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***cables1* Is Required for Embryonic Neural Development: Molecular, Cellular, and Behavioral Evidence From the Zebrafish**

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SUMMARY

In vitro studies have suggested that the *Cables1* gene regulates epithelial cell proliferation, whereas other studies suggest a role in promoting neural differentiation. In efforts to clarify the functions of *Cables1* in vivo, we conducted gain- and loss-of-function studies targeting its ortholog (*cables1*) in the zebrafish embryo. Similar to rodents, zebrafish *cables1* mRNA expression is detected most robustly in embryonic neural tissues. Antisense knockdown of *cables1* leads to increased numbers of apoptotic cells, particularly in brain tissue, in addition to a distinct behavioral phenotype, characterized by hyperactivity in response to stimulation. Apoptosis and the behavioral abnormality could be rescued by co-expression of a morpholino-resistant *cables1* construct. Suppression of p53 expression in *cables1* morphants partially rescued both apoptosis and the behavioral phenotype, suggesting that the phenotype of *cables1* morphants is due in part to p53-dependent apoptosis. Alterations in the expression patterns of several neural transcription factors were observed in *cables1* morphants during early neurulation, suggesting that *cables1* is required for early neural differentiation. Ectopic overexpression of *cables1* strongly disrupted embryonic morphogenesis, while overexpression of a *cables1* mutant lacking the C-terminal cyclin box had little effect, suggesting functional importance of the cyclin box. Lastly, marked reductions in p35, but not Cdk5, were observed in *cables1* morphants. Collectively, these data suggest that *cables1* is important for neural differentiation during embryogenesis, in a mechanism that likely involves interactions with the Cdk5/p35 kinase pathway.

INTRODUCTION

The CDK5 and ABL1 Enzyme Substrate 1 (*Cables1/ik3-1*) gene, a member of the cyclin superfamily, was identified in two independent screens for genes that interact with the cyclin-dependent kinase (Cdk) family of serine/threonine protein kinases (Matsuoka et al., 2000; Zukerberg et al., 2000). In a yeast-two hybrid screening assay, Cables1 protein interacted strongly with Cdk5 (a non-cell cycle-associated kinase required for neural differentiation), while weaker interactions were also observed with cell cycle-associated kinases Cdk2 and Cdk3. Subsequent in vitro studies utilizing COS7 cells, E15 mouse brain lysates and cultured primary rat cortical neurons suggested that Cables1 can function in two distinct cellular contexts: Firstly, Cables1 promoted protein-protein interactions among c-ABL kinase, Cdk5 and p35 that enhanced tyrosine phosphorylation of Cdk5 (Zukerberg et al., 2000); secondly, Cables1 could negatively regulate cell proliferation, by augmenting inhibitory phosphorylation cascades that suppress cyclin-dependent kinase activity (Wu et al., 2001). While informative, these in vitro studies have yet to provide definitive insights into the functions of the *Cables1* gene in vivo, particularly during early development.

Although *Cables1* knockout mice are viable, early (non-congenic) strains were notably subfertile, with evidence of fetal resorption in pregnant dams (Zukerberg et al., 2004). These observations suggest that embryonic development is compromised in *Cables1* knockouts. Interpretation of these data remains difficult, however, due to the potential redundancy of a related gene (*Cables2*) in mice. In light of multiple studies reporting a loss of Cables1 expression in selected tumor types (Wu et al., 2001; Dong et al., 2003; Tan et al., 2003; Zukerberg et al., 2004; DeBernardo et al., 2005; Zhang et al., 2005; Kirley et al., 2005a; Park do et al., 2007) there remains a strong rationale to clarify the in vivo functions of the *Cables1* gene.

To broaden our understanding of *Cables1* functions in vivo, we have performed gain- and loss-of-function studies of the zebrafish *Cables1* ortholog (*cables1*; NM_001105665.1). Importantly, the lack of a functional paralog in this species permitted a direct investigation of *cables1* functions in vivo. The data from these studies support the notion that *cables1* is critically important for neural development in the zebrafish embryo. The developmental impact of *cables1* suppression appears substantially more dramatic than that observed in murine or avian models (Zukerberg et al., 2000; Rhee et al., 2007). In the zebrafish, knockdown of *cables1* results in disruptions in the expression of multiple neural transcription factors, increased apoptosis in embryonic neural tissues, and subsequent behavioral defects characterized by hyperactivity in response to stimulation. In addition, we present evidence suggesting that the embryonic functions of *cables1* likely involve interactions with the Cdk5/p35 kinase pathway. Collectively, these data from the zebrafish model provide in vivo evidence supporting an essential functional role for *cables1* during embryonic neural differentiation.

