Identification of p53 regulators by genome-wide functional analysis

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The p53 tumor-suppressor protein is a critical mediator of cellular growth arrest and the induction of apoptosis. To identify proteins involved in the modulation of p53 transcriptional activity, a gain-of-function cellular screen was carried out with an arrayed matrix of $\approx 20,000$ cDNAs. Nine genes previously unknown to be involved in regulating p53 activity were identified. Overexpression of seven of these genes (*Hey1*, *Hes1*, *TFAP4*, *Osr1*, *NR2F2*, *SFRS10*, and *FLJ11339*) resulted in up-regulation of p53 activity; overexpression of two genes (*M17S2* and *cathepsin B*) resulted in down-regulation of p53 activity in mammalian cells. HES1, HEY1, and TFAP4, which are members of the basic helix-loop-helix transcription family, and OSR1 were shown to activate p53 through repression of HDM2 transcription. Ectopic expression of these basic helix-loop-helix transcription factors in both zebrafish and avian developmental systems activated p53 and induced apoptosis *in vivo*, resulting in a phenotype similar to that of p53 overexpression. Furthermore, *ras*- and *myc*-mediated transformation of mouse embryonic fibroblasts was abrogated by expression of HEY1 in a p53-dependent manner. These results suggest that these transcription factors are members of an evolutionarily conserved network that governs p53 function.

The p53 tumor suppressor protein is a transcription factor that negatively regulates cell proliferation. It is involved in a number of cell-signaling pathways, including cell cycle, programmed cell death, and DNA repair. Activation of the p53 tumor suppressor protein occurs in response to a number of cellular stress signals such as DNA damage, UV light, and oncogene activation, and initiates a transcriptional program that can result in cell cycle arrest, senescence, or apoptosis (1, 2). The central importance of this transcription factor in these cellular processes is underscored by the high mutational frequency of the p53 genetic locus in human cancers (3). In $\approx 50\%$ of all human cancers, p53 is inactivated directly as a result of point mutations in the p53 gene (3). In many other malignancies that retain WT p53 gene, tumors acquire other mechanisms to compromise the p53 pathway in response to oncogenic stimuli (1). Thus, inactivation of p53 pathway is a common feature in tumor development.

Regulation of the cell cycle and tumor suppressor activities of p53 is known to be controlled by various mechanisms such as subcellular localization, phosphorylation, acetylation, and degradation (1, 2). Although p53 activity is regulated by various molecules (4–7), a common feature is the control of p53 protein stability. One key molecule in this regulatory function is HDM2, the human homolog of MDM2, which directs the nuclear export and proteasomal degradation of p53 in a ubiquitin-dependent fashion (8, 9). Many cellular stresses such as DNA damage stabilize p53 protein by mechanisms that block the HDM2-mediated degradation of p53 (10). For example, in response to oncogene activation, p14^{ARF} activates p53 by inhibiting the ubiquitin ligase activity of HDM2 and relieving HDM2-dependent inhibition of p53 acetylation (11).

To identify additional gene products that might be involved in the response of this tumor suppressor protein to cellular stress, a genome-scale gain-of-function cellular screen was carried out. Here, we report seven proteins that enhance p53 activity and two proteins that inhibit the tumor suppressor. Among the activators, five act at the posttranslational level, and two function at the transcriptional level. A number of the activators belong to the basic helix-loop-helix (bHLH) superfamily of transcription factors, three of which (HEY1, HES1 and TFAP4) are shown to activate p53 through transcriptional modulation of HDM2 expression. Furthermore, HEY1 and HES1 consistently recapitulate p53-induced phenotypes when ectopically expressed in zebrafish and chick embryos and can function as p53-dependent suppressors of transformation in mouse embryo fibroblasts.

