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Mitosis-Independent *Survivin* Gene Expression *In vivo* and Regulation by p53

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Abstract

Survivin is an essential mitotic gene, and this has been speculated to reflect its primary function in development and cancer. Here, we generated a knock-in transgenic mouse (SVVp-GFP) in which a green fluorescent protein (GFP) reporter gene was placed under the control of the survivin promoter that regulates transcription at mitosis. The expression of endogenous survivin was widespread in mouse tissues during development and shortly after birth. In contrast, GFP reactivity was undetectable in transgenic mouse embryos, and was largely limited postnatally to mitotic cells in the testes. Double transgenic mice generated in the tumor-prone Min/+ background exhibited intestinal adenomas that strongly expressed endogenous survivin, but only isolated GFP-positive cells. Conversely, dysplastic adenomas (16%) stained intensely for GFP, and revealed focal reactivity for mutant, but not wild-type, p53. The expression of GFP was increased by ~10-fold in $p53^{-/-}$ as opposed to $p53^{+/+}$ HCT116 colorectal cancer cells, and reintroduction of p53 in $p53^{-/-}$ cells abolished GFP expression. Therefore, the mitotic transcription of the *survivin* gene is highly restricted *in vivo*, and unexpectedly negatively regulated by p53. Contrary to a commonly held view, the dominant function(s) of survivin in development and tumor ontogeny are largely cell cycle–independent. (Cancer Res 2006; 66(7): 3392-5)

Introduction

Survivin is a unique member of the inhibitor of apoptosis (*IAP*) gene family (1) with dual functions in the control of mitosis and preservation of cell viability (2). It has been difficult, however, to determine which of these two roles is dominant *in vivo*, and conditional knockout studies in mice produced conflicting phenotypes with only defects of cell division (3), only exaggerated apoptosis (4), or both (5). Insights into the function of survivin may come from analysis of gene expression. Accordingly, the cell cycle–dependent transcription of the *survivin* gene at mitosis (6), coupled with the localization of the protein to the mitotic apparatus and the multiple mitotic defects ensuing from survivin loss/targeting (2), have been taken to suggest that this pathway operates predominantly, if not exclusively, in the control of mitosis (7, 8). Conversely, cell cycle–independent mechanisms of *survivin* gene expression have been described, and linked to the inhibition of apoptosis in various cell types (2). Elucidating how the *survivin* gene is regulated *in vivo* is important for the indispensable role of this pathway during development (4), and the sharp differential expression of survivin in cancer as opposed to normal tissues, which has been pursued for novel cancer diagnostics and therapeutics (2).

To address these questions, we have generated a knock-in transgenic mouse that expresses a green fluorescence protein (GFP) reporter gene under the control of the proximal survivin promoter (9). This DNA region has been characterized for maintaining cell cycle periodicity at mitosis via CDE/CHR G_1-repressor elements and Sp1-type boxes (9). Unexpectedly, we found that the expression of survivin during development and tumor formation is largely cell cycle–independent.

Materials and Methods

Plasmid construction and generation of transgenic mice. A mouse cDNA containing the proximal 830 nucleotide of the survivin promoter (mS) upstream of the translational initiation codon (9) was inserted into *Bam*HI and *Hin*dIII sites of pBluescript II KS. The construct was fused to a full-length cDNA encoding an enhanced GFP (EGFP) gene at the 3′ end, plus SV40 splice and polyadenylation sequences to generate pBS-mS-830-GFP. A control pEGFP-C1 plasmid was obtained from BD Biosciences Clontech (Palo Alto, CA). All experiments involving animals were approved by an institutional animal care and use committee. The pBS-mS-830-GFP targeting vector was purified, confirmed by DNA sequencing, and microinjected (200 ng/μL) into C57BL/6 embryos, which were transferred into pseudopregnant females. Littermates were screened by PCR of tail genomic DNA using primers (10 pmol) corresponding to mS (5′-AAGTTGGGCTGCAG-GAATTCCCGCT-3′) and GFP (5′-CTTGTCGGCCATGATATAGACGTTG-TG-3′). Colonies from three independent transgene-positive founder mice (SVVp-GFP), or control littermates were maintained as described (10). C57BL/6J-APC^{Min}/J breeder mice were purchased from Jackson Laboratory (Bar Harbor, ME) and crossed with SVVp-GFP mice. Littermates were screened by PCR of tail genomic DNA with primers (10 pmol) corresponding to pMin (5′-TTCTCGTTCTGAGAAAGACAGAAGTTA-3′) and pCOM (5′-TTTATATTCCACTTTGGCATAAGGC-3′), and double transgene-positive mice were maintained as described (10).