RESULTS

Embryonic *Cables1* mRNA Expression

cables1 mRNA was detected by RT-PCR throughout embryogenesis (Fig. 1A), including one cell-stage embryos (0 hr post-fertilization, hpf), indicating that *cables1* mRNA is provided to embryos as a maternal transcript. By in situ hybridization, *cables1* mRNA was detected most robustly in anterior neural tissues (Fig. 1B,C). Expression in neural tissues was confirmed by cryosectioning embryos after in situ hybridization (Fig. 1D).

Brain Necrosis in *cables1* Morphants

Embryos microinjected at the one-cell stage with a control morpholino oligonucleotide (MO) were indistinguishable from wild-type (non-injected) embryos (Fig. 2A). By contrast, microinjecting a morpholino oligonucleotide targeting zebrafish *cables 1* (cabMO; 2 ng/embryo) led to visible developmental defects, most notably affecting anterior (neural) tissues. By 24 hpf, the most readily observable phenotype was the appearance of cloudy (necrotic) tissue in midbrain and hindbrain (Fig. 2B); in a representative experiment, this phenotype was observed in 81% (58/72) of morphants, in a pattern that was highly consistent among injected embryos.

To ensure target specificity of cabMO, rescue experiments were performed using a MO-resistant mRNA construct (*cab^{RESC}*) encoding the complete open reading frame of *cables1*. Co-injection of *cab^{RESC}* (15 pg/embryo) into cabMO-injected embryos markedly attenuated the brain phenotype; substantially fewer embryos (25/78, 32%) showed evidence of brain necrosis (Fig. 2C), while the proportion of embryos resembling control-injected embryos more than doubled (29/78; 37%, vs. 17% for cabMO-injected embryos). The remaining embryos showed mild overexpression phenotypes (described below).

Increased Apoptosis in *cables1* Morphants

It was previously reported that *cables1* can modulate apoptosis, via both p53-dependent and p53-independent pathways (Tsuji et al., 2002). To determine whether or not the necrotic appearance of brain tissues in *cables1* morphants resulted from increased apoptosis, embryos were assayed for DNA fragmentation at 18 hpf using the terminal dUTP nick-end labeling (TUNEL) assay. Relative to control-injected embryos (Fig. 3A), a significant increase was observed in the mean number of TUNEL-positive cells (TUNEL index, see Materials and Methods Section) in *cables1* morphants (Fig. 3B; $P < 0.001$), with the highest intensity of TUNEL-positive cells observed in regions corresponding to areas of necrotic degeneration (Fig. 2B).

We next examined whether or not the increased apoptosis could be rescued by co-expressing *cab^{RESC}*. As shown (Fig. 3C,E), the number of TUNEL-positive cells in cabMO-injected embryos co-injected with *cab^{RESC}* was significantly reduced relative to embryos injected with cabMO alone ($P < 0.001$). The TUNEL index of *cab^{RESC}*-injected embryos also did not differ significantly from the mean TUNEL index in control-injected embryos ($P > 0.05$), indicating a robust rescue of the phenotype.

To determine if the increased number of TUNEL-positive cells in cabMO-injected embryos was mediated by a p53-dependent apoptotic pathway, endogenous p53 expression was suppressed in cabMO-injected embryos by co-injection with a MO targeting p53 (p53MO). Co-injection of p53MO (Fig. 3D) markedly rescued apoptosis in cabMO-injected embryos, with significantly fewer TUNEL-positive cells detected relative to embryos injected with cabMO alone ($P < 0.01$). The mean TUNEL index of p53MO co-injected embryos was similar to the mean TUNEL index of *cables1* morphants co-injected with cab^{RESC} ($P > 0.05$), but was still significantly greater ($P < 0.05$) than the mean TUNEL index of control-injected embryos. These data indicate a significant, but incomplete, rescue of apoptosis by suppressing p53 expression.

