Perturbation of rRNA Synthesis in the *bap28* Mutation Leads to Apoptosis Mediated by p53 in the Zebrafish Central Nervous System*\(^2\)\(^3\)

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Zebrafish is a powerful vertebrate model system for using forward genetics to elucidate mechanisms of early development. We have used chemical mutagenesis to screen for mutants that show defects in the CNS. Here we describe the isolation of the *bap28* mutation that leads to abnormalities in the brain starting at mid-somitogenesis stages. Mutant embryos display excess apoptosis primarily in the central nervous system (CNS) and die by days 6–7 after fertilization. The mutation was positionally cloned and shown to affect a gene that encodes a large protein with high similarity to the uncharacterized human protein BAP28 and lower similarity to yeast Utpt10. Utpt10 is a component of a nucleolar U3 small nucleolar RNA-containing RNP complex that is required for transcription of ribosomal DNA and for processing of 18 S rRNA. We show that zebrafish Bap28 likewise is required for rRNA transcription and processing, with a major effect on 18 S rRNA maturation. We suggest that *bap28* is required for cell survival in the CNS through its role in rRNA synthesis and processing. Inhibition of p53 protein expression in *bap28* mutants led to embryos with morphologically normal appearance, suggesting that p53 is involved in triggering apoptosis in the *bap28* mutant CNS. The *bap28* mutation provides a genetic approach to study the role of ribosome biogenesis in the development of a vertebrate embryo.

Cell growth and proliferation in vertebrates must be synchronized with differentiation because cells usually withdraw from the cell cycle before terminal differentiation. Deregulation of these processes can lead to hypertrophy, apoptosis, or cancer. Such coordinate control requires the production of proteins in proportion to the rate of proliferation and increases in cell size. Thus, ribosome biogenesis is a tightly regulated process that involves many events including rRNA transcription, processing, modification, and folding, the coordinated synthesis of ribosomal proteins, and the assembly and export of the ribosomal particles. This process has been studied most extensively in yeast because of the powerful genetic and biochemical tools available in this organism.

The steps for rRNA synthesis and maturation are complicated processes that are regulated by protein complexes at different levels. RNA polymerase I and cofactors transcribe the monocopy rRNA genes to produce a large rRNA precursor that contains the 18 S, 5.8 S, and 28 S rRNA sequences as well as spacer regions. External transcribed spacers (ETS)\(^3\) are located at both the 5′ and 3′ ends of the pre-rRNAs, and two internal transcribed spacers (ITS) lie between the 18, 5.8, and 28 S sequences. The pre-rRNA undergoes multiple modifications and cleavages to generate the mature forms. This overall pattern of rRNA synthesis and processing is well conserved among eukaryotes, but the specific order of processing steps and the length of spacers and to a lesser extent of mature rRNAs varies considerably. In *Saccharomyces cerevisiae*, cleavage in the ETS followed by cleavages in the ITS1 generate precursors to the mature 18 S and 25 S rRNAs (1). In *Xenopus laevis*, rRNA processing follows two alternate pathways, one leading from a large precursor directly to 18 S rRNA, and the other including an 18 S rRNA-specific intermediate named 20 S RNA (2).

rRNA maturation requires several RNP complexes containing small nucleolar (sno) RNAs and many proteins (3, 4). U3 snoRNA complexes are prominently involved in the production of 18 S rRNA and assembly of the small ribosomal subunit (2, 5). In *S. cerevisiae* a 90 S complex has been identified many years ago as a precursor to 40 S and 60 S subunits (6–8). Pre-ribosomes are assembled and processed in the nucleolus, transferred to the nucleoplasm as pre-60 S and pre-40 S intermediates, and exported to the cytoplasm to form mature 60 S and 40 S ribosome subunits. Recently, several pre-ribosomal complexes have been isolated, and their protein composition has been elucidated (9). Nascent pre-rRNA appears to assemble co-transcriptionally with a protein complex, as indicated by the “terminal knobs” seen at the 5′ end of pre-rRNAs in Miller spreads (10). These knobs may correspond to RNP particles named the small ribosomal subunit (SSU) processome or 90 S pre-ribosome; these particles appear to be precursors to the 40 S ribosomal subunit as they contain U3 snoRNA and known small subunit processing factors but few components involved in large subunit processing (9, 11). A component of the SSU processome named Utpt10, a protein that is essential for survival in yeast, is located in the nucleolus and contains the Huntington-elongation-A subunit-TOR (HEAT) repeat designated as a protein interaction domain (12). Utpt10 also is a component of a distinct U3 snoRNA-containing complex that associates with the 5′-ETS of pre-rRNA (13). Thus, Utpt10 is implicated in processing events that lead to mature 18 S rRNA and the 40 S ribosomal subunit in yeast.

