Loss of p53 synthesis in zebrafish tumors with ribosomal protein gene mutations

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Zebrafish carrying heterozygous mutations for 17 different ribosomal protein (*rp*) genes are prone to developing malignant peripheral nerve sheath tumors (MPNSTs), a tumor type that is seldom seen in laboratory strains of zebrafish. Interestingly, the same rare tumor type arises in zebrafish that are homozygous for a loss-of-function point mutation in the tumor suppressor gene *p53*. For these reasons, and because *p53* is widely known to be mutated in the majority of human cancers, we investigated the status of *p53* in the *rp*^{+/-} MPNSTs. Using monoclonal antibodies that we raised to zebrafish *p53*, we found that cells derived from *rp*^{+/-} MPNSTs are significantly impaired in their ability to produce *p53* protein even in the presence of a proteasome inhibitor and γ -irradiation. Although the coding regions of the *p53* gene remain wild type, the gene is transcribed, and overall protein production rates appear normal in *rp*^{+/-} MPNST cells, *p53* protein does not get synthesized. This defect is observed in all MPNSTs we examined that were derived from our 17 zebrafish lines with *rp* gene mutations. To date, studies of *p53* in malignancies have focused predominantly on either *p53* gene mutations or the aberrant posttranslational regulation of the *p53* protein. Our results show that the appropriate amount of numerous ribosomal proteins is required for *p53* protein production *in vivo* and that disruption of this regulation most likely contributes to tumorigenesis.

MPNST | protein synthesis

Among several hundred insertional mutants isolated in a screen for zebrafish genes essential for early development were mutations in 28 different ribosomal protein (*rp*) genes (1–4). Although the homozygous *rp* mutations are embryonic lethals, fish with heterozygous mutations develop into adults normally, albeit with a slight growth defect (5), as is seen in other animal models of heterozygous *rp* gene mutations (6, 7). However, heterozygous mutation of 17 of these 28 *rp* genes resulted in the development of malignant peripheral nerve sheath tumors (MPNSTs) as early as 8 months of age (1). The *rps* that when mutated give rise to MPNSTs include S3a, S5, S7, S8, S11, S15a, S18, S28, S29, L7, L13, L14, L19, L23a, L35, L36, and L36a, whereas those that do not include SA, S12, S15, acidic LP1, L3, L6, L9, L11, L12, L24, and L28 (5). The MPNST is a very rare tumor type only observed in wild-type zebrafish in our colony with a frequency of $\approx 1:2,000$ fish (A.A., unpublished data). Surprisingly, a homozygous loss-of-function point mutation in the zebrafish *p53* gene (*p53*^{M214K/M214K}) results in tumors that appear similar (8). This observation led us to consider the possibility that heterozygosity at certain *rp* loci results in the aberrant regulation of the *p53* pathway.

The tumor suppressor *p53* is one of the most intensely studied proteins in biology because it has been shown that between 50% and 70% of all human tumors contain mutations in the *p53* gene (9). Furthermore, it is thought that most of the remaining tumors that do not harbor *p53* gene mutations contain other gene amplifications or deletions that result in the down-regulation of the *p53* protein. When regulated under normal conditions, *p53* is usually kept at low levels in cells by binding to the MDM2 protein, which rapidly ubiquitinates *p53* and targets it for degradation by the proteasome (10, 11). DNA damage is one of several signals that results in the disruption of the association of MDM2 with *p53*, an event that

triggers *p53* stabilization and transcriptional activation of *p53* target genes that instigate cell cycle arrest and/or apoptosis (12, 13). *p53* can be similarly stabilized experimentally via inhibition of the proteasome with drugs such as MG132 (14). Deregulation of *p53* protein by constitutive ubiquitination has been shown to occur in tumor cells that preserve wild-type copies of the *p53* gene yet contain gene amplifications of *mdm2* or deletions of the MDM2 inhibitor *Arf* (15, 16). Thus, in addition to the vast number of *p53* gene mutations found associated with tumors, it is safe to say that many, if not all, cancerous cells contain some impairment of the *p53* pathway. Notably, zebrafish maintain the core components of the *p53* pathway. As mentioned above, *p53*^{M214K/M214K} zebrafish develop tumors (8); although there is no known *arf*, an *mdm2* ortholog has been identified. There also is genetic confirmation that the epistasis between *mdm2* and *p53* evident in mammals is conserved: *mdm2* knockdown in zebrafish embryos causes widespread apoptosis, and this phenotype is rescued by simultaneous knockdown of *p53* (17).