Materials and Methods

High-Throughput Transfection and Reporter Assay. High-throughput (retro)transfections of the 20,704-member arrayed cDNA library were carried out essentially as described (12). Briefly, to each well of 384-well plates containing 62.5 ng of a distinct cDNA was added 20 μ l of serum-free medium containing FuGENE 6 (Roche) and the p53 response element reporter p53-luc (CLONTECH). Forty microliters of 20% FBS DMEM media containing 10⁴ HCT116 cells were plated in each well. After 48 h at 37°C in 5% CO₂, 40 μ l of the luciferase assay reagent BrightGlo (Promega) was added to each well, and luminescence was immediately analyzed with an Acquest Plate Reader (LJL Biosystems, Sunnyvale, CA). Relative light intensity was normalized on a per-plate basis, and genes were ranked according to median activation. Detailed specifications of the cDNA library can be found at <http://function.gnf.org>. To compare the distribution of replicate assays, a Q-Q plot was generated by using the inverse function of the empirical cumulative distribution function (quantiles) of replicate samples as ordered pairs in the graph (13). Deviation from a straight line through the upper and lower quartiles of these ordered pairs is indicative of differences in the distributions.

Microinjection of Retrovirus in Chick Embryos. Chick embryos were obtained from MacIntyre Poultry (San Diego) and staged as described (14). Full-length cDNAs were cloned into RCAS vector and transfected into embryonic chick fibroblasts by using Lipofectin (GIBCO/BRL). Retrovirus production, harvesting, and concentration were carried out as reported (15). Concentrated virus was microinjected into the right lateral plate of stage 14 chick embryos. The left side was used as control. After injection, some embryos were incubated to stage 24–25, harvested, inspected for phenotypical alterations, and then stained

Abbreviations: bHLH, basic helix-loop-helix; MEF, mouse embryonic fibroblast; eGFP, enhanced GFP; β -gal, β -galactosidase.

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for cell death with neutral red (16). Other embryos were incubated to stage 35–36, fixed in 5% trichloroacetic acid overnight, and stained for cartilage with 0.1% Alcian green. Skeletal morphology was analyzed after dehydrating and clearing the embryos in methylsalicylate.

Microinjections of RNA in Zebrafish Embryos. WT zebrafish (AB line) were maintained at 28.5°C and bred to obtain fertilized eggs following standard methods (17). Capped RNAs were synthesized by using the mMessage mMachine kit (Ambion, Austin, TX). RNA (100 pg) was microinjected into one-cell-stage zebrafish embryos. Groups of embryos were fixed at different time points between 12 and 48 h postfertilization and processed for *in situ* hybridization (18). Cell death was analyzed in live embryos by vital staining with acridine orange following published methods (18).

In Situ Hybridization. EST clones MPMGp609O1914 and MPMGp609B127, representing zebrafish *p53*, were obtained through RZPD Deutsches Ressourcenzentrum für Genomforschung (Berlin) and used to synthesize antisense RNA probes. Whole-mount *in situ* hybridization of zebrafish embryos was performed as described (19).

Foci Formation Assay. Phoenix A cells (1.5×10^6) were plated on a 60-mm plate, and 5 μ g of plasmid containing *ras*, *myc*, or cDNAs from the screen were transfected with Lipofectamine 2000 (Invitrogen). Cells were incubated at 37°C in 5% CO₂ and medium was changed 12 h after transfection; supernatant containing virus was collected 48 h and 72 h after transfection and filtered through a 0.45- μ m filter. WT mouse embryonic fibroblasts (MEFs) or *p53*^{−/−} MEFs were plated in six-well 35-mm plates (10^5 cells per plate) and incubated overnight at 37°C in 5% CO₂. Equal amounts of viruses containing *ras*, *myc*, or cDNAs from the screen (0.4 ml each) along with 1 ml of DMEM and 8 μ g/ml (final concentration) polybrene were added to each well. Plates were centrifuged for 90 min at 2,500 $\times$ g. Medium was changed 12 h postinfection, and foci were stained with crystal violet 10–14 days postinfection. The medium was aspirated, the cells were washed with PBS, fixed in 5% formaldehyde/PBS for 5 min, and then stained for 30 min at room temperature in a solution of 0.1% crystal violet, 20% aqueous methanol. After thorough washing with water to remove unincorporated dye, the plates were dried completely at room temperature and photographed.

