Supporting Information

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SI Methods

Axolotl Procedures. Axolotls (*Ambystoma mexicanum*) were obtained from Neil Hardy Aquatica (Croydon, United Kingdom) and maintained in individual aquaria at ~18 °C. Animals were anesthetized in 0.1% tricaine before amputation at the mid-humerus level. Animals were allowed to regenerate at 20 °C. Axolotl regeneration stages were defined as previously described (1, 2). To test the effect of p53-modulating compounds on limb regeneration, axolotls were amputated, kept under normal aquarium conditions overnight, and then treated with 5 μM (−) nutlin3a (dissolved in 100% ethanol; Cayman), 5 μM α-pifithrin (dissolved in 100% DMSO; Sigma), and either ethanol or DMSO at the equivalent concentrations as control treatments. All compounds were administered directly to the aquarium water at the desired concentrations. Solutions were changed every day for the duration of the experiment. Pifithrin-α treatments were performed in the dark, as well as their corresponding DMSO controls. Reversibility was assessed by transferring treated axolotls to normal aquarium conditions until complete regeneration was achieved.

Salamander Electroporation. For electroporation of limbs and blastemas, newts and axolotls were anesthetized in 0.1% (wt/vol) tricaine and observed under a stereomicroscope (Nikon SMZ800). The target limb was then held with a pair of electrodes in a recessed chamber under amphibian PBS. Customized platinum disk electrodes were used, modeled on those previously described (3). One or 3 μL of DNA solution (5 μg/μL) was injected under the epidermis of the blastema or limb, respectively, through a glass needle with a 0.2-μm-diameter tip using a Picospritzer III pressure injector (Intracel) with 5-ms pulse length. Electroporation was carried out by administration of 10 electric pulses (duration: 100 ms; voltage: 80 V/cm) using a SD9 electroporation device (ETL). Animals were allowed to recover in sterile water with 0.5% sulfamerazine for 12–24 h.

Luciferase Assay. A1 and AL1 cells were lysed at 36 h post-transfection using the passive lysis buffer provided in the dual-luciferase reporter assay kit following the Manufacturer's instructions (Promega). For luciferase assays performed using limb and blastema samples, blastemas at different stages of regeneration and their contralateral limbs were collected 48 h after electroporation and immediately homogenized using passive lysis buffer (Promega).

Cell lysates were subjected to a freeze–thaw cycle, centrifuged to remove debris, and assayed according to the dual luciferase reporter protocol. Assays were performed in duplicate in a 96-well plate format and luminescence measured using a FLUOstar Optima detector (BMG labtech). The activities of the p53-Luc and MG15 mutant reporters were normalized to the activity of an internal *Renilla* control and expressed as relative luciferase activity (Firefly/*Renilla*).

Quantitative Real-Time PCR. RNA was isolated from newt and axolotl limbs, blastemas and salamander tissue culture cells using Tri Reagent (Sigma) and random primed cDNA synthesized using SuperScript II (Invitrogen). Gene expression was determined by quantitative real time PCR with the indicated primers (Table S2) and iQ SYBR Green supermix (Bio-Rad) on a Chromo 4 instrument running Opticon 3 software (Bio-Rad). All reactions were run in triplicate and at least two independent RNA preparations were analyzed for each sample.

Cell Culture. A1 cells were previously derived from newt limb mesenchyme (4). Axolotl AL1 cells were obtained from S. Roy (University of Montreal, Montreal, QC, Canada). A1 and AL1 cells were grown on gelatin-coated plastic dishes in MEM (Gibco) supplemented with 10% (vol/vol) heat-inactivated FBS (Gibco), 25% (vol/vol) H_2O, 2 nM L-Glutamine (Gibco), 10 μg/mL insulin (Sigma), and 100 U/mL penicillin/streptomycin (Gibco) in a humidified atmosphere of 2.5% CO_2 at 25 °C. Routine cell subculture was performed as previously described (5, 6).

Cloning and Vector Construction. p53Luc reporter vector was a kind gift from T. Hupp (Edinburgh Cancer Research UK Centre, Edinburgh, United Kingdom), and its corresponding mutant vector, mutant-Luc (MG15Luc), was purchased from Addgene. pGL3 basic, SV40 (pGL3 promoter vector) and pRenilla-Luc were purchased from Promega. Axolotl growth arrest and DNA damage 45 (*gadd45*), axolotl p73, newt Gadd45, and newt p53 partial sequences were amplified by real-time PCR (RT-PCR) using degenerate primers based on annotated sequences in different species. Full-length coding sequences were obtained by 5′ and 3′ primer extension using Smart RACE Kit (Clontech) and RACE-ready (Clontech) axolotl or newt cDNA. Axolotl p53 full-length sequence was already available (accession no. DQ848588). All sequences were cloned into pBluescript KS(+) (Stratagene). Axolotl p53 and ΔNp73 sequences were myc-tagged by RT-PCR and cloned into the expression vector pEGFPN2 (Clontech), replacing the eGFP sequence with the desired DNA. To generate the ΔNp73-GFP fusion construct, the axolotl ΔNp73 sequence was amplified and cloned between the XhoI and EcoRI sites of pGFPN2, in frame with the eGFP sequence. The axolotl p73 genomic sequence upstream of exon 3′ was isolated using the universal Genome Walker kit (Clontech) with axolotl forelimb genomic DNA as template. The construct encoding the p53 dominant-negative DDp53, containing a C-terminal fragment of hsp53, as previously described (7), was purchased from Addgene. All primers used are indicated in Table S1.