Although there has been no direct study to show that in humans the heterozygous loss of a ribosomal protein leads to cancer, there are several lines of intriguing evidence to suggest such a link may exist. One is the disease Diamond-Blackfan anemia (DBA). It has been shown that >25% of all patients with this disease carry heterozygous mutations in ribosomal protein genes *rpS19*, *rpS24*, or *rpS17* (18–20). It also is known that a significant number of DBA patients develop leukemia, lymphomas, or solid tumors later in life (21, 22). Additionally, the ribosomal protein gene *rpS14* has been identified as one of the nonbiallelic deletions in 5q syndrome, a subtype of myelodysplastic syndrome (MDS) known to predispose individuals to both acute and chronic myeloid leukemia (23). Very recent evidence suggests that the knockdown of the *rpS14* gene in hematopoietic progenitor cells recapitulates the phenotype of MDS and that exogenous expression of *rpS14* rescues the phenotype in MDS patient bone marrow samples (24). However, for both DBA and MDS, the role of the ribosomal protein gene mutations in the manifestation of disease and the progression to cancer remains unknown.

There also have been a number of cellular studies that link the *rps* to *p53* regulation. Several previous reports have shown that *rpL11*, *rpL5*, *rpL23*, and *rpS7* are able to bind to the *p53* inhibitor MDM2 in cells, resulting in *p53* stabilization (25–29). Furthermore, there is evidence that *rpL26* is able to bind to *p53* mRNA and influence its rate of translation (30). *rpL22* also has been implicated as being able to affect the biosynthesis of *p53* (30, 31).

In this article, we investigate how heterozygosity of numerous *rp* genes might contribute to the formation of tumors in zebrafish. This analysis reveals that there is a widespread link

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between *rp* gene dosage and the regulation of p53 protein synthesis. Specifically, we show that, irrespective of the affected *rp* gene, heterozygous *rp* gene mutant zebrafish tumor cells maintain wild-type copies of the *p53* gene and mRNA transcript, yet do not translate this transcript into p53 protein. This article provides *in vivo* evidence that alterations in *rp* gene dosage impair p53 protein synthesis and promote tumorigenesis.

Results

We have previously shown that *rp*^{+/-} mutant zebrafish develop MPNSTs. However, the molecular basis for this increased tumorigenicity was unclear (1). Subsequent studies showed that *p53*^{M214K/M214K} zebrafish developed histologically similar tumors (8), raising a possible relationship between *rp* heterozygosity and p53 regulation. To further explore this notion, we first used expression array profiling to determine whether the *rp*^{+/-} mutant tumors were indeed the same tumor type as those arising in the *p53*^{M214K/M214K} zebrafish. mRNAs used for profiling were isolated from tumor cells dissected from *p53*^{M214K/M214K} (three tumors) and *rp*^{+/-} mutants (six tumors from four different lines representing *rps* that participate in both large and small ribosomal subunits). Based on gene expression analysis, the MPNSTs from *rp*^{+/-} and *p53*^{M214K/M214K} mutant fish coclustered rather than segregating by genotype. To provide a context for the clustering, we also analyzed mRNAs from seminoma tumors (a commonly occurring tumor type in zebrafish) and compared these data with samples from other publicly available datasets representing a variety of cell types and developmental stages [supporting information (SI) Fig. S1]. We found that one of the *rp*^{+/-} samples (hi258.3) clustered most closely with a heart sample likely because a subset of the MPNSTs are highly vascularized and contain a high number of blood cells. With the exception of this single outlier, the MPNST samples are more closely related to one another than they are to any other available sample, including the seminoma tumor samples. This finding is consistent with the notion that the *rp* and *p53* mutant tumors share common origins.