Immunoblots and RT-PCR. HCT116 cells (5×10^5) were plated 12 h before transfection in six-well 35-mm plates. Cells were transfected with 3 μ g of plasmid per well with FuGENE 6 and cultured at 37°C in 5% CO₂. Cell extracts were prepared and immunoblots were performed as described (20). The blots were incubated with primary antibodies, anti-p53 (Ab-6), anti-p21 (Ab-1), and anti- β -tubulin monoclonal antibodies from Calbiochem, anti-hDM2 from Pharmingen, and subsequently, with the secondary HRP-conjugated antibody (Calbiochem). Signals were detected with an ECL plus kit (Pharmacia). For RT-PCR, cells were collected 36 h posttransfection, and RNA was purified by using RNeasy Kit (Qiagen, Valencia, CA). The amount of template and number of PCR cycles were optimized to ensure reactions were in the linear range of amplification. Equal amounts of each PCR reaction were loaded on a 1% agarose gel and photographed.

Results and Discussion

High-Throughput Screen of Arrayed cDNA Library. A cDNA expression library containing 20,704 arrayed full-length cDNAs in the mammalian expression vector pCMVSPORT6 (GIBCO) was screened for regulators of p53 activity. Briefly, by means of a

high-throughput transfection methodology, individual genes along with the p53 reporter construct, p53-luc, were introduced into human colon cancer HCT116 cells. The p53 reporter construct contains a luciferase gene under the control of 14 tandem p53 response elements (tgcttgacttgcttgcc). This reporter will identify not only genes that regulate p53 at the transcriptional level but also genes that regulate p53 activity at the posttranslational level. Single-well luminescence was used to detect luciferase activity across the cDNA array (Fig. 1*A* and *B*). The reproducibility for the screening data set was assessed by calculating quantile distributions derived from duplicate assays and plotting these values against the quantiles of a normal distribution (Fig. 1*C*). Only minor deviations from a straight line were observed, indicating a high degree of reproducibility for independent experiments. The experimental “noise” of the assay was determined by carrying out an independent screen in a p53-deficient background. A comparison of the activity plots from the corresponding assays (Fig. 1*D*) suggests that only a small fraction of the observed activation or inhibition of p53 can be attributed to nonspecific effects.

Two known activators of p53 function, HIF1 α and p14^{ARF}, were among the cDNAs identified by this screen, suggesting that the results from the screen are valid. Interestingly, the oncogene *myc* did not induce p53, probably due to p14^{ARF} defect in HCT116 cells. Seven proteins, including HEY1, OSR1, HES1, TFAP4, NR2F2, SRFS10 and a hypothetical protein FLJ11339, were found to be potent activators of p53 function, and, conversely, M17S2 and cathepsin B were identified as inhibitors of the tumor suppressor. Among the genes identified in the screen, *Hey1*, *Hes1*, and *TFAP4* are members of the bHLH transcription factor superfamily. *Hey1* and *Hes1* can also be grouped into the class C subfamily based on sequence comparison and can serve as transcriptional repressors or activators in a cell type-dependent manner (21). OSR1 is a human homolog of murine odd-skipped related protein that plays an important role in embryonic development (22). NR2F2 is a nuclear receptor that is involved in the modulation of steroidogenic enzymes in human ovary (23), and SFRS10 is a splicing factor involved in the splicing of calcitonin (24). Intriguingly, both negative regulators have been previously linked to tumorigenesis. M17S2 is a surface marker in ovarian cancer (25), and the expression of M17S2 was found to be inversely correlated to the survival in renal cell carcinoma patients (26). Cathepsin B is a lysosomal cysteine protease, and the overexpression of cathepsin B has been associated with ovarian cancer, adenocarcinoma, and other tumors (27). The ability of these proteins to inhibit p53 activity may underlie these correlations to oncogenesis.

