Yaf2 Inhibits Caspase 8-mediated Apoptosis and Regulates Cell Survival during Zebrafish Embryogenesis

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Rybp (DEDAF) is a member of the Rybp/Yaf2 protein family and has been shown to encode pro-apoptotic functions and to be essential for mouse embryogenesis. The related Yaf2 protein has not been studied extensively at the cellular or organismal levels. Here we describe zebrafish yaf2 (zyaf2) and show that it is widely expressed during early embryogenesis, with subsequent enrichment of transcripts in the anterior head region. Depletion of zYaf2 during embryogenesis using specific morpholinos activates a wide-spread program of apoptosis and causes developmental arrest before the one somite stage. Partial depletion of Yaf2, achieved by injecting lower dosages of morpholino, circumvents the early arrest but leads to CNS degeneration associated with excessive apoptosis. These phenotypes can be rescued by treatment with a pan-caspase inhibitor or a caspase 8-specific inhibitor. Finally, the observed activation of caspase 8 in the morphants is in accord with the ability of Yaf2 to inhibit caspase 8-mediated apoptosis in cultured cells. Our findings implicate Yaf2 as a survival factor during early zebrafish development and organogenesis. This may suggest that Yaf2 and Rybp can encode opposing functions in the regulation of apoptosis.

Programmed cell death affects many different aspects of development including sculpting structures, controlling cell number, and eliminating abnormal, misplaced, or harmful cells (reviewed in Ref. 1–3). It has been suggested that the default for developing cells is cell death, and that trophic factors (e.g. NGF, GDNF) and morphogens (e.g. Shh and Wnt) are needed to keep cells alive by inhibiting apoptotic pathways and activating cell survival pathways (reviewed in Ref. 4). In the adult organism, apoptotic pathways are essential to eliminate cells at risk for neoplastic transformation (reviewed in Refs. 5 and 6).

Because of the important role that apoptotic pathways play in normal and neoplastic development, relevant protein components are expressed constitutively, and their activity is maintained by many regulatory proteins (reviewed in Refs. 3 and 7). Well-known regulatory proteins include the Bcl2 protein family, the IAPs, SMAC/DIABLO, and the ubiquitin/proteasome pathway (reviewed in Refs. 7 and 8). A recently characterized, putative regulator of apoptosis is Rybp (also known as DEDAF), a zinc finger-containing protein that belongs to a family of proteins that also includes Yaf2 (9, 10). Rybp has been shown to interact with death effector domain (DED)-containing proteins including FADD, caspase 8, caspase 10, and DEDD (11). The first three of these interacting proteins are components of the extrinsic or death receptor apoptotic pathway, while DEDD is a nuclear protein that promotes apoptosis in the nucleolus as well as in the cytosol after its translocation (reviewed in Ref. 12; also see Ref. 13 and references therein). A pro-apoptotic role for Rybp was suggested because it could promote formation of the DISC (death-inducing signaling complex comprising Fas, FADD, and pro-caspase 8) in 293T cells, and enhance Fas- and caspase 10 DED-mediated apoptosis in lymphoma cell lines (11). In a separate study, Rybp was shown to interact with the viral protein Apoptin, and to enhance apoptosis in transformed cells but not primary cells (14). Finally, Rybp has been shown to be up-regulated (with a corresponding decrease in a miRNA that may target it) in breast cancer cells that have been treated with a pro-apoptotic dose of the HDAC inhibitor LAQ824 (15).

Mice homozygous null for Rybp died around the time of implantation, and exhibited embryonic and extraembryonic defects (16). Of note, rybp+/− embryos were unable to trigger full decidualization in vivo, as evidenced by the lack of a normal apoptotic response following the initiation of implantation. Beyond this phenotype, a subset of the Rybp heterozygous null mice exhibited an exencephalic phenotype because of disrupted neural tube closure. This mouse model demonstrated that the effects of Rybp loss are dose-dependent, and that Rybp has a role in

The abbreviations used are: DED, death effector domain; AO, acridine orange; BAC, bacterial artificial chromosome; CNS, central nervous system; hf, hours post-fertilization; MO, morpholino; Pcs, Polyc Comb Group; S5, splice site; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling; zETD-fmk, benzyloxycarbonyl-Ile-Glu-Thr-Asp-fluoromethyl ketone; zLETD-aminoluciferin, benzylozycarbonyl-Leu-Glu-Thr-Asp-aminoluciferin; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone; PBS, phosphate-buffered saline; HA, hemagglutinin; PFA, paraformaldehyde.
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Early development as well as in brain organogenesis. Moreover, even though Yaf2 is structurally and functionally related to Rybp, it cannot fully compensate for Rybp loss (16). To date, Yaf2 has not been studied in the context of a developing organism, and a link between Yaf2 and apoptosis has not been described.

In this study, we characterized the zebrafish ortholog of yaf2 and employed antisense morpholino oligomers to knock-down Yaf2 expression during zebrafish embryogenesis. Yaf2 morphant embryos exhibited a dramatic increase in the level of caspase-dependent cell death that was causal to a developmental arrest prior to the one somite stage. With decreased dosage of the morpholinos, the morphological defects became less severe and an essential role for Yaf2 in maintaining the anterior central nervous system (CNS) was revealed. Once again, the disruptions in the CNS resulted at least in part from excessive apoptosis. These findings implicate Yaf2 as a survival factor during early development and organogenesis. This function of Yaf2 may relate in part to its newly recognized ability to inhibit caspase 8-mediated apoptosis.

EXPERIMENTAL PROCEDURES

Fish Husbandry and Documentation

Zebrafish embryos were maintained at 28.5 °C and staged as described (17). The wild-type fish strain used was an AB/TUB hybrid; both the AB and Tübingen fish were acquired from the Zebrafish International Research Center (Eugene, OR). All of the injections and embryo observations were performed on a Nikon SM1500 dissecting microscope. The bright field, in situ hybridization, and TUNEL microscopy/photography were performed on a Nikon SM1500 fluorescent microscope with a Diagnostic Instruments Insight Firewire 2 digital camera; the pictures were captured with SPOT advanced software.

The one somite arrest morphants were scored based on the (i) lack of somite development and (ii) darkened, rounded up cells around the yolk. Moreover, the Yaf2 morphant embryos remained in this arrested state, until dying by 30 hpf. The hypomorphic embryos were first scored at the 10 somite stage where a dark band of dying cells could be seen beneath the eyes. In later stages, the hypomorphic phenotype was easily scored by the progressive anterior brain degeneration.