Because *p53* gene mutations occur so frequently in human tumors, we first sequenced the coding exons of the *p53* gene in genomic DNA isolated from *rp*^{+/-} MPNSTs. In 18 of 18 samples we examined from several different lines of *rp*^{+/-} mutants, the *p53* gene was found to be wild type. As an added control, we also examined the sequence of *p53* mRNA transcripts isolated from *rp*^{+/-} MPNST cells. We cloned and sequenced the coding and noncoding regions of the *p53* cDNA from six different MPNSTs (originating from different mutant lines). In every case, the *p53* cDNA sequence in the MPNSTs was found to be identical to the published sequence of zebrafish *p53* (data not shown). This result would suggest that the transcription of the *p53* gene in *rp*^{+/-} MPNST cells does not introduce genetic mutations, and the *p53* mRNA transcript in these cells remains wild type. Because there has been no prior investigation of spontaneous *p53* mutations in zebrafish tumors, we also sequenced the *p53* gene in 12 seminoma samples from non-*rp* mutant backgrounds (data not shown). We found that three of these tumors had missense mutations (V124L, H161Y, and L286S) that were not germ-line mutations, as judged by comparative sequencing of tail DNA (which remained wild type). Importantly, the amino acid H161 is analogous to H193 in human p53, which also is found mutated in a number of different cancers (IARC TP53 Mutation Database, www.p53.iarc.fr). These data suggest that *p53* gene mutations do occur spontaneously in zebrafish and may contribute to tumorigenesis. However, we did not observe *p53* mutations in *rp*^{+/-} MPNSTs.

Given the absence of *p53* gene mutations in the *rp*^{+/-} MPNSTs, we next sought to determine whether the p53 protein in these cells was deregulated. To this end, we raised monoclonal antibodies to the full-length zebrafish p53 protein. Several of these antibodies

Fig. 1. Monoclonal antibodies specifically recognize zebrafish p53. (A) 293 cells were transiently transfected with HA-tagged zebrafish p53 and immunoprecipitated with monoclonal antibodies raised against zebrafish p53 (LLzp53-9E10, LLzp53-12E6, and LLzp53-16H12), followed by Western blotting with αHA. (B) Zebrafish embryos that were either uninjected or injected with p53MO or p53misMO, γ-irradiated with 20 Gy, and Western blotted with αp53.

recognize zebrafish p53 in ELISA, Western blot, immunoprecipitation, and whole-mount immunohistochemistry assays (data not shown). Three select αp53 antibodies, LLzp53-9E10, LLzp53-12E6, and LLzp53-16H12, were shown to immunoprecipitate HA-tagged zebrafish p53 expressed in transfected 293 cells (Fig. 1A). (From this point on, LLzp53-16H12 is used for Western blots and LLzp53-9E10 is used for immunoprecipitations.) Using the αp53 antibody for Western blotting, we determined that p53 levels are undetectable in zebrafish embryos 24 h after fertilization (Fig. 1B). This finding is entirely consistent with the known instability of human and murine p53 protein in the absence of DNA damage. Accordingly, after treatment of the embryos with γ-irradiation, the αp53 antibody detected two bands of 49 kDa, the predicted size of full-length zebrafish p53, and 44 kDa. To verify that these species were zebrafish p53, we injected embryos with either a translation-blocking morpholino directed to the start of the *p53* transcript (p53MO) or a similar morpholino with four base mismatches (p53misMO) at the one-cell stage. Both protein bands were lost exclusively when the p53MO was injected, confirming both the identity of these protein species as p53 and the specificity of the αp53 antibody (Fig. 1B). Based on significant mobility differences, the 44 kDa does not appear to be the previously reported truncated zebrafish p53, Δ113 (32). Thus, its identity is currently unclear. Notably, beyond embryogenesis, we only detect the presumptive full-length 49-kDa p53 species.