p53 Activators Function Through a p53-Dependent Mechanism. To confirm that the activities of the cDNAs identified in the reporter assay were specifically dependent on p53 function, cDNAs were cotransfected with p53-luc into isogenic HCT116 p53^{+/+} and p53^{−/−} cells, which are genetically identical except for p53 status. In WT (p53^{+/+}) HCT116 cells, the seven activators consistently led to a 3- to 8-fold induction in reporter activity; M17S2 and cathepsin B inhibited p53 activity by 60% and 50%, respectively (Fig. 2*A* and *B*). When expressed in a p53^{−/−} genetic background, none of the encoded proteins activated or inhibited transcription of the luciferase reporter gene (Fig. 2*A* and *B*). These results strongly suggest that the observed activities are mediated by direct or indirect regulation of p53 activity. To further demonstrate that the cDNAs function through a p53-dependent mechanism, a reporter vector devoid of the p53 response elements (tgcttgacttgcttgcc) was cotransfected with the cDNAs into HCT116 cells. The transfected cDNAs had no effect on luciferase activity, indicating that these genes are not functioning as general activators or repressors of transcription.

Fig. 1. Genome-wide screen of novel p53 regulators. (A) Scheme illustrating high-throughput screen. Approximately 20,000 cDNAs were cotransfected into 384-well plates with p53 reporter plasmid p53-luc. After a 48-h incubation at 37°C in 5% CO₂, cells were lysed and luciferase activity were determined. (B) Single-well luminescence signals. Red indicates high luciferase activity; blue indicates low luciferase activity. (C) Reproducibility of the screening data was assessed by calculation of quantile distribution derived from duplicate assays and plotting these values against the quantile of a normal distribution. (D) Comparison of p53 activity plot from the screens in isogenic HCT116 p53^{+/+} and HCT116 p53^{-/-} cells.

The induction of endogenous p53 expression by the putative activators HEY1, OSR1, HES1, TFAP4, NR2F2, SFRS10, and FLJ11339 along with positive control p53 was confirmed by immunoblot analysis. Ectopic expression of these genes led to a significant accumulation of the p53 protein product, when

compared with steady-state protein levels in HCT116 cells (Fig. 2C). These results demonstrate that these genes are bona fide agonists of the p53 transcriptional network.

p53 activity is known to be regulated at both the transcriptional level and posttranslational levels (28). To distinguish between these mechanisms, we profiled the activities of these putative p53 modulators by using p53-1-luc, a reporter vector that contains a luciferase gene under the control of genomic sequences corresponding to the p53 promoter region. To circumvent any possible autoregulatory effects, cDNAs were co-transfected into HCT116 p53^{-/-} cells, and reporter gene activities were measured after 48 h. Expression of *NR2F2* and *SFRS10* genes resulted in a dramatic increase in reporter activity, suggesting that these two proteins increase levels of p53 transcription. *NR2F2* is a transcription factor that likely binds the p53 promoter region and increases p53 expression directly, whereas *SFRS10*, a splicing factor, may affect p53 transcriptional regulators indirectly. The remaining five putative modulators, HEY1, HES1, OSR1, TFAP4, and FLJ11339, had no significant effect in this reporter assay, suggesting that these regulators likely alter p53 activity through posttranslational mechanisms (Fig. 2D).

HEY1, HES1, TFAP4, and OSR1 Inhibit HDM2 Expression. Among these five posttranslational modulators, three genes, *Hey1*, *Hes1* and *TFAP4*, are members of the bHLH transcription factor family (21). One possibility is that these genes regulate p53 activity by modulating the expression of HDM2. HDM2, the human homologue of MDM2, is a major regulator of p53 function at the posttranslational level. To test this notion, a reporter construct (hDM2luc01) containing a luciferase gene under the control of HDM2 promoter region, was cotransfected with each of these bHLH genes, as well as an unrelated bHLH transcriptional repressor *DCP1*, into HCT116 p53^{-/-} cells. The use of HCT116 p53^{-/-} cells circumvents the autoregulatory feedback loop between p53 and HDM2. All three proteins HEY1, HES1, and TFAP4 inhibited the HDM2 reporter activity 3- to 8-fold

Fig. 2. Novel p53 regulators activate or suppress p53 expression activity in mammalian cells. p53-positive regulators and control HIF1 α (A) or negative regulators and control HDM2 (B) were transfected with FuGENE 6 into isogenic HCT116 p53^{+/+} and p53^{-/-} cells with the p53 reporter and a plasmid overexpressing β -galactosidase (β -gal). After 48 h incubation, cells were lysed, and luciferase activity was normalized based on β -gal activity. (C) Cell lysate from HCT116 cells transfected with p53 regulators were probed with antibodies against p53. (D) Reporter plasmid p53-1-luc was transfected into HCT116 p53^{-/-} cells with p53 activators and β -gal plasmid. After incubating for 48 h, cells were lysed, and luciferase activity was normalized based on β -gal activity.

Fig. 3. p53 regulators activate p53 by an HDM2-dependent mechanism. (A) A reporter plasmid containing the HDM2 promoter sequence was transfected into HCT116 p53^{-/-} cells along with *Hey1*, *Hes1*, *TFAP4*, or *Osr1* and β-gal plasmid. After incubating for 48 h, cells were lysed, and luciferase activity was detected and normalized based on β-gal activity. (B) Cell lysates from HCT116 p53^{-/-} cells transfected with *Hey1*, *Hes1*, *TFAP4*, or *Osr1* and immunoblotted with anti-HDM2 antibody. (C) mRNAs from HCT116 p53^{-/-} cells transfected with *Hey1*, *Hes1*, *TFAP4*, or *Osr1* were extracted, and RT-PCR was performed by using primers corresponding to HDM2 sequence.

whereas DCP1 expression had no inhibitory activity (Fig. 3A). Interestingly, the protein OSR1 also showed a similar inhibitory effect (Fig. 3A). Immunoblot analysis of cellular extracts confirmed that HDM2 protein levels are significantly decreased after the transfection of these four genes (Fig. 3B). Semiquantitative RT-PCR analysis also confirmed that expression of these bHLH proteins inhibit HDM2 expression at the transcriptional level (Fig. 3C).

Among these genes, *Hey1* and *Hes1* are members of the class C subfamily of bHLH transcription repressors and are related to *Drosophila* hairy and Enhancer of split (E(spl)) (21). These transcription factors are known to repress transcription by two mechanisms. First, these proteins bind to specific DNA sequences, such as E box (CANNTG), N box (CACNAG), and class C (CACGNG) sites, and recruit other transcriptional corepressors to inhibit target gene expression through the C-terminal tetrapeptide or Orange domain/helix3–helix4 motifs. Second, these proteins may act as passive repressors of transcription by sequestering bHLH activator proteins through formation of nonfunctional heterodimers. We identified three E box sequences (CACGTG, CAGGTG, CAGCTG), one N box (CACTAG), and one class C box sequence (CACGGG) in the HDM2 promoter region. These DNA sequences were synthesized, and nuclear extracts from HCT116 and HEK293 cells overexpression of HEY1 and HES1 were prepared and incubated with the duplexes with or without specific antibodies against HEY1 or HES1. Electrophoretic mobility-shift assay was performed to test the ability of HEY1 and HES1 to bind elements of HDM2 promoter. Under the conditions we used, HEY1 and HES1 do not associate with any identified E box, N box, or class C binding elements in the HMD2 promoter region, suggesting that HDM2 regulation by these proteins may depend on other activities not present in the extract or, alternatively, may be independent of their DNA-binding activities.