Expression Plasmids

The full-length IMAGE clone of zebrafish yaf2 was obtained from Open Biosystems. The full-length ORF of zyaf2 was amplified with the following primers: forward primer 5'-GGG-GATCCGGAGAAATGGCTACTACAAGGAGCATGAC- AAGGGGACAAAGGGAGTGGATCCGGACAG-3' (introduced a BamHI site and an in-frame N-terminal FLAG tag) and reverse primer 5'-CCGAATTTCCTAGTGAATTCGGGTTT- TGGAG-3' (introduced an EcoRI site). The FLAG-tagged zebrafish yaf2 insert was then subcloned into the pcS2+ and pcDNA3.1 expression vectors. Human YAF2 cDNA was obtained from Dr. Te-Chueung Lee, a C-terminal FLAG tag was introduced by PCR, and the PCR product was subcloned into pcDNA3.1. For the rescue experiments, mutations in the wobble position were made in amino acids 2–4 of the hYAF2 coding region by PCR mutagenesis on an hYAF2-FLAG template using the following primer, which also introduced a BamHI site: 5'- CGGGATCCCAGGCAAGCCATGGGCGATATAAAGACGCC- CACCAGG-3' (the changed nucleotides are in bold). The 3' primer was directed to the pcDNA3.1 vector (5'-CATGTGGCTGCAACTAG-3'). The hYAF2-FLAG PCR product was then subcloned into pcS2+. Mouse rybp was cloned with a C-terminal FLAG tag by RT-PCR with 5' primer, 5'-GAATTCATCATCAGTGC- TGAATGTGCG-3' (introduced an EcoRI site) and 3' primer, 5'-GAATTCATCATCAGTGC-3' (introduced an EcoRI site), and the PCR product was subcloned into pcDNA3.1 that carried a FLAG tag. Human caspase 8 cDNA was cloned into the pcDNA 3.1 vector using primers that added an N-terminal HA tag with the forward primer, 5'-GGCAAGCTTTGGCATGGTGATACACCATCACGTCCATCAGCAG-3' (introduced a HindIII site) and reverse primer, 5'-CGGGATCCCAGGTCATTGCTTCTTT- CATTGTAAC-3' (introduced a BamHI site) and added a stop codon after amino acid 216. Human caspase 8 was cloned into pRES2 EGFP (Clontech) by PCR with forward primer, 5'-ACCGTGCACATGGACTCCAGAATCTCTT- TATG-3' (introduced a Sall site) and reverse primer, 5'-GGGG- GATCCGTCAATCAGAAGGAGAAGACAG (introduced a BamHI site). Zebrafish caspase 8 was cloned by RT-PCR from embryonic zebrafish mRNA using the forward primer, 5'-CATGACATATGGTTGATACCCAGTCATGAC- CGGGACCTAAGGAG-3' (introduced an EcoRI site) and reverse primer, 5'-CGGAATTCATCATCAGTGC-3' (introduced an EcoRI site) and the PCR product was subcloned into the pcDNA 3.1 expression vector. Additional details regarding construct generation are available upon request.

Whole Mount In Situ Hybridization

Embryos were fixed in 4% paraformaldehyde (PFA, Sigma) in PBS overnight and then dechorionated and stored in 100% methanol. Embryos older than 24 hpf were treated with 0.003% phenylthiourea (Sigma) to prevent pigmentation. Before probing, embryos were rehydrated and, if older than 24 hpf, treated with proteinase K (10 μg/ml) at room temperature for 5–45 min depending upon stage. The embryos were then refixed in 4% PFA for 1 h, and prehybridized in a 57% formamide hybridization buffer for 1 h at 70 °C. The buffer was replaced with 57% formamide hybridization buffer containing 1 ng/μl of the digoxigenin-labeled probe, and hybridization was performed at 70 °C overnight. Embryos were exposed to sheep anti-digoxigenin Fab antibody conjugated to alkaline phosphatase (Roche Applied Science) for 2 h at room temperature. The signal was
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detected using alkaline phosphatase NBT/BCIP staining, and the embryos were then cleared in a BB:BA 2:1 solution.

To generate the probes, the expression constructs were linearized and purified by phenol/chloroform extraction. Antisense RNA was generated using an in vitro transcription kit (Promega) and labeled with a digoxigenin NTP mix (Roche Applied Science). The probes were purified over G-50 Sephadex RNA columns (Roche Applied Science) and the concentration determined by spectrophotometry. The zyaf2 probe comprised the full open reading frame subcloned into pCS2+. The zCaspase 8 probe in pCS2+ contained its open reading frame from amino acid 131 to the end (amino acid 476). SK-zkopl1 was provided by Dr. Hazel Sive, pCS2+ zotx2 was provided by Dr. Shuo Lin, KS+ zkrox20 was provided by Dr. Yu Chen, and pSPORT1 zpax2.1 was obtained from the ZFIN zebrafish data base (ID: ZDB-EST-021126-10).

**Morpholinos**

Two phosphorodiamidate morpholino oligomers were generated (by Open Biosystems and GeneTools) against the Yaf2 genomic locus. BLAST searches predicted that the morpholinos would only affect the predominant isoform that is the focus of this report. A morpholino (5'-CGTGGAGTCTCTTCATGG-3') is complementary to -1 thru +24 relative to the zYaf2 translation initiation codon. The splice site morpholino (SS MO) (5'-ATTGCAGCTTCACCT-3') is targeted to the splice donor in the long intron following the penultimate exon in the zebrafish yaf2 genomic locus. BLAST searches predicted that the morpholinos were specific for yaf2. The three zyaf2 mRNA isoforms that have been identified would all be targeted by the splice site morpholino, which gives the same phenotype as the ATG morpholino that would only affect the predominant isoform that is the focus of this report. A morpholino (5'-ATATCCATCACACTGGCGGC-3'), based on the pBluescript vector (Stratagene), was used as a control in the injections. Morpholinos were injected into fertilized eggs at the 1–2 cell stage in a total volume of 2.3 nl.

**Reverse Transcription-PCR**

RNA was isolated using Trizol reagent (Invitrogen) and purified by phenol/chloroform extraction. First-strand cDNA was generated using the Superscript First-strand PCR kit (Invitrogen) with random hexamer primers, and then amplified by PCR using Platinum Taq (Invitrogen). The primers used to amplify yaf2 were the same primers used for the subcloning into pCS2+. The same first strand cDNA samples were amplified with primers for the housekeeping gene β-actin: forward primer, 5'-AAGCAGGATCGAGTGTCCCTC-3' and reverse primer, 5'-GGCTAACGCTCTGGAATGACA-3'.

**Apoptosis Assays and Caspase Inhibitor Treatment**

**TUNEL Assay**—Injected embryos were staged and fixed overnight in 4% PFA. After washing with PBST, they were dechorionated and dehydrated into 100% methanol. Prior to staining, the embryos were rehydrated into PBST, post-fixed for 1 h in 4% PFA, blocked for 1 h in 1× TdT buffer, and incubated with a mixture of the TdT enzyme (150 units/ml; Invitrogen) and digoxigenin-labeled dUTP (0.5 μM; Roche Applied Science) overnight at room temperature. The embryos were then washed first in PBST/1 mM EDTA at 65 °C for 2 h and then in PBST/bovine serum albumin. Embryos were exposed to anti-digoxigenin Fab antibody conjugated to alkaline phosphatase (Roche Applied Science) overnight at 4 °C. The signal was detected using alkaline phosphatase NBT/BCIP staining and the embryos were then cleared in a BB:BA 2:1 solution.

**zVAD-fmk and zIETD-fmk Treatment**—Four hours after injection with the morpholinos, a hole was made in the chorions using watchmaker’s forceps, and the embryos were transferred to a 60-mm glass Petri dish of E3 containing 200 nM zVAD-fmk per Ref. 18 or 200 nM zIETD-fmk. They were then either staged and fixed for the TUNEL assay or monitored for developmental defects by microscopy.

**Acridine Orange/Ethidium Bromide**—HEK293T cells were plated at a density of 1 × 10^5 cells per 10-cm dish and transfected with 3 μg of each expression construct using calcium phosphate transfection. After 24 h, cells were washed with PBS and fixed with 4% PFA/PBS for 20 min at 4 °C. After being rinsed in PBS, the cells were incubated with 50 μg/ml acridine orange/Ethidium Bromide in PBS for 2 min at room temperature. Then the cells were washed three times with PBS and mounted with 50% glycerol/PBS. The nuclei were imaged in a Leica confocal microscope.

**FIGURE 1.** The zYaf2 protein sequence is highly conserved with those of its mammalian homologues in the Rybp/Yaf2 family. A, pile-up of zebrafish (z), mouse (m), and human (h) Yaf2, and mouse (m) and human (h) Rybp protein sequences is shown. Dots represent identity to the zebrfish sequence, and dashes represent gaps. The N-terminal zinc finger motif (ZnF) is marked by a bracket. Note that the zYaf2 isoform shown corresponds to NCBI accession no. CAL11643. Two other zYaf2 isoforms, arising from the zyaf2 genomic clone (GenBankTM accession no. AL929504) can be found in the Protein Data Base (NCBI accession no. CAL11644 and NCBI accession no. CAL11645). These putative isoforms differ only in their extreme N termini. Based on EST representation the zYaf2 isoform shown here is the predominant one. B, phylogenetic tree illustrating the relationship between the Yaf2 and Rybp proteins. Note that zYaf2 clusters with the mammalian Yaf2 proteins. The pile-up and tree were compiled using the Multalin program. PAM, point accepted mutation model (a reflection of the distance between protein sequences).
phosphate precipitation. The cells were harvested after 24 h by trypsinization (Invitrogen), resuspended in 1 ml of PBS, and then a 50-μl aliquot was removed and labeled in a double blind manner. The aliquots were stained by adding 4 μl of an acridine orange (100 μg/ml)/ethidium bromide (100 μg/ml) solution. 10 μl of each sample was dotted on a glass slide, covered with a coverslip, and counted using ×180 magnification on a Nikon SM1500 fluorescent microscope per Ref. 19. A minimum of 200 cells were counted per point, and grouped into four categories based on nuclear morphology and stain: viable (green with intact nuclei), necrotic (orange with intact nuclei), early apoptotic with intact membrane (green with fragmented nuclei), and late apoptotic with damaged membrane (orange with fragmented nuclei). Whole cell lysates were made from the remaining cells to ensure expression of the introduced proteins.

Caspase 8-Glo Assay—At the appropriate stage, 20 injected embryos were dechorionated and quick frozen in liquid nitrogen for at least 24 h. The embryos were then thawed, suspended in 100 μl of lysis buffer (20 mM Hepes-KOH, pH 7.5, 250 mM saccharose, 50 mM KCl, 2.5 mM MgCl₂, and 1 mM dithiothreitol) and passed through a 26.5-gauge needle. The embryo lysate was high power dorsal views, focused on the brain, with anterior to the left. The magnification in panels a–g is ×144, and panels h–i is ×180.

FIGURE 2. Expression pattern of zebrafish yaf2. Whole mount in situ hybridization was performed using a yaf2-specific antisense riboprobe on embryos at the following stages: panel a, 64-cell stage; panel b, 30% epiboly; panel c, tailbud; panel d, one somite; panel e, 10 somites; panels f and h, 24-h post-fertilization (hpf), and panels g and i, 50 hpf. The embryos in panels a and b are lateral views with the animal pole toward the top. The embryos in panels c–g are lateral views, with dorsal toward the top and anterior to the left. Panels h and i are high power dorsal views, focused on the brain, with anterior to the left. The magnification in panels a–g is ×144, and panels h–i is ×180.

4 °C and probed with a 1:3000 dilution of the horseradish peroxidase-conjugated anti-mouse secondary antibody (Amersham Biosciences) for 1 h at room temperature. Bound antibodies were detected by chemiluminescence (Amersham Biosciences). Titration of hYAF2 mRNA injected into embryos revealed that injection of up to 200 pg did not cause any phenotypic changes. For rescue, a mixture of 5 ng of ATG MO, 200 pg of hYAF2 mRNA, and 100 mM KCl or 2.5 ng of ATG MO, 200 pg of hYAF2 mRNA, and 100 mM KCl was injected into fertilized eggs at the one cell stage, and the injected embryos were observed for appearance of the morphant phenotypes.

Immunoprecipitations

Capped hYAF2 mRNA was made by linearizing the pCS2+ hYaf2 FLAG construct with NotI and transcribing with Sp6 using the mMessage Sp6 kit (Ambion), according to the manufacturer’s instructions. To confirm the integrity of the capped mRNA, in vitro translations were performed with rabbit reticulocyte lysate (Promega) and resolved by SDS/PAGE followed by Western blotting. The membranes were incubated overnight with primary antibody (1:1000 FLAG antibody (Sigma, M2)) at

Results

yaf2 Is Highly Conserved and Its Expression Becomes Localized to the Brain during Somitogenesis—Searching the zebrafish genome for orthologs of the mammalian Yaf2 proteins revealed a BAC clone3 4 capable of encoding a 182-amino acid protein that displayed marked homology (76% amino acid identity and 86% similarity) to mouse Yaf2 (Fig. 1, A and B). The putative

The amino acid sequences of the human, mouse, and zebrafish Yaf2 proteins can be accessed through the NCBI Protein Database under NCBI accession no. Q81YS7; NCBI accession no. AAH02192; and NCBI accession no. CAI11643, respectively. The amino acid sequence for human and mouse Rybp can be accessed through the NCBI Protein Database under NCBI accession no. NP_036366 and NCBI accession no. NP_062717, respectively.
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To determine the expression pattern of zyaf2, whole mount in situ hybridization was performed upon staged embryos using a digoxigenin-labeled antisense riboprobe encompassing the zyaf2 coding region. As shown in Fig. 2, high levels of maternally expressed zyaf2 can be seen at the 64 cell and early epiboly stages (Fig. 2, panels a and b). Zygotic yaf2 expression is first clearly observable at the 80% epiboly stage (data not shown), and the signal intensifies from the tailbud to one somite stage (Fig. 2, panels c and d). Ubiquitous expression is observed until the 10 somite stage, at which point the signal becomes concentrated in anterior regions of the embryo (Fig. 2, panels f–i).

Embryos Blocked for Yaf2 Expression Undergo an Early Developmental Arrest—To determine the physiological role of Yaf2 in vivo, we designed a translation interference antisense morpholino and injected it into embryos at the 1–2 cell stage. Seventy percent of embryos derived from fertilized eggs injected with 5 ng of this morpholino arrested development before somitogenesis (∼16 hpf) (Fig. 3A, ATG MO). These embryos developed normally through gastrulation, but failed to progress to the one somite stage (for staging see Ref. 21). The developmental arrest was preceded by the appearance of “dark regions” within the embryo (Fig. 3B) that have been shown previously to indicate the presence of dying cells (22). Of the morphant embryos that did not arrest, many displayed a less severe phenotype similar to that seen consistently with injection of lower doses of morpholino (described below). Injection of control morpholinos did not cause any abnormal phenotypes (Fig. 3, A and B).

To confirm the specificity of this phenotype, and to control for the possibility that the ATG MO might unexpectedly target another transcript, a second morpholino was designed against a zyaf2 splice junction (the splice site morpholino, or SS MO) and used similarly for injection into fertilized eggs. Injection of the SS MO arrested many of the embryos (20 ng of SS MO in comparison to 5 ng of ATG MO). Seventy percent of embryos derived from fertilized eggs injected with 5 ng of this morpholino arrested development before somitogenesis (∼16 hpf) (Fig. 3A, ATG MO). These embryos developed normally through gastrulation, but failed to progress to the one somite stage (for staging see Ref. 21). The developmental arrest was preceded by the appearance of “dark regions” within the embryo (Fig. 3B) that have been shown previously to indicate the presence of dying cells (22). Of the morphant embryos that did not arrest, many displayed a less severe phenotype similar to that seen consistently with injection of lower doses of morpholino (described below). Injection of control morpholinos did not cause any abnormal phenotypes (Fig. 3, A and B).

FIGURE 3. Early arrest phenotype of embryos derived from fertilized eggs injected with Yaf2 morpholinos. A, pre-somatic arrest is observed in 69.6% of embryos (averaged from three experiments, n = 758) injected with Yaf2 ATG MO (5 ng) and 66.5% of embryos (averaged from two experiments, n = 597) injected with Yaf2 SS MO (20 ng). This is compared with 3.5% of embryos (averaged from two experiments, n = 175) injected with control MO (10 ng). B, representative embryos from the experiments tabulated in A are shown at 16 hpf. Dark regions of cells along the yolk in the morphants indicate increased cell death (lateral views, left column, anterior to the left and dorsal top). From the dorsal views (right column, anterior at the top), the cells surrounding the notochord (marked by arrowheads) exhibit a pebbled appearance, and the first somite is not formed in the morphants (one of the first pair of somites is marked in the control-injected embryo by an arrow). C, by reverse transcription–(RT–)PCR, yaf2 transcripts are detected in uninjected embryos at the one somite stage (lane 5) but not in the Yaf2 SS MO-injected embryos exhibiting pre-somatic arrest (lane 6). Injection of a control morpholino does not alter yaf2 expression (compare lanes 7 and 8). β-Actin RT-PCRs were used to control for RNA integrity (lanes 1–4). D, in situ hybridization analysis with a zyaf2 antisense riboprobe shows that uninjected (uninj) embryos at the one somite stage exhibit ubiquitous yaf2 expression, whereas the arrested SS MO morphants show no yaf2 staining. The magnification in B and D is ×144.

zebrafish Yaf2 protein is more similar to its mammalian Yaf2 orthologs than to the mammalian Rybp proteins (Fig. 1B), and bears an N-terminal zinc finger motif characteristic of members of the Rybp/Yaf2 family (bracketed and labeled ZnF in Fig. 1A). An IMAGE clone capable of encoding this full-length putative zebrafish Yaf2 (zyaf2) protein was subcloned to include a FLAG epitope tag and expressed in HEK293T cells. Western blotting of the lysates confirmed that this clone encodes a protein of the expected size of 26 kDa (see Fig. 8 below).

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performed using primers that amplify the entire yaf2 coding region and flank the splice junction to which the SS MO is targeted. These assays showed the absence of the normal yaf2 message (Fig. 3C, compare lane 6 to lane 5), a result that was confirmed by in situ hybridization assays on morphant embryos that had been injected with the SS MO (Fig. 3D). The fact that two morpholinos of entirely distinct sequences, and that function by distinct mechanisms, generate identical phenotypes indicates that the specific loss of Yaf2 is incompatible with normal embryonic development.