Behavioral Abnormalities in *cables1* Morphants

A distinct behavioral phenotype was subsequently observed in *cables1* morphants allowed to develop to the hatching stage (~48 hpf). At this stage of development, wild-type embryos exhibit a rapid and short-lived motor response (burst-swimming) following stimulation (Granato et al., 1996). In contrast to control-injected and wild-type embryos, *cables1* morphants exhibited a notably sustained motor response following stimulation (not shown). To investigate this behavioral phenotype in greater detail, we employed an automated behavioral assay (Photomotor Response, PMR) that provides a quantitative readout of behavior (movement) in response to photostimulation. Activity measurements were made over a 3-sec interval within the excitation phase, as previously described (Kokel et al., 2010).

Control-injected embryos exhibited a PMR (Fig. 4A; Supplementary Movie 1) that is typical of equivalent stage wild-type embryos at all stages examined (Kokel et al., 2010). In marked contrast, *cables1* morphants (Fig. 4B,D; Supplementary Movie 2) displayed a significantly increased PMR relative to control-injected embryos ($P < 0.001$).

To determine whether the aberrant PMR could be attributed to a loss of *cables1* expression or not, PMR measurements were made in *cables1* morphants co-injected with mRNA encoding cab^{RESC}. As shown (Fig. 4C,D), the mean PMR of cabMO-injected embryos co-injected with cab^{RESC} was significantly reduced relative to embryos injected with cabMO alone ($P < 0.05$), and did not differ significantly from the PMR of control-injected embryos ($P > 0.05$). Collectively, these data (Fig. 4D), strongly suggest that the aberrant behavioral phenotype is attributable to a loss of *cables1* expression.

We next sought to determine whether or not the aberrant behavioral phenotype in *cables1* morphants was associated with the observed increased in p53-dependent cell death, by measuring the PMR in *cables1* morphants co-injected with p53MO. Similar to the above experiment, injection of cabMO (Fig. 4F) resulted in a significant increase ($P < 0.001$) in the mean PMR relative to control-injected embryos (Fig. 4E). Co-injection with p53MO (Fig. 4G) significantly reduced the mean PMR ($P < 0.05$) relative to cabMO-injected embryos. However, the mean PMR of p53MO co-injected embryos was still significantly higher ($P < 0.01$) than the mean PMR of control-injected embryos, suggesting that the behavioral defects in *cables1* morphants are only partially attributable to an increase in p53-dependent apoptosis. These data are summarized in Figure 4H.

Neural Tube Patterning Defects in *cables1* Morphants

In efforts to narrow down the origins of the neural defects in *cables1* morphants, we examined the expression of a selection of marker genes by in situ hybridization. To ensure that p53-dependent cell death did not contribute to any observed changes in marker gene expression, control and morphant embryos were co-injected with p53MO. A minimum of 20 embryos (10 treatments and 10 controls) were examined for each marker gene. As shown (Fig. 5), distinct alterations in the expression of selected neural transcription factors were observed in *cables1* morphants. We consistently observed reductions in the expression of *emx3* (presumptive forebrain), *eng2a* (midbrain/hindbrain boundary), and *gsc* (prechordal plate, diencephalon), but increased expression of *otx2* (anterior neural plate). In contrast, the expression patterns of *pax2a* (midbrain/hindbrain boundary) and *ntl* (axial mesoderm) were unchanged among control-injected embryos and *cables1* morphants.

Cyclin Box Confers Biological Activity to *Cables1*

To further investigate the in vivo biological activity of *cables1*, we performed ectopic overexpression experiments in zebrafish embryos. Injection of synthetic mRNA (100 pg/embryo) encoding full-length *Cables1* protein potently disrupted embryonic morphogenesis (Fig. 6). In contrast to embryos injected with control mRNA (GFP, 100 pg/embryo; Fig. 6B), of which only 4/48 (8%) showed mild phenotypic defects, embryos injected with full-length *cables1* mRNA had a very high frequency (39/45, 87%) of morphological defects. These ranged from mild/moderate axis defects (Fig. 6C,D) to complete dysmorphogenesis (Fig. 6E).

The region of the *Cables1* protein most highly conserved among species is the C-terminal cyclin box (CB; amino acid residues 413–501 of zebrafish *Cables1*), which is presumed to mediate its interactions with cyclin-related proteins. To determine whether or not this region confers biological activity to zebrafish *Cables1*, we microinjected synthetic mRNA (100 pg/embryo) encoding a truncation mutant of *Cables1* protein (ΔCB) that lacks the cyclin box. Overexpression of ΔCB in zebrafish embryos resulted in a distribution of phenotypes that differed markedly from embryos-injected with full-length *cables1* mRNA (Fig. 6F); 73% (33/45) of embryos developed completely normally, 20% (9/45) had very mild phenotypic defects, and only 4% (2/45) showed severe effects.

p35 Expression Is Reduced in *cables1* Morphants

The potent biological activity of the *cables1* cyclin box suggests that interactions with cyclin-related proteins are an important aspect of *cables1* function. The neural defects in *cables1* morphants are further suggestive of an interaction with Cdk5, with which *cables1* was first shown to interact in a yeast two-hybrid screen (Zukerberg et al., 2000). To determine if a loss of *cables1* affects the Cdk5/p35 kinase pathway in vivo, we used Western immunoblot analysis to examine protein levels of both Cdk5 and p35 in whole embryo lysates after *cables1* knockdown. No significant differences were detected in Cdk5 protein levels between control-injected embryos and *cables1* morphants (Fig. 7); by contrast, levels of p35 in *cables1* morphants showed a significant (~65%) reduction relative to control-injected embryos ($P < 0.01$).

Cell Proliferation Is Unaffected in *cables1* Morphants

Previous studies have suggested an inhibitory function for *cables1* in epithelial cell proliferation (Wu et al., 2001; Kirley et al., 2005b). In our knockdown studies, *cables1* morphants progressed through early stages of embryonic development (blastula, epiboly, gastrulation) at a similar rate as control-injected embryos, as evidenced by their simultaneous onset of somitogenesis (Fig. 8A,B). While this observation suggests that cell cycle progression is not affected in *cables1* morphants, we opted to examine cell proliferation dynamics in more detail during early development. The number of mitotic cells was quantified in control-injected and *cables1* morphants at the shield stage (6 hpf), using immunohistochemical staining for phosphorylated histone H3 (PH3) as a marker of proliferating cells (Fig. 8C,D). No significant differences were detected in the number of PH3-positive cells between control-injected and *cables1* morphants (Fig. 8E; $P > 0.05$).

DISCUSSION

Using the zebrafish as an experimental model, this study provides molecular, cellular, biochemical, and behavioral evidence that suggest an important functional role for *cables1* in embryonic neural development. Knockdown of *cables1* in zebrafish embryos led to increased apoptosis in embryonic neural tissues, enhanced neural excitability in response to stimulation, significant reductions in p35 protein levels, and alterations in the expression of neural transcription factors during early neurulation.

It is important to note that not all transcription factors examined were affected equally. We observed consistent decreases in *emx3*, *eng2a*, and *gsc* expression in *cables1* morphants, whereas *otx2* expression was consistently increased. However, no changes were detected in the expression patterns of *pax2a*, or the axial mesoderm marker *ntl*. These observations suggest an underlying deregulation of neural differentiation pathways, rather than simply global shifts in gene expression. The down-regulated expression of some transcription factors also cannot be explained by increased p53-dependent cell death during differentiation, as these observations were made in embryos in which p53-dependent apoptosis had been suppressed. Taken together, these data suggest that *cables1* is functionally important during neural tube differentiation, such that a lack of *cables1* expression results in neural tube patterning defects. These findings are in agreement with in vitro studies in the chick model, where *Cables1* was shown to link the Slit/Robo complex to the N-cadherin pathway, leading to β -catenin phosphorylation, nuclear translocation and increased transcription of genes involved in CNS patterning (Rhee et al., 2007).

The behavioral response to a loss of *cables1* is interesting, although the underlying mechanism for this is not entirely clear. The rescue experiments suggest that this phenotype is linked partly to an increase in p53-dependent cell death, but the precise mechanism underlying the behavioral phenotype remains unclear. Notably, hyper-excitability is not observed in all cases of excess cell death in the embryonic CNS. For example, knockdown of an insulin-like growth factor receptor (*Igf1rb*) led to widespread neural apoptosis in the zebrafish embryo, but a distinct loss of motor activity (Schlueter et al., 2006). It is noteworthy that the aberrant behavior was only partially rescued by suppressing p53 expression, whereas a complete rescue was achieved by co-expressing full-length *cables1*.

In mammals, *Cables1* has been reported to influence both p53-dependent and p73-dependent cell death pathways, functions that were attributed to distinct functional domains within the Cables1 protein (Tsuji et al., 2002). Further studies are required to determine whether or not the zebrafish ortholog of Cables1 also contains discrete domains with distinct functional properties.