p53 Activators Up-Regulate p53 and Induce Apoptosis in Zebrafish. Next, we investigated the activity of the p53 by using the developing zebrafish embryo as a model system. Up-regulation of p53 in zebrafish embryos by UV irradiation or by down-

regulation of zebrafish homologs of *mdm2* has been shown to produce readily recognizable phenotypes such as growth retardation and an increase of apoptosis (29). Consistent with these results, ectopic overexpression of p53 mRNA induced a generalized growth arrest and failure to develop anterior and posterior structures in 82% of embryos ($n = 120$), concurrent with an increased number of apoptotic cells throughout the embryo (Fig. 4E). Interestingly, microinjection of RNAs encoding the p53 activators into one-cell-stage zebrafish embryos resulted in various degrees of developmental arrest, which could be grouped in two phenotypic classes. Injection of mRNAs encoding *Hes1*, *Hey1*, or *NR2F2* induced defects indistinguishable from those produced by p53 overexpression in 89%, 92%, and 82% of the embryos with $n = 170$, 175, and 120, respectively (Fig. 3B and F). Overexpression of *Osr1* or *SFRS10* resulted in less severe phenotypes in which the development of anterior structures was affected in 87% and 90% of the embryos with $n = 200$ and 150, respectively (Fig. 4C and G). Consistent with these results, we found that overexpression of *Hes1*, *Hey1*, or *NR2F2* caused generalized apoptosis throughout the embryo (Fig. 4D and F) whereas injection of *Osr1* or *SFRS10* mRNA led to an increased number of apoptotic cells only in regions of normal programmed cell death (compare Fig. 4G and D).

The pattern of endogenous p53 expression was then analyzed by whole-mount *in situ* hybridization of zebrafish embryos overexpressing the transcripts of interest, or a control mRNA, enhanced GFP (eGFP). Notably, all of the genes tested were able to induce p53 expression although *Hes1*, *Hey1*, and *NR2F2* resulted in stronger and more widespread induction than *Osr1* or *SFRS10* (Fig. 4A–C). Our results indicate that HEY1, HES1, and NR2F2 are sufficient to induce p53-mediated apoptosis in zebrafish whereas OSR1 and SFRS10 seem to require additional cofactors to induce p53 expression. These results are consistent with our *in vitro* findings that HEY1 and HES1 inhibit HDM2 transcription because inhibition of *mdm2* homolog in zebrafish embryos results in up-regulation of p53 expression (29).

In Vivo Activity of p53 Activators in Chick Embryos. The early developmental arrest induced by overexpression of some p53 regulators in zebrafish embryos prevented analysis of later phenotypes and raised the possibility that some effects might be secondary to a general block in embryo development. For these reasons, *in vivo* experiments were also carried out with chick embryos, which permit misexpression experiments in a temporally and spatially restricted manner. Retroviral vectors (RCAS) expressing *Hey1*, *Hes1*, or *Osr1*, as well as either a positive (p53) or a negative eGFP control, were injected into the presumptive wing field of stage-14 chick embryos, and wing development was monitored for changes. Injection of RCAS-p53 led to a significant truncation of the wings in 33% of the embryos ($n = 24$). Morphological examination of the truncated wings after cartilage staining revealed that limb elements were truncated at later stages of development. The most common phenotypes were hypoplasia or absence of zeugopodal elements, typically the radius, and the absence of some anterior digits (Fig. 4K). These phenotypes closely resemble alterations induced by misexpression of other proapoptotic genes, such as *death inducer-obliterator-1* (*DIO-1*; ref. 30) and are consistent with increased apoptosis in the apical ectodermal ridge and/or underlying mesenchyme during development. Importantly, retroviral delivery of *Hey1*, *Hes1*, or *Osr1* induced similar distal truncations in 26%, 31%, and 36% of embryos with $n = 21$, 26, and 28, respectively (Fig. 4I and L). Consistent with the mild phenotype induced by *SFRS10* overexpression in zebrafish embryos, injection of RCAS-*SFRS10* into the presumptive wing field of chick embryos did not result in noticeable wing defects (100% of embryos, $n = 24$). Embryos injected with a retrovirus expressing eGFP did not display any obvious phenotype (100% of embryos,

