quinucleate apoptosis is rare. We also show that neural development proceeds in the absence of RacGAP1, even if cells are multinucleate, but primary motorneurons in particular fail to survive and sensory neurons exhibit cytoskeletal defects. Our studies indicate that while RacGAP1 function is more crucial for the final stages of cytokinesis, it is also critical for some aspects of post mitotic neural development and survival.

2. Material and methods

2.1. Zebrafish strains

All experiments except antisense morpholino injections were performed in embryos derived from crosses of identified ogre<sup>as2a</sup> heterozygotes. For following the cell cycle, the dual FUCCI transgenic line (Bouldin and Kimelman, 2014) was also bred into the ogre mutant background. Morpholino injections were performed in embryos derived from wild type, the hrp<sup>as25</sup> allele of Em1 (Riley et al., 2010) or the zom<sup>606</sup> allele of Cdc20 (D.A.K. unpublished). The ogre, hrp and zom mutant phenotypes are only visible when homozygous and segregate 1:3 as a normal Mendelian recessive trait. Because there is no discernable heterozygous phenotype, throughout the paper we refer to wild-type siblings (the remaining three quarters of the progeny) we are always referring to their phenotype and not their genotype (which could be either homozygous for the wild-type allele or heterozygous for the mutant allele).

2.2. Mapping and genotypic characterization

The ogre<sup>as2a</sup> allele, recovered in a large-scale ENU mutagenesis screen (Haffter et al., 1996), was initially outcrossed to the polymorphic WIK (L11) strain of wild-type fish for mapping by half tetrad analysis (Johnson et al., 1995) using single-stranded polymorphism of closely linked microsatellite markers (Knüpik et al., 1998) as in McFarland et al. (2005). This mapped ogre to one arm of Chromosome 23. Finer resolution on the Sanger map was further obtained using a haploid panel, which identified five potential candidate genes in a 6.2 Mb interval. To identify which gene was ogre we used RT-PCR, this revealed a T to A transversion in racgap1 at nucleotide 1173 changing the reading frame from a cysteine to a stop. The mutation was confirmed by further sequence analysis using genomic DNA isolated from individual mutant and wild-type embryos.

2.3. Antisense morpholino and mRNA injections

Morpholinos (Gene Tools, LCC) and mRNA were injected into 1- to 4-cell stage embryos. 4 nanograms of the exon8-intron8 splice blocking morpholino (5'-AGGAAAATATCTTACCAGTCTGCT-3') was injected per embryo to recapitulate the ogre mutant phenotype and 5 nanograms of the racgap1 mRNA was injected per embryo to rescue the ogre mutant phenotype. The racgap1 coding sequence was purchased from (Open Biosystems) and transcribed with the SP6 mMessage mMACHINE (Ambion).

2.4. Lineage tracing

Cells were labeled at the 1000–2000-cell stage using protocols previously described in Warga et al. (2013) and Warga and Nüsslein-Volhard (1999) with the following modification: embryos were injected with a 5% solution of neutral rhodamine-dextran (10,000 MW; Thermofisher Scientific, formerly Molecular Probes).

2.5. Time-lapse analysis

Embryos were mounted and recorded in multi-plane as previously described in Warga and Kane (2003) except for experiments with the Dual FUCCI transgene or lineage tracer where we used a Nikon C2 confocal microscope. For embryos labeled with lineage tracer cells were recorded every 3 min from sphere until early gastrulation. For Dual FUCCI embryos, cells were recorded every 5 min from midgastrulation until early somite stages. After each time-lapse, individual embryos were unmounted and allowed to develop to determine its phenotype (mutant or wild type). Recordings were analyzed using Cytos Software as in Warga and Kane (2007) or NIS elements viewer (Nikon). Images were imported into Adobe Photoshop and pseudo-colored to aid in presentation.

2.6. Cell dissociation and percent of multinucleate cells

Individual embryos were digested from 5 to 8 min (depending on stage) as in Riley et al. (2010) and resuspended in 1-part 4% paraformaldehyde containing DAPI (0.1 μg/ml) and 4-parts Hohlfreter’s buffer. Dissociated cells were immediately mounted on a hemocytometer and inspected at 400x magnification to determine the number of nuclei for each cell. Typically, 100–300 cells were counted per embryo. Statistics were done using contingency tables of the total or partial results from individual embryos using Pearson’s chi-squared test (without Yate’s correction) and calculating probabilities from the binomial distribution. Often these results were crosschecked using a simple Wilcoxon rank sum.

2.7. In situ hybridization, antibody, acridine orange, and DAPI staining

Whole-mount RNA in situ hybridization was carried out using digoxigenin-labeled riboprobes following the protocol in Thisse and Thisse (2008).
Whole-mount antibody staining was carried out using an anti-active Caspase 3 antibody (BD Biosciences, 1:200), an anti-Acetylated Tubulin antibody (Sigma, 1:2000) or an anti-Phospho-Histone H3 antibody (Santa Cruz Biotechnology, Inc., 1:1000) and detected with an AP-conjugated or peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc. 1:1000). For visualization of microtubules at 1000 × magnification with anti-Acetylated Tubulin, we used an Alexa Fluor 488-conjugated secondary antibody (Invitrogen 1:500).

Acridine orange staining was carried out by placing live dechorionated embryos in 10⁻⁵ M acridine orange with 1% DMSO in the dark for 30 min, washing briefly in fish H₂O several times and examining immediately with fluorescence.

DAPI staining was obtained by placing an embryo between coverslips in 4% paraformaldehyde containing DAPI (0.1 μg/ml) and crushing gently. This rendered the embryo a loose association of 2–3 cell layers, greatly increasing optical clarity of DAPI-stained nuclei. Photographs were taken immediately at 400 × on a Zeiss Axioskop 2 of typical cells.