Having verified the specificity of our antibodies in zebrafish embryos, we next examined p53 response in zebrafish tumors. Initially, we examined the levels of p53 in control tumors, specifically seminomas from non-*rp* mutant backgrounds. For these experiments, the tumor cells were plated and then left untreated or treated either with γ-irradiation, the proteasome inhibitor MG132, or both. In the absence of treatment, p53 protein is present at low or undetectable levels in most of the seminoma samples (Fig. 2A–C). Either γ-irradiation or MG132 increased zebrafish p53 levels, and treatment with both led to further p53 stabilization (Fig. 2A–C), exactly as is observed in mammalian cells. Notably, in a small subset of the seminomas, we observed similar, high levels of p53 protein in either the absence or presence of treatment with γ-irradiation and MG132 (Fig. 2B). This finding is entirely consistent with our finding that a subset of seminomas carries *p53* mutations and the fact that mutant p53 protein is often stabilized in human tumors. In agreement with this notion, we also see high levels of p53 protein expressed in *p53* mutant tumor cells (*p53*^{M214K/M214K}

Fig. 2. $rp^{+/-}$ MPNST cells from many mutant rp lines do not express p53 protein. (A–F) Plated seminoma cells from non- rp mutant backgrounds, $p53^{M214K/M214K}$ MPNST cells, or $rp^{+/-}$ MPNST cells were exposed to 20 Gy γ -irradiation and/or overnight incubation with 20 μ M MG132 and Western blotted with α p53.

MPNST cells) in the absence of DNA damage and proteasome inhibition (Fig. 2 D–F).

Having established that both proteasome inhibition and DNA damage are capable of inducing p53 protein in zebrafish seminoma cells, we next examined the $rp^{+/-}$ MPNST cells. Remarkably, although these cells retain wild-type $p53$, we detected little or no p53 protein in plated $rp^{+/-}$ MPNST cells even after treatment with γ -irradiation and MG132 (Fig. 2). Importantly, we examined one or more MPNSTs from 17 different tumor-prone rp mutant zebrafish lines, and in every single case we observed a similar absence of p53 in the presence of DNA damage and proteasome inhibition (7 of 17 lines are shown in Fig. 2; 10 lines are data not shown). This finding shows that loss of p53 is a universal feature of tumors that result from rp haploinsufficiency, which is in stark contrast to the high expression of p53 in $p53^{M214K/M214K}$ MPNST cells, ruling out the possibility that peripheral nerve sheath cells per se are unable to synthesize p53 protein (Fig. 2 D–F).

To determine whether the loss of p53 protein in the $rp^{+/-}$ MPNSTs was due to a loss of transcription of the $p53$ gene, we measured the $p53$ mRNA in tumor cells by Northern blot analysis. Tumor cells from seminomas, $p53^{M214K/M214K}$ MPNSTs, and $rp^{+/-}$ MPNSTs were plated and subjected to γ -irradiation and MG132 as described above. Before cell lysis, the samples were divided, and a fraction of each sample was then used for either northern or Western blot analysis. $p53$ mRNA is transcribed by seminoma cells in the absence of DNA damage and proteasome inhibition (Fig. 3A). This level of $p53$ mRNA significantly increases upon treatment of the cells with γ -irradiation and MG132 most likely due to the reported p53-dependent induction of $p53$ mRNA in response to γ -irradiation (8). $p53$ mRNA also is present in MPNST cells from $rp^{+/-}$ and $p53^{M214K/M214K}$ mutants (Fig. 3A). However, in contrast to seminoma cells, neither of these tumor samples exhibit an increase of $p53$ mRNA upon γ -irradiation, suggesting that, although $p53$ is transcribed in both MPNST samples, both reveal a loss of p53 protein function.

$p53$ mRNA is consistently found at lower levels in $rp^{+/-}$ MPNST cells compared with seminoma cells, as seen in Fig. 3A. Therefore, to determine that the amount of $p53$ mRNA expressed in the $rp^{+/-}$ MPNSTs was sufficiently high to be translated into enough protein to be within the detection limits of the α p53 antibody, we compared the $p53$ mRNA and p53 protein levels in an $rp^{+/-}$ MPNST sample to a serial dilution of both total protein and RNA in seminoma cells exposed to both DNA damage and proteasome inhibition. The amount of $p53$ mRNA was similar between the MPNST sample and the 4-fold dilution of the seminoma sample. At this dilution of the seminoma sample, p53 protein was still detectable by the α p53 antibody (Fig. 3B). In contrast, the equivalent amount of $p53$ mRNA in $rp^{+/-}$ MPNSTs did not result in any detectable p53 protein despite the application of DNA damage and proteasome inhibition. This finding suggests that, although an adequate level of $p53$ mRNA exists in $rp^{+/-}$ MPNST cells to produce detectable protein, the process does not occur.