The tumor suppressor gene product p53 protects cells from transformation and tumorigenesis by responding to various stresses to activate genes that induce apoptosis, cell growth arrest, and senescence (14, 15). Normally, p53 protein is maintained at a low level by a negative feedback...
mechanism. Mdm2, a ring finger containing E3 ubiquitin ligase, plays a major role in this negative regulation by ubiquitinating p53 to trigger its degradation (15). Ribosomal biogenesis stress leads to effects on the cell cycle in a p53-dependent manner (16), and perturbation of rRNA synthesis by actinomycin D or by injection of anti-upstream binding factor antibody stabilizes p53 protein (17, 18). In addition, ribosomal proteins L5, L11, and L23 form a complex with MDM2, and thus, free ribosomal proteins may activate p53 by preventing its proteosomal degradation (19). Thus, activation of p53 is one mechanism through which impairment of ribosome biogenesis may affect cell behavior.

We report here the isolation and characterization of a mutant in zebrafish that affects development of the early nervous system. Increased apoptosis leads to diminished populations of neural cells with a notable deficit in neuronal precursors, whereas the overall patterning of the nervous system is not changed. We have identified the locus disabled in this mutation as encoding Bap28, a vertebrate homolog of Utp10 whose function has not been studied previously. In mutant zebrafish embryos, processing of rRNA and especially of 18 S rRNA is reduced, providing a molecular basis for the phenotype that we observed. Knockdown of p53 through an antisense morpholino in bap28 mutant embryos led to a significant reduction of apoptotic cell death, indicating that impairment of bap28 activates p53. The bap28 mutant allows the study of developmental consequences of compromised ribosome biogenesis in a vertebrate embryo.

**EXPERIMENTAL PROCEDURES**

*Zebrafish Maintenance—The Oregon AB* line was used for mutagenesis. Heterozygous mutants were crossed to the WIK line for mapping (20). Embryos were kept at 28.5 °C and staged according to Kimmel et al. (21).

*Mutagenesis—Wild type sperm was collected by gently squeezing the abdomen of male fish 8–14 months old. Approximately 5 μl of sperm in 350 μl of isotonic buffer (116 mM NaCl, 23 mM KCl, 6 mM CaCl₂, 2 mM MgSO₄, 29 mM NaHCO₃, 0.5% fructose) was mixed with 4,5 m°C. FtCCGTGAAAGTC-3’ and 5’-CGAGGAGTTGAGGAAGAGC-3’); ITS1 (5’-CTCGGAACCGTGAACTCG-3’ and 5’-GTGCTTGTTCAGGGTCCG-3’); ITS2 (5’-CTTAAACGGGAGCAGGC-3’ and 5’-AGGCGTCCTGGAACCTGATC-3’); and 18S (5’-CCCGTAGAGGTGAAATTTCA-3’ and 5’-CAGTTTGGCAACATCATCAC-3’). Embryos were kept at 28.5 °C and staged according to Kimmel et al. (21).

*Whole Mount in Situ Hybridization—Embryos were analyzed by whole-mount in situ hybridization by the protocol of Strahle et al. (23) with the following modification: 1) 0.3% CHAPS was added to all wash solutions, and 2) after hybridization, samples were washed twice with 0.2 × SSC (1 × SSC = 0.15 μM NaCl and 0.015 M sodium citrate), 0.3% CHAPS at 65 °C for 0.5 h. The bap28 probe contained the 3.3-kilobase region from the 3’ end of the cDNA.

*TUNEL Assay—For detection of apoptotic cells, the TUNEL assay was performed as described by Kawahara and Dawid (24).

*Morpholino Oligonucleotide Injection—Control, BAP28, and p53 morpholinos were obtained from Gene Tools, LLC. Sequences are: control morpholino (MO), 5’-CCTCTTACCTCAGTTACAAATTTATA-3’; BAP28-MO, 5’-GATGCCTAAGCGTATCTCCCTGG-3’. The sequence complementary to the initiation codon is underlined. The sequence of p53 morpholino was described by Langeheinrich et al. (25). 0.5 or 3 ng of morpholinos against BAP28 or p53 were injected into the yolk region of one-to-two-cell embryos.

*Northern Blotting—Total cellular RNA was prepared from 18 somite stage embryos using RNA wizard (Ambion). Total RNA (1.5 μg per lane) was separated by electrophoresis on a 6% formaldehyde, 1.2% agarose gel and blotted onto a nylon membrane (Hybond-N, Amersham Biosciences). RNA blots were fixed to the membrane by UV irradiation and probed with

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**RESULTS**

With the aim of obtaining mutations that affect the development of the CNS, we evaluated the potential of the DNA cross-linking reagent TMP as a mutagen in zebrafish. TMP has been used as a mutagen in *Escherichia coli* and is known to induce small deletions in *Caenorhabditis elegans* (28, 29). In zebrafish, no tectal neuron (ntn) is a TMP-induced mutant that has been shown to carry a 143-bp deletion (30–32). Among a small number of mutants obtained we chose a line that showed defects in brain development for further analysis. For reasons that will be explained below we named the affected locus *bap28*.

*bap28 Mutants Exhibit Abnormalities in the Midbrain-Hindbrain Boundary and the Forebrain—Zebrafish embryos homozygous for the bap28 allele, which is recessive, showed an abnormal structure and dark, rough tissue in the midbrain-hindbrain boundary by 12–18 somite stages, whereas the trunk region including the somites and notochord appeared to be normal (Fig. 1, A and B). From this stage onward, the bap28 mutant embryos showed delayed development. At 25 hpf, enlargement of tectal and hindbrain ventricles was observed in the mutant, whereas the trunk and tail still appeared normal (Fig. 1, C–F), and by 2 days post-fertilization (dpf), the head and eye were smaller in bap28 embryos as compared with the wild type. The heart and brain region in mutant embryos displayed severe edema by 4 dpf, and blood flow was slow and often accompanied by shunts in major vessels. The mutant embryos died between 6 and 7 dpf.

*Neuronal Progenitor Cells Are Reduced in the bap28 Mutant—To reveal the cell types affected in the bap28 mutant, we performed in situ hybridization with marker genes characteristic for various cell types in the CNS. The expression of the neuronal progenitor markers zash1a and neurogenin1 (ngn1) (33, 34) was reduced in the mutant. Zash1a was significantly reduced in the retina of bap28 mutant embryos by the 18 somite stage (Fig. 2, A and B), and this reduction became more apparent in the entire brain at 25 hpf (Fig. 2, C and D). Likewise, ngn1 expression was reduced in the midbrain and hindbrain at the 18 somite stage, and the deficit became more prominent at 25 hpf (Fig. 2, E–H). Although the
expression level of these genes was strongly reduced, their pattern of expression seemed unaffected, suggesting that cell fate is not changed in the mutant. The expression of other markers including shh and krox20 was not affected in the mutant embryos except for a general delay in development noted above (Fig. 2, I–L). Thus, it appears that the mutant embryos have normal anterior-posterior and dorsal-ventral patterning in the brain.

Increased Apoptosis in the CNS of bap28 Mutants—Our results imply that neuronal progenitor cells are initially formed in bap28 mutants but fail to give rise to a normal number of cells in subsequent development. Thus, these neuronal progenitor cells might be blocked in their development, leading to apoptosis. We tested this notion by checking for apoptotic cell death by TUNEL assay at different developmental stages. Similar levels and patterns of TUNEL-positive cells were detected in wild type embryos as reported by Cole and Ross (35), but DNA fragmentation in the bap28 mutant appeared to increase at the 10 somite stage. At the 18 somite stage, when morphological defects become apparent, cell death increased dramatically within the mutant brain and spinal cord (Fig. 3, A and B). Cell death in the spinal cord was unexpected because the morphology of trunk and tail appears to be normal. Sections of the trunk region of bap28 mutant embryos showed that dying cells are localized within the spinal cord, and no bias was observed with respect to localization of degenerating cells at specific dorsoventral levels (data not shown). Slightly elevated levels of cell death were also observed in the tail bud. Thus, mutation in the bap28 gene results in increased apoptosis primarily in the CNS, which can account for the compromised development of the brain in the mutant embryos. This bap28 mutant phenotype appeared to be similar to the mutant named gumowy described in a large scale ethylnitrosourea screen (36), but we have not tested complementation between these two lines.