3. Results

3.1. Cells in the ogre mutant undergo apoptosis

Identified in the Tübingen zebrafish screen for early morphogenetic mutants, the mutant ogre was characterized as an early arrest phenotype with massive cell death and is represented by a single recessive allele ta52a (Kane et al., 1996b). The earliest visible phenotype is a shorter body once somites begin to form (11 h), and by 6-somites (12 h) the eye placode is smaller than normal and

![Image of embryos with different staining and labeling](https://example.com/image)

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**Fig. 1.** The ogre mutant phenotype. (A and B) Live embryos; (C) after acridine orange staining to show cell death; and (D) after DAPI-squash to show nuclei. (E-H) anti-Active Caspase 3 staining showing progression of apoptosis; and with dlA expression (I) marking neural tissue in the brain and spinal cord, with myoD expression (K) marking somite muscle in the trunk, and with gata1a expression (M) marking blood in the posterior blood island. (J, L, N) Are high magnification cross-sections through the areas indicated with lines and tissues are circled while arrows indicate apoptotic cells; abbreviations: nt, neural tissue; nc, notochord. (O-Q) Expression of: (O) tal1 mRNA marking blood cells (arrows) and (P) Acetylated Tubulin marking trigeminal (trg) and Rohon-Beard sensory (rb) neurons (arrows). (Q) High magnification of mutant axons, showing abnormal varicosities (arrows).
there is a general loss of optical transparency (Fig. 1A). By 9-somites (13.5 h) ogre mutants are easy to identify because of cell death in the head (Fig. 1B and C), and by 18-somites (18 h) cell death is also evident within the trunk and tail (Fig. 2I). Further analysis using anti-active Caspase 3 staining shows that cell death is largely the result of apoptosis which begins at about 4-somites (11.3 h) more or less randomly, but by 6-somites is prevalent in the head and anterior trunk (Fig. 1E and F). By 9-somites apoptosis has also begun to occur within the posterior trunk and tail (Fig. 1G) preceding the in vivo appearance of cell death in roughly the same sequence; by 12-somites (15 h) apoptosis is rampant everywhere (Fig. 1H). To determine if apoptosis is largely restricted to the nervous system, we made cross-sections through embryos co-expressing tissue specific markers (Fig. 1I–N). This revealed that

Fig. 2. ogre is a mutation in racgap1. (A) Genetic map of chromosome 23 showing number of recombinants and calculated distance for microsatellite markers and genes. (B) Schematic of the racgap1 cDNA showing location of termination codon in exon 10 and (C) sequence trace data. (D) Schematic of the RacGAP1 protein. MKLP1 binding region; CC, coiled-coiled domain; ECT2/FIP3 binding region; C1, atypical C1 domain; GAP, GTPase-activating domain. (E–H) Expression of racgap1 mRNA: (E) maternal expression at 1 h; and (F–H) zygotic expression at: (F) 7 h; (G) 18 h; and (H) 24 h. (I–J) Anti-sense knockdown of racgap1 mRNA by morpholino (MO) phenocopies ogre; left side: embryo, right side: DAPI-stained nuclei. (K–P) racgap1 mRNA rescues the ogre mutant phenotype. (K) racgap1 injected and (L) kaede control embryos. (M) Quantification of multinucleate cells at 10-somites. Differences between kaede and racgap1 injected mutant embryos are significantly different (p < 0.01, Wilcoxon rank sum); racgap1 injected wild type and mutant embryos are also significantly different (p < 0.01, Wilcoxon rank sum). (N–P) dlA expression in neuroblasts: (N) racgap1 injected and (O) kaede control embryos and (P) their average number in ogre mutants.

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apoptosis occurs in a rostral-caudal gradient not only in nervous tissue but also in mesodermal tissues such as muscle and blood. In summary, it seems cell death occurs in any tissue with actively dividing cells.

Previously we reported that cells in the ogre mutant exhibit double nuclei as somites begin to form (Kane et al., 1996b). Consistent with the idea that defective cytokinesis may be responsible for cell death, a high proportion of mutant cells are binucleate by 9-somites (Fig. 1D). Regardless, many cell types develop in ogre mutants including those that maintain actively dividing cell populations such as blood and neurons (Fig. 1O and P). However, their differentiated progeny at 24 h are far fewer in number and often larger in size compared to wild type (arrows in O and P). Moreover, the axons of sensory neurons are more disorganized and appear to overgrow as well as exhibit abnormal swellings along their length (Fig. 1P and Q).

3.2. ogre is a mutation in racgap1

We mapped ogre via half-tetrad analysis to chromosome 23 and using a haploid mapping panel fine-mapped ogre to the interval between the simple sequence length polymorphisms z5141 and z31657 (Fig. 2A). This area was further refined using the nearby linked gene graspl (grp1) that had a size polymorphism that could be visualized on an agarose gel, placing ogre near the zebrafish homolog of racgap1. Sequencing of the mutant coding sequence revealed a nonsense mutation in exon 10 (Fig. 2B and C) that occurs within the predicted atypical C1 domain and before the catalytic GAP domain (Fig. 2D), domains that are highly critical for cytokinesis (Basant et al., 2015; Bastos et al., 2012; Canman et al., 2008; Lekomtsev et al., 2012; Loria et al., 2012). In situ analysis showed that zebrafish racgap1 mRNA is expressed maternally (Fig. 2E), consistent with the idea that maternal transcripts must be depleted before cell division defects appear (Kane et al., 1996b), as well as zygotically (Fig. 2F–H). In the gastrula, expression was ubiquitous, but by 1 day expression was highest in the nervous system, particularly the brain. Investigation of ogre mutants revealed that racgap1 mRNA was mostly depleted by 18-somites (18 h; Fig. 2G) and not detectable by 1 day (Fig. 2H) in keeping with depletion of mRNAs transcripts that are subjected to nonsense-mediated degradation.

To confirm the ogre phenotype results from a mutation in racgap1, we injected a splice-blocking morpholino against racgap1 transcripts. Antisense inhibition completely recapitulated the morphological and cytological defects of the ogre mutant (Fig. 2I and J). Next we injected in vitro synthesized mRNA for racgap1 or a control mRNA encoding the photoconvertible fluorescent protein Kaede (Hatta et al., 2006). This demonstrated that wild-type racgap1 mRNA delayed onset of the ogre phenotype (Fig. 2K and L), partially rescued the cytokinesis defect (Fig. 2M), increased the number of neural cells (Fig. 2N–P) and even rescued motor function in mutant embryos (Supplementary Fig. 1). We conclude from these experiments that ogre is a null allele and a complete loss of racgap1 morpholo.