RT-PCR. Total RNA was prepared from total mouse embryos collected at E12, E14, or E18, from individual tissues isolated from 6- to 12-week-old mice or at days 1, 3, or 5 postnatally using RNeasy Mini Kit (Qiagen, Valencia, CA), and cDNA was synthesized using a First-Strand Synthesis System (Invitrogen, Carlsbad, CA), according to the manufacturer's recommendations. mRNA expression was determined by RT-PCR amplification with the following primers: pGFP-1, 5′-AGCTGACCCTGAAGTTCATCTG-3′; pGFP-2, 5′-GATCTTGAAGTTCACCTTGATGC-3′; pM-Survivin-1, 5′-GCAGCTG-TACCTCAAGAACTACC-3; pM-Survivin-2, 5′-TGCAGTCTCTTCAAAC-TCTTTTTG-3; pGAPDH-1, 5′-ACGGATTTGGTCGTATTGGGCG-3; pGAPDH-2, 5′-CTCCTGGAAGATGGTGATGG-3.

Cell culture, antibodies, and Western blotting. Cervical carcinoma HeLa, and $p53^{+/+}$ or $p53^{-/-}$ HCT116 colorectal adenocarcinoma cells were maintained in culture as described (11). Transient transfection experiments were carried out using LipofectAMINE (Invitrogen), according to the manufacturer's recommendations. Cell cycle synchronization using

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thymidine block and release was carried out as described (6). Cells were lysed in 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 5 mmol/L EDTA, 50 mmol/L NaF, 0.5% Nonidet P40 plus protease inhibitors (Roche Applied Science), and protein-normalized extracts (40 μg) were processed for Western blotting, as described (11). The following antibodies to survivin (Novus Biologicals, Littleton, CO), GFP (BD Biosciences), mutant/wild-type p53 (ab 26, Abcam), wild-type p53 (ab16776, Abcam, Cambridge, MA), or β-actin (Sigma-Aldrich, St. Louis, MO) were used.

Immunohistochemistry. Tissue expression of survivin, p53 or GFP was determined with the Histostain-Plus (Zymed, South San Francisco, CA) system after antigen retrieval by pressure cooking, as described (10).

Results and Discussion

To determine whether cell cycle regulation of survivin accounts for its expression in development and tumor formation, we generated a knock-in transgenic mouse that expresses GFP under the control of an 830 nucleotide genomic fragment encoding the core *survivin* promoter, and with SV40 regulatory sequences at the 3′ end (Fig. 1*A*). The proximal region of this promoter (~ 300 nucleotides) contains cell cycle elements for transcription at mitosis (Fig. 1*B*), which were previously confirmed by site-directed mutagenesis and promoter activity (9). Transfection of the pBS-mS-830-GFP targeting construct (Fig. 1*A*) in synchronized HeLa cells resulted in strict cell cycle–regulated GFP expression (Fig. 1*C*), with undetectable levels in interphase, and peak activity 8 to 12 hours after thymidine release, which coincides with progression through mitosis, by DNA content analysis and flow cytometry (Fig. 1*D*). Endogenous survivin was also regulated in a cell cycle–dependent manner peaking at mitosis in synchronized HeLa cells (6), albeit high levels of expression were also found in interphase and asynchronous cultures (Fig. 1*C*).

Confirmed transgenic mice containing the pBS-mS-830-GFP targeting construct (SVVp-GFP) were next analyzed for the expression of GFP and endogenous survivin during development. Previous data suggested that survivin is abundantly and ubiquitously found in embryonic and fetal development, progressively down-regulated postnatally, and largely undetectable in most adult tissues (12). Consistent with this, endogenous survivin was strongly expressed in 14-day-old embryos of SVVp-GFP transgenic mice or nontransgenic littermates (Fig. 2*A*), and was present in the thymus, lung, spleen, and kidney at postnatal day 5 as determined by RT-PCR (Fig. 2*B*). In contrast, GFP expression was undetectable in 14-day mouse embryos of SVVp-GFP transgenic mice (Fig. 2*A*), and was faintly present in brain and kidney, but not in other tissues at postnatal day 5, as determined by RT-PCR (Fig. 2*B*). In developed mouse tissues (6-12 weeks of age), GFP or endogenous survivin was undetectable with the exception of brain, in agreement with recent observations (13). Conversely, both GFP and endogenous survivin were highly expressed in the testes of SVVp-GFP transgenic mice as determined by RT-PCR (Fig. 2*A*) and Western blotting (Fig. 2*C*).

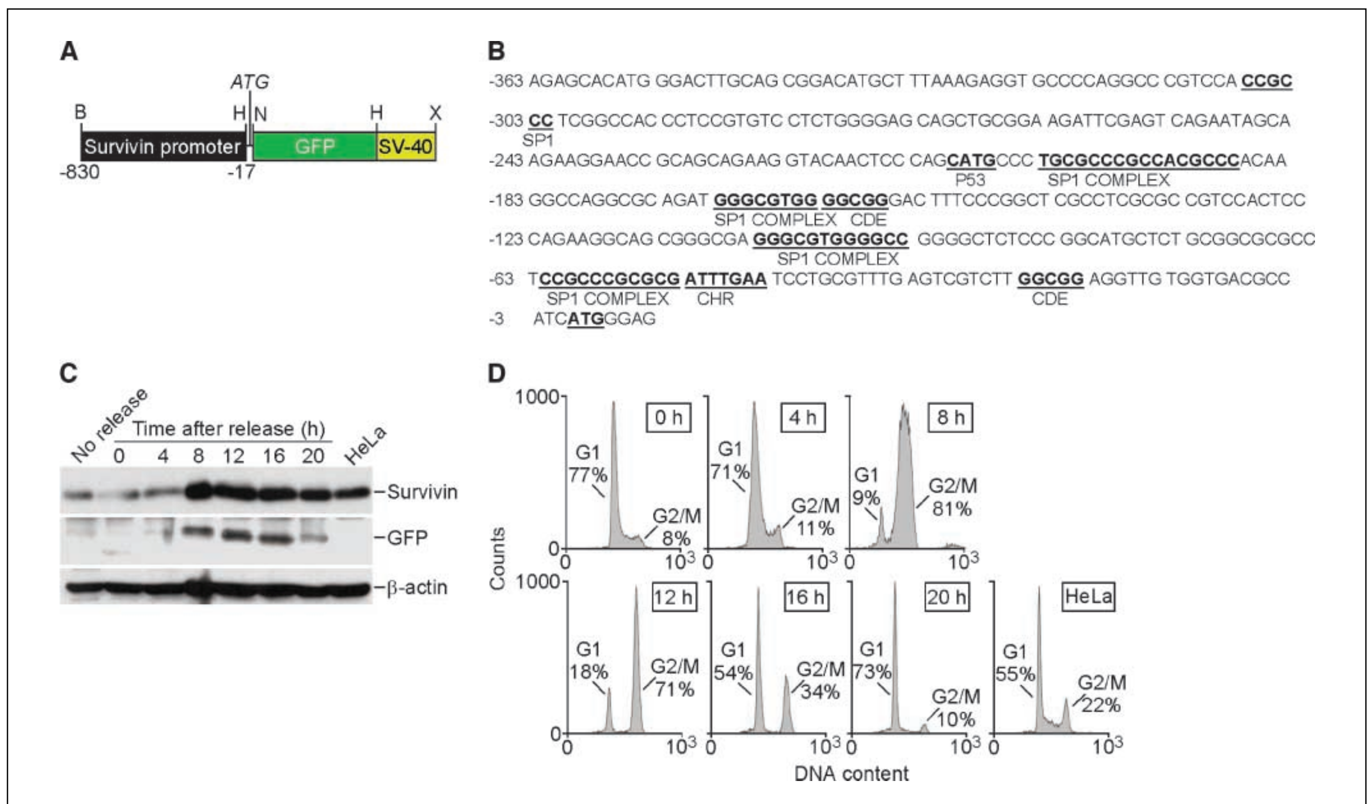

Figure 1. Requirements for mitotic survivin gene expression. *A,* targeting construct (pBS-mS-830-GFP). The positions of restriction sites and a translational initiation codon (*ATG*) are indicated: (*B*) *Bam*HI, (*H*) *Hin*dIII, (*N*) *Nco*I, (*X*) *Xho*I. *B,* proximal *survivin* promoter sequence. Cell cycle–regulated CDE, CHR, and Sp1-like boxes are indicated. A putative p53-binding site is underlined. *C,* cell cycle periodicity. HeLa cells transfected with the pBS-mS-830-GFP targeting vector were synchronized by thymidine block, harvested at the indicated time