The Early Arrest of Yaf2 Morphant Embryos Is Associated with Increased Apoptosis—To elucidate the cellular basis of the developmental arrest observed in the Yaf2 morphant embryos, we analyzed whether there was a reduction in cell proliferation or an increase in cell death. From 80% epiboly through the one somite stage, we did not find a significant change in the morphant embryos in the levels of cell proliferation, as assessed by immunofluorescence using an antibody that recognizes phosphorylated histone H3 (data not shown). In contrast, loss of Yaf2 did lead to increased levels of cell death, as determined by TUNEL assays on embryos derived from fertilized eggs injected with the Yaf2-specific morpholinos (Fig. 4A; embryos in untreated columns). The increased levels of apoptosis in the morphants were most evident beginning at the tailbud stage, before the time at which changes in cellular morphology were apparent. This is in contrast to the low level of normal programmed cell death observed in wild-type embryos from the gastrula period through the one somite stage.

As also suggested by acridine orange studies (data not shown), the TUNEL positivity was associated with caspase-dependent apoptosis and thus was significantly reduced by treating the injected embryos with 200 nm pan-caspase inhibitor zVAD-fmk (Fig. 4A; embryos in 200 nm zVAD columns). zVAD-fmk treatment also had an impact upon the developmental arrest phenotype of the morphants (Fig. 4B). At 16 hpf, 80% of the untreated morphant embryos were arrested at the pre-somatic stage, whereas only 28.5% of the zVAD-fmk-treated embryos were arrested (Fig. 4B, left graph). The rescue achieved by caspase inhibition was partial, because it did not entirely eliminate the pre-somatic arrest, and since the majority of the treated morphants that did survive exhibited developmental abnormalities (Fig. 4B, right graph; hypomorphic phenotype discussed next). Nevertheless, taken together our findings suggest that the phenotype caused by yaf2 depletion can be attributed at least in part to a significant increase in the level of caspase-dependent apoptosis.

A Dose-response Assay with the Morpholinos Reveals a Hypomorphic Phenotype—As alluded to above, 13% (n = 522) of embryos derived from fertilized eggs injected with the ATG MO or 4.5% (n = 597) of embryos derived from fertilized eggs injected with the SS MO survived the pre-somatic arrest and displayed instead what we term a “hypomorphic phenotype” (Fig. 5A, left graph). This phenotype became more predominant

FIGURE 4. The developmental arrest phenotype results from increased caspase-dependent cell death. A, TUNEL assays showing increased cell death in embryos derived from fertilized eggs injected with the morpholinos (5 ng of Yaf2 ATG MO or 20 ng of Yaf2 SS MO) relative to control-injected embryos. Treating the embryos with the pan-caspase inhibitor zVAD-fmk decreases the level of cell death in the morphants at the tailbud and one somite stages. Views are dorsal, anterior up. The magnification is ×112. B, inhibiting caspase-mediated apoptosis in the ATG-MO morphants with 200 nm zVAD-fmk allowed these embryos to develop further, as can be seen by the increased number of embryos in pre-somatic arrest at 16 hpf and the increased number of morphants showing the hypomorphic phenotype (at 29 hpf). At 16 hpf, 80% of the untreated morphants (averaged from two experiments, n = 52) arrested just prior to the one somite stage, compared with 28.5% of the zVAD-fmk treated embryos (averaged from two experiments, n = 52). At 29 hpf, 73% of zVAD-fmk-treated embryos progressed to the hypomorphic phenotype as compared with 17.5% of untreated morphant embryos. Similar results were obtained for the SS MO-injected embryos (data not shown). There were no control-injected embryos arresting prior to the one somite stage or displaying the hypomorphic phenotype (n = 49 for untreated and n = 42 for treated control-injected embryos).

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A Dose-response Assay with the Morpholinos Reveals a Hypomorphic Phenotype—As alluded to above, 13% (n = 522) of embryos derived from fertilized eggs injected with the ATG MO or 4.5% (n = 597) of embryos derived from fertilized eggs injected with the SS MO survived the pre-somatic arrest and displayed instead what we term a “hypomorphic phenotype” (Fig. 5A, left graph). This phenotype became more predominant
when the dosages of the morpholinos were halved (Fig. 5A, right graph; 75.7% with 2.5 ng of Yaf2 ATG MO (n = 229) and 73.0% with 10 ng of Yaf2 SS MO (n = 155)). The hypomorphic phenotype is first evident around the 10 somite stage. Whereas the posterior somites and tail region appear to have developed normally in these morphants, the anterior region is malformed and marked by dark cells with pebbled morphology (Fig. 5B, compare panels c and d to a and h; SS MO hypomorphic embryos not shown). This dark region of apparent cell death expands as the embryo develops, and by 29 hpf an overall deterioration of the brain/head structures can be seen (Fig. 5B, compare panels g and h to e and f). Although there is a range in expressivity, the characteristic defects include malformed forebrain, eyes, and ears, and disrupted/contorted neural tube and notochord. There are also frequent disruptions in the periderm around the yolk (data not shown). Most of these hypomorphic embryos do not move, fail to hatch, and die around 72 hpf.

The Hypomorphic Phenotype Particularly Affects the Forebrain Region and Is Associated with Increased Levels of Apoptosis—A battery of neural markers was examined to determine if the hypomorphic phenotype localizes to particular regions of the brain. As expected from the apparent lack or low level of expression of zyaf2 in the hindbrain (Fig. 2), the control-injected embryos and ATG morphants displayed identical patterns of krox20 expression from the 10 somite stage to 20 hpf (Fig. 6A; krox20 staining marks rhombomeres 3 and 5 of the hindbrain). Similarly, the controls and ATG morphants showed the same patterns of pax2.1 expression (Fig. 6A; pax2.1 staining marks the midbrain/hindbrain junction, the otic placode, the developing eyes, and the nephroplacodal). Differences between the controls and morphants became apparent when more anterior brain markers were tested. At the 10 somite stage and 20 hpf, there was reduced forebrain and midbrain signal in the ATG morphant embryos for opl1 as compared with the control-injected embryos, but the rhombomeric staining appeared unaffected (Fig. 6A, opl1). Similarly, levels of otx2, a marker for diencephalon, were reduced in the ATG morphants at both stages as compared with control-injected embryos (Fig. 6A, otx2).

The SS morphants displayed a profile of neural marker expression similar to that of the ATG morphants (data not shown). The fact that the forebrain appears particularly sensitive to Yaf2 deficiency is consistent with the enriched yaf2 expression in this region (see Fig. 2 above). Finally, we suspect that the changes in expression of the forebrain markers would be even more pronounced at 29 hpf, but because of the fragility of the hypomorphic embryos at this stage it is difficult to perform the hybridizations to assess this.

To determine whether changes in the level of apoptosis underlie the phenotypes of the hypomorphic embryos, we performed TUNEL assays on embryos that had been injected with...
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The hypomorphic phenotype particularly affects the forebrain region and is associated with increased levels of apoptosis. A, Whole mount in situ hybridization analyses showing unaltered expression patterns in the morphants (derived from half dose Yaf2 ATG MO injections) for krox20 and pax2.1, but reduced intensity and fields of expression opl1 and otx2 at both the 10 somite and 20 hpf stages panels (marked by arrowheads and arrows). The views are lateral with anterior to the left and dorsal up. The magnification for the krox20 panels is ×180, for the pax2.1 panels is ×112, and for the opl1 and otx2 panels is ×144. Five to ten embryos were examined for each marker at each stage. B, TUNEL assays showing increased cell death in embryos derived from fertilized eggs injected with the lower dosages of the morpholinos (2.5 ng of Yaf2 ATG MO or 10 ng of Yaf2 5S MO [data not shown]) compared with stage-matched embryos derived from fertilized eggs injected with 10 ng of control MO. Staining the hypomorphic embryos with acridine orange confirmed that the cell death in the anterior region was indeed apoptosis (data not shown). The magnification for the left panels at each stage is ×144 (views are lateral with anterior to the left) and for the right panels at each stage is ×180 (views are dorsal with ventral down).

FIGURE 6. The hypomorphic phenotype particularly affects the forebrain region and is associated with increased levels of apoptosis. A, Whole mount in situ hybridization analyses showing unaltered expression patterns in the morphants (derived from half dose Yaf2 ATG MO injections) for krox20 and pax2.1, but reduced intensity and fields of expression opl1 and otx2 at both the 10 somite and 20 hpf stages panels (marked by arrowheads and arrows). The views are lateral with anterior to the left and dorsal up. The magnification for the krox20 panels is ×180, for the pax2.1 panels is ×112, and for the opl1 and otx2 panels is ×144. Five to ten embryos were examined for each marker at each stage. B, TUNEL assays showing increased cell death in embryos derived from fertilized eggs injected with the lower dosages of the morpholinos (2.5 ng of Yaf2 ATG MO or 10 ng of Yaf2 5S MO [data not shown]) compared with stage-matched embryos derived from fertilized eggs injected with 10 ng of control MO. Staining the hypomorphic embryos with acridine orange confirmed that the cell death in the anterior region was indeed apoptosis (data not shown). The magnification for the left panels at each stage is ×144 (views are lateral with anterior to the left) and for the right panels at each stage is ×180 (views are dorsal with ventral down).

the lower morpholino dosage in comparison to controls. Indeed, there was a significant increase in cell death in the anterior region of the hypomorphic embryos from the 10 somite stage through 29 hpf (Fig. 6B). This increase was apparent even at 20 hpf, when there is a normal developmental surge in apoptosis in the brain related to neurogenesis (23) (data not shown). Taken together with the gross morphology and the marker gene expression patterns (Figs. 5B and 6A), our findings suggest that depletion of Yaf2 causes increased apoptosis resulting in degeneration of anterior structures of the zebrafish embryo.

The Early Arrest and Hypomorphic Phenotypes Can Be Rescued by Co-injection of Human YAF2—to demonstrate that the morphant phenotypes are due specifically to Yaf2 depletion, we carried out rescue experiments by coinjecting the ATG MO with in vitro-generated mRNA encoding human YAF2 (Fig. 7). Titration studies showed that injection of up to 200 pg of the human YAF2 mRNA did not cause an overexpression-induced loss of Yaf2 results in developmental defects and increased apoptosis, and that the molecular and cellular functions of Yaf2 are highly conserved throughout evolution.

Yaf2 Interacts with Caspase 8 and Inhibits Caspase 8-mediated Apoptosis—Our data from the zebrafish Yaf2 morphants suggest that Yaf2 may function normally to inhibit apoptosis/promote cell survival. However, there are repeated examples in the literature of apoptotic regulators that when deregulated lead to unexpected apoptotic phenotypes (for example see Ref. 24). As a first step toward addressing whether Yaf2 could be anti-apoptotic, we examined the relationship between Yaf2 and caspase 8; the rationale for this stemmed from the previously reported interactions of the Yaf2-related family member Rybp with caspases 8 and 10 (11). As shown in Fig. 8A, co-immunoprecipitation experiments were performed upon lysates from HEK293T cells transfected with an HA-tagged version of the human caspase 8 DED domain and Flag-tagged human Yaf2, zebrafish Yaf2, mouse Rybp, or control proteins. As expected, a phenotype (Fig. 7). As an aside, when 500–1000 pg of human YAF2 mRNA was injected into the embryos, a hypermorphic phenotype emerged that included perturbations to the head and forebrain, as well as an inability of the fish to move or hatch from their chorion (data not shown).

Co-injection of 200 pg of hYAF2 with 5 ng of the ATG MO rescued the developmental arrest; only 26.9% of the embryos (n = 93) arrested prior to the one somite stage as compared with 80% when the ATG MO was injected alone at this dosage. Of note, we did not observe complete rescue of morphant embryos to survival under these rescue conditions, as the majority presented as hypomorphs (Fig. 7A). Finally, coinjection of 200 pg of human YAF2 mRNA with 2.5 ng (“half-dose”) of the ATG MO was able to rescue partially the hypomorphic neural phenotype (Fig. 7B). Specifically, this coinjection gave rise to 36% of embryos displaying the hypomorphic phenotype and dying by 72 hpf, 48.5% of embryos with normal heads and surviving up through 5 days (but displaying cardiac edema), and 9.4% of embryos appearing normal (n = 203). This is in contrast to 76% of embryos developing the hypomorphic phenotype when the half-dose of the ATG MO is injected alone (Fig. 7B). Taken together, the findings of these rescue studies suggest that the specific
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A strong interaction was seen for Rybp and the caspase 8 DED (Fig. 8A, lane 5). Strong interactions with the caspase 8 DED also were observed for human and zebrafish Yaf2, respectively (Fig. 8A, lanes 1 and 3). This is consistent with the fact that the regions on Rybp/DEDAF important for interaction with the DED of caspase 10 (human DEDAF amino acids 24–45 and 144–180; (11)) are highly conserved (~77% identity) within Yaf2 (corresponding residues in zYaf2 are amino acids 22–43 and 102–137; see Fig. 1A).

In the previous report describing the Rybp/caspase interaction, it was shown that Rybp could enhance apoptosis mediated by factors in the extrinsic pathway (11). Given this and the interactions just described, we next assessed the effects of Yaf2 on caspase 8-mediated apoptosis. In the study shown in Fig. 8B, left graph, HEK293T cells were transfected with either empty vector, human caspase 8 alone, or human caspase 8 with mouse Rybp, human Yaf2, or zebrafish Yaf2. Apoptosis was determined after 24 h by nuclear morphology using an acridine orange/ethidium bromide assay; similar results were obtained using FACs with annexin V staining (data not shown). Whereas Rybp did not affect the level of caspase 8-induced apoptosis in these assays, human and zebrafish Yaf2 led to a significant and reproducible reduction (Fig. 8B, left graph). A very similar pattern was seen when zebrafish caspase 8 was used to induce the apoptosis, albeit that zebrafish Yaf2 was slightly more effective in inhibition than human Yaf2 (Fig. 8B, right graph). Of note, this study represents the first functional characterization of zebrafish caspase 8, which bears 56% similarity on the amino acid level to human caspase 8 (zebrafish caspase 8 was also recognized in Ref. 25). Whole mount in situ studies of zcaspase8 expression in the developing embryo showed that it is maternally and zygotically expressed, and can be found in the brain in older embryos (supplemental Fig. 51).

The Zebrafish Yaf2 Morphant Phenotype Is Caspase 8-mediated—Encouraged by the findings from the cell culture-based assays suggesting that Yaf2 can inhibit caspase 8-mediated apoptosis, we returned to the zebrafish to assess whether the apoptosis seen in the Yaf2 morphants was linked to caspase 8. First, a caspase 8 activation assay was performed upon lysates made from the morphants, using a synthetic caspase 8-specific substrate (LETD) conjugated to luciferin. As shown in Fig. 9A, left graph, there was a 3.7-fold increase in luciferin (indicative of caspase 8 activation) in the Yaf2 morphant embryos injected with 5 ng of ATG MO at 14 hpf as compared with stage-matched control-injected embryos. When these morphants were sampled at slightly later time points, this increase in caspase 8 activity was less impressive (~1.6-fold), suggesting that the caspase 8 activation is an early event (data not shown; see also Ref. 26). For the hypomorphic phenotype, when embryos that had been injected with the half dose of the Yaf2 ATG MO were sampled at the 18 somite stage, there was a 2.3-fold increase in luciferin as compared with stage-matched control-injected embryos (Fig. 9A, right graph). The caspase 8 activation also appears to be an early event in the hypomorphs, because at 29 hpf there was minimal caspase 8 activation observed (data not shown).

Finally, to further demonstrate that the apoptosis seen in the Yaf2 morphant embryos is caspase 8-mediated, we treated these embryos with a caspase 8-specific competitive inhibitor, zIETD-fmk (Fig. 9B). These studies showed that zIETD-fmk treatment rescues the phenotype resulting from Yaf2 deficiency (from the one somite arrest to the hypomorphic phenotype) to the same extent as zYAD-fmk treatment (compare with Fig. 4B). Taken together with the results of Fig. 8, these results suggest that Yaf2 plays a role in inhibiting caspase 8-mediated apoptosis, which may in turn relate to the cellular and organismal phenotypes observed in the morphants.

**DISCUSSION**

In this study, we have described for the first time the zebrafish Yaf2 ortholog and have shown that it is essential for normal zebrafish development. Embryos that have been depleted for Yaf2 by morpholino injection exhibit a dose-dependent phenotype. The developmental arrest before the one somite stage in morphants that have received the full dose (Fig. 3) likely reflects a null phenotype. The CNS perturbations in morphants that have received half-doses (Fig. 5) presumably result from a partial Yaf2 depletion. This theory is also supported by our studies in which co-injection of the two distinct morpholinos at their half doses led to pre-somatic arrest (data not shown). We firmly believe that the observed gross phenotypes and associated increased level of cell death result from the specific depletion of Yaf2. This stems from the facts that two independent morpholinos (acting by distinct mechanisms) yielded identical phenotypes, and that the defects were rescued to a considerable degree by co-injection of human Yaf2 mRNA (Fig. 7).

The pre-somatic arrest phenotype of Yaf2 morphant embryos classifies zyaf2 among the “early arrest” genes that when mutated give rise
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FIGURE 8. Yaf2 interacts with caspase 8 and inhibits caspase 8-mediated apoptosis. A, HEK293T cells were transfected with the indicated plasmids, and lysates were immunoprecipitated (IP) with the HA antibody, and Western-blotted with the FLAG antibody. hYaf2, zYaf2, and mRybp were recovered in caspase 8 DED+ immuno precipitates. Controls that were negative for the interaction included empty pCDNA vector and FLAG-tagged mMxi1-SRα (a member of the Myc/Max/Mad network that should not associate with caspase 8). Lysates probed with the FLAG and HA antibodies are shown below the IP blot. Molecular mass is shown in kDa on the left. B, HEK293T cells were transfected with human caspase 8 (hC8) alone or with either mRybp, hYaf2, or zYaf2 (left graph) or with zebrafish caspase 8 (zC8) alone or with either mRybp, hYaf2, or zYaf2 (right graph). Apoptosis was determined by nuclear morphology using an acridine orange/ethidium bromide assay. The amount of apoptosis (early + late) is graphed relative to that obtained with caspase 8 only, which is taken to be 100%. At least 200 cells per sample were counted, and the studies were performed in a double blind fashion. Error bars represent S.D. for two independent experiments. m, mouse; h, human; z, zebrafish.

to general phenotypes during the first day of development (22). These mutants have been subdivided into two classes. Class I mutants have increased cellular abnormalities (e.g. lysis) before morphological defects are observed. Class II mutants have morphological defects before cell death, and many of these exhibit extensive degeneration of the CNS by 30 hpf. Because dying (i.e. dark, spherical, acidine orange-, and TUNEL-positive) cells are observable in the Yaf2 morphant embryos at the tailbud stage prior to the arrest, we assign zyaf2 to the Class I group along with other genes required for general cell maintenance (22). This assignment is also supported by the finding that the cellular death appears causal to the embryonic death since treatment with either the pan-caspase inhibitor zVAD-fmk or the caspase 8-specific inhibitor zIETD-fmk allows the embryos to survive the pre-somatic arrest (Figs. 4B and 9B).

Our findings suggest that Yaf2 functions during early embryogenesis to inhibit apoptotic cell death. The timing of the developmental arrest of embryos depleted for Yaf2 may indicate that this function is required for somitogenesis, when cellular differentiation initiates (21). Notably, somitogenesis begins after a pre-patterning phase involving cyclical gene expression and repression (reviewed in Ref. 27). However, we cannot rule out that Yaf2 can also have a function prior to somitogenesis, and this function may be carried out by residual maternal Yaf2 stores (see Fig. 2). One must also consider that apoptosis in the zebrafish embryo does not normally occur before the tailbud stage, after cell cycle checkpoints have been put into place at the mid-gastrula transition (28–30). This delayed apoptosis in the normal zebrafish embryo could dictate the timing of the Yaf2 deficiency-induced phenotype, which may then not relate to the onset of somitogenesis per se.

A later developmental role for Yaf2 in cell survival in the CNS was uncovered in our studies employing lower dosages of the morpholinos. Beginning at the 10 somite stage when brain morphogenesis is initiating and continuing through 29 hpf when the brain is partitioned into its individual lobes (21), morphological changes in the head region of Yaf2 hypomorphs are apparent including the presence of dark patches of dying cells (Fig. 5B). TUNEL analysis indicated that these changes correlate with significantly increased levels of apoptosis in the hypomorphs in comparison to stage-matched controls (Fig. 6B). The end result of this massive apoptosis is the degeneration of anterior head structures including the anterior brain (for other reports on zebrafish neural degeneration mutants see Refs. 31–33). It is interesting to note that a dose-dependent role in the CNS for the Yaf2-related protein Rybp was uncovered in Rybp-deficient mice (16). Specifically, a subset of the Rybp heterozygous null mice showed an exencephalic phenotype due in part to defective neural tube closure. In addition, in rybp−/− ↔ rybp+/+ diploid embryo chimeras, the presence of Rybp-deficient cells in the developing murine CNS resulted in chaotic forebrain overgrowth, among other abnormalities (16).

Our study provides support for a possible physiological mechanism of Yaf2 action in cell survival that relates, at least in part, to its ability to inhibit caspase 8-mediated apoptosis (Fig. 8B). It also appears that caspase 8 pathways are relevant to Yaf2 function in the morphants because (i) caspase 8 activation
Conversely, 69.6% of the embryos injected with 5 ng of ATG MO and treated with 200 nM zIETD-fmk continued to develop and showed the hypomorphic phenotype, whereas only 22.5% of the embryos injected with 5 ng of Yaf2 ATG MO and left untreated developed the hypomorphic phenotype. None of the embryos injected with 10 ng of control MO showed abnormal phenotypes associated with zIETD-fmk treatment (n = 46 for untreated and n = 45 for 200 nM zIETD-fmk treated control-injected embryos).

appears to be an early event therein and (ii) specific inhibition of caspase 8 by zIETD-fmk treatment rescues the morphant phenotype (Fig. 9). Notably, this rescue was similar to that seen with pan-caspase inhibition (Fig. 4B), as well as that seen with coinjection of YAF2 mRNA (Fig. 7). Whereas the caspase 8 inhibition studies suggest that Yaf2 inhibits apoptosis at the level of caspase 8 itself, we cannot rule out that Yaf2 also may inhibit apoptosis upstream of caspase 8 or even other types of apoptosis.

From the studies reported to date, the Rybp protein appears to encode pro-apoptotic functions (11). Specifically, Rybp was shown to enhance the degree of cell death in a variety of cell types (11, 14). Moreover, although perhaps more indirect, loss of Rybp in mouse embryos leads to their failed implantation associated with dramatically diminished apoptosis during decidualization (16). Taken together with the findings presented here, the possibility exists that Yaf2 and Rybp may encode opposing functions in the regulation of cell death processes. The precise molecular roles of these two proteins as they relate to apoptosis/survival remain to be further elucidated and compared. Clues along this line may lie in the reported interaction of Rybp/DEDAF with several DED-containing proteins (11), and our findings of Yaf2 interaction with the DED of caspase 8 (Fig. 8A). However, our interaction data must be interpreted with some degree of caution until Yaf2 antibodies are generated and the existence of endogenous complexes is demonstrated.

Although our data implicate Yaf2 as a survival factor during early zebralfish development and organogenesis and suggest that Yaf2 can influence caspase 8 pathways, it is important to note that Yaf2 (and Rybp) most often have been reported to interact with transcriptional regulators. These regulators include DNA-binding proteins as well as Polycomb Group (PcG) transcriptional repressors (9, 10, 34–41). Originally classified as regulators of differentiation during embryogenesis, PcG proteins have now been implicated in the processes of cell renewal, cell cycle control, and cell survival (for recent reviews see Refs. 42–46). We have shown that, in addition to binding to caspase 8, zYaf2, like its mammalian counterpart (37, 41), is capable of interacting with the mammalian Ring1A and Ring1B PcG proteins (data not shown). Accordingly, the apoptosis resulting from Yaf2 deficiency may relate in part to the Yaf2 interaction with PcG proteins and their involvement in these cellular processes. The contributions of Yaf2-PcG interactions to the observed morphant phenotypes remain to be determined. Finally, it is possible that functions of Yaf2 in apoptosis and in Polycomb group protein biology could interrelate. One model that warrants further investigation is that Yaf2 (and Rybp) may be playing more general cellular roles in regulating processes such as post-translational modifications and/or subcellular localization.

**Acknowledgments**—We thank members of the Schreiber-Agus and Evans laboratories for stimulating discussions and helpful advice on the study. Fish husbandry was provided by Spartak Kalinin.

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