3.3. Mutant cells exhibit defects in abscission before defects in cleavage furrow ingression

RacGAP1 has a well-studied role as a component of the centralspindlin complex which itself has several critical functions during cytokinesis including positioning of the cleavage furrow, ingestion of the cleavage furrow and abscission. To better understand how cells in the mutant become multinucleate we video time-lapsed embryos from 4 to 10 h of development. We chose this period because mutants that affect the cell cycle are likely to exhibit their phenotypes during this time once maternal transcripts no longer compensate for zygotic transcripts (Kane et al., 1996a, 1996b). Beginning after the onset of gastrulation (6 h), 3 h after the onset of zygotic transcription (Kane and Kimmel, 1993) and when the embryo has segregated into three spatially distinct cellular domains (Kane et al., 1992), we found that the behavior of cells dividing in the deep cell domain of the blastoderm (the embryo proper) was markedly different between mutant and wild type. Upon cleavage furrow ingression daughter cells in the mutant separated from each other as in wild type but then slowly coalesced back together as if they were unable to physically sever the cytoplasmic bridge between each other (compare Fig. 3B to A). This cell behavior persisted until 75% epiboly (8 h; Fig. 3C, blue cell) whereupon a more abbreviated version of cytokinesis appeared. Here a cleavage furrow formed, but it rapidly regressed immediately coalescing the future daughter cells back together (Fig. 3C, purple cell). Both these cell behaviors are illustrated in Fig. 3E. By 90% epiboly (9 h; Fig. 3D), some binucleate cells were now beginning to divide. Here cells exhibited almost no cleavage furrow, but underwent karyokinesis. As nuclei reappeared they were often unequal in size suggesting possible defects in aneuploidy. Notably, cells that failed at furrow ingression also no longer rounded up during mitosis, instead, they wrinkled exhibiting tiny projections all over their surface (Fig. 3C and D). A similar phenotype is observed in cultured human cells expressing a mutated GAP domain of RacGAP1, a defect attributed to improper cell adhesion at the cleavage plane of the dividing cell (Bastos et al., 2012). We conclude that falling levels of RacGAP1 first compromise abscission and then cytokinesis entirely.

A related cellular behavior was observed in the extraembryonic domain of the blastoderm, where cells normally cycle more slowly (Kane et al., 1992). However, rather than cytokinesis failing, it was unusually delayed. Starting around the onset of epiboly (4.3 h), some enveloping layer cells entering mitosis completed nuclear division, but like deep cells later, did not complete cytoplasmic division and fused back together (Fig. 3F). Usually, before gastrulation, most of these binucleate cells rounded up and exhibited violent protrusive activity (Fig. 3F, panel 1:17:00) that may be akin to the cell projections seen later all over dividing deep cells, before subsequently dividing into two mononucleate daughter cells that possibly are tetraploid rather than diploid. Though transient, this phenotype was 100% penetrant regardless of zygotic genotype, demonstrating it to be a maternal dominant phenotype. Nevertheless, during early gastrulation, this marked behavior disappeared in all but the homozygous mutant where a cell cycle later cytokinesis stopped altogether in enveloping layer cells. Hence, rising zygotic levels of RacGAP1 return cytokinesis to normal.

3.4. Mutant cells reenter the cell cycle after becoming binucleate

To characterize in more detail mutant cytokinesis, we dissociated embryos derived from heterozygous mutant carriers at different stages of development. Because cell number in the mutant does not increase due to defective cytokinesis, this allowed us to calculate the percent of cells that were mononucleate, binnucelar or quadnucleate for a single embryo (Fig. 4A–C and Supplementary Fig. 2) and thus illustrate how many times a cell attempted to divide. Octonucleate cells were also observed, but only rarely. In agreement with above, by 70% epiboly (7 h) and well before we could identify mutants morphologically, a quarter of the dissociated embryos exhibited from 11% to 23% binucleate cells (Supplementary Fig. 2A). The average number of binucleate cells in these embryos (14%), contrasted sharply to the remainder of the embryos where few cells on the average were binucleate (3%) consistent with this portion being phenotypically wild type (Fig. 4D) and likely reflecting normal cells caught in cytokinesis.

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From tailbud (10 h) to the 4-somite stage (11.3 h), the average number of quadnucleate cells rose from 4% to 18% in the portion of dissociated embryos we deemed mutant based upon the frequency of multiple nuclei in our counts (Fig. 4D and Supplementary Fig. 2B and C). Simultaneously, the average number of binucleate cells in these embryos rose to 62% while the average number of mononucleate cells fell to 20%. Hence by early somite stages much of the embryo is multinucleate and many cells have attempted to divide at least once after cytokinesis is blocked. After the 4-somite stage, when ogre mutants can be unambiguously identified before dissociating (Supplementary Fig. 2D and E), the average number of quadnucleate cells fell to 9% and the average number of mononucleate cells rose to 55% (Fig. 4D). As this correlates to a period when apoptosis is marked (Fig. 1F–H) and there is no reciprocal increase in octonucleate cells, we conclude that attrition of quadnucleate cells between 4- and 10-somites (14 h) is due to apoptosis and that as a result mononucleate cells become more highly represented. Later, from 10- to 20-somites (19 h) the average number of quadnucleate cells rose again (28%) and the average number of mononucleate cells fell once more (38%), while during this time the average number of binucleate cells remained more or less the same (~34%; Fig. 4D). Therefore it seems that most cells reenter the cell cycle and attempt to divide at least once after cytokinesis is blocked. As with our observations earlier, in embryos that were phenotypically wild type, cells were almost exclusively mononucleate for all time periods (Fig. 4D).
of mitotic cells between wild type and mutant at 4-somites (Fig. 4E); at this time mitotic mutant cells were either mono-nucleate or binucleate (inset). However, by 10-somites ogre mutants had notably fewer mitotic cells compared to wild-type embryos (Fig. 4F). This was confirmed by counting the number of cells in rhombomere 4, outlined by expression of the zinc-finger transcription factor egr2b (Oxtoby and Jowett, 1993). On the average there were 16.9 ± 1.0 mitotic cells in wild type versus 4.1 ± 0.3 mitotic cells in mutants (Fig. 4H). By now the majority of mitotic cells in mutants were binucleate (69%; Fig. 4I), but many were quadnucleate (21%), suggesting that perhaps they die because of entering mitosis. Not many mitotic cells were mononucleon (10%), however because these cells did not divide earlier and waited till now to enter mitosis, likely explaining their reduction between 10- and 20-somites (Fig. 4D), this suggests there may be a population of cells that have a very long cell cycle. At one day few mitotic cells were apparent in ogre (Fig. 4G) and these were largely abnormal. Hence cells in ogre mutants continue to cycle after cytokinesis is blocked, but once a cell has four nuclei it is detrimental and further attempts to divide are likely lethal.