intervals after release, and analyzed by Western blotting. *No release,* thymidine-arrested cells (G_1-S); *HeLa,* asynchronous cultures. *D,* DNA content analysis. Synchronized HeLa cells were harvested at the indicated time intervals after thymidine release and analyzed by propidium iodide staining and flow cytometry. The percentage of cells in G_1 or G_2-M phase is indicated for each time point. *HeLa,* asynchronous cultures.

By immunohistochemistry of 8-week-old testes, GFP-positive cells in SVVp-GFP transgenic mice were identified morphologically as mitotic cells, preferentially at the metaphase transition (Fig. 2*D, inset*, and *E*) whereas nontransgenic animals had no GFP reactivity in the testes (Fig. 2*F* and *G*). Identical results were obtained with colonies from independent SVVp-GFP founder mice.

We next studied the mitotic regulation of *survivin* gene expression during tumor development, and we generated double transgenic mice expressing SVVp-GFP on the tumor-prone Min/+ background. In this model, a mutation in the mouse homologue of the adenomatous polyposis (*APC*) gene results in the formation of multiple intestinal adenomas (14), which are histologically characterized by epithelial hyperplasia and disorganized glandular architecture, as determined by H&E staining (Fig. 3*A*). Adenomas in double transgenic mice stained intensely for endogenous survivin (Fig. 3*B*), whereas GFP reactivity was restricted to isolated cells (Fig. 3*C*), and a control IgG was negative (Fig. 3*D*). In contrast, 2 out of 12 adenomas (16%) formed in double transgenic mice exhibited morphologic features of dysplasia, which coincided with extensive labeling for GFP (Fig. 3*F*), and focal reactivity for mutant (Fig. 3*G*), but not wild-type, p53 (Fig. 3*H*). A control IgG gave no staining in dysplastic adenomas (Fig. 3*E*).