The significant reduction in p35 protein levels in *cables1* morphant embryos is consistent with previous in vitro data suggesting that *cables1* regulates neural differentiation via interactions with the Cdk5/p35 kinase pathway (Zukerberg et al., 2000). Unlike related cyclin-dependent kinases, Cdk5 is not activated by cyclins and does not function in cell cycle regulation. Rather, its kinase activity is confined to postmitotic (differentiating) neurons, via interactions with p35 and p39, that exhibit neural-specific expression (Ohshima et al., 1996; Ko et al., 2001). p35 is a highly labile protein that is rapidly degraded by the ubiquitin-proteasome pathway (Patrick et al., 1998; Kerokoski et al., 2002; Saito et al., 2003), and this degradation is dependent upon phosphorylation by Cdk5 (Kerokoski et al., 2002; Saito et al., 2003). The marked reduction of p35 protein levels in *cables1* morphants is therefore suggestive that *cables1* may function to stabilize p35, similar to its reported function in stabilizing p63 (Wang et al., 2010).

The results of overexpressing a C-terminal truncation mutant lacking the cyclin box suggest that the cyclin box confers potent biological activity to *cables1*. This observation is consistent with the model involving interactions between *cables1* and Cdk5/p35, as the cyclin box is the domain predicted to interact with the Cdk5/p35 complex. While deletion of the cyclin box substantially reduced the activity of *cables1* overexpressed in vivo, this could potentially be explained by precocious degradation (e.g., nonsense-mediated decay) of the CB mRNA (Wen and Brogna, 2008). However, our data are consistent with in vitro studies showing that the cyclin box is essential to mediate important functions of *cables1*, including p53-dependent cell death (Tsuji et al., 2002).

Lastly, the lack of a significant difference in the number of phosphorylated histone H3-positive cells among *cables1* morphants and controls suggests that during early embryogenesis, *cables1* does not directly regulate cell proliferation. This differs from what has been reported in other biological contexts. For example, *CABLES1* over-expression in human endometrial cells markedly slowed the rate of cell proliferation (Zukerberg et al., 2004), while embryonic fibroblast from *Cables1*^{-/-} mice proliferated more rapidly than wild-type cells (Kirley et al., 2005b). It is unclear whether these differences reflect species-specific variations in gene function, or reflect the different experimental or biological contexts in which the genes were studied.

In summary, this study provides in vivo evidence that *cables1* is critically important for neural development in the zebrafish embryo, and suggests that the observed defects may be due in part to its interactions with the Cdk5/p35 kinase pathway during neural differentiation. Future studies using conditional gene ablation approaches are required to determine if *cables1* adopts other biological functions after the embryonic period. The mounting evidence of a role for this gene in early development and possibly tumorigenesis (Dong et al., 2003; Zukerberg et al., 2004; Zhang et al., 2005; Kirley et al., 2005a,b; Lee et

al., 2007; Park do et al., 2007; Sakamoto et al., 2008) suggests that much remains to be learned about its functions in humans and other vertebrates.

MATERIALS AND METHODS

Experimental Animals

Wild-type zebrafish (Tubingen AB strain) were maintained in standard conditions, and staged as described (Kimmel et al., 1995). Embryos were generated by natural crosses, and reared at $28 \pm 1^\circ\text{C}$ in embryo medium.

Chemicals and Reagents

Reagents for RT-PCR (Superscript II reverse transcriptase, *Taq* DNA polymerase, dNTPs) were purchased from Invitrogen (Carlsbad, CA). FailSafe™ PCR buffers were purchased from Epicenter Biotechnologies, and antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA; rabbit polyclonal anti-Cdk5 [C-8]; mouse monoclonal anti-p35), ThermoFisher Scientific (Waltham, MA; mouse monoclonal anti-Actin, pan Ab-5) and Millipore (Billerica, MA; rabbit anti-phosphorylated histone H3, #06.570). Morpholino oligonucleotides were purchased from Gene Tools LLC (Philomath, OR). TUNEL analyses were performed using the In situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN). All other reagents were from Sigma, St. Louis, MO or ThermoFisher unless otherwise stated.