3.5. The cell cycle is longer in ogre mutant cells

Our proceeding study indicates that the cell cycle may be longer in a population of mutant cells. It is well known that cells respond to cellular stress by delaying their cell cycle (reviewed in: Clarke and Allan (2009), Rieder and Maiato (2004)) as might occur because these cells did not divide earlier and waited till now to enter mitosis, likely explaining their reduction between 10- and 20-somites (Fig. 4D), this suggests there may be a population of cells that have a very long cell cycle. At one day few mitotic cells were apparent in ogre (Fig. 4G) and these were largely abnormal. Hence cells in ogre mutants continue to cycle after cytokinesis is blocked, but once a cell has four nuclei it is detrimental and further attempts to divide are likely lethal.

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As expected, in wild-type embryos, erythrocytes progressively increased in number every 5–6h from ~316 ± 21 cells at 15 h to ~920 ± 25 cells at 32 h (Fig. 5G). In ogre mutants, because most cells already had at least two nuclei to start with, there were initially half as many erythrocytes than in wild type (~158 ± 21) and a cell cycle later at 22-somites (20 h), their number was almost the same, indicating cells were not dying. Two cell cycles later (26 h), there were fewer erythrocytes (~87 ± 6 cells), but a cell cycle after that (32 h) there was little change (Fig. 5G). Therefore between 15 and 32 h, mutant erythrocytes appear to cycle only once whereas wild-type erythrocytes appear to cycle mostly twice. In support of this view, at 26 h when mutant erythrocytes begin to die a cell cycle after they should, they now possessed mostly four nuclei (Fig. 5D). Other blood cells, macrophages and neutrophils, normally have a much longer cell cycle than erythrocytes, but still divide at least once during this time interval (Warga et al., 2009). We counted these cells as well (Fig. 5G) using lcp1 (Fig. 5E), an actin binding protein expressed in differentiating macrophages (Herbomel et al., 1999) and mpo (Fig. 5F), a peroxidase expressed in differentiating neutrophils (Bennett et al., 2001; Lieschke et al., 2001). In contrast to wild type, there was little change in their number over this time span in ogre.

To investigate this further, we visualized the cell cycle in real time using the Fluorescence Ubiquitin Cell Cycle Indicator (FUCCI). This is a two-color system that makes use of oscillating levels of two key cell cycle proteins: Cdt1, which accumulates during G1 and Geminin, which accumulates during S, G2, and early M (reviewed in: Callat and Peraklis (2012), Ziieke and Edgar (2015)). In zebrafish, a single transgene expresses a Cdt1-degron tagged with mCherry and a Geminin-degron tagged with Cerulean (Bouldin and Kimelman, 2014) to report the different phases of the cell cycle as illustrated in Fig. 5H. Using this transgene we performed...
Fig. 5. The cell cycle is longer in ogr. (A, B) Expression of: (A) gata1a and (B) hbbe2 in erythrocytes. (C, D) High magnification view of erythrocytes at 15 h (C) and at 26 h (D); numbers beside cells indicate their number of nuclei (dotted), not all nuclei are visible in the plane of focus. (E, F) Expression of: (E) lcp1 in macrophages and (F) mpx in neutrophils. (G) The average number of blood cells at different times of development. For erythrocytes we used gata1a expression at 15 and 20 h and hbbe2 expression at 26 and 32 h, for macrophages we used lcp1 expression and for neutrophils we used mpx expression, which comes up later. We examined 5 wild type and 5 mutant embryos per stage. (H) Schematic of the dual Fluorescence Ubiquitin Cell Cycle Indicator (FUCCI) with respect to the cell cycle. (I–K) Selected frames from three confocal time-lapses used to quantitate how long cells spent in late M and cytokinesis (when there is no signal) and the G1/S transition (when nuclei are pink). For each nucleus and its daughter nuclei, the stage of the cell cycle is indicated. Embryos (I) and (J) are siblings and were recorded simultaneously. Note that in mutant (J) cells take not only longer to transition through mitosis and G1/S, but that the Cdt1 degron takes longer than the Geminin degron to build expression in the G1/S transition. Note also that in mutant (K) the two daughter nuclei do not cycle together. (L) Quantification of G1/S transition and mitosis; n indicates the number of cells and in all but one case only one cell was used per embryo. Respectively, differences are significant at p < 0.005 and p < 0.002 (Wilcoxon Rank Sum).
time-lapse confocal microscopy beginning at 75% epiboly (8 h) to quantify the length of time mutant cells and their wild-type counterparts spent in the G1/S transition (when cells are pink due to the presence of both Geminin and Cdt1) and in late M and cytokinesis (when no signal is seen because Geminin is degraded). Here we focused on enveloping layer cells because they do not undergo as extensive morphogenetic movements as the deeper cells of the embryo (Fig. 5I and J). Notably even though mutant cells had only one nucleus to start with, the average length of time mutant cells spent in late M and cytokinesis was considerably longer than wild-type cells and the same was true for the G1/S transition (Fig. 5L). Thus lengthening of the cell cycle in ogre mutants may have little to do with the number of nuclei and more to do with absence of RacGAP1 itself. However, we also observed that after mitosis not all daughter nuclei cycled together (Fig. 5K), not unusual for normal daughter cells (Kane et al., 1992; Kimmel and Warga, 1986, 1987; Kimmel et al., 1994; Warga and Kimmel, 1990), but perhaps contributing to slowing of the cell cycle once cells are multinucleate. These results and those above demonstrate that different cellular domains, both embryonic (blood) and extraembryonic (enveloping layer cells) cycle more slowly in ogre.