Fig. 4. Phenotypic characterization of cDNAs in zebrafish, chicken, and MEFs. (A–M) *Hey1*, *Hes1*, *Osr1*, *NR2F2*, and *SFRS10* induce p53-mediated apoptosis *in vivo*. (A–G) Zebrafish embryos were injected at one-cell stage with mRNAs encoding eGFP (control), *p53*, *Hey1*, *Hes1*, *Osr1*, *NR2F2*, or *SFRS10* (B, C, F, and G), and analyzed at 30 h postfertilization (hpf) for p53 expression or at 40 hpf for cell death. Shown are representative images for different treatments and assays. (A–C) Detection of endogenous zebrafish *p53* (*zp53*) by whole-mount *in situ* hybridization. Embryos injected with mRNAs encoding eGFP (A, control), *Hey1* (B), or *Osr1* (C) revealed strong induction of *p53* transcripts, more widespread for *Hey1* (B) than for *Osr1* (C). Note the severe anterior and posterior defects induced by *Hey1* overexpression (B). (D–G) Detection of apoptotic cells by acridine orange staining showed massive cell death in embryos injected with mRNAs encoding *p53* (E) or *Hes1* (F) whereas overexpression of *SFRS10* (G) resulted in increased apoptosis in areas where normal programmed cell death takes place (D). Also note the severe anterior and posterior defects induced by *p53* (E) or *Hes1* (F) overexpression. All views are lateral, anterior to the left and dorsal to the top. (H–M) Chick embryos were injected in the prospective right wing field at HH14 (stage 14) with retroviruses (RCAS) encoding eGFP, *p53*, *Hey1*, *Hes1*, *Osr1*, *NR2F2*, or *SFRS10* and analyzed at HH25 for cell death (M) or at HH35 for morphological wing alteration (H–L). Shown are representative images for different treatment and assays. (H and I) Injection of retroviruses encoding *Hes1* (I) induced distal truncations in the right (injected) wing (arrowhead), compared with control-injected (H) or uninjected wings. (J–L) Cartilage staining showing typical skeletal patterns: embryos injected with RCAS-eGFP (J) showed a normal wing with three digits in the autopod, two well developed bones in the zeugopod, and one well developed bone in the stylopod. Embryo injected with RCAS-*p53* (K) showed loss of digit III (arrowhead) and severe hypoplasia of digit II and IV.

$n = 21$; Fig. 4 H and J). Moreover, when the injected embryos were analyzed at earlier stages, before any morphological alteration was evident, increased apoptosis was detected in wing buds injected with RCAS-*p53*, RCAS-*Hey1*, or RCAS-*Hes1* or RCAS-*Osr1* when compared with the contralateral, noninjected wing bud (Fig. 4M). The combined results from these experiments using the zebrafish and chicken embryo models confirm that the regulators of p53 identified in this study function to induce p53-mediated apoptosis *in vivo*.

Inhibition of Transformation in Mouse Embryonic Fibroblasts. Given the role of p53 in tumor suppression, we tested the ability of these genes to inhibit the transformation of MEFs by forced coexpression of the *ras* and *myc* proto-oncogenes. Transformation of MEFs by oncogenes results in foci formation and immortalization of the cells (11). Previous studies have demonstrated that inhibition of p53 is required for *ras* transformation in MEFs (11), and reactivation of p53 can inhibit this event (11). To test their ability to inhibit oncogene transformation in MEFs, the p53 activators identified in the screen were cloned into the retroviral vector pBabe, and viruses were collected on transfection of these constructs into Phoenix A cells. Retroviruses encoding *ras* and *myc* were coinfecting with each of the p53 regulators in both *p53*^{+/+} and *p53*^{-/-} MEFs, and foci formation was used to determine the ability of these genes in transformation inhibition. Expression of *Hey1*, and to a lesser extent *Hes1*, significantly inhibited foci formation by *ras/myc* in WT MEFs (Fig. 4N). However, in *p53*-deficient cells, neither *Hey1* nor *Hes1* had any effect on the transformation activities of *ras/myc*, demonstrating that the suppression of transformation by these bHLH proteins is mediated through p53 activation (Fig. 4N). The rest of the cDNAs were also tested and did not show transformation inhibition in MEFs, suggesting that the activation of p53 by these cDNAs is cell-type-dependent.