To test a role of p53 in negatively regulating mitotic *survivin* gene expression, we used $p53^{+/+}$ and $p53^{-/-}$ HCT116 colorectal cancer cells (11). Transfection of control pEGFP resulted in the strong expression of GFP, which was indistinguishable in $p53^{+/+}$ or $p53^{-/-}$ cells, as determined by fluorescence microscopy (Fig. 4*A*) and Western blotting (Fig. 4*B*). In contrast, transfection of the SVVp-GFP targeting construct pBS-mS-830-GFP resulted in a ~10-fold increased GFP expression in $p53^{-/-}$ as compared with $p53^{+/+}$ HCT116 cells, as determined by fluorescence microscopy (Fig. 4*A*) and Western blotting (Fig. 4*B*). Reintroduction of wild-type p53 in $p53^{-/-}$ HCT116 cells largely abolished the expression of the pBS-mS-830-GFP targeting construct, but did not affect pEGFP reactivity (Fig. 4*C*).

In summary, the mitotic regulation of the *survivin* gene is highly restricted *in vivo*, and does not account for the widespread expression of endogenous survivin in development and tumor formation. Our results counter a commonly held view that the primary function of survivin is at cell division (7, 8), and argue that *survivin* gene expression *in vivo* is largely mediated by cell cycle–independent mechanisms. This may reflect the ubiquitous role of survivin in apoptosis inhibition, which contributes to tumorigenesis (15), and, at least in certain tissues, is indispensable for development (4). Unexpectedly, the mitotic component of *survivin* gene expression is negatively regulated by p53, in a pathway that may involve direct transcriptional silencing via putative p53-responsive elements (16), or indirect promoter squelching (17). Inhibition of mitotic *survivin* gene expression by p53 may cooperate with checkpoint functions at the G_2-M phase, especially p53-dependent apoptosis (18), as acute loss of survivin in dividing cells results in massive cell death (19). Conversely, loss of p53 during tumor progression may cooperate with cell cycle–independent mechanisms to further deregulate

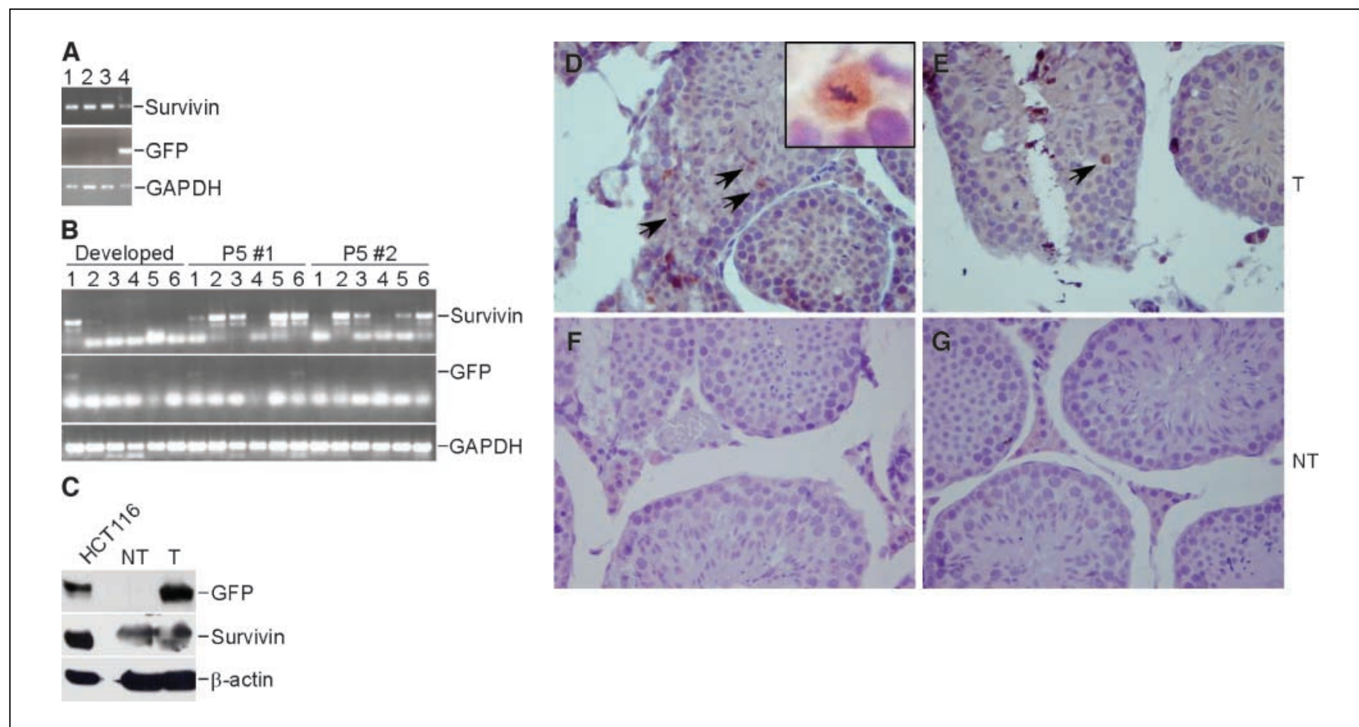

Figure 2. Mitotic *survivin* gene expression during development. *A,* expression in mouse embryos. Two independent embryos (*lanes 2* and *3*) from SVVp-GFP transgenic mice were harvested at E14, and analyzed by RT-PCR for expression of endogenous survivin, GFP or GAPDH. A transgene-negative embryo (*lane 1*), or testes isolated from 4-week-old transgenic mice (*lane 4*) were used as controls. *B,* postnatal expression. Tissues isolated from SVVp-GFP transgene-positive developed mice (6-12 weeks of age) or at day 5 postnatally (*P5 1* and *P5 2*) were harvested, and analyzed for expression of endogenous survivin, GFP or GAPDH, as determined by RT-PCR. The tissues used are: *lane 1,* brain; *lane 2,* thymus; *lane 3,* lung; *lane 4,* liver; *lane 5,* spleen; and *lane 6,* kidney. *C,* Western blotting. Testes were isolated from 4-week-old nontransgenic (*NT*) or transgenic (*T*) mice, and analyzed by Western blotting. HCT116 cells transfected with SVVp-GFP were used as control. *D–G,* immunohistochemistry. Testes from (*D, E*) transgenic (*T*) or (*F, G*) nontransgenic (*NT*) mice (4 weeks old) were stained with an antibody to GFP. *Arrows*, GFP-positive cells (magnification, ×200). *Inset,* mitotic morphology of a GFP-positive cell (magnification, ×400).