3.6. Stopping cytokinesis prevents RacGAP1-dependent apoptosis

Cells also respond to cellular stress by initiating a program of cell death through the process of apoptosis (reviewed in: Clarke and Allan, 2009; Fulda et al. (2010)). To determine if defective cytokinesis is causing mutant cells to enter apoptosis we prevented cell division from occurring in embryos injected with the racgap1 morpholino (Fig. 6). Here we took advantage of a novel cell cycle mutant harpy (hrp; Fig. 6A), carrying a mutation in the gene emi1 (Kane et al., 1996b; Rhodes et al., 2009; Riley et al., 2010; Zhang et al., 2008). Upon running out of maternal product around the onset of gastrulation (6 h), cells in the homozygous zom mutants arrest after the chromosomes condense in either prophase or metaphase (Fig. 6B), but like hrp, there is little apoptosis (Fig. 6C, compare row 2 and 3). Knockdown of RacGAP1 in the zom mutant resulted in a more severe morphological phenotype (Fig. 6D) that exhibited a range of nuclear phenotypes (Fig. 6E) as the zom mutant phenotype occurs later than the hrp mutant phenotype and RacGAP1 depleted cells mostly do not arrest in mitosis until after becoming binucleate. Typically both nuclei were in some state of mitotic arrest. Usually both were in prophase or in metaphase (red arrowheads), but infrequently one of the nuclei was in prophase and the other in metaphase (red arrow) agreeing with our observation above that not all daughter nuclei are in synchrony with one another (Fig. 5K). However, there were also cells where both nuclei had not yet arrested (yellow arrowhead) or only one (yellow arrow). Significantly, loss of zom function decreased RacGAP1-dependent apoptosis (Fig. 6F, compare row 1 and 3) demonstrating that cells lacking RacGAP1 will not enter apoptosis, even with excess chromosomes and two nuclei, if cytokinesis is blocked. zom however, was not as effective as hrp at preventing apoptosis (Fig. 6F, compare row 2 and 3) likely because some binucleate cells that were not arrested divided once more. In sum these results demonstrate that apoptosis is not due to lack of RacGAP1 per se, but rather to defective cytokinesis that results when RacGAP1

![Fig. 6. RacGAP1 depleted cells survive when cytokinesis is prevented. Row (1) phenotypic wild type, row (2) homozygous hrp mutants, and row (3) homozygous zom mutants. Row 1 and 2 are siblings. Column (A-C) uninjected embryos; column (D-F) morpholino (MO) knockdown embryos. (A, D) Live embryos. (B, E) DAPI stained nuclei: red arrows indicate sister nuclei where both nuclei are in prophase; red arrow indicates two sister nuclei where one nuclei is in prophase and the other nuclei is in metaphase; yellow arrowheads indicate sister nuclei where both nuclei are in interphase; and yellow arrow indicates two sister nuclei where one nuclei is in interphase and the other nuclei is in prophase, and (C, F) anti-active Caspase 3 stained embryos.](http://dx.doi.org/10.1016/j.ydbio.2016.06.021)
function is lost. These results also once again demonstrate that going from two nuclei to four is disadvantageous.

3.7. The ogre mutation causes reductions in later born neurons and eventual loss of primary motoneurons

Our initial analysis of the ogre mutation suggested that some neural cells differentiate (Fig. 1P). The nervous system is a complex organ with a multitude of cell types and for most cell cycle mutants in zebrafish, its initial patterning is rather normal and it is only later proliferating cell types that are affected (Fischer et al., 2007; Johnson et al., 2014; Riley et al., 2010; Song et al., 2004). Therefore to determine how normal the nervous system in ogre is we examined the notch1b receptor (Fig. 7A), expressed in proliferating neural cells (Bierkamp and Campos-Ortega, 1993), and dlA its ligand (Fig. 7B), which is briefly expressed in newly specified neuroblasts or in post mitotic neurons (Appel et al., 2001). We found that by 9-somites (13.5 h) the number of cells expressing either of these markers in ogre mutants was decidedly less than normal. Furthermore, by 17-somites (17.5 h) their number was even more diminished and by 1 day no notch1b proliferating cells were evident nor almost any dlA neural precursors. We expected fewer cells over time to express notch1b, due to the cytokinesis defect and cells becoming specified as neurons or dying, however we did not expect for the number of cells expressing dlA to fall so rapidly as these are cells that have decided to leave the cell cycle and in other cell cycle mutants their number is relatively unchanged (Riley et al., 2010). Thus, we examined how many nuclei dlA-positive cells had using DAPI, a nuclear dye (Fig. 7C and D). We found that most cells specified as neurons in ogre mutants were already binucleate by 10-somites (14 h; Fig. 7G) much like we observed with erythrocytes at this time (Fig. 5C). Corresponding to

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**Fig. 7.** The ogre nervous system lacks later born neurons. (A, B) Expression of: (A) notch1b in proliferating cells and (B) dlA in neuroblasts. (C, D) High magnification view of neuroblasts visualized with dlA and DAPI. Numbers beside circled dlA-positive cells indicate their number of nuclei or if the cell was apoptotic (apop), white bar shows the boundary between somites 3 and 4. (E) Expression of islet1 in primary motoneurons (pmn) and Rohon-Beard sensory neurons (rb). Inset (E’’) expression of col2a in floorplate neurons (fp) and hypochord cells (hc). (F) Expression of: pax2a and egr2b in CoSA interneurons (CoSA) and inset (F’’) high magnification of CoSA interneurons. Numbers beside cells indicate their number of nuclei. (G) Quantification of multinucleate neuroblasts between somites 2 and 8; n indicates the number of embryos at different stages. Differences are significant compared between 14 h and 17 h (p < 0.001) and compared between 17 h and 21.5 h (p < 0.05; contingency tables/Pearson’s chi square).

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their steady decline in number (Fig. 7B), there was an increasing tendency for specified neurons to appear apoptotic rather than multinucleate after 10-somites (Fig. 7G) in keeping with the idea that not all neuroblasts have yet left the cell cycle and made the switch from proliferation to differentiation before expressing dIa (Appel et al., 2001). These likely are the cells that are dying.

To see which cells in the nervous system differentiate we examined the expression of two post mitotic markers: isl1 (isl1) and pax2a (Fig. 7E and F). Here we concentrated on the spinal cord because the brain of ogre mutants is almost nonexistent by one day (Fig. 7F; compare brain expression between mutant and wild type). In the spinal cord, the LIM/homeodomain transcription factor isl1 is expressed in primary motor and sensory neurons at the time these cells differentiate (Appel et al., 1995; Inoue et al., 1994; Korzh et al., 1993) while the paired-box homeodomain transcription factor pax2a is expressed in CoSA interneurons (Krauss et al., 1991; Milkola et al., 1992). We found that at 12-somites (15 h), the expression of isl1 and pax2a was remarkably similar between mutant and wild type. However at one day, their expression indicated that only the earliest born neurons differentiate in ogre mutants for no more Rohon-Beard sensory or CoSA interneurons were visible than earlier, much like happens in other cell cycle mutants (Johnson et al., 2014; Riley et al., 2010; Song et al., 2004). Therefore early born neurons differentiate in ogre, even though many possess multiple nuclei (Fig. 7P), but later born neurons such as those posterior in the embryo (Kimmel et al., 1994) do not, in agreement with our results above that later specified neurons tend to die. Unexpectedly, primary motoneurons no longer were apparent by one day (Fig. 7E). Thus they died sometime after differentiating, even though adjacent floorplate neurons (identified by col2a1a expression) still were present (Fig. 7E). As no other mutation in the cell cycle has a phenotype where cells differentiate and later die before the embryo (Johnson et al., 2014; Riley et al., 2010; Song et al., 2004), this shows that some cell types such as primary motoneurons must require RacGAP1 post mitotically to survive.

### 3.8. Microtubule organization is aberrant in mutant sensory axons

In *Drosophila* when either the fly homolog of RacGAP1 or MKLP1 is mutated or depleted (del Castillo et al., 2015; Goldstein et al., 2005), axons overgrow. ogre mutants have a similar phenotype and their axons exhibit visible swellings along their length (Fig. 1P and Q). To look at this morphology in more detail, we examined the peripheral processes of trigeminal sensory and Rohon-Beard neurons using acetylated tubulin staining and fluorescent microscopy. High magnification revealed that the microtubule organization in mutant axons was impaired, although this phenotype was not completely penetrant possibly because these sensory neurons are among the earliest neurons to differentiate (9–10 h) and send out processes (4–5 h later; Lewis and Eisen, 2003; Metcalfe et al., 1990). Thus it is likely that in these cells maternal products have not yet been completely exhausted (Fig. 2G).

In wild type (Fig. 8A), peripheral axons of trigeminal and Rohon-Beard neurons branch extensively over the lateral surface of the embryo (Metcalfe et al., 1990). When processes bifurcate the microtubules within a process defasciculate and fan out before bundle back together into one or the other branch (Fig. 8B). Occasionally loops of microtubules also occur along processes (Fig. 8C) that resemble the architecture of a presynaptic varicosity (Chang and balice-Gordon, 2000; Conde and cáceres, 2009). In mutants (Fig. 8D and G), peripheral axons were more disorganized, frequently wandering back and forth, and their microtubule organization at branch points was rarely normal (Fig. 8E). More often at bifurcations the microtubules were tangled around each other (Fig. 8F), a phenotype also observed with possible presumptive presynaptic swellings that tended to be larger, more tangled and more frequent than normal (Fig. 8H and I). The large tangles of microtubules along peripheral axons likely explains the conspicuous swellings we observed at lower magnification (Fig. 1P and Q) a phenotype that would be enhanced by using an enzymatic reaction. We conclude from these results that RacGAP1 is not only required for organizing the cytoskeleton in dividing cells but also for organizing the cytoskeleton in post mitotic neurons.

### 4. Discussion

Here we describe a zebrafish mutation in racgap1, one of two components of the centralspindlin complex essential for orchestrating cytokinesis in all animal cells. We show that with decreased maternal RacGAP1 there is a marked delay between nuclear division and cell division, whereas in the absence of zygotic RacGAP1 abscission fails and then finally cleavage furrow ingression. Cells that no longer complete cytokinesis continue to cycle, albeit more slowly, and can become quad or even octonucleate. However, while many mutant cells differentiate containing multiple nuclei—and survive—more often they enter apoptosis. By preventing mutant cells from re-entering the cell cycle we demonstrate that apoptosis is not due to lack of RacGAP1 per se, but rather cumulative rounds of defective cytokinesis. We also demonstrate that RacGAP1 is not only required for cytokinesis, but also for survival of some post mitotic neurons as well as the cytoskeletal organization of axons.

#### 4.1. ogre is a loss of function maternal zygotic mutation

We demonstrate by antisense knockdown and wild-type mRNA rescue that the ogre mutant phenotype is unequivocally the result of a premature termination (nonsense) codon in exon 10 of racgap1. If translated, mutant mRNAs would produce a shorter protein having a truncated atypical C1 domain and no catalytic GAP domain—each highly critical for cytokinesis (Basant et al., 2015; Bastos et al., 2012; Canman et al., 2008; Lekomtsev et al., 2012; Loria et al., 2012). However, in situ analysis shows that racgap1 transcripts are present in lesser amounts in mutant embryos and eventually lost in keeping with these mRNAs being targeted for degradation through the process of nonsense-mediated mRNA decay (reviewed in: Chang et al. (2007)). Therefore, these results indicate that ogre is most probably a loss of function mutation.

In zebrafish, racgap1 mRNA is expressed maternally and zygotically and like other model organisms where racgap1 is mutated, cells in homozygous ogre mutants become binucleate. Excepting the dominant maternal phenotype which is transient, binucleate cells do not appear until long after the onset of zygotic transcription (Kane et al., 1996b), consistent with the idea maternal stores of cell cycle gene products regulate cytokinesis during the first 15 or so cell cycles of zebrafish (Kane and Kimmel, 1993). The transient maternal phenotype, where extraembryonic cells become binucleate before eventually dividing likely is due to half as much maternal wild-type mRNA. Hence there are distinct maternal and zygotic features to the cytokinesis defect.

#### 4.2. RacGAP1 and cytokinesis

Numerous studies have examined the role of centralspindlin—a protein complex consisting of the Rho family GTPase-activating protein RacGAP1 and its partner the kinase MKLP1—for its various functions during cytokinesis including ingress of the cleavage furrow, formation of the midbody and abscission (reviewed in: Davies and Canman (2012), Glotzer (2013), Green et al. 2014; Johnson et al., 2014; Riley et al., 2010; Song et al., 2004). This shows that some cell types such as primary motoneurons must require RacGAP1 post mitotically to survive.

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However few of these studies have used live imaging analysis to characterize the cytokinesis defects in an intact embryo and to date the only animal where this has been done extensively is in *C. elegans* during the first cleavages. While studies in cultured cells and fixed *Drosophila* tissue depleted of RacGAP1 or MKLP1 homologs by RNAi or mutation indicate cells do not appear to form a cleavage furrow (Adams et al., 1998; D’Avino et al., 2006; Lekomey et al., 2012; Mishima et al., 2002; Somers and Saint, 2003; Sommi et al., 2010; Zavortink et al., 2005), live imaging of *C. elegans* eggs carrying equivalent mutations show that the first cleavage furrow forms correctly and ingresses extensively, but then regresses before a stable midbody is established (Canman et al., 2008; Davies et al., 2014; Jantsch-Plunger et al., 2000; Powers et al., 1998; Raich et al., 1998). Our live imaging studies of cytokinesis in an intact vertebrate embryo carrying a presumptive null mutation show cytokinesis decays over time due to the elimination of maternal *racgap1* transcripts. Cytokinesis defects first appear in all embryos derived from a heterozygous *ogr* female shortly after the onset of epiboly (4.3 h), just 1.3 h after the zebrafish mid blastula transition (Kane and Kimmel, 1993; Kane et al., 1992). Although embryos recover, cytokinesis is abnormally delayed relative to karyokinesis. Defects begin to appear in homozygous mutants shortly after the onset of gastrulation (6 h), now 3 h after the zebrafish mid blastula transition. Initially cytokinesis progresses nearly as far as abscission, before the intercellular bridge between dividing cells regresses and the two daughter cells fuse back together, similar to studies in *C. elegans*. Eventually, however, mutant cells do not complete cleavage furrow ingression before fusing back together, and finally no...
cytokinesis occurs. 

Centralspindlin is only functional as a complex, not as its individual components (Mishima et al., 2002; Pavicic-Kaltenbrunner et al., 2007), and it is essential for centralspindlin to oligomerize and form higher order clusters before becoming active (Bassat et al., 2015; Hutterer et al., 2009). Hence, as maternal wild-type RacGAP1 is eliminated over time and not replaced by zygotic wild-type product, the effective concentration of centralspindlin falls offering a unique perspective to study which aspects of cytokinesis are most sensitive to centralspindlin levels. We know of no other study that has looked at the result of centralspindlin depletion over time and our studies indicate that the end of cytokinesis rather than the beginning of cytokinesis is exquisitely sensitive to the amount of centralspindlin available.

4.3. Apoptosis in ogre mutants

Loss of function mutations in the homologs of racgap1 or mklp1 in Drosophila and C. elegans are embryonic lethal when homozygous (Adams et al., 1998; Jantsch-Plunger et al., 2000; Powers et al., 1998; Somers and Saint, 2003; Unhavaithaya et al., 2013; Zavortink et al., 2005). However, while the cytokinesis defect has been much studied in these mutants the cause of lethality has been largely ignored perhaps because perturbations in cytokinesis are apparent well before cells start to die. In contrast, the mutation in the zebrafish homolog of racgap1 was identified initially because of massive cell death before it was determined there were earlier defects in cytokinesis (Kane et al., 1996b). Here we show that cell death is due to apoptosis and this is a direct consequence of defective cytokinesis for RacGAP1 depleted cells do not die if cytokinesis is prevented using mutants of the cell cycle. While apoptosis is also observed in the brain of mice and rats carrying a mutation in citron kinase (Di Cunto et al., 2000; Mitchell et al., 2001; Sarkisian et al., 2002), a contractile ring component needed for successful cytokinesis (Bassi et al., 2013; Gruneberg et al., 2006), and in the brain of zebrafish carrying a mutation in aurora B kinase (Yabe et al., 2009), a chromosomal passenger protein important for the completion of cytokinesis (Bassat et al., 2015; Guse et al., 2005) it has never been demonstrated that apoptosis ensues because cytokinesis fails. Our results show it does.

Why might cells undergoing defective cytokinesis die? In normal development it is not uncommon for certain cell types to become polyploid either as a result of endoreplication or programmed cytokinesis failure thereby resulting in tetraploid or even much greater cell ploidy (reviewed in: Celton-Morizur et al. (2010), Fox and Duronio (2013), Orr-Weaver (2015)). And although cells often die as a result of chromosome missegregation, an ad hoc mechanism when this occurs is for regression of the cleavage furrow yielding a binucleate tetraploid cell rather than two mononucleate aneuploid cells (Shi and King, 2005). Thus in principle, ogre cells should not die as a result of their ploidy, which in some instances has been shown to be an adaptive strategy to modulate gene expression (Anastaskaya and Vinogradov, 2007; Raslova et al., 2007). However, except for a syncytium where cells fuse together to become multinucleate, most vertebrate cells that become polyploid rarely have more than two nuclei (reviewed in: Celton-Morizur et al. (2010), Fox and Duronio (2013), Orr-Weaver (2015)), whereas in ogre they do. Frequently mutant cells go from two to four nuclei and, less frequently, from four to eight (Fig. 4C). But many, possibly most, mutant cells enter apoptosis during or after that division. For, as we show, the majority of mitotic cells are binucleate rather than quadnucleate (Fig. 4I) and there is a tendency over time for apoptotic cells to out number quadnucleate cells (Fig. 7G). Indeed, conditional deletion of RacGAP1 in the mouse germline where mutant cells stop dividing once they reach two or four nuclei does not cause apoptosis (Lores et al., 2014). Hence it seems having four or eight nuclei, within perhaps a constrained geometry, compromises the cell and triggers apoptosis. Perhaps because mispairing of centromesomes is now more likely to occur leading to defects in cell polarity or a multipolar spindle that does not satisfy the spindle assembly checkpoint (reviewed in: Bornens (2008), Chavali et al. (2014)) either of which can trigger apoptosis and also contribute to a longer cell cycle (McCaffrey and Macara, 2011; Rieder and Maiato, 2004).