Salamander Cell Transfection. A1 and AL1 cells were transfected by nucleofection using the Amaxa Nucleofector II (Lonza) combined with the cell line nucleofector solution V (Lonza). For the luciferase assays, a cell suspension (2×10^5 cells in 0.1 mL nucleofection solution) was mixed with 3 μg p53-Luc or MG15 mutant reporter, 1 μg *Renilla* DNA, and the indicated amounts of p53 expression vector and nucleofected according to manufacturer's instructions. For standard vector nucleofections, 2–4 μg of DNA were used. Electroporation was carried out using program T-030 and the nucleofected cells were recovered in 1.5 mL supplemented MEM (Gibco) and incubated at 25 °C for 10 min before plating into gelatin-coated dishes.

In Situ Hybridization. A 688-kb fragment of axolotl Gadd45β (tctcgagGCAAGGATTGGCATATCAC, tctagaGAGACCGAAGGCACCCACGTG) was cloned into pciNEO vector (Promega) and the resulting construct was linearized with either XbaI or XhoI. The respective linearized templates were transcribed with T3 or T7 RNA polymerases to generate digoxigenin-UTP labeled antisense or sense riboprobes following the manufacturer's protocol (Roche). In situ hybridization of cultured newt A1 cells with the digoxigenin-UTP labeled riboprobes was performed as previously described (8).

Western Analysis. Protein extracts were prepared by resuspending cells in 0.02 M Hepes (pH 7.9), 0.2 mM EDTA, 1.5 mM $MgCl_2$,

0.42 M NaCl, 25% glycerol, incubating for 30 min at 4°C, and clearing the debris by centrifugation. The resulting extracts were analyzed by SDS/PAGE and transferred to a nitrocellulose membrane (Whatman), which was incubated in Odyssey blocking buffer (Licor) and incubated with the indicated antibodies overnight (Table S3). The membrane was then washed twice in TBS, incubated with IR-labeled secondary antibodies, AlexaFluor680, and AlexaFluor800, against the corresponding species (Licor) and analyzed with an Odyssey scanner (Licor). Western blots were quantified using ImageGauge V4.21 (Fuji Photo Film Co.).

Tissue Sectioning and Immunofluorescence. For analysis of protein expression, red fluorescent protein (RFP) imaging and TUNEL assays in axolotl limbs, regenerating or intact limbs were collected by amputation and fixed in 4% (wt/vol) ice-cold paraformaldehyde (PFA) for 16–18 h at 4 °C, washed twice in PBS, and embedded in Tissue Tek-II. The blocks were serially sectioned longitudinally in a cryostat (Leica) at 12 μm. Sections were collected in Superfrost slides and stored at −30 °C until use.

Antibody staining of tissue sections was performed as described previously (9). For staining of cultured cells, these were fixed in 2% PFA for 1 min, followed by a 5-min incubation in cold 100% methanol and processed as described previously (10). Both cells and tissue sections were incubated with the indicated primary antibodies (Table S2) overnight. In all cases, anti-mouse or anti-rabbit AlexaFluor488 and AlexFluor594 antibodies (Invitrogen) were used for secondary staining. Hoechst 33258 (2 μg/mL) was used for nuclei counterstaining.

Samples were observed under a Zeiss Axiskop2 microscope and images were acquired with a Hamamatsu Orca camera using Openlab (Improvision) software. Whenever comparative analyses between intact and regenerating limb samples were performed, all images were acquired with identical camera settings and illumination control. Image processing (contrast enhancement) was equally applied to all matched experimental and control samples using Openlab software. For the quantification of $BrdU^+$ and pRb^+ myotubes, 160 myotubes were analyzed per replica, and the data presented are representative of six replicas.

TUNEL Assay. Axolotl tissue sections were reacted with fluorescein-dUTP using the In Situ Cell Death Detection Kit (Roche) following the manufacturer's protocol with minor modifications. The sections were incubated for 4 h at room temperature with fluorescein-dUTP reagent. Sections were mounted in DAKO mounting media and imaging was performed under a Zeiss Axiskop2 microscope coupled to a Hamamatsu Orca camera.

Myotube Formation Assay. Myogenesis was induced in confluent A1 cells by lowering the FCS concentration from 10 to 0.25%. Cells were incubated in a humidified atmosphere of 2.5% CO_2 at 25 °C. After 5 d, >90% of cells fused into multinucleate myotubes as previously described (5).

BrdU Analysis. Cells and myotubes were labeled for 2 and 24 h, respectively, by adding 1 μL/mL BrdU to the growth media. Following the corresponding incubation period, cells were fixed in 4% PFA for 1 min followed by 100% methanol for 5 min, and stained for BrdU, as previously described (11, 12).

