

Repression of *Puma* by *Scratch2* is required for neuronal survival during embryonic development

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Although *Snail* factors promote cell survival in development and cancer, the tumor-suppressor p53 promotes apoptosis in response to stress. p53 and *Snail2* act antagonistically to regulate p53 upregulated modulator of apoptosis (*Puma*) and cell death in hematopoietic progenitors following DNA damage. Here, we show that this relationship is conserved in the developing nervous system in which *Snail* genes are excluded from vertebrate neurons and they are substituted by *Scratch*, a related but independent neural-specific factor. The transcription of *scratch2* is induced directly by p53 after DNA damage to repress *puma*, thereby antagonizing p53-mediated apoptosis. In addition, we show that *scratch2* is required for newly differentiated neurons to survive by maintaining *Puma* levels low during normal embryonic development in the absence of damage. *scratch2* knockdown in zebrafish embryos leads to neuronal death through the activation of the intrinsic and extrinsic apoptotic pathways. To compensate for neuronal loss, the proliferation of neuronal precursors increases in *scratch2*-deficient embryos, reminiscent of the activation of progenitor/stem cell proliferation after damage-induced apoptosis. Our data indicate that the regulatory loop linking p53/*Puma* with *Scratch* is active in the vertebrate nervous system, not only controlling cell death in response to damage but also during normal embryonic development.

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When activated by cellular stress, p53 acts as a crucial tumor suppressor, acting through a very intricate signaling network involving multiple positive and negative regulatory feedback loops.¹ Indeed, its activity is modulated by its own transcriptional targets, p53 upregulated modulator of apoptosis (*Puma*) and murine double minute gene 2 (*Mdm2*), which transduce the apoptotic response or trigger p53 degradation, respectively.² Accordingly, irreparable damage results in apoptosis, whereas mild stress may elicit a p53 survival response associated with DNA repair.³ Hence, the effects of p53 are defined by its affinity for response elements (REs) in different target genes, the cell context and the nature and duration of the stress signal.

Scratch and *Snail* are transcriptional repressors from independent gene families that originated by duplication of the ancestral *snail* gene.⁴ They are prominently expressed during embryonic development, occupying complementary territories in vertebrates. As such, *Snail* genes are expressed in mesodermal and mesenchymal cells,⁵ whereas *Scratch* family members are neural specific.^{6,7} Although *Snail* factors have received much attention in cancer research and developmental biology,⁸ the functional analyses of *scratch* genes *in vivo* are scarce^{9,10} and still missing in vertebrates.

Although *Snail* factors drive the conversion of epithelial cells to migratory cells,⁸ *scratch* genes are excluded from epithelial and mesenchymal cells, which makes them unlikely to

participate in epithelial–mesenchymal transition. However, members of the *Snail* family^{11,12} and the *Caenorhabditis elegans* *Scratch* homolog, cell death specification 1 (*ces-1*), have been implicated in conferring resistance to cell death.¹⁰ Expression of the *C. elegans* *puma* homolog, *egl-1* (egg laying defective 1), is repressed by *ces-1*,¹³ and *Puma* is the main effector of p53-induced cell death in vertebrates.² Indeed, *Snail2* can antagonize p53-mediated apoptosis by repressing *Puma* in hematopoietic precursors following DNA damage.¹²

The only *Snail*-like sequence found in the *C. elegans* genome is thought to encode a non-functional protein.⁴ Hence, we wondered whether *ces-1*, the sole *Scratch* homolog, had co-opted the role of *Snail* in repressing *Puma* in the nematode. Alternatively, *Puma* repression might be an ancestral function associated to both *snail* and *scratch* genes before their divergence. Therefore, we investigated the role of *Scratch* in vertebrate cell survival using zebrafish, a suitable model to study cell death and p53 signaling.^{14–16} We characterized three zebrafish *scratch* genes that were specifically expressed in the nervous system and we found that *scratch2* acts as a survival factor in neurons *in vivo*. We show that *scratch2* is directly upregulated by p53 and that it represses *puma* transcription after DNA damage. More importantly, we show that *scratch2* protects neurons from apoptosis by maintaining *Puma* levels low during normal embryonic development.

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Abbreviations: *Puma*, p53 upregulated modulator of apoptosis; *Mdm2*, murine double minute gene 2; *ces-1*, cell death specification 1; h.p.f., hours post-fertilization; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling; CPT, Campthothecin; ChIP, chromatin immunoprecipitation; RT-PCR, reverse transcription PCR; FLIP, FLICE-inhibitory protein; *bcl2*, B-cell lymphoma 2; pH3, phosphohistone 3

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Results

Expression of zebrafish *scratch* genes. Two *Scratch* genes exist in vertebrates, *Scratch1* and *Scratch2*, and although an extra genome duplication gave rise to additional genes in the teleost lineage,¹⁷ only two *scratch1* (*scratch1a* and *1b*) and one *scratch2* genes make up the *scratch* family in zebrafish.⁴ We obtained the three full-coding regions of zebrafish *scratch* genes that encode Scratch proteins with strong similarity to those predicted from other teleosts (Supplementary Figure 1) and vertebrates (Supplementary Figure 2). The three sequences contain the diagnostic Scratch domains: five Scratch-type C2H2 zinc-fingers and the Scratch and Snail1/GFI domains.¹⁸

The *scratch1* genes were first expressed at the 21 somite stage in the developing central nervous system of zebrafish embryos (Figures 1a and d). Although *scratch1a* transcripts were detected in the telencephalic dorsorostral cluster¹⁹ (drc) and hindbrain (h, Figