Gas2l3 is essential for brain morphogenesis and development

Yaara Sharaby a,b, Roxane Lahmi a,b, Omer Amar a,b, Idan Elbaz a,d, Tali Lerer-Goldshtein a,d, Aryeh M. Weiss c, Lior Appelbaum a,d, Amit Tzur a,b,*

a The Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan 5290002, Israel
b Faculty of Engineering, Bar-Ilan University, Ramat-Gan 5290002, Israel
c The Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan 5290002, Israel
d Leslie and Susan Gonda Multidisciplinary Brain Research Center, Bar-Ilan University, Ramat-Gan 5290002, Israel

ABSTRACT

Growth arrest-specific 2-like 3 (Gas2l3) is a newly discovered cell cycle protein and a cytoskeleton orchestrator that binds both actin filament and microtubule networks. Studies of cultured mammalian cells established Gas2l3 as a regulator of the cell division process, in particular cytokinesis and cell abscission. Thus far, the role of Gas2l3 in vivo remains entirely unknown. In order to investigate Gas2l3 in developing vertebrates, we cloned the zebrafish gene. Spatiotemporal analysis of gas2l3 expression revealed a ubiquitous maternal transcript as well as a zygotic transcript primarily restricted to brain tissues. We next conducted a series of loss-of-function experiments, and searched for developmental anomalies at the end of the segmentation period. Our analysis revealed abnormal brain morphogenesis and ventricle formation in gas2l3 knockdown embryos. This signature phenotype could be rescued by elevated levels of gas2l3 RNA. At the tissue level, gas2l3 downregulation interferes with cell proliferation, suggesting that the cell cycle activities of Gas2l3 are essential for brain tissue homeostasis. Altogether, this study provides the first insight into the function of gas2l3 in vivo, demonstrating its essential role in brain development.

© 2014 Elsevier Inc. All rights reserved.

Introduction

Cell division ends as the cytoplasm is halved by the process of cytokinesis and two individual daughter cells are formed. This process requires an orderly rearrangement of the cytoskeletal networks and, ultimately, ensures accurate partitioning of the cell's genetic material, organelles, and biomass (Eggert et al., 2006; Fededa and Gerlich, 2012). Such a maneuver involves many regulatory components that coordinate cytoskeletal elements with membrane structures. This dynamic backbone sets the cell center and division axis, reforms the two daughter cells and, eventually, safely disconnects them.

Growth arrest-specific 2-like protein 3 (Gas2l3) is a newly discovered member of the growth arrest-specific 2 (Gas2) family of cytoskeletal orchestrators. Gas2l3, much like Gas2 (Brancolini et al., 1992) and the other two family members – Gas2l1 and Gas2l2 (Goriou nov et al., 2003) – carries a conserved calponin homology (CH) actin-binding domain and a Gas2-related (GAR) microtubule-binding domain. Relying on these two domains and additional sites located at the protein’s C-terminus, Gas2l3 directly interacts with both actin filaments and microtubules (Stroud et al., 2011). This ability to cross-link different cytoskeletal networks resembles that of spectraplakins – a group of large proteins that associate with all three elements of the cytoskeleton (F-actin, microtubules, and intermediate filaments) and coordinate a wide range of fundamental cellular processes ranging from cell division, migration, and polarization to neuronal pathfinding and overall tissue integrity. Indeed, spectraplakins and other cytoskeleton orchestrators are vital in tissues that preserve elaborate and dynamic cytoskeletal networks, certainly the nervous system (Suozzi et al., 2012).

Gas2l3 is upregulated in proliferating cells and strictly controlled by the cell cycle at the transcriptional and post-translational levels (Pe’er et al., 2013). Gas2l3 associates with the mitotic spindles at metaphase and with the midzone microtubules at telophase (Pe’er et al., 2013; Wolter et al., 2012). By the time the intercellular bridge connecting the daughter cells is formed in late cytokinesis, Gas2l3 is primarily localized to the midbody at two specific spots called constriction sites (Pe’er et al., 2013).