Reverse-Transcriptase PCR (RT-PCR)

Total RNA was purified from whole embryos at desired stages using Tri Reagent (20 embryos/ml), resuspended in nuclease-free water and quantified by absorbance at 260 nm. First-strand synthesis was performed as previously described (Sang et al., 2008). Briefly, 500 ng of total RNA was reverse-transcribed using Superscript III reverse-transcriptase, according to the manufacturer's protocol. Aliquots of first-strand cDNA were then PCR-amplified, using the following gene-specific primers: *cables1*-F2: 5'-TGCTCTCTCATTCTCAGCA-3'; *cables1*-R4: 5'-GGCAGGATTCCCTGAGTGTA-3'. This primer pair amplified a 345 base-pair fragment corresponding to nucleotides 81–425 (ATG start codon designated as position 1). PCR conditions were as follows: 94°C for 5 min; 30–35 cycles of $94^\circ\text{C} \times 1$ min, $58^\circ\text{C} \times 30$ sec, and $72^\circ\text{C} \times 30$ sec; $72^\circ\text{C} \times 5$ min. Each reaction (25 μl) contained 2 μl of first-strand cDNA, 0.8 mM of each primer and 100 U of Platinum *Taq* polymerase, diluted in $1\times$ FailSafe™ PCR Buffer D. PCR products were electrophoretically separated in 1.5% agarose gels, and products were visualized under UV light. Amplification of *ornithine decarboxylase 1* (*odc1*) served as an internal control for RNA and cDNA quality, using the following primer pair: *odc*-F1: 5'-CAGAAGACGCTCAACCAAACC-3'; *odc*-R1: 5'-ATCCCATCTCTTTACGTCC-3'.

Morpholino Oligo-Mediated Gene Knockdown

The MO targeted against zebrafish *cables1* (cabMO: 5'-AAGTCAGCGGAGACTAAAGGTGTTC-3') is complementary to a 25-mer region in the 5'-UTR (−52 to −28 relative to ATG codon); a standard control MO designed by the manufacturer was used to control for the effects of morpholino microinjection. A

commercially available MO targeting p53 (p53MO) has been previously described (Robu et al., 2007).

Synthetic mRNA Overexpression

Three expression constructs were made to overexpress variants of *cables1* in zebrafish embryos. pCS2 + *cab*^{ORF} corresponded to the complete open reading frame (525 amino acids) of zebrafish *cables1*. pCS2 + *cab*^{RESC} also encoded the complete open reading frame, but excluded the morpholino (*cab*MO) target region. pCS2 + *CB* encoded a truncated variant of *cables1*; this was generated by RT-PCR using primers that introduced a stop codon (TAA) at amino acid residue 407, thereby encoding a 406 amino acid protein (*CB*) lacking the C-terminal cyclin box.

Each fragment was amplified by RT-PCR using total RNA purified from adult brain tissue as template. The PCR products were subcloned into pCRII-TOPO (Invitrogen), sequenced to confirm accuracy, digested with *Eco*RI and ligated into pCS2+. After identifying clones containing the appropriate inserts, they were sequenced to ensure accuracy and used for synthetic mRNA synthesis, as described (Turner and Weintraub, 1994).

Synthetic mRNA was generated using the SP6 mESSAGE mACHINE (Sang et al., 2008), precipitated in LiCl, washed extensively in nuclease-free 70% ethanol and resuspended at the desired concentration in sterile 1× Danieau microinjection buffer.

Embryo Microinjection

Approximately 1 nl of each solution (morpholino, synthetic mRNA) was microinjected into 1-cell stage zebrafish embryos, as previously described (Sang et al., 2008). Microinjection volumes were calibrated using the oil drop method (Yuan and Sun, 2009). For rescue experiments, synthetic mRNA (*cab*^{RESC}) was simultaneously co-injected with *cab*MO (2 ng/embryo). Synthetic mRNA encoding GFP (100 pg/embryo) was injected into a subset of embryos to confirm the quality of in vitro-derived synthetic mRNA.

TUNEL Analyses

TUNEL analyses for DNA fragmentation were performed on paraformaldehyde-fixed whole embryos, as previously described (Schlueter et al., 2007). For quantification purposes, a mean TUNEL index was generated by counting the number TUNEL-positive cells in five random-selected, non-overlapping fields of view per embryo. This method of quantification was replicated in each of five embryos per treatment group. The observer was blinded with respect to the identity of each embryo during quantification.

In Situ Hybridization

Embryos were fixed in buffered paraformaldehyde (4%) and stored in methanol for a minimum of 24 hr prior to analysis. The methods used for in situ hybridization analyses have been previously described (White et al., 2009). Plasmid templates for target genes were generously provided by Iain Drummond (Department of Nephrology, Massachusetts General Hospital, Charlestown, MA), and Igor Dawid (NIH, Bethesda, MD). Cryosectioning was performed as previously described (Wood et al., 2005).