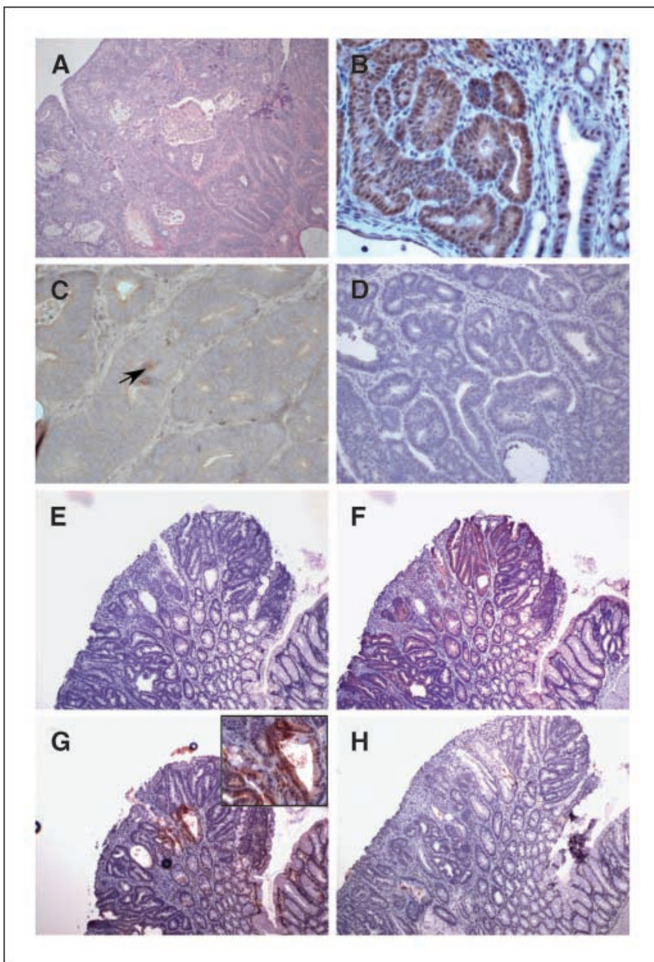

Figure 3. Mitotic *survivin* gene expression during tumor formation. *A-H,* immunohistochemistry. Intestinal adenomas without (*A-D*) or with (*E-H*) dysplasia formed in Min/+-SVVp-GFP double transgenic mice were analyzed by H&E (*A*), or stained with antibodies to survivin (*B*), GFP (*C* and *F*), control IgG (*D* and *E*), mutant p53 (*G*), or wild-type p53 (*H*). Magnification, ×100. *Inset,* focal reactivity for mutant p53 in dysplastic adenomas (magnification, ×400).

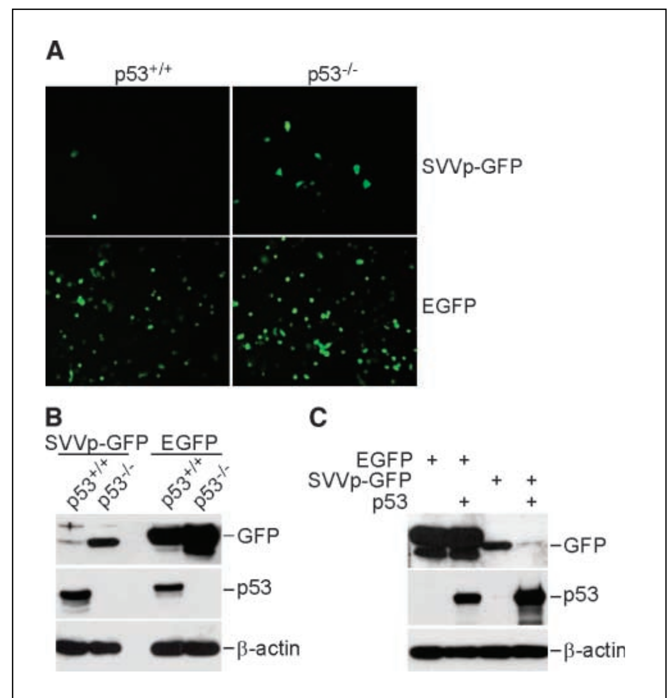

Figure 4. Negative regulation of mitotic *survivin* gene expression by p53. *A,* fluorescence microscopy. $p53^{+/+}$ or $p53^{-/-}$ HCT116 cells were transfected with the targeting construct p-BS-mS-830-GFP (SVVp-GFP) or pEGFP, and analyzed by fluorescence microscopy. The percentage of labeled cells in each condition was: SVVp-GFP/$p53^{+/+}$, 1.5%; SVVp-GFP/$p53^{-/-}$, 10.8%; pEGFP/$p53^{+/+}$, 46.5%; pEGFP/$p53^{-/-}$, 48.6% (n = 2). *B,* Western blotting. The experimental conditions are as in (*A*), except that cells were harvested and analyzed by Western blotting. *C,* p53 transfection. $p53^{-/-}$ HCT116 cells were cotransfected with a plasmid encoding wild-type p53 and pEGFP or pBS-mS-830-GFP (SVVp-GFP), and analyzed by Western blotting.

survivin gene expression *in vivo*, ablating mitotic checkpoint mechanisms and promoting resistance to apoptosis of cells traversing mitosis (19).

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