The Functions of HEY1 and HES1 as p53 Activators. Taken together, the above results indicate that both HEY1 and HES1 activate p53 in mammalian cells, zebrafish, and chicken embryos and can behave as tumor suppressors. Five other cDNAs identified in this screen activate p53 in a species or cell-type-dependent manner. Both HEY1 and HES1 belong to class C subfamily of bHLH transcription factor family (31) and are involved in various physiological events in the developmental process. HEY1 plays a role in blood vessel formation and is involved in proliferation, migration, and network formation of endothelial cells (32). It is induced during endothelial cell tube formation and down-regulates vascular endothelial growth factor (VEGF) receptor 2 in endothelial cells, suggesting that it regulates VEGF signals and the response to those signals (32). HES1 plays an essential role in preventing the differentiation of neurons from neural precursor cells (33). Deletion of *Hes1* in mice leads to premature differentiation of neurons in the telencephalon (34). Although mice that lack *p53* are developmentally normal, deletion of *p63*, a *p53* homologue that can act as a dominant negative factor toward *p53* (35), leads to striking limb truncation (36). This phenotype is similar to what we observed in chick embryos when *Hey1* or *Hes1* is overexpressed and *p53* is subsequently induced,

Embryos injected with RCAS-*Hey1* (L) showed loss of digit II and defects in the zeugopod (arrowhead). (M) Staining with neutral red detected increased cell death in the distal mesenchyme of the wing bud injected with RCAS-*Osr1* (arrowhead), compared with the contralateral, uninjected bud. (N) HEY1 suppresses *ras/myc* transformation in MEFs. MEFs from WT and *p53* knockout mice were infected with retroviruses containing *ras*, *myc*, and *Hey1* or vector. After 12 days of incubation, foci were stained with crystal violet and photographed.

suggesting the role of HEY1 and HES1 may be conserved across species.

Both HEY1 and HES1 have been shown to be regulated by the Notch-signaling pathway (37, 38). Notch has been shown to act as an oncogene or a tumor suppressor in a cell-type-dependent manner (39, 40). It has been shown that in T cells overexpression of Notch suppresses lymphocyte differentiation and promotes cell proliferation, causing T cell lymphoblastic leukemia (39, 41); in contrast in mammalian keratinocytes, Notch suppresses tumorigenesis (40). The fact that HEY1 and HES1 modulate p53 activity and are regulated by Notch signaling supports a model for cell-type-specific crosstalk between the Notch and p53 signal transduction pathways in which Notch activates p53 by up-regulating target genes such as *Hey1* and *Hes1*. This finding provides an explanation for the tumor suppressor effect of Notch in some cell types. Indeed, preliminary results show that transfection of the Notch intracellular domain, a constitutive active *notch*, results in an increase of p53 activity in HeLa cells. Efforts are underway to further elucidate the interaction between Notch and p53 pathways to better understand their roles in development and malignancy progression.

Interestingly, it has been shown that HES1, p53, and MDM2 oscillate with a 2- to 3-h cycle (42–44). Based on our observation that HES1 regulates HDM2 and p53, these three molecules and possibly other bHLH members may form a molecular clock

network that is distinct from the circadian clock. This molecular clock may regulate critical biological processes such as embryogenesis and DNA repair; oscillation in the clock may control the timing of somite segmentation or allow DNA repair in cells without the risk of excessive p53 activation.

Our experiments suggest that HEY1 and HES1 are behaving in an analogous mechanism to that of p14^{ARF}. Specifically, these proteins modulate p53 signaling by inhibiting HDM2 function. However, the activities of HEY1 and HES1 are distinct from those of the p14^{ARF} tumor suppressor because they act to repress HDM2 function at the transcriptional, rather than the posttranslational level. Regulation of HDM2 by HEY1 and HES1 points to a previously uncharacterized mechanism of p53 activation that is apparently conserved between vertebrate species. The elucidation of this transcriptional cascade suggests that these bHLH Class C proteins may participate in regulating aspects of gene expression that are linked to cell-cycle control and apoptosis.

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