Immunohistochemistry

Immunohistochemical staining was performed on paraformaldehyde-fixed embryos after dehydration and storage (-30°C) in methanol. Embryos were rehydrated by serial washes in phosphate-buffered saline containing 0.1% Tween (PBST), incubated in blocking buffer (PBST containing 2% serum, 1% DMSO, 1% BSA), and incubated in primary antibody solution overnight at 4°C . Embryos were then washed extensively in PBST, and incubated overnight (4°C) in biotinylated secondary antibody diluted (1:500) in blocking buffer. After washing in PBST, embryos were incubated in Vectastain ABC reagents for 30 min at RT, washed in PBST and incubated in DAB solution (DAKO) to complete the color reaction.

Western Immunoblot Analyses

A total of 20 embryos from each sample group were suspended in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Hank's balanced salt solution (HBSS), deyolked by passage through a narrow-bore pipette, and washed briefly in fresh HBSS. The carcasses were then reconstituted in SDS sample buffer (5 μl /embryo) and homogenized for 1 min in a Mini-Beadbeater-8 (Biospec Products, Inc., Bartlesville, OK), using 1.0 mm glass beads. The homogenates were then heated for 15 min at 75°C , centrifuged for 1 min at 14,000g, and aliquots of the soluble fraction (20 μl homogenate/lane) were electrophoretically separated in 4–12% Bis-Tris polyacrylamide gels, with MOPS running buffer. Proteins were transferred to PVDF membrane, washed briefly in Tris-buffered saline containing 0.1% Tween-20 (TBST), and blocked for a minimum of 2 hr at room temperature (RT) in blocking buffer (10% non-fat dry milk in TBST). Primary antibody was diluted (1:1,000) in blocking buffer and applied overnight (4°C) with gentle agitation. After extensive washes in TBST, membranes were incubated in HRP-conjugated secondary antibody solution (diluted 1:5,000 in blocking buffer) for 1 hr at RT, and target proteins were detected using the Enhanced Chemiluminescence system (ECL, GE Healthcare, Milwaukee, WI). Proteins were visualized by exposure to X-ray film and quantified by densitometry on a FluorChem ChemImager 5500.

Photomotor Response (PMR)

PMR measurements were conducted in a 96-well plate format, using 8–10 embryos per well, similar to methods previously described (Kokel et al., 2010). PMR measurements were performed on embryos at 40–42 hpf. Reported PMR values correspond to the 3rd quartile of the Motion Index, for a measurement interval lasting 3 sec after the light pulse. Mean PMR data represent the average of three replicate measurements, with each replicate derived from a separate group of 8–10 embryos.

Data Analyses

Data are presented as mean values $\pm$ standard error of the mean (SEM). Mean values for TUNEL, PMR, and densitometry data were statistically compared by analysis of variance (ANOVA), followed by the Student–Newman–Keuls multiple comparison test. An unpaired Student's *t*-test was used to compare mean values for phosphorylated histone H3 staining. Differences between mean values were considered statistically significant if $P < 0.05$.

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Abbreviations

Cdk	cyclin-dependent kinase
hpf	hours post-fertilization
MO	morpholino oligonucleotide
PMR	photomotor response
TUNEL	terminal dUTP nick-end labeling

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Figure 1.

A: RT-PCR showing expression of *cables1* mRNA throughout embryogenesis in zebrafish. Numbers indicate hours post-fertilization (hpf). **B:** In situ hybridization showing neural expression of *cables1* mRNA at 24 hpf; **(C)** dorsal view of 24 hpf embryo; **(D)** cryosectioned embryo (24 hpf) showing neural expression of *cables1* mRNA; **(E)** control (sense probe) showing no signal at 24 hpf.

Figure 2.

Phenotypes of MO-injected embryos: (A) Control-injected embryo at 24 hpf; (B) cabMO-injected embryo (*cables1* morphant) showing evidence of necrotic degeneration in brain tissues (arrows); (C) *cables1* morphant embryo co-injected with MO-resistant *cables1* mRNA (*cab^{RESC}*), showing rescue of brain necrosis; (D) relative proportions of each phenotype in wild-type (WT), control-injected (Control), *cables1* morphant (*cabMO*) and *cables1* rescued (*cab^{RESC}*) groups. KDP, knockdown phenotype (brain necrosis); OEP, over-expression phenotype (see Fig. 6).