Another possible reason cells with four or eight nuclei might die is that after cells have two nuclei DNA damage due to re-replication of previously replicated DNA or premature chromosomal condensation is likelier to occur. This is a common occurrence and triggers apoptosis during nuclear transfer if the donor nucleus and the recipient cytoplasm are not in phase with each other (reviewed in: Campbell et al. (1996), Gurdon et al. (2003), Shufaro and Reubinoff (2011)). Notably, we see sister nuclei in different phases of the cell cycle using the dual FUCCI cell cycle indicator (Fig. 5K) or in cdc20/racgap1 mutant cells (Fig. 6E). Therefore, in some mutant cells, each nucleus is progressing through the cell cycle and its individual checkpoints at different rates. However, DNA damage is the least likely explanation for ogre mutant cells dying: homozygous p53 mutants, that do not exhibit apoptosis upon DNA damage (Berghmans et al., 2005), do so when injected with the racgap1 morpholino (R.M.W. unpublished).

4.4. RacGAP1 and cellular development

While critical for cytokinesis, little is known of the role of RacGAP1 in other cellular processes. The neuronal defects we observe in ogre are especially interesting because they suggest that RacGAP1 is needed in various aspects of neuronal development. We know from studies in Xenopus when cell division is chemically blocked early in development that patterning of the nervous system is surprisingly normal and cells even grow axons properly, which elicit typical motor reflexes (Harris and Hartenstein, 1991; Rollins and Andrews, 1991). Therefore it should perhaps not come as a shock that neurons (and blood) might develop with multiple nuclei as we show. Clearly some defects in ogre stem from defective cytokinesis, such as the gradual attrition of neuroblasts and stem cells over time, which is why in most cell cycle mutants the earliest born neurons (primary neurons) are present and later born neurons are absent (Fischer et al., 2007; Johnson et al., 2014; Riley et al., 2010; Song et al., 2004).

In general, GTase-activating proteins similar to RacGAP1, negatively regulate Rho-mediated signals by stimulating GT P hydrolysis of small G-proteins in the Ras superfamily, specifically: RhoA, Rac1 and Cdc42. While the major targets of these Rho- GTPases are the actomyosin and microtubule cytoskeletons (thus their importance for cytokinesis) by doing so they mediate a diverse variety of cellular processes that include not only cell cycle progression, but also cell movement, cell signaling, and cell polarity (reviewed in: Chircop (2014), Citi et al. (2014), Mack and Georgiou (2014)). Accordingly, deregulation of Rho GT Pases are linked to a number of neurodegenerative disorders (reviewed in: Stankiewicz and Linseman (2014)). In particular, loss of Rac1 activity has been shown to be a contributing factor in several forms of early onset amyotrophic lateral sclerosis (ALS), a devastating neuromuscular disorder characterized by loss of motoneurons. Because the GAP activity of RacGAP1 is specific for Rac1 and not RhoA (Bastos et al., 2012; Canman et al., 2008; D’Avino et al., 2004; Toure et al., 1998), it is intriguing that while primary motoneurons begin to differentiate in ogre, they later die. Other differentiated cells in the spinal cord of the mutant continue to survive including floorplate neurons (Fig. 7E), which lie directly adjacent to primary motoneurons (Lewis and Eisen, 2003). Therefore, lack of RacGAP1 likely explains their specific demise. If so, too much Rac1 activity

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may be as great a risk to motoneuron survival as too little suggesting either is a crucial contributing factor to early onset of ALS. Unclear is whether motoneuron survival in zebrafish requires RacGAP1’s association with MKLP1. However in Drosophila, depleting motoneurons of Pavarotti (the fly homolog of MKLP1) by RNAi once motoneurons have begun to differentiate does not appear to affect their survival (del Castillo et al., 2015) suggesting that in this context RacGAP1 acts independently.

On the other hand, there is growing evidence that the centrospindlin complex has additional functions in axon outgrowth fitting with our observations that axon axes overgrow and their microtubules are disorganized and tangled (Fig. 8). In flies, when the homolog of RacGAP1 is lost, as it is in the tumbleweed mutant, axons overgrow (Goldstein et al., 2005), much like we see in ogre. Notably cytokinesis is mostly normal in tumbleweed mutants (Goldstein et al., 2005), therefore longer axes seems specific to loss of RacGAP50C itself. Likewise, if Pavarotti is depleted by RNAi post mitotically (avoiding possible defects from cytokinesis) axes are also longer (del Castillo et al., 2015). Recently it has been shown that Drosophila neurons initially extend axes by a mechanism involving sliding of microtubules against one another (Lu et al., 2013) and if either Pavarotti or RacGAP50C are depleted this stimulates microtubule sliding resulting in longer processes (del Castillo et al., 2015). Therefore centrospindlin appears to act as a negative regulator of microtubule dynamics perhaps as suggested by del Castillo et al. (2015) by crosslinking the microtubules as occurs during cytokinesis. While microtubule sliding has yet to be demonstrated in vertebrates, conserved mechanisms for axon outgrowth likely operate as depletion of MKLP1 by RNAi in cultured rat neurons also causes longer axes with a greater number of mobile microtubules (Lin et al., 2012). Our results extend this study by showing RacGAP1 also is important for vertebrate axon development providing further evidence that the mechanisms for axon outgrowth are conserved across species.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2016.06.021.

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Mayo Clinic, Rochester, Minnesota, USA.

Mogre

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Mogre

Mogre’s association with MKLP1. However in del Castillo et al., 2015). Therefore centralspindlin appears to act as a negative regulator of microtubule dynamics perhaps as suggested by del Castillo et al. (2015) by crosslinking the microtubules as occurs during cytokinesis. While microtubule sliding has yet to be demonstrated in vertebrates, conserved mechanisms for axon outgrowth likely operate as depletion of MKLP1 by RNAi in cultured rat neurons also causes longer axes with a greater number of mobile microtubules (Lin et al., 2012). Our results extend this study by showing RacGAP1 also is important for vertebrate axon development providing further evidence that the mechanisms for axon outgrowth are conserved across species.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2016.06.021.

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