Statistical Analysis. Statistical analyses were performed with Prism 4.0 and unpaired two-tailed *t* tests were applied unless otherwise stated. Paired two-tailed *t* tests were carried out to analyze RT-PCR experiments. Ratio *t* test analyses were performed on relative luciferase activity ratios to determine whether the behavior of the p53Luc reporter was equivalent in 0-d postamputation (dpa) and 15-dpa samples, and in axolotlp53-N2 electroporated samples. The ratio *t* test was performed as described in the Prism 4.0 statistics guide and the *P* values determined (13).

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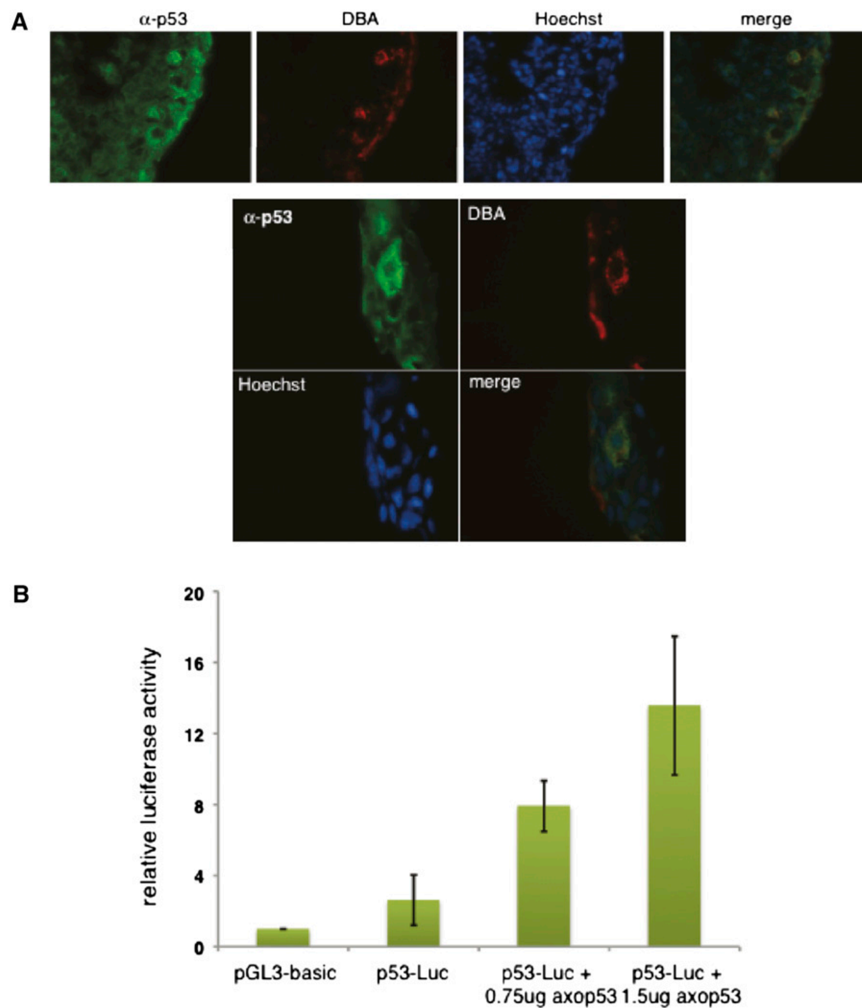

Fig. S1. (*A*) p53 protein levels are elevated in glandular Leydig cells underneath the wound epidermis. Immunofluorescence images of a normal mid-bud blastema section, stained with antibodies against axolotl p53 (green), the Leydig cell marker Dolichos Biflorus Agglutinin–rhodamine conjugated (red) and Hoechst 33258 (blue). (*Lower*) A single Leydig cell within the epidermis. (Magnification: *Upper*, 40×; *Lower*, 80×.) (*B*) p53Luc transactivation by axolotl p53 is dose-dependent. Luciferase activity assay in AL1 cells, 48 h after transfection with the control vector pGL3 or the p53 reporter p53Luc plus 0, 0.75 or 1.5 μg of axop53N2 vector. Cells were cotransfected with a *Renilla* luciferase vector. The activity of each construct was normalized to that of *Renilla* luciferase, and expressed in relation to pGL3-basic. Values represent the mean ± SD of at least four independent experiments.

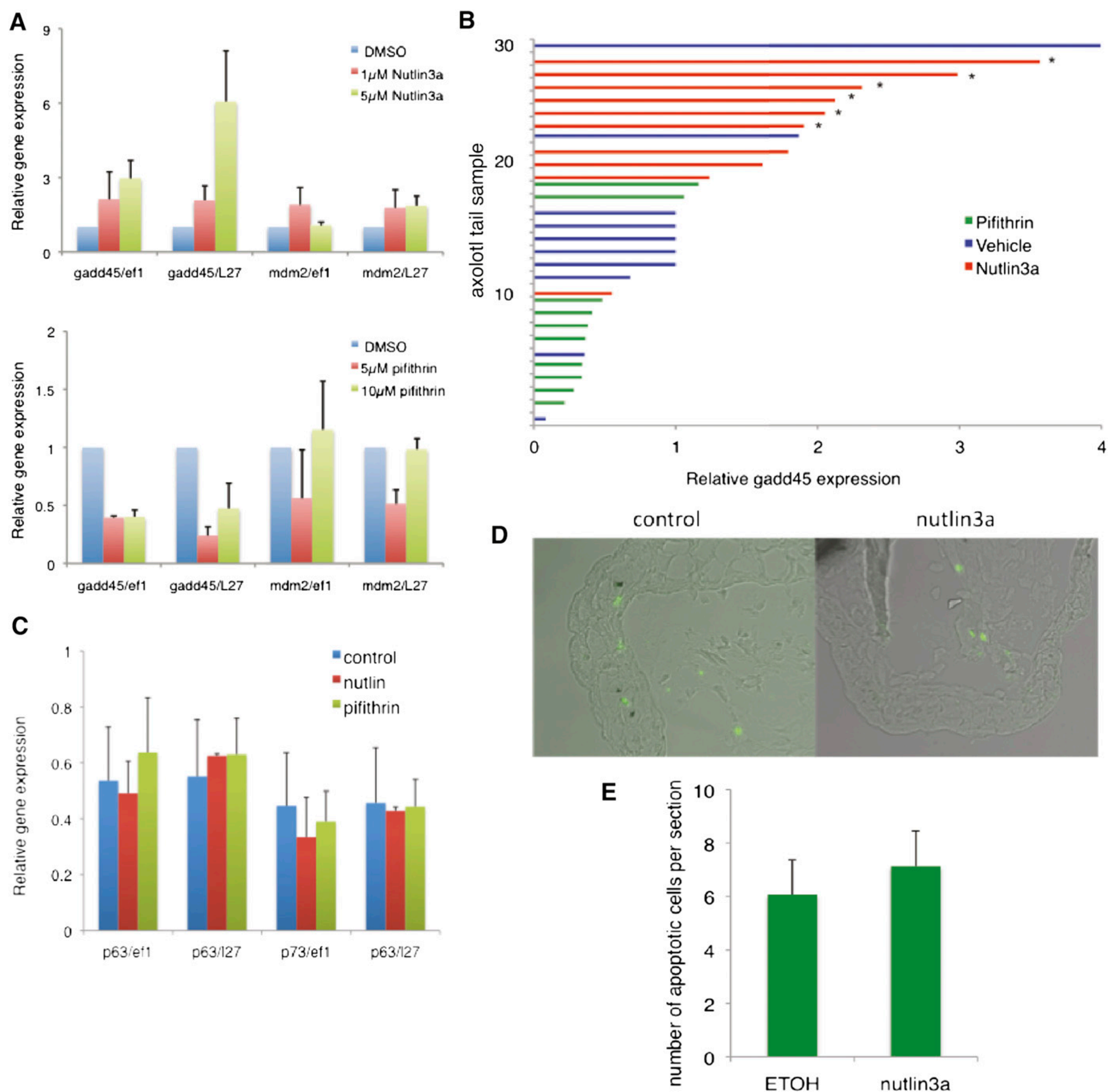

Fig. S2. (*A*) Treatment with the p53 inhibitor α-pifithrin or the p53 stabilizer nutlin3a leads to changes in the expression levels of Gadd45 and mouse double minute 2 (*mdm2*) in AL1 cells. Quantitative RT-PCR (qRT-PCR) analysis of axolotl Gadd45 and Mdm2 expression levels in AL1 cells treated with nutlin3a (*Upper*) or pifithrin (*Lower*). Gene expression levels were normalized to those of Ef1-α or L27. Values represent the mean ± SD of at least three independent experiments. (*B*) Axolotls treated with α-pifithrin and nutlin3a display changes in the expression levels of the p53 target gene Gadd45. Micrograph depicting 30 individual axolotl tail samples (collected after 9-d treatment with the indicated compounds, 10 samples per treatment), ranked according to their relative Gadd45 expression levels. Gadd45 expression levels were determined by qRT-PCR and normalized to those of Ef1-α. Similar results were obtained normalizing to L27. These tail samples are taken from a subset of the axolotls analyzed in Fig. 2. An asterisk indicates tail samples corresponding to axolotls treated with nutlin3a which showed impaired regeneration. All α-pifithrin samples correspond to axolotls with impaired or delayed regeneration. (*C*) Axolotls treated with α-pifithrin and nutlin3a do not display changes in the expression levels of the p53 family members p73 and p63. qRT-PCR analysis of p73 and p63 expression levels in individual axolotl tail samples taken after a 9-d treatment with either α-pifithrin, nutlin3a, or DMSO. Gene-expression levels were normalized to those of Ef1-α. Similar results were obtained normalizing to L27. (*D* and *E*) Treatment with nutlin3a does not lead to an increase in apoptosis in axolotl blastemas. (*D*) Representative sections of control or nutlin3a-treated blastemas following TUNEL staining (green). (Magnification: 50×.) (*E*) The number of apoptotic cells per blastema section was quantified following TUNEL staining. A total of 10 sections were analyzed for each replica. Values represent the mean ± SD of six independent experiments.

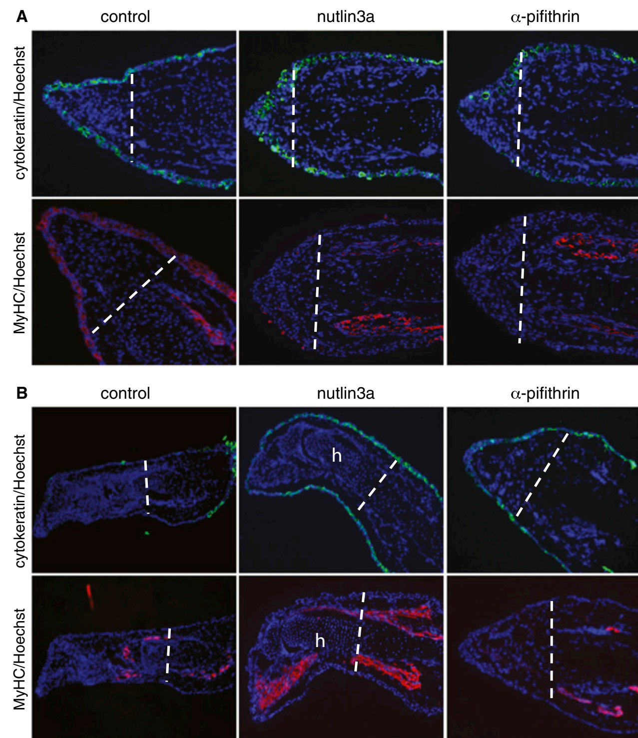

Fig. S3. Treatment with nutlin3a or α-pifithrin leads to defects in bastema formation and disrupts the redifferentiation phase. Representative sections of axolotl blastemas treated with nutlin3a, α-pifithrin, or vehicle for 10 d during the blastema formation phase (*A*) or during the redifferentiation phase (*B*), following immunohystochemical staining to detect muscle (MyHC antibody) or epidermis (pan-cytokeratin antibody). Note that wound-healing has occurred but extension is arrested following both α-pifithrin and nutlin treatments in *A*, relative to the control axolotls, and that treatment with α-pifithrin leads to impaired morphogenesis and muscle regeneration (*B*). The sections corresponding to nutlin-treated axolotls in *B* show the humerus (h) and joint of a fully regenerated limb. Sections are representative of six pairs of axolotls for each treatment. (Magnification: 30×.)

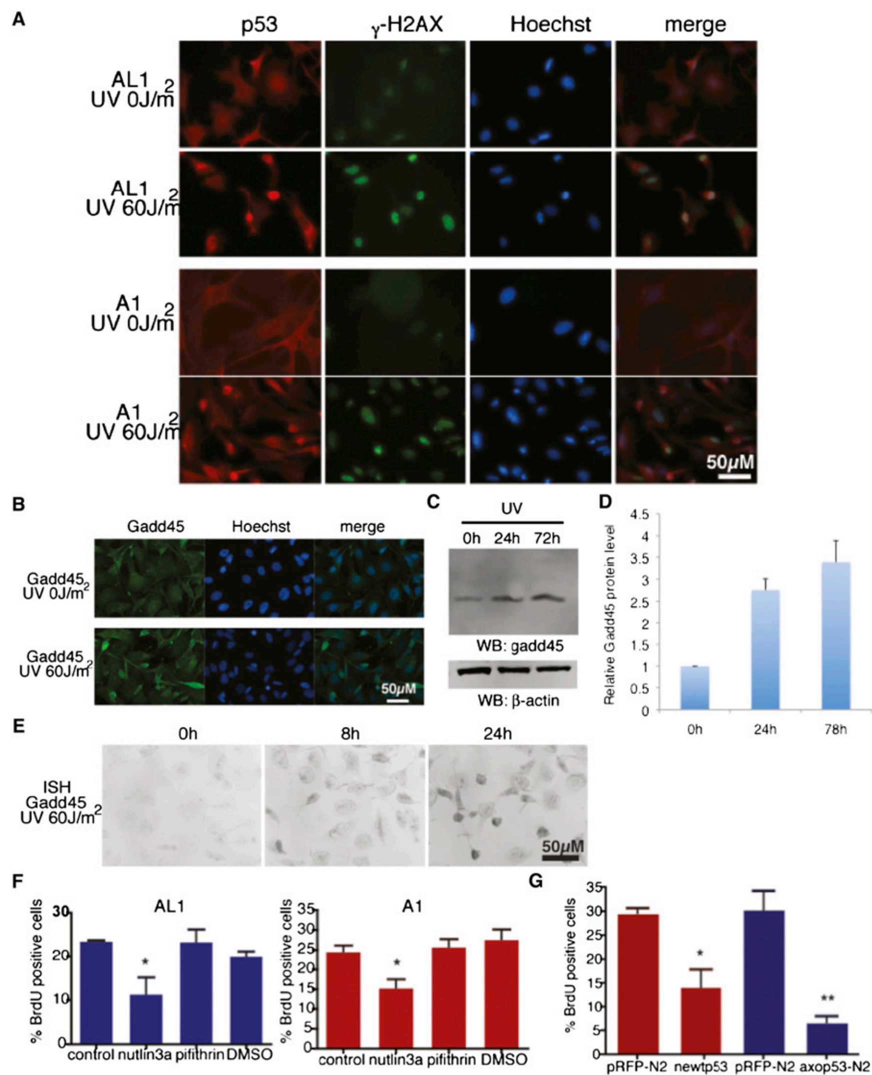

Fig. S4. The genome-protective functions of p53 are conserved in salamanders. (*A*) Immunofluorescence images from AL1 or A1 cells 12h after UV irradiation stained with antibodies against axolotl p53 (red), phospho γ-H2AX (green), and Hoechst 33258 (blue). (*B*) Immunofluorescence images from AL1 cells 48h post UV stained with antibodies against axolotl gadd45 (green) and Hoechst 33258 (blue). (*C*) Representative Western blot of Gadd45 and β-actin protein levels in AL1 cells following UV damage (60 J/m^2). (*D*) UV-dependent induction of salamander Gadd45. ImageGauge Western blot quantification of Gadd45 protein levels relative to β-actin. (*E*) In situ hybridization analysis of Gadd45 mRNA expression in A1 cells following UV damage. (*F* and *G*) BrdU incorporation assay in AL1 and A1 cells, 48 h after treatment with 1 μM nutlin3a, 10 μM α-pifithrin or DMSO (*F*), or following transfection of the indicated vectors (*G*). All values represent the mean ± SEM, $n = 6$ ($*P < 0.05$, $**P < 0.01$).

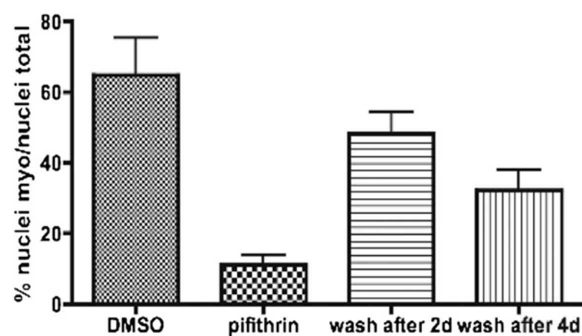

Fig. S5. The effect of inhibiting p53 during myogenesis is reversible. Graph representing the percentage of myotube formation. A1 cells were incubated for 4 d in 0.25% FCS in the presence of DMSO or 10 μM α-pifithrin. To test reversibility, A1 cells treated with α-pifithrin for 2 or 4 d were changed to 0.25% FCS without pifithrin and incubated for 3 additional days before evaluating myotube formation (number of nuclei within myotubes relative to total nuclei expressed as percentage). Values represent the mean ± SEM of four independent experiments.

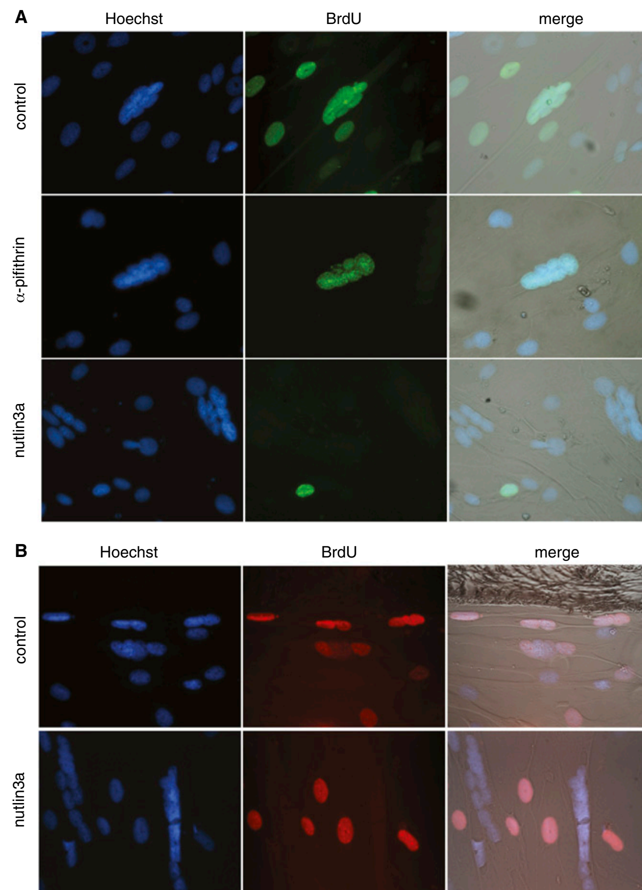

Fig. S6. Regulation of p53 activity is necessary for myotube cell cycle reentry. (*A*) Representative images of A1 myotubes at 3 d postinduction in high serum in the presence of the indicated compounds, following BrdU incorporation. Myotubes were stained with antibodies against BrdU and Hoechst 33258. (*B*) Representative images of A1 myotubes after 2 d in high serum in the presence of the indicated compounds, stained with antibodies against p-RB$^{S807/811}$ and Hoechst 33258. Merge panels include bright-field micrographs. (Magnification: 100×.)

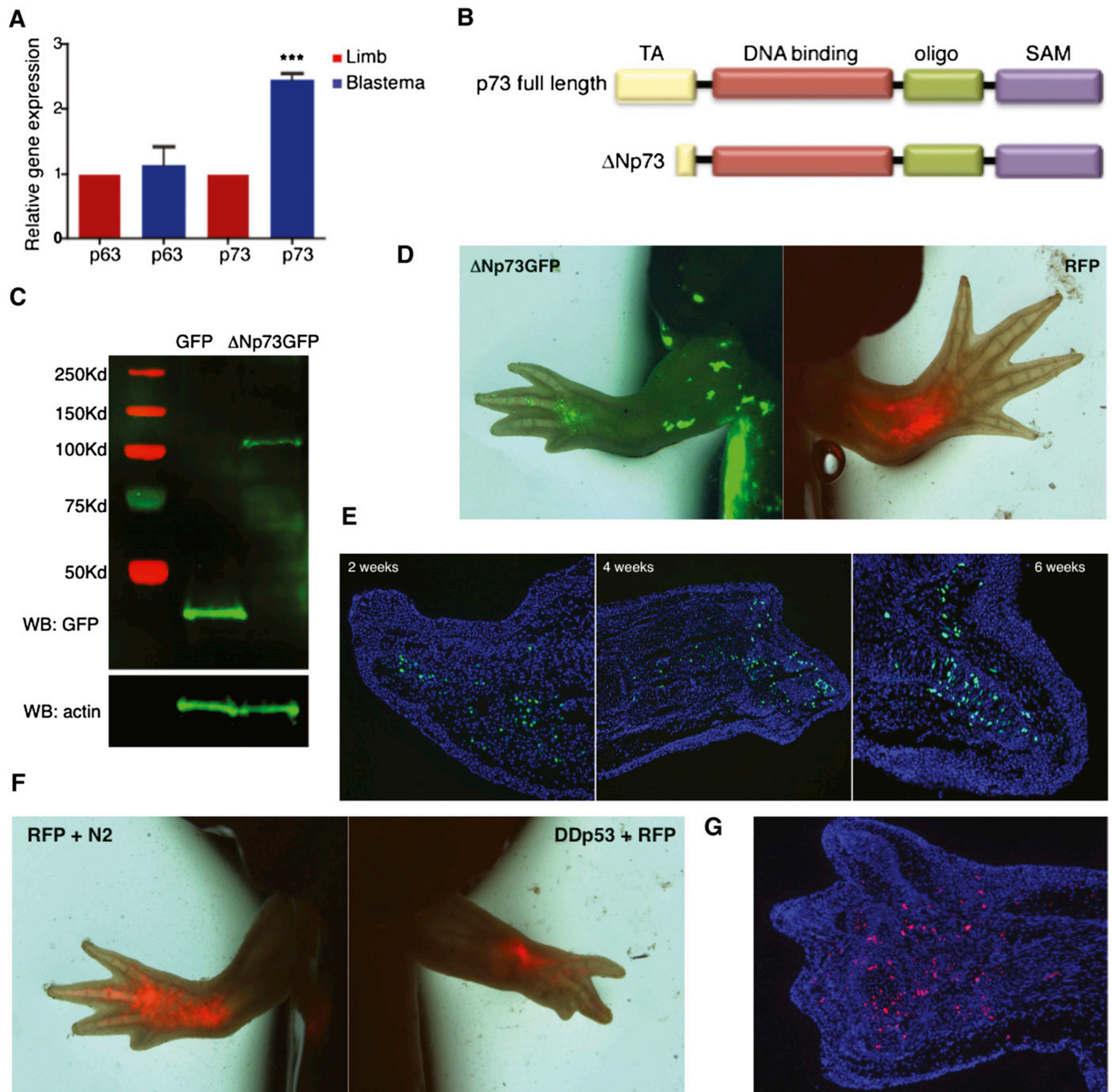

Fig. S7. (*A* and *B*) Gene expression of the p53 family members in regenerating limbs. (*A*) qRT-PCR analysis of axolotl p63 and p73 expression levels in mid bud blastemas compared with limbs, after normalization to Ef1-α. (*B*) Domain structure of full-length or ΔN p73 proteins. (*C*–*E*) Sustained expression of ΔNp73-gfp in axolotl regenerates following electroporation. (*C*) Western blot analysis of GFP proteins and β-actin in whole cell extracts from blastemas, 3 d after electroporation of GFPN2 or ΔNp73-GFPN2 constructs. Note that the ΔNp73-GFP protein is detectably expressed within the axolotl blastema. (*D*) Regenerated limbs, 4 wk after coelectroporation of pair-matched mid-bud blastemas with either RFPN2 or ΔNp73-GFPN2. (*E*) Representative sections of regenerated limbs electroporated with ΔNp73-GFPN2, at 2, 4, and 6 wk postamputation. Note that the construct continues to be expressed. (*F* and *G*) Overexpression of the p53 dominant-negative construct DDp53 during the redifferentiation phase causes defects in limb regeneration. (*F*) Regenerated limbs, 4 wk after coelectroporation of pair-matched mid-bud blastemas with either RFPN2 or DDp53-RFPN2. In 25% of all cases analyzed, the overexpression of DDp53 led to impaired cartilage morphogenesis, as observed in the corresponding image. (*G*) Representative sections of regenerated limbs at 6 wk postamputation showing the distribution of RFPN2/DDp53^{+} cells. (Magnification: *E*, 15×; *G*, 30×.)

Table S1. Cloning primer sequences

Primer	Sense	Antisense
Axolotl Gadd45 full-length	GTGGATTACTTGCTGCCAGAA	AAGGCACCCACGTGGTTCTT
Newt Gadd45 full-length	CTGTCTCGAGCGATCACCGT	TTTCTCTAGATGTGTGGCATCCT
Newt p53 full-length	CCCTCGAGTGAATCTGGCCTGGTCGAAT	GCTCTAGACAGCAGACAAGCACTAGCG
Axolotl p53 C-terminal myc-tagged	GATCCGCTAGCATGGATTCACTA	GTTGCGCGGCCGCCTACAGATCTTCTTCAGAAATAAGTTT TTGTTCATCAGAGTCCTGCGTCTCAT
Axolotl ΔNp73 C-terminal myc-tagged	GGCCTCGAGTCTCCCGCCGCCACCATGGACC CGGGCATCGGCAGCCGT	CAAGCGCGGCCGCCTACAGATCTTCTTCAGAAATAAGTT TTTGTTCGTTCATCTCATTCTCCGAAAACTCT
Axolotl p73 genomic sequence upstream exon 3′	CTGTAAACCTGCCAATGGCCACCAGCAGA	GCAGACCATTCAGGAGACAGTAACACAGA
Axolotl ΔNp73 GFP fusion	GGCCTCGAGTCTCCCGCCGCCACCATGGACCCGGGCATCGGCAGCCGT	GCGGAATTCGTTCATCTCATTCTCCGAAAACT

Table S2. RT-PCR primer sequences

Primer	Sense	Antisense
Axolotl Gadd45-β	CGTGCACCTTACTTGGGACT	ATGTCATTGTCGCAGCAAAA
Axolotl Mdm2	CGTCTACATCTGGCAGCATC	TATAGGTTGCCTGCAAACAG
Axolotl p53	CATGTGGCAGAGGTAGTCAAACGC	TTAATCAGAGTCCTGCGTCTCAT
Axolotl p73 common	AACGATGCCCCAGCTGGGCT	CTGCTGAGGGACCAGCTCCA
Axolotl p73 full length	GTCGTCGAGGCGAGGTCAA	GACATGGCGTCGAAGGTGGA
Axolotl ΔNp73	TGTCCTCACCTGCAGTCCCA	TGCTGGAAGGTGACTTCGAA
Axolotl p63	CAACACTGACCATGCTCAGAA	CTTTGAGAGTGGTGCTTACCGTC
Axolotl Ef1-α	AACATCGTGGTCATCGGCCAT	GGAGGTGCCAGTGATCATGTT
Axolotl L-27	CATCAGATCAAGCAAGCAGTA	CCAATGCAGCAGTTTAGATG
Axolotl Cytochrome-c	CGATGCGGGAAGGACTGTATTAC	GGGAAGAATGATGGCAGCAATC
Newt Gadd45-β	AGGGCACAGGAAAGAAGATG	TCATTGTCGCAGCAGAAGG
Newt L-27	TACAACCACTTGATGCCA	CAGTCTTGTATCGTTCCTCA

Table S3. Antibodies and reagents

Antibody	Origin	Clonality	Species	Dilution WB	Dilution IHC
Axolotl p53*	Custom, raised against peptide	Polyclonal	Rabbit	1:1,000	1:500
Axolotl Gadd45*	Custom, raised against peptide CLL ATD EED EGD IAL Q	Polyclonal	Rabbit	1:200	1:100
γ-H2AX	Upstate	Monoclonal	Mouse	N/A	1:1,000
c-myc	Sigma	Monoclonal	Mouse	1:5,000	1:1,000
BrdU	Sigma	Monoclonal	Mouse	N/A	1:3,000
pRB S807/811	Cell signaling	Polyclonal	Rabbit	1:1,000	1:500
DBA-rhodamine	Vector laboratory.	N/A	N/A	N/A	1:100
Pan-Keratin	Abcam	Monoclonal	Mouse	N/A	1:100
GFP	Roche	Monoclonal	Mouse	1:500	1:300

IHC, immunohistochemistry; N/A, not applicable; WB, Western blot.
*Affinity-purified.