Molecular Nature of the bap28 Mutant—To identify the gene responsible for the mutation, we mapped bap28 by bulk segregant analysis with simple sequence length polymorphism markers (37) to chromosome 12 (data not shown). Linkage was confirmed by genotyping of individual mutant embryos with the flanking simple sequence length polymorphism markers Z23536, Z1473, and Z10806. Fine mapping allowed us to restrict the critical interval harboring the mutation between markers Z23536 and Z10806 (Fig. 4A). To establish a physical contig starting from Z23536, BAC clones were isolated using Z23536 sequences as probe (see “Experimental Procedures”). We obtained BAC clones zC90G3 and zC100M12 that overlap with Z23536. These BAC clones overlap with ctg11003 from the Sanger Center zebrafish genome.
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FIGURE 4. Identification of the mutated gene. A, meiotic and physical map of the interval carrying bap28 on chromosome 12. Markers that were meiotically mapped are shown at the top; the number of recombination events and number of meiosis tested is shown below the line. BAC clones Zc090G3 and Zc100M12 were picked using Z23536 and are shown in blue. Ctg11003 is an overlapping sequence in the Sanger Institute genome data base (Assembly version 2). Using the sequences of ctf11003, BAC clones Zc146F4, Zc277H15, and Zc68H15 were identified (shown in red). The BAC clone Zc146F4 was entirely sequenced by the Sanger Center, but only end sequences were available for the other clones. Aragonase and bap28 are genes identified in the sequenced region. A, B, and C are RFLP markers generated from the available sequences in this region. Recombination mapping showed that the mutation maps between markers A and C. The bap28 gene falls within the critical interval and contains marker B, which showed no recombination with the mutation. cen, centromere; tel, telomere. B, sequencing of cDNAs obtained from wild type (wt) and mutant (mut) embryonic RNA revealed a 5-bp deletion within the coding region of the bap28 gene. The red and blue bar or arrowheads represent the two possible deletion sites that lead to the same sequence. C, the amino acid sequence at the deletion site. The 5-bp deletion at amino acid 92 leads to a frameshift followed by a stop codon. The predicted 22-amino acid missense peptide and stop codon (*) are shown in red. D, summary of Bap28 protein structure. HEAT repeats predicted by the EMBL motif search are shown as black lines, with those also predicted Pfam motif search identified by asterisks. The position of the mutation at residue 92 is indicated by the triangle. E, injection of morpholino oligonucleotide specific for bap28 (B28MO) phenocopies the mutant, as seen at 24 hpf; contMO, morpholino of unrelated sequence.

data base (Assembly version 2), and contig sequences were used to identify BAC clones Zc146F4, Zc277H15, and Zc68H15 in the fpc website of the Sanger Institute (Fig. 4A, red). Three RFLP markers, named A, B, and C, were designed from the sequences in the latter BAC clones. All three RFLP markers showed strong linkage to the mutant: A (2 recombinations in 904 meiosis), B (0/1526), and C (1/1104), with the critical interval falling between A and C (Fig. 4A).

The RFLP marker B that showed no recombination with the mutation is located within a gene with high sequence similarity to a named but uncharacterized human gene, BAP28 (direct submission GenBank™ XM375853); similarity between the zebrafish and human proteins is distributed along the entire length of the protein (zebrafish bap28 gene, GenBank™ NM199900) (Fig. 5A; supplemental Fig. S1). To test whether this gene is affected in the zebrafish mutant, the cognate cDNA was isolated and sequenced from wild type and mutant embryos (Fig. 4, B–D). bap28 cDNA from mutant embryos contained a 5-bp deletion at amino acid 92 that results in a frameshift adding 22 unrelated residues before a stop codon is encountered (Fig. 4C). The mutant is expected to produce an N-terminal peptide corresponding to less than 5% of the entire Bap28 sequence and, therefore, almost certainly represents a null mutation. A search for a possible domain structure using two independent motif search databases revealed the presence of HEAT repeats, considered to be protein-protein interaction modules. The 5-bp deletion in the mutant introduces a stop codon before the first of these HEAT repeats (Fig. 4D).