Figure 3.

A–C: TUNEL staining to identify apoptotic cells in zebrafish embryos at 18 hpf; **(A)** control-injected embryo; **(B)** *cables1* morphant (cabMO-injected) embryo; **(C)** *cables1* morphant embryo co-injected with *cab^{RESC}*; **(D)** *cables1* morphant co-injected with p53MO; **(E)** quantitative estimation of TUNEL-positive cells in each embryo group; y-axis represents mean TUNEL index, as described in the Materials and Methods Section. Different letters denote statistically significant differences ($P < 0.05$); results of individual pair-wise comparisons are provided in the Results Section. Arrows in (B) indicate high density of TUNEL-positive cells.

Figure 4.

Photomotor response (PMR) data demonstrating behavioral abnormalities in *cables1* morphant embryos. **A–C:** Representative PMR plots from (A) control-injected embryos, (B) cabMO-injected embryos, and (C) cabMO-injected embryos co-injected with *cab^{RESC}* mRNA. **D:** Quantitative summary of PMR data in (A–C). Values are means $\pm$ SEM of the Motion Index, corresponding to a 3 sec measurement interval following the light pulse (see Materials and Methods Section). Different letters denote statistically significant differences ($P < 0.05$). **E, F:** Representative PMR plots from (E) control-injected embryos, (F) cabMO-injected embryos, and (G) cabMO-injected embryos co-injected with p53MO; **(H)** quantitative summary data in (E–G). Different letters denote statistically significant differences ($P < 0.05$); results of individual pair-wise comparisons are provided in the Results Section. Arrows in PMR plots indicate timing of 1 sec light pulse; horizontal bar above each PMR plot indicates time interval used for quantification.

Figure 5.

In situ hybridization analyses showing marker gene mRNA expression in representative control-injected embryos (left column) and *cables1* morphants (right column). Reduced expression of *emx3* (A, B), *eng2a* (C, D), and *gsc* (E, F), and increased expression of *otx2* (G, H) was observed in *cables1* morphants. No changes were detected in the expression of *pax2a* (I, J) or the axial mesoderm marker *ntl* (K, L). Results shown are representative images from a minimum of 10 embryos examined per treatment group.

Figure 6. Phenotypes of (A) wild-type, (B) control-injected (GFP, 100 pg/embryo), and (C–E) treatment embryos, injected with synthetic mRNA encoding full-length zebrafish Cables1 protein. *cables1* mRNA-injected embryos exhibited a range of overexpression phenotypes, ranging from mild (C) or moderate (D) axis defects, to severe dysmorphogenesis (E); (F) quantitative summary of embryo phenotype proportions, pooled from two independent experiments. WT, wild-type (uninjected) embryos; GFP, embryos injected with synthetic mRNA encoding GFP; *cab*^{ORF}, embryos injected with synthetic mRNA encoding the complete open reading frame (ORF) of *cables1*; CB, embryos injected with synthetic mRNA encoding a truncation mutant of *cables1* that lacks the C-terminal cyclin box. OEP; overexpression phenotype.

Figure 7.

Western immunoblot analyses of pan-Actin, Cdk5 and p35 protein levels in de-yolked embryo lysates from *cables1* morphant embryos at 24 hpf. Inset shows representative Western immunoblot; plot shows protein levels, expressed as a ratio of intensities (measured by densitometry) between control-injected and *cables1* morphant groups. Values shown are the means ($\pm$ SEM) of three experimental replicates. p35 protein levels were significantly lower (**) in *cables1* morphants relative to control-injected embryos ($P < 0.01$).

Figure 8.

A: Control-injected, and **(B)** *cables1* morphant embryos at 12 hpf; images demonstrate simultaneous onset of somitogenesis (arrows) among embryos, indicating no gross disruptions to early development, or notable differences in developmental rate; **(C)** control - injected, and **(D)** *cables1* morphant embryos (shield stage, 6 hpf) immunohistochemically stained for phosphorylated histone H3 (PH3) to identify proliferative cells (brown dots); **(E)** quantitative representation of the number of PH3-positive cells in shield stage embryos; values represent the mean density ($\pm$ SEM) of PH3-positive cells in 6–8 random fields per embryo. No significant differences were detected in the mean densities of PH3-positive cells between control-injected and *cables1* morphant embryos at this stage of development ($P > 0.05$).