These data strongly suggest that the $p53$ mRNA in the $rp^{+/-}$ MPNSTs is not being translated into protein. Wild-type $p53$ mRNA is sufficiently present in the cells. Moreover, the nature of our assay suggests that alterations in the expression and/or activity of upstream p53 regulators, like Arf and MDM2, cannot account for the observed p53 protein loss. Specifically, if elevated ubiquitination were responsible for the lack of p53 protein either by $mdm2$ gene amplification or through deletion of Arf (or some other negative regulator of MDM2 because Arf

Fig. 3. $rp^{+/-}$ MPNST cells transcribe sufficient $p53$ mRNA to detect p53 protein, but fail to induce $p53$ transcription upon DNA damage. (A) Plated seminoma cells from non- rp mutant backgrounds, $p53^{M214K/M214K}$ MPNST cells, or $rp^{+/-}$ MPNST cells were exposed to 20 Gy γ -irradiation and/or overnight incubation with 20 μ M MG132. Cells were then split, with one part lysed for Western blot analysis with α p53 and α actin and the other part for Northern blot analysis with $p53$ and $actin$ probes. (B) The whole-cell protein lysate from seminoma cells treated with 20 Gy γ -irradiation and overnight incubation with 20 μ M MG132 was serially diluted for Western blot analysis with α p53 compared with the lysate from undiluted $rp^{+/-}$ MPNST cells. Total RNA from the same seminoma cells was diluted as for the protein lysates, and the level of $p53$ mRNA was measured by Northern blot analysis compared with undiluted total RNA from the $rp^{+/-}$ MPNST cells. Phosphorimaging quantification of the $p53$ transcript levels in the Northern blot is shown. Asterisks denote lanes of approximately equivalent levels of $p53$ mRNA.

We favor the notion that *rp* haploinsufficiency selectively impairs p53 translation. However, our data do not establish whether this defect reflects a direct effect on the translation machinery or is operating through a more indirect mechanism. For example, rps might directly or indirectly interact with RNAi machinery. It is well established that microRNAs (miRNAs) are capable of down-regulating the expression of specific proteins, and it has been proposed that translational silencing of mRNAs by miRNAs is possible with or without a detectable decrease in the levels of the target transcripts (34–36). It is entirely possible that the *p53* transcript in *rp*^{+/-} MPNST cells is being specifically regulated by miRNAs not active in seminomas or *p53*^{M214K/M214K} MPNSTs. It is not clear how stoichiometry of rps could result in the expression of *p53* specific miRNAs in the *rp*^{+/-} MPNST cells, but one possibility is that the scarcity of a single *rp* impairs ribosome biogenesis and results in excess rRNA that is processed into miRNAs. Interestingly, a number of short RNAs cloned have been found to contain rRNA sequences (Phillip Sharp, personal communication), although their function remains unknown. It would be interesting to try to map *cis*-acting elements in either the 3' or 5' UTRs of the zebrafish *p53* gene and then determine whether they show a relationship to target sequences of any detectable miRNAs. Identification of such elements also would allow investigation of other possible mechanisms, such as the possibility of direct regulation by the binding of individual ribosomal proteins like that described for human L26 (30).

Our finding that the *rp*^{+/-} MPNST cells do not synthesize p53 protein provides a new mechanism for p53 regulation and perhaps a new mechanism for tumorigenesis. Adding to the quantity of data linking *p53* gene mutations and faulty posttranslational regulation of p53 protein to tumor formation, we provide evidence for a loss of p53 protein synthesis in tumors. It will be of great interest to determine whether this mechanism is evident in human tumors and if it explains why tumors arise in some cells that maintain wild-type copies of the *p53* gene and no detectable gene amplifications of *mdm2* or deletions of *Arf*.