Important progress has recently been made toward understanding the roles played by Gas2l3 in mammalian cells. Down-regulation experiments in cultured cells identified Gas2l3 as a cytokinesis regulator (Wolter et al., 2012), whereas complementary overexpression studies suggested that this protein is involved in cell abscission, i.e., the cutting of the intercellular bridge at the...
constriction sites (Pe’er et al., 2013). The role of Gas2l3 in the physiological context of a developing embryo or mature organism has never been investigated. Utilizing the zebrafish, a well-established model organism for development and human diseases (Kabashi et al., 2011; Kettleborough et al., 2013; Kimmel et al., 1995; Lowery and Sive, 2004), we now show that Gas2l3 is essential for brain morphogenesis and development in vertebrates.

Results

Gas2l3 is functionally conserved in vertebrates

Gas2l3 regulates basic cellular processes, including cytoskeleton organization and cytokinesis. However, the role of this protein in the whole animal is unknown. We, therefore, tested the function of Gas2l3 in zebrafish embryos. To date, the zebrafish gas2l3 orthologue has not been isolated, therefore, we first cloned the coding sequence of zebrafish gas2l3 (accession no. JX013520) (throughout the text, zebrafish gas2l3 will be referred to as gas2l3). gas2l3 open reading frame (ORF) consists of 1650 base pairs, encoding a 550 amino acids (aa) protein with a highly conserved N-terminus in vertebrates (human-to-fish identity/similarity is 66.6% and 81.3% respectively) and overall human-to-fish identity of 39.6% (Fig. 1A). Following acceptable categorization, human Gas2l3 N-terminus (aa 1–290) carries the CH domain and most, if not all, of the GAR domain. Gas2l3 C-terminus exhibits 20.7% human-to-fish identity (33.4% similarity). Despite this relatively low conservation, this part of the protein seems important for microtubule association (Stroud et al., 2011). Although we could not unequivocally specify Gas2l3 orthologues in invertebrates, Gas2l3 N-terminus could be found in a wide range of lower metazoans, including sponges. This observation indicates high selectivity/similarity of Gas2l3 C terminus, which seems to be a “recent” addition – shared by vertebrates – to an ancient N-terminus fragment.

Mammalian Gas2l3 protein associates with both microtubules and F-actin networks during interphase. More specifically, Gas2l3 is localized to the constriction sites in late cytokinesis. The latter is a unique feature shared by only a few proteins (Pe’er et al., 2013). We tagged gas2l3 with EGFP in order to monitor the subcellular localization of the zebrafish protein in cells. Human HeLa cells were transfected with EGFP-gas2l3 and fixed. The cells were also labeled with either anti-Tubulin or phalloidin for visualizing the microtubules and F-actin, respectively. In Fig. 1, we show that the zebrafish Gas2l3, much like the human protein, is colocalized with both the microtubule (Fig. 1B) and F-actin (Fig. 1C) networks. Furthermore, the distribution of the conserved Gas2l3 N-terminus carrying both the CH and GAR domains closely resembled only that of actin filaments (Fig. 1D, left panels). In contrast, Gas2l3 C-terminus appeared mostly nuclear, nevertheless maintained a week non-active distribution that partly overlaps with microtubule structures (Fig. 1D, mid and right panels).

We next examined whether Gas2l3 association with the constriction sites is preserved across vertebrates. The constriction sites are the narrowest spots across the intercellular bridge. Once the midbody is formed, human Gas2l3 at the constriction sites is flanked by the cytokinesis regulator Aurora B (Pe’er et al., 2013). At these specific sites, Aurora B (as well as Tubulin) is faintly labeled, perhaps because of the overly packed microtubules at these locations that may limit antibody accessibility (Hu et al., 2012; Pe’er et al., 2013). In practice, this labeling pattern marks the constriction sites (Pe’er et al., 2013). We show that zebrafish Gas2l3, when fused to EGFP at either N-terminus (Fig. 1F) or C-terminus (Fig. S1) maintained its unique property as a constriction site-associated protein. These results suggest that Gas2l3 is functionally conserved across vertebrates, at least at the cellular level.

Gas2l3 is a maternal gene

During earlier embryonic development, the cell cycle is rapid and synchronous. At this stage, the transcription machinery is turned off, thus, all transcripts are maternally inherited. Once embryos pass the mid-blastula transition (MBT), zygotic transcription commences (Newport and Kirschner, 1982a,b). Gas2l3 regulates the basic process of cell division; nevertheless, as proven for other global, cell cycle regulators (e.g., Cdh1 (Lorca et al., 1998)), this may not guarantee its presence pre-MBT.