To further test the conclusion that impairment of the bap28 gene is sufficient to cause the mutant morphology, an antisense morpholino oligonucleotide overlapping the start codon of bap28 RNA was injected into wild type embryos. Injection of 0.5 ng of the morpholino was sufficient to phenocopy the mutant (Fig. 4E), indicating that lack of Bap28 protein production is the cause of the phenotype. We, therefore, named the mutant bap28.

Because the function of the mammalian Bap28 protein has not been characterized, we searched for similar proteins in other species. These searches indicated low but substantial sequence similarity along much of their entire length between Bap28 and the Utp10 proteins of S. cerevisiae (GenBank™ NC001142) and Schizosaccharomyces pombe (GenBank™ CAA18872) (supplemental Fig. S1). We believe that the Utp10 proteins are the orthologs of zebrafish and more gener-
ally vertebrate Bap28 because no more similar gene exists in either yeast genome, both of which are fully sequenced. It is notable that \textit{S. cerevisiae} Utp10 is about as similar to zebrafish Bap28 as it is to \textit{S. pombe} Utp10; in contrast, the zebrafish and human Bap28 proteins are much more closely related (Fig. 5, A and B; supplemental Fig. S1).

The spatial expression of zebrafish \textit{bap28} was analyzed by whole-mount \textit{in situ} hybridization (Fig. 6). \textit{Bap28} mRNA was expressed maternally and distributed globally until the bud stage (Fig. 6, A–C). Subsequently, high levels of expression were concentrated in the forebrain primordium, eye, midbrain, and midbrain-hindbrain boundary (Fig. 6, D–H). This distribution is consistent with the domains that showed phenotypic abnormalities during somitogenesis stages in the \textit{bap28} mutant.

\textbf{rRNA Synthesis and Maturation Are Impaired in the bap28 Mutant—}\textit{S. cerevisiae} Utp10 has been identified as a component of a U3 snoRNA-containing nucleolar complex involved in rRNA maturation that was named the SSU processome (11). Utp10 is essential for growth in yeast; its elimination leads to cessation of the production of 18 S rRNA, whereas 28 S rRNA is reduced but not eliminated. Based on the sequence similarity of Bap28 and Utp10, we hypothesized that Bap28 may regulate rRNA maturation in zebrafish. Among eukaryotes, ribosomal gene structure is highly conserved, but details of the processing pathways are more variable. To generate an overview of zebrafish rRNA processing, we generated probes from the external and both internal transcribed spacers (ETS and ITS) based on the available genomic sequence (Fig. 7B). Although sequence similarity allows prediction of mature rRNA regions and, thus, ITS domains in zebrafish, the extent of ETS regions is not known. Northern blots of RNA isolated from 18 somite-stage zebrafish embryos using 5’-ETS, ITS1, and ITS2 as probes (see B). Each panel represents two independent sets of experiments comparing the processing pattern between RNA samples derived from different batches of wild type and mutant embryos. w, wild type; m, mutant. a–e, rRNA intermediates as outlined in B; asterisk, unidentified rRNA molecules. A Northern blot of 18 S rRNA, which is mostly maternal, is shown as a loading control. B, schematic diagram showing rRNA processing pathway based on the results in A. The dashed bars represent the uncertain regions of 5’-ETS and 3’-ETS in zebrafish pre-rRNA. Probes used in Northern blotting are indicated. The drawing is not precisely to scale. The 5.8 S rRNA sequence is shown as part of intermediate (c) by analogy to other species but has not been assayed here.

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\textbf{FIGURE 6. Embryonic expression of bap28.} Lateral (A–D and F) and dorsal (E, G, and H) views of embryos stained with antisense \textit{bap28} RNA. \textit{bap28} is expressed maternally and continues to be expressed ubiquitously through the end of gastrulation. A, 1-cell embryo; B, shield stage; C, bud stage. During somitogenesis, the expression of \textit{bap28} mRNA is broad but becomes prominent in the forebrain primordium, eyes, midbrain, and midbrain-hindbrain boundary. D and E, 18 somite stage; F and G, 24 hpf; H, 33 hpf.