Materials and Methods

Zebrafish. The *rp*^{+/-} and *p53*^{M214K/M214K} zebrafish (*Danio rerio*) have been described previously (2–4, 8).

Microarray Analysis. Total RNA from zebrafish tumors was isolated with TRIzol (Invitrogen), and cRNA was hybridized to zebrafish microarrays (Affymetrix). Summarization and normalization were done with robust multiarray analysis with sequence-based (GC) background adjustment (gcRMA, www.bioconductor.org). The samples included were the 11 tumor samples from this study (GSE11493), a heart sample (GSM112796), a retina sample (GSM113859), a 16-cell stage embryo sample (GSM95612), a 10-somite embryo sample (GSM209542), a 5-day postfertilization (dpf) sample (Niss.wt1; A5005 wt1.CEL, unpublished data), and a 90-dpf sample (Emb.zon; Tue-90d-1.CEL, unpublished data). Hierarchical clustering of samples was done by using pvclust (37). Full datasets are publicly available at www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc = GSE11493.

Genomic Sequencing. Genomic DNA was isolated from tumor cells and tail tissue and used as the template in PCR using primers designed to amplify the coding exons of the *p53* gene. After gel isolation, the PCR products were sequenced with nested primers. All primer sequences are available upon request. Chromatograms were carefully observed for double peaks, indicating one wild-type copy and one mutant copy.

Antibody Development. Murine monoclonal antibodies were raised against full-length zebrafish p53 protein purified from bacteria transformed with zebrafish *p53* gene cloned into the pQE-30 vector (Qiagen) as described previously (38). Positive cell lines were identified by ELISA, single cell cloned, and tested for immunoreactivity against zebrafish p53 by Western blot, immunoprecipitation, and whole-mount immunohistochemistry.

Transfections/Immunoprecipitations. Briefly, 293 cells were transfected with 1 µg of HA-tagged zebrafish *p53* cloned into pCDNA3.1 (Invitrogen) using Lipofectamine (Invitrogen) according to the manufacturer's protocol. Immunoprecipitations were conducted with 1,000 µg of total protein and either an equal volume of the antibody supernatant or 5 µl of nonimmunized mouse serum or serum extracted from the mice immunized with zebrafish p53 protein.

Western Blotting. Tumors were dissected from zebrafish, finely diced, treated with dispase, plated on coatings of 0.2% gelatin in DME + 10% FCS, and incubated at 28°C + 5% CO₂. MG132 (Calbiochem) incubations were done for either 6 h (for Western blots with corresponding Northern blots) or 18 h (for all other samples) at a concentration of 20 µM. Cells were treated with 20 Gy γ -irradiation 4 h before lysis. Then 40 µg of total protein was run on a 10% SDS/PAGE gel for Western blotting of the tumor cells. Embryos were injected with p53MO and p53misMO morpholinos at the 1–2 cell stage as previously described (17), treated with 20 Gy γ -irradiation at 1-dpf, and lysed 4 h later. The protein from two embryos per lane was run on a 10% SDS/PAGE gel. Western blotting was performed with supernatant from α p53 hybridoma cell lines at a 1:1 dilution in TBST blocking solution (+5% powdered milk) or a 1:1,000 dilution of α -actin (Santa Cruz Biotechnology). For Western blotting of immunoprecipitations from transfected 293 cells, a 1:1 dilution of anti-influenza hemagglutinin antibody (α HA) 12CA5 supernatant was used.

Northern Blotting. RNA was isolated with TRIzol (Invitrogen), and 5–10 µg of RNA was electrophoresed through a formaldehyde denaturing agarose gel, blotted, and probed as described previously (39) using radiolabeled probes against the coding sequence of zebrafish *p53* or *actin*. Blots were exposed to a phosphorimaging screen (Molecular Dynamics) overnight and analyzed using ImageQuant software.