As a first step toward elucidating the function of gas2l3 in vivo, we examined the gene’s transcription pre- and post-MBT using whole-mount in situ hybridization with gas2l3-specific antisense and sense (control) probes. In zebrafish, MBT occurs at the tenth cell cycle, approximately 2.75 h post-fertilization (hpf) (Kane and Kimmel, 1993). We showed that 8-cell stage embryos are positively stained for gas2l3 transcripts, indicating that gas2l3 is a maternally expressed gene (Fig. 2A).

Gas2l3 is essential for brain morphogenesis and development

We next studied the pattern of gas2l3 expression in post-MBT embryos. A strong and specific signal indicating zygotic gas2l3 transcript was observed in 3-hpf embryos and during the gastrulation stages (6- and 9-hpf). At these three stages, gas2l3 expression was ubiquitous (Fig. 2B). Interestingly, starting at 13 hpf (arrow) and throughout the segmentation period, gas2l3 transcript was found to be highly enriched in the three main sections of the brain (forebrain [FB], midbrain [MB] and hindbrain [HB]); however, it seemed undetectable in the spinal cord or elsewhere. gas2l3 expression was more pronounced in the telencephalon (TL), the region of the olfactory placodes (OP), and along the cranial neural tube surrounding the brain ventricles (Fig. 2C). gas2l3 expression remained primarily brain-specific also in 48-hpf embryos (data not shown). This spatiotemporal pattern suggests a hitherto unappreciated role of gas2l3 in brain development.

In order to address the in vivo function of Gas2l3, we performed loss-of-function experiments using Morpholino antisense oligonucleotide (MO). We designed an MO that blocks splicing at the intron 2/exon 3 junction of the gas2l3 pre-mRNA, resulting in aberrant intron 2 at the mature mRNA and, consequently, a premature stop codon that terminates translation at the tenth amino acid (Fig. 3A). MO ability to specifically block gas2l3 splicing was validated by PCR (Figure S2). Zebrafish embryos were injected with gas2l - or control MO at the one-cell stage and examined for developmental abnormalities. At 24 hpf, gas2l3 morphants exhibited abnormal head morphology. This phenotype could be attributed to profound brain malformations. We categorized malformations as mild or severe (representative embryos of each phenotype class are depicted in Fig. 3B). Several characteristic phenotypes were observed: (1) crooked, narrow, and asymmetric neural tubes (panels vii–ix), evidence of the defective neural tube morphogenesis; (2) reduced ventricular space in the three main parts of the brain (panels i–vi) (note the moderate and extreme reduction in brain ventricular space in the representative mild and severe morphants); and (3) abnormal protrusion in the region of the telencephalon (black arrows).

While all control embryos exhibited normal morphology at 24 hpf, nearly 70% of the gas2l3-MO-injected embryos exhibited the characteristic brain malformations (Fig. 3C). Doubling the amount of injected MO from 1 to 2 pmol increased the proportion of morphants to over 83%. Importantly, co-injecting gas2l3 MO with gas2l3 mRNA or EGFP-gas2l3 mRNA, but not with EGFP mRNA,
successfully lowered the frequency of embryos displaying signature phenotypes by more than two fold (Fig. 4C; p < 0.001, ANOVA, Bonferroni). The ability of gas2l3 mRNA to rescue the phenotype in MO-injected embryos strongly suggests that the observed brain malformations were specific to gas2l3 loss-of-function. Consequently, these results implicate gas2l3 in the regulation of brain morphogenesis and development. An analogous set of brain malformations was observed in embryos injected with gas2l3(ATG)MO, which blocks the translation of gas2l3 transcripts (Fig. S3). Similar to the splicing gas2l3 MO, this phenotype was specific and could be rescued by both gas2l3- and EGFP-gas2l3 mRNAs (Fig. S3B).

Reduced ventricular size was a phenotype shared by all gas2l3 morphants (Fig. 3). Further analysis of the forebrain and hindbrain ventricles in 24-hpf embryos demonstrated abnormal ventricle formation. Often, the ventricles barely opened or remained entirely closed across a large portion of the cranial neural tube (Fig. 4A). We measured the width of the ventricle at maximum opening and used this value as a metric to approximate the ventricle-formation defect. As shown in Fig. 4B, gas2l3 knockout embryos exhibit over a 50% reduction in forebrain and hindbrain maximum ventricle opening. This two-fold reduction, as measured one-dimensionally, reflects a larger reduction in the actual ventricle space (cubic ratio). Moreover, because some sections across the neural tube opened slightly or remained entirely closed in typical gas2l3 morphants (Fig. 4A), the actual reduction of the ventricular space in gas2l3 morphants is, in practice, even higher.