\textbf{FIGURE 7. The ablation of Bap28 leads to the reduction of rRNA precursors.} A, Northern blotting of RNA isolated from wild type and \textit{bap28} mutant embryos using 5’-ETS, ITS1, and ITS2 as probes (see B). Each panel represents two independent sets of experiments comparing the processing pattern between RNA samples derived from different batches of wild type and mutant embryos. A Northern blot of 18 S rRNA, which is mostly maternal, is shown as a loading control. B, schematic diagram showing rRNA processing pathway based on the results in A. The dashed bars represent the uncertain regions of 5’-ETS and 3’-ETS in zebrafish pre-rRNA. Probes used in Northern blotting are indicated. The drawing is not precisely to scale. The 5.8 S rRNA sequence is shown as part of intermediate (c) by analogy to other species but has not been assayed here.
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To assess the possible function of Bap28 in rRNA maturation, we compared the processing patterns between sibling phenotypically wild type (homozygous wild type plus heterozygous) and homozygous bap28 mutant embryos at the 18 somite stage. It is important to note that this is the earliest stage at which the mutants can be securely identified and before widespread cell death occurs in the CNS of the affected embryos. Mutant embryos showed strongly reduced rRNA precursors and intermediates (Fig. 7A, compare mutant (m) with wild type (w) lanes). This overall reduction of rRNA production without the accumulation of intermediates implies that Bap28, like its homolog Utp10, might directly regulate rDNA transcription in addition to being an essential component for rRNA processing (38). However the extent of reduction was different for 18 S and 28 S rRNA precursors. Intermediates leading to 18 S rRNA such as RNAs (d) and (e) were almost entirely absent in the mutants, whereas 28 S rRNA intermediates such as RNA (c) were reduced but still present. This result is consistent with the effect of the elimination of Utp10 in yeast where 18 S rRNA is more strongly reduced than 25 S rRNA (11) and suggests that Bap28 is a component of the U3 snoRNA-containing complex that is involved in 18 S rRNA maturation. We conclude that the zebrafish mutation we have described involves the loss of the Bap28 protein, which results in compromised rRNA processing in the embryo leading to apoptosis and developmental malformations.

Increased Apoptotic Cell Death in the CNS of bap28 Mutant Embryos Is Mediated by p53—The possible involvement of p53 in apoptosis elicited by the bap28 mutation was tested by suppressing p53 expression with the aid of an antisense morpholino (25). The results are summarized in Fig. 8 and Table 1. Although injection of a control morpholino had no effect on the frequency of the bap28 phenotype at 24 hpf among the progeny of an intercross of heterozygous fish, injection of p53 morpholino reduced the frequency of abnormal progeny to 12% or half the Mendelian level (Table 1, Phenotype). A subset of embryos from the un.injected, control morpholino-injected, and p53 morpholino-injected embryos was subjected to genotyping. Fig. 8B shows that among the phenotypically normal p53 morpholino-injected embryos, some were found that carried the bap28 mutation in their genome (Fig. 8B); one such embryo is pictured in Fig. 8A (lower panel). Table 1 presents the results for all embryos that were genotyped. All phenotypically normal control embryos tested were wild type or heterozygotes, and almost all phenotypically abnormal un.injected or control MO-injected embryos were homozygous mutant. In contrast, 20% of phenotypically normal p53 morpholino-injected embryos that were genotyped represented homozygous bap28 mutants (Table 1). Abnormal p53 MO-injected embryos were not genotyped because they would not be informative. All of the p53 morpholino-injected bap28 mutants eventually developed edema in the heart region and died between 5 and 8 dpf (data not shown). These data show that inhibition of p53 expression leads to a suppression of early apoptosis in mutant embryos, indicating that p53 is a mediator of the pathway that signals a deficit in ribosome biogenesis to the apoptotic machinery in the zebrafish embryo.

**TABLE 1**

| Genotyping of phenotypically normal and abnormal embryos |  |
|----------------------------------------------------------|--|
| **Phenotype**                                           |  |
| Norm Abnorm                                             | Norm Abnorm          |
| Uninjected                                              | p53 MO               |
| Wt Mut                                                  | Wt Mut               |
| 32 0                                                    | 3 30                 |
| 30 0                                                    | 33 NT                |
| **Genotyping of p527 and 391**                         |  |
| **Wt Mut**                                              |  |
| 527 131                                                 | 391 132              |
| 80% 20%                                                 | 75% 25%              |
| **Norm Abnorm**                                         | **Norm Abnorm**      |
| **Wt Mut**                                              | **Wt Mut**           |
| 527 131                                                 | 391 132              |
| 80% 20%                                                 | 75% 25%              |
| **Norm Abnorm**                                         | **Norm Abnorm**      |

a Of these, eight had atypical phenotype.

b 42 had atypical phenotypes.

c Six had atypical phenotypes.

DISCUSSION

In this paper we report the identification of Bap28 as the protein affected in a developmental mutant in zebrafish that is associated with increased apoptosis in the CNS, malformations in the brain, and lethality by 6–7 days of development. Tight genetic linkage, the presence of a 5-bp deletion close to the beginning of the bap28 open reading frame, and a phenocopy obtained by an antisense morpholino lead us to conclude that we have identified the gene responsible for the mutation. The affected protein is the apparent ortholog of the named but uncharacterized human protein BAP28 and of the yeast protein Utp10; we, thus, named the mutation bap28. S. cerevisiae Utp10 has been characterized...
as a component of a pre-ribosomal RNP complex and is required for rRNA production in yeast (11). We show that rRNA maturation is compromised in the zebrafish bap28 mutant, and we propose that this deficit in rRNA production constitutes the molecular basis of the observed phenotype.

A Role for Bap28 in Ribosome Formation—No functional characterization of an animal Bap28 protein has been reported, and motif searches revealed only HEAT repeats that point to a role in protein-protein interaction. However, the apparent S. cerevisiae ortholog of Bap28, Utp10, has been identified as a component of the ribosome maturation machinery. Ribosome biogenesis in yeast involves large nucleolar precursor particles that contain precursor 35 S rRNA, U3 snoRNA, and many processing and ribosomal proteins. Utp10 is a component of the SSU processome and the 90 S pre-ribosome, nucleolar RNP complexes of largely overlapping composition (11, 39). The 90 S pre-ribosome/SSU processome contains components associated with 40 S subunit processing and small subunit ribosomal proteins but lacks 60 S subunit processing components (9, 11, 39). Thus, these particles may form through the co-transcriptional assembly of pre-40 S factors onto the 35 S rRNA before the bulk of pre-60 S factors are recruited. In their characterization of the SSU processome, Dragon et al. (11) showed that depletion of Utp10 causes a reduction in the level of rRNA that is pronounced with respect to 18 S rRNA, consistent with the presence of the protein in the particle associated with small subunit synthesis. This large particle may be composed of smaller subunits, and UTP10 is associated with one of these sub-particles named UTP-A, where it copurifies with both U3 snoRNA and 5' -ETS-rRNA sequences (13). Furthermore, ablation of Utp10 is lethal in yeast (11).

Based on sequence relationships, we hypothesized that Bap28 in vertebrate animals is involved in ribosome assembly and in the maturation of rRNA, in particular 18 S rRNA. Processing of rRNA precursors and assembly of ribosomal particles in vertebrate animals proceed similarly as summarized above. In particular, U3 snoRNA and large protein complexes are involved in the process (40). Therefore, we tested the abundance of rRNA precursors and processing intermediates in mutant embryos by comparison to their wild type siblings and found all to be reduced. Furthermore, the reduction was more pronounced in intermediates leading to 18 S rRNA than in those leading to 28 S rRNA. This observation is consistent with the association of the complexes containing the Bap28 homolog Utp10 with the 5'-ETS region of pre-rRNA and the involvement of these complexes in small subunit maturation (11, 13, 39, 40). Thus, we suggest that the loss of Bap28 compromises the function of nucleolar RNP complexes involved in 40 S subunit formation and 18 S rRNA maturation.

Compromised rRNA Maturation Affects the Survival of Neuronal Progenitor Cells in the CNS—The production of ribosomes is an essential function in all cells, but the bap28 mutant shows a restricted phenotype in the brain. The requirement for Bap28 function is fully evident at the 18 somite stage, when apoptosis is greatly increased in the CNS of mutant embryos. We observed no difference in the domain of neuronal progenitor cell formation between wild type and mutant embryos, suggesting that early specification of neuronal fate does not depend on zygotically supplied Bap28. Subsequently there is a reduction in neuronal progenitor cells as seen by the expression of marker genes such as ngl1 and zash1, but some mutant cells are able to continue their development to form a neuronal network. The restriction of the phenotype to later phases of neural development may be explained in at least two non-exclusive ways. The primary reason for unaffected development in the mutant embryos during early stages is very likely the large store of mature ribosomes provided to the embryo from maternal sources.

Maternal ribosome accumulation has been studied in Xenopus a long time ago (41, 42), and whereas less is known in zebrafish, it is clear that the egg contains a large store of ribosomes. Thus, we postulate that initial development of the nervous system and of other regions and tissues in the embryo relies on maternal ribosomes. The regionally specific effect of the bap28 mutation may indicate that the brain is the first tissue to “run out” of maternal ribosomes.

A second potential explanation for the regional phenotype is that loss of Bap28 may be compensated by another factor or factors in different tissues. The expression level of bap28 mRNA was prominent in regions such as eye, midbrain, and neural keel that showed high levels of cell death in the mutant, suggesting that bap28 expression is regulated in a spatial manner. This observation further supports the view that these domains demand a high level of ribosome production, consistent with the rapid development of the brain and its large relative size in the embryo. Specifically, at these stages a majority of cells in the CNS still undergo proliferation before exiting the cell cycle to proceed to terminal differentiation. Furthermore, ribosomes are abundant in growth cones to support the process of axon projection (43, 44). Thus, Bap28 may be more highly expressed in the CNS than elsewhere, and the need for it may be more acute in this tissue.

Rather, few mutations affecting ribosome synthesis have been described in vertebrates. The anucleolate mutation, affecting rRNA genes, was instrumental in identifying the nucleolus as the site of ribosome production (45). More recently, pescadillo (pes) was identified as a developmental mutation in zebrafish, showing defects distinct from those in bap28 (46). pes mutant embryos exhibit small eyes, reduced brain and visceral skeleton, shortened fins, and liver and gut defects. The Pescadillo homolog in yeast, Nop7p, was shown subsequently to be involved in 60 S ribosome subunit synthesis (47, 48), and mouse Pescadillo is likewise essential for nucleolar assembly and ribosome formation. Pescadillo and the fibrillarin mutation, which also affects ribosome biogenesis, led to arrest of preimplantation development in the mouse (42, 43). This early effect of ribosome biogenesis mutants in the mouse is likely caused by the absence of a store of maternal ribosomes in this embryo.

The Impairment of rRNA Processing Induces Apoptosis—The analysis of the bap28 mutant implies a link between ribosome synthesis and apoptosis in the CNS. This relationship is reminiscent of the human X-linked recessive disease dyskeratosis congenita that displays bone marrow failure, nail dystrophy, mucosal leukoplaia, interstitial fibrosis of the lung, and increased susceptibility to cancer. This disease is caused by mutations in DKC1, a gene encoding dyskerin, a component of snoRNP, and the telomerase RNP (49). Ruggero and coworkers have elegantly shown that the pathogenesis of dyskeratosis congenita is due to impaired ribosome synthesis after reduced pseudouridylation caused by mutated dyskerin (50). Important in the present context is the fact that embryonic fibroblasts isolated from the Dkc1 mutant were more sensitive to translation inhibitors, as manifested by increased rates of apoptosis compared with wild type. In this study, the bap28 mutation resulted in perturbation of rRNA synthesis as well as 18 S pre-rRNA processing that appeared to be the cause of apoptotic cell death in CNS. This phenotype was rescued by inhibiting p53 protein expression (Fig. 8), suggesting that apoptotic cell death involved a p53 response to a deficit in ribosome biogenesis. It is possible that the reduction in pre-rRNA and 18 S rRNA leads to elevated levels of free ribosomal proteins including L5, L11, and L23, which are known to bind to MDM2, thus activating p53 protein (19). A different possibility is that Bap28 directly suppresses p53 mRNA levels. The yeast homolog of Bap28, UTP10, has been demonstrated to be a component of transcriptional regulatory complex of rRNA genes (38), and this
could hold for Bap28 in animal cells. The study of mutants such as bap28 should contribute to improved understanding of ribosome assembly and of consequences of its disruption during vertebrate development.

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