Pulse Labeling/Immunoprecipitations. Tumors were dissected and treated as for Western blotting. Then 2.5 h after γ -irradiation, the media were changed to DME without methionine or cysteine (Sigma); 45 min later, 200 µCi of [³⁵S]methionine/cysteine (PerkinElmer) was added to each well for 45 min. Cells were lysed and 1,200 µg of protein was incubated with an equal volume of α p53 antibody supernatant at 4°C overnight and precipitated with Protein A/G Plus agarose beads (Calbiochem) for 1 h at 4°C. Samples were run on a 10% SDS/PAGE gel that was fixed and incubated in Autofluor (National Diagnostics) for 2 h before being dried and exposed to autoradiography film (Kodak). For assessment of protein synthesis, cells were plated and labeled with 500 µCi [³⁵S]methionine/cysteine for 30 min, and then an equal number of cells ($\approx 2 \times 10^6$) from each sample was removed, lysed in equal volumes of lysis buffer, and loaded on a 10% SDS/PAGE gel that was treated for autoradiography as described above. Immunoprecipitations of actin were done with 1 µg of actin antibody (Santa Cruz Biotechnology) and lysate from an equal number of cells ($\approx 4 \times 10^7$) labeled with 500 µCi [³⁵S]methionine/cysteine for 30 min. TCA precipitations were done by labeling an equal number of cells ($\approx 1 \times 10^6$) with 100 µCi [³⁵S]methionine/cysteine for 30 min, samples were lysed, and protein was precipitated with 0.25 volumes of TCA, filtered by vacuum, and ³⁵S cpm calculated with a scintillation counter.

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- Amsterdam A, et al. (2004) Many ribosomal protein genes are cancer genes in zebrafish. *PLoS Biol* 2:E139.
- Golling G, et al. (2002) Insertional mutagenesis in zebrafish rapidly identifies genes essential for early vertebrate development. *Nat Genet* 31:135–140.
- Amsterdam A, et al. (2004) Identification of 315 genes essential for early zebrafish development. *Proc Natl Acad Sci USA* 101:12792–12797.
- Amsterdam A, et al. (1999) A large-scale insertional mutagenesis screen in zebrafish. *Genes Dev* 13:2713–2724.

- Lai K (2006) A cancer screen in zebrafish identifies many ribosomal proteins as haploinsufficient tumor suppressor. PhD dissertation (Massachusetts Institute of Technology, Cambridge, MA).
- Oliver ER, Saunders TL, Tarle SA, Glaser T (2004) Ribosomal protein L24 defect in belly spot and tail (Bst), a mouse Minute. *Development (Cambridge, UK)* 131:3907–3920.
- Marygold SJ, et al. (2007) The ribosomal protein genes and Minute loci of *Drosophila melanogaster*. *Genome Biol* 8:R216.