Low cell proliferation rate during brain development will naturally result in a shortfall of cell number and, eventually, reduced brain size, a characteristic phenotype of the gas2l3 morphants at 24 hpf (Fig. 3). Moreover, stalled cell proliferation at the neural tube is known to induce developmental delay and abnormal brain ventricle formation (Lowery and Sive, 2005), another characteristic phenotype of the gas2l3 morphants (Fig. 4A and B). Gas2l3 is a canonical cell cycle protein, with proven roles in mammalian cell division (Pe’er et al., 2013; Wolter et al., 2012). We, therefore, asked whether Gas2l3 deficiency interferes with cell proliferation in vivo, particularly at the neural tube.
High-resolution images of the developing brain of gas2l3 morphants expressing CAAX-GFP (a cell membrane marker) confirmed the abnormal ventricular formation phenotype observed by lower-resolution imaging (Fig. 4A and C). More importantly, the cells along the cranial neural tube of gas2l3 morphant were fewer in number, elongated, and seemed larger in size (as estimated by cell area, not volume) compared to the control embryos (Fig. 4C). See, for example, the magnified regions A and B at the mid- and hindbrains of a representative control embryo and gas2l3 morphant (Fig. 4C, lower panels). Cells that were located at both sides of the neural tube in regions that failed to open appeared interconnected (see magnified region B).

We next tested the mitotic index in gas2l3 morphants, focusing on 2 brain sections at 24 hpf: one section included the forebrain and a portion of the midbrain while the second section included the hindbrain. To this end, whole-mount immunocytochemistry using anti-phosphorylated-histone H3 (PH3) antibodies, was performed to label mitotic cells (Hendzel et al., 1997; Hong and Dawid, 2011) (Fig. 4D and S4). The number of mitotic cells (normalized to area) in the 2 brain sections of gas2l3 morphants was significantly lower compared to that in the control embryos (2-fold reduction in the fore/mid brain and 1.5-fold reduction in the hindbrain; \( p < 0.02 \); Mann–Whitney U test (Fig. 4D and E)). The mitotic index was also determined for the eye. Similar to the brain, eyes are early-developed organs. Nevertheless, despite the close proximity to the brain, eye morphology appeared nearly unaffected in both mild and severe gas2l3 morphants (Fig. 3). As suspected, the mitotic index in the eyes of gas2l3 morphants was insignificantly different from that of the control group (\( p > 0.2 \); Mann–Whitney U test) (Fig. 4E). These results emphasize the specific and critical role gas2l3 plays in brain tissue homeostasis and development.

Cell proliferation balanced by cell death determines tissue homeostasis. We, therefore, tested whether gas2l3 loss-of-function also influences cell death during early brain development. A TUNEL assay was performed in 24-hpf embryos following injection with either gas2l3- or control MO. Images of representative embryos are shown (Fig. 4F). A noticeable reduction in apoptotic cells appeared anterior to the mid-hindbrain boundary in all control embryos and gas2l3 morphants (marked by a white bracket). This observation is in agreement with the literature (see, for example, (Lowery and Sive, 2005)) and, in fact, indicates that neither gas2l3- nor control MO induces ubiquitous non-specific cell death in zebrafish embryos. The anterior margin of this brain section (yellow dotted line) marked the posterior boundary for the analysis. The apoptotic index ratio between gas2l3 morphants and control embryos was found to be 1.16. This insignificantly different (\( p > 0.38 \)) suggests that cell death is unlikely to be a central aspect of the gas2l3-mediated regulation of brain tissue homeostasis.

Altogether, these results present gas2l3 as an indispensable factor for tissue homeostasis in the developing brain, and are in agreement with the protein cell cycle activity, its expression pattern in vivo (Fig. 2), and its function in brain morphogenesis (Figs. 3 and 4).
Gas2l3 is essential for brain morphogenesis and development. (A) The zebrafish gas2l3 gene consists of 9 exons interspersed with 8 introns. Black square indicates target sequence of gas2l3 splicing-block MO. Gray arrow indicates transcript direction. Black arrows indicate forward and reverse primers designed to test MO efficiency (see Fig. S2 for more information). (B) One-cell stage embryos were injected with 2 pmol gas2l3 MO or standard control MO. Representative images of MO-injected embryos (mild or severe morphants), alongside an uninjected embryo (normal), were taken at 24 hpf. Panels ii, iv, and vi are magnified images of panels i, iii, and v respectively. Matching dorsal views are depicted in panels vii, viii, and ix. FB, forebrain; MB, midbrain; HB, hindbrain. Black arrows point toward abnormal forebrain protrusions. (C) Embryos were injected with gas2l3 MO (1 or 2 pmol) or control MO (2 pmol) in combination with mock, gas2l3 RNA, EGFP-gas2l3 RNA, and EGFP RNA (100 pg). Injected embryos were categorized as normal or abnormal according to their brain morphology at 24 hpf. Values are shown as mean normal or abnormal percentages ± SEM of abnormal. We used the ANOVA Bonferroni test in order to determine the statistical significance of the results; Means with distinct letters on top of the chart are significantly different ($p < 0.001$, $F=25.846$, $df=5$, 31). Following microinjection, dead, mechanically damaged embryos or embryos with unspecified morphology were excluded from the analysis.
Discussion

In this study, we determined the first in vivo activity of gas2l3 in vertebrates. We studied the first day of development, focusing on 24 hpf. During this period, there is intensive proliferation in neuronal tissues that is balanced by tightly regulated cell death. At the cellular level, Gas2l3 is a cell cycle protein with a significant role in mammalian cell division (Pe'er et al., 2013; Wolter et al., 2012). RNAi experiments also suggested that Gas2l3 might promote irradiation-mediated apoptosis (Asai et al., 2012). Focusing on developing brain tissues, we did not detect an abnormal apoptotic index in gas2l3 knockdown embryos. However, the mitotic index in brain tissues was significantly reduced, in particular in the forebrain (Fig. 4). This phenotype is in agreement with the noticeable enrichment of the gas2l3 transcript in this particular brain section (Fig. 2). It is plausible that the role of gas2l3 in brain development is attributed to its cell cycle activity per se. That said, the established link between cell cycle proteins, such as Gas2l3, and brain...
development, seems to go beyond the trivial implications of the cell cycle and cell proliferation in tissue homeostasis. Geminin is an example of a canonical cycle protein (McGarry and Kirschner, 1998) that functions as a neutralizing factor (Caronna et al., 2013; Kroll et al., 1998; Seo et al., 2005; Yellajoshyula et al., 2012). Geminin also demonstrates that maternal proteins can be dispensable pre-MBT despite a fundamental role in cell cycle regulation (Kroll et al., 1998; McGarry, 2002). Perhaps a more relevant example for the diversified roles of cell cycle proteins in brain development is MCPH proteins; at the cellular level, this family of proteins regulates the chromosome and centrosome cycles, whereas at the whole organism level, MCPH genes are susceptibility alleles for autosomal recessive primary microcephaly (Mahmood et al., 2011). Interestingly, like Gas2l3, MCPH proteins (e.g., microcephalin and Stil), as well as Geminin, are regulated by mitotic phosphorylation, and a proteasome-mediated degradation, as regulated by the anaphase-promoting complex/cyclosome (APC/C) ubiquitin ligase (Arquint et al., 2012; Dephoure et al., 2008; McGarry and Kirschner, 1998; Ooi, 2008; Pe’er et al., 2013). These recent findings highlight an important relationship between general cell cycle mechanisms and brain morphogenesis.

The phenotype of abnormal ventricles in gas2l3 knockdown embryos is particularly intriguing considering the role cytokinesis plays in the opening and expansion of brain ventricles in zebrafish (Buckley et al., 2013; Ciruna et al., 2006) and the fact that Gas2l3 is a cytokinesis regulator (Pe’er et al., 2013; Wolter et al., 2012). At this juncture, it is also important to note the role of cytokinesis in asymmetric division, which set the balance between progenitor and differentiated cells with relevance to neuronal tissue patterning and morphogenesis (Alexandre et al., 2010; Shitamukai and Matsuzaki, 2012). Perhaps Gas2l3, much like the cytokinesis regulator RhoA (Rosko et al., 2006), contributes to the delicate balance between symmetric and asymmetric cell division so critical for neural tube formation.

As a cytoskeleton orchestrator, Gas2l3 is also likely to regulate cytoskeleton dynamics post-mitotically. This set of nonproliferative activities can be relevant for processes such as cell movement. In this context, gas2l3 expression in the olfactory placode region (Fig. 2) caught our attention because of the limited cell proliferation and intense cell rearrangement characterizing these emerging organs (Whitlock, 2004). Furthermore, the APC/C, which regulates Gas2l3 levels in proliferating cells (Pe’er et al., 2013), also mediates post-mitotic neuronal activities, such as differentiation, plasticity, and connectivity (Yang et al., 2010). Whether post-mitotic regulation of Gas2l3 coordinates cellular and tissue dynamics in the developing and mature brain is currently unknown.

Material and methods

Plasmids and RNA constructs

Total RNA from 24 hpf TL (wt) embryos was extracted (Qiagene) for cDNA preparation (Superscript III, Invitrogen). zfgas2l3 ORF was amplified by PCR using primers 5′-CTATGATGCCCCTTACAG-3′ (forward) and 5′-CTTAGTCCTCCTCCTTC-3′ (reverse). The PCR product was cloned into a pGEM T- Easy vector (Promega) and fully sequenced. The gas2l3 ORF sequence is deposited in NCBI (XM13520). gas2l3 ORF was amplified with primers flanked by FseI (forward) and Ascl (reverse) sites, and cloned into the pCS2-FA vector. gas2l3 ORF was also amplified with primers flanked by FseI (forward) and BamHI (reverse) sites or XhoI (forward) and Ascl (reverse) sites to generate gas2l3-EGFP and EGFP-gas2l3 in a pCS2-FA vector; respectively, gas2l3 constructs encoding for Gas2l3-C (aa 1–298) and N- (aa 293–550) termini were cloned into pCS2-EGFP vector using Xhol and Ascl sites. pCS2-GFP and pCS2-GFP-CAAX were a gift from Dr. Marc Kirschner (Harvard Medical School, Boston, MA).

HeLa cells were maintained in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco), penicillin, and streptomycin (Gibco) at 37 °C, 5% CO2. Immunoﬂuorescence experiments were conducted as follows: HeLa cells were grown on coverslips (1.5), transfected using metafectene® (Biontex), and ﬁxed in 4% paraformaldehyde (PFA) for 20 min at RT. The ﬁxed cells were then blocked (0.1% BSA, 0.1% Triton X-100, in phosphate-buffered saline [PBS]) for 1 h, and incubated overnight with primary antibodies (mouse monoclonal anti-α Tubulin [DSHB, 12G10]; rabbit polyclonal anti-Aurora B [Abcam, ab2254]) at 4 °C or, alternatively, for 60 min at RT. After washing, coverslips were incubated with the secondary antibodies: Alexa Fluor A11029 or A11008 (Molecular Probes). Coverslips were mounted on slides with Immu-Mount mounting solution (Thermo Shandon Limited) and DAPI (5 μg/ml, Sigma-Aldrich), and sealed. Actin ﬁlaments were detected in ﬁxed cells using phallolidin CF 594 (Biotium), following a standard labeling protocol. We used AxioImager.Z1 upright and AxioObserver.M1 inverted ﬂuorescence microscopes (Carl Zeiss, Inc.) equipped with 100× oil immersion lens objectives (NA 1.4) for imaging, and AxioVision Rel 4.8 (Carl Zeiss, Inc.) and ImageJ (NIH) software for image analysis.

Zebrafish husbandry

Adult AB (wt) zebrafish were raised and maintained in fully automated housing systems (Aquazone, Israel; temperature 28 ± 0.5 °C, pH 7.0, conductivity 300 μS) under 14 h light/10 h dark cycles, and fed twice a day. Embryos were generated by natural spawning and raised in egg water at 28 ± 0.5 °C. All animal protocols were reviewed and approved by the Bar-Ilan University Bioethics Committee.

Whole-mount in situ hybridization

Probe preparation: Linear pGEM-T-Easy-gas2l3 construct served as a template to transcribe Digoxigenin-labeled sense (sp6) or antisense (T7) mRNA probes (DIG RNA labeling kit [Roche, Basel, Switzerland]).

Dechorionated embryos were ﬁxed in 4% PFA (20 min, RT; 8-cell-stage- and 3-hpf- embryos; overnight, 4 °C; 6-, 9-, and 24-hpf-embryos), washed in PBS, and stored in 100% methanol. The embryos were then rehydrated with a reduced methanol concentration, and incubated in 10 μl/ml proteinase K (PK) for 2–10 min (8-cell stage- and 3-hpf-embryos were not treated with PK). All samples were prehybridized at 60- (9–48 hpf) or 70 °C (8-cell stage and 3 hpf) for 1 h, and incubated overnight with 1.6 ng/ml DIG-labeled sense or antisense riboprobes at prehybridization temperature. We next rinsed the samples twice with 50% formamide in 2 × SSC (30 min), once with 2 × SSC (15 min), and twice with 0.2 × SSC (30 min). The embryos were blocked (1 h, RT) with 5% normal goat serum in PBT buffer (0.1% tween 20 in PBS), and incubated for 4 h with DIG antibody (Roche) conjugated to alkaline-phosphatase, diluted 1:2500 (0.06 u final). Samples were rinsed twice (10 min) with staining buffer (Roche), and incubated with BM purple solution (Roche) at 37 °C in a dark incubator. Once the staining was revealed, samples were rinsed three times with PBT (5 min), and stored in 100% glycerol at 4 °C. Imaging was performed using...
an M165 FC stereomicroscope (Leica, Wetzlar, Germany), and image processing was carried out using ImageJ (NIH).

Morpholino design, preparation, and injection

Gene knockdown experiments were performed using MO modified antisense oligonucleotides (MO, Gene Tools, LLC). gas2/3(I2E3)MO (5′-accaagctggaaagagggaga-3′) was designed to interfere with the splicing of the second intron/exon junction, thus introducing a premature stop codon. Splicing interference was validated using cDNA (Superscript III, Invitrogen) from gas2/3 (I2E3)MO- and standard control (GeneTool, LLC) MO-injected embryos as templates (Fig. S2). In all knockdown experiments, one-cell stage wild-type embryos (wt, AB) were microinjected with 1–2 pmol of gas2/3(I2E3)MO solution. In rescue experiments, 100 pg of in-vitro transcribed gas2/3, EGFP-gas2/3, or EGFP mRNAs were co-injected with gas2/3(I2E3)MO. Embryos were microinjected 2–24 h post-injection by an M167FC stereomicroscope (Leica). For high resolution imaging of living embryos, 2 pmol gas2/3(I2E3)MO and 100 pg GFP-CAAX RNA co-injected embryos were anesthetized with Tricaine (0.01%), placed in low-melting agarose (0.5–1.0%) wells present on a glass-bottom dish, and imaged by a Zeiss LSM780 upright confocal microscope (Zeiss).

Throughout this study, we used a micromanipulator and a PV830 pneumatic pico pump (World Precision Instruments) for microinjections, ImageJ software (NIH) for image processing and analysis, and the one-way analysis of variance (ANOVA) with Bonferroni post-hoc test for determining the statistical significance of the results (unless otherwise is indicated).

Whole mount immunostaining and TUNEL assay

Fish embryos were dechorionized, fixed (4% PFA) overnight at 4 °C, washed with PBST (3×5 min), permeabilized (proteinase K [TUNEL] or acetone, −20 °C [immunostaining]), and washed again. For monitoring the mitotic index, embryos were incubated (2 h, RT) in blocking serum (20% normal goat serum, 0.5% triton in PBS), and labeled (ON, 4 °C) with anti-phosphorylated-Histone H3 antibodies (Cell Signaling; #9706; 1/200 dilution in blocking solution) and Alexa Fluor 594 secondary antibodies (Jackson Immuno-Research; #115-585-003). For monitoring the apoptotic index, the fixed embryos were incubated with TUNEL (Tdt-mediated dUTP nick end labeling) solution (In Situ Cell Death Kit, TMR red [Roche Diagnostics]) for 1 h at 37 °C, and washed 3 times with PBS. Fixed embryos were placed on coverslips for confocal imaging (Zeiss LSM780 upright confocal microscope; 20× lens). Images were processed using ImageJ software. The mitotic index was determined from the mid z-section. The apoptotic index was determined from the z-section with the highest number of dead cells.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.08.006.

References


Ooi, L.Y., 2008. The role of microcephalin in cell cycle regulation and embryonic development. Harvard University, Boston, MA, USA.