8. Berghmans S, *et al.* (2005) tp53 mutant zebrafish develop malignant peripheral nerve sheath tumors. *Proc Natl Acad Sci USA* 102:407–412.
9. Lane DP (2005) Exploiting the p53 pathway for the diagnosis and therapy of human cancer. *Cold Spring Harb Symp Quant Biol* 70:489–497.
10. Momand J, Zambetti GP, Olson DC, George D, Levine AJ (1992) The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell* 69:1237–1245.
11. Honda R, Tanaka H, Yasuda H (1997) Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. *FEBS Lett* 420:25–27.
12. Kastan MB, Onyekwere O, Sidransky D, Vogelstein B, Craig RW (1991) Participation of p53 protein in the cellular response to DNA damage. *Cancer Res* 51:6304–6311.
13. Lu X, Lane DP (1993) Differential induction of transcriptionally active p53 following UV or ionizing radiation: Defects in chromosome instability syndromes? *Cell* 75:765–778.
14. Maki CG, Huibregtse JM, Howley PM (1996) *In vivo* ubiquitination and proteasome-mediated degradation of p53(1). *Cancer Res* 56:2649–2654.
15. Oliner JD, Kinzler KW, Meltzer PS, George DL, Vogelstein B (1992) Amplification of a gene encoding a p53-associated protein in human sarcomas. *Nature* 358:80–83.
16. Zhang Y, Xiong Y, Yarbrough WG (1998) ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. *Cell* 92:725–734.
17. Langheinrich U, Hennen E, Stott G, Vacun G (2002) Zebrafish as a model organism for the identification and characterization of drugs and genes affecting p53 signaling. *Curr Biol* 12:2023–2028.
18. Drapchinskai N, *et al.* (1999) The gene encoding ribosomal protein S19 is mutated in Diamond-Blackfan anaemia. *Nat Genet* 21:169–175.
19. Gazda HT, *et al.* (2006) Ribosomal protein S24 gene is mutated in Diamond-Blackfan anemia. *Am J Hum Genet* 79:1110–1118.
20. Cmejla R, Cmejlova J, Handrkova H, Petrak J, Pospisilova D (2007) Ribosomal protein S17 gene (RP517) is mutated in Diamond-Blackfan anemia. *Hum Mutat* 28:1178–1182.
21. Willig TN, Gazda H, Sieff CA (2000) Diamond-Blackfan anemia. *Curr Opin Hematol* 7:85–94.
22. Lipton JM, *et al.* (2001) Osteogenic sarcoma associated with Diamond-Blackfan anemia: A report from the Diamond-Blackfan Anemia Registry. *J Pediatr Hematol Oncol* 23:39–44.
23. Boultonwood J, Lewis S, Wainscoat JS (1994) The 5q-syndrome. *Blood* 84:3253–3260.
24. Ebert BL, *et al.* (2008) Identification of RP514 as a 5q-syndrome gene by RNA interference screen. *Nature* 451:335–339.
25. Lohrm MA, Ludwig RL, Kubbutat MH, Hanlon M, Vousden KH (2003) Regulation of HDM2 activity by the ribosomal protein L11. *Cancer Cell* 3:577–587.
26. Marchal V, Elenbaas B, Piette J, Nicolas JC, Levine AJ (1994) The ribosomal L5 protein is associated with mdm-2 and mdm-2-p53 complexes. *Mol Cell Biol* 14:7414–7420.
27. Dai MS, Lu H (2004) Inhibition of MDM2-mediated p53 ubiquitination and degradation by ribosomal protein L5. *J Biol Chem* 279:44475–44482.
28. Chen D, *et al.* (2007) Ribosomal protein S7 as a novel modulator of p53-MDM2 interaction: Binding to MDM2, stabilization of p53 protein, and activation of p53 function. *Oncogene* 26:5029–5037.
29. Jin A, Itahana K, O'Keefe K, Zhang Y (2004) Inhibition of HDM2 and activation of p53 by ribosomal protein L23. *Mol Cell Biol* 24:7669–7680.
30. Takagi M, Absalon MJ, McLure KG, Kastan MB (2005) Regulation of p53 translation and induction after DNA damage by ribosomal protein L26 and nucleolin. *Cell* 123:49–63.
31. Anderson SJ, *et al.* (2007) Ablation of ribosomal protein L22 selectively impairs alpha-beta T cell development by activation of a p53-dependent checkpoint. *Immunity* 26:759–772.
32. Chen J, *et al.* (2005) Loss of function of def selectively up-regulates Delta113p53 expression to arrest expansion growth of digestive organs in zebrafish. *Genes Dev* 19:2900–2911.
33. Bortoluzzi S, d'Alessi F, Romualdi C, Danieli GA (2001) Differential expression of genes coding for ribosomal proteins in different human tissues. *Bioinformatics* 17:1152–1157.
34. O'Donnell KA, Wentzel EA, Zeller KI, Dang CV, Mendell JT (2005) c-Myc-regulated microRNAs modulate E2F1 expression. *Nature* 435:839–843.
35. Poy MN, *et al.* (2004) A pancreatic islet-specific microRNA regulates insulin secretion. *Nature* 432:226–230.
36. Jackson RJ, Standart N (2007) How do microRNAs regulate gene expression? *Sci STKE* 2007:re1.
37. Suzuki R, Shimodaira H (2006) Pvcust: An R package for assessing the uncertainty in hierarchical clustering. *Bioinformatics* 22:1540–1542.
38. Trimarchi JM, Fairchild B, Wen J, Lees JA (2001) The E2F6 transcription factor is a component of the mammalian Bmi1-containing polycomb complex. *Proc Natl Acad Sci USA* 98:1519–1524.
39. Gaiano N, *et al.* (1996) Insertional mutagenesis and rapid cloning of essential genes in zebrafish. *Nature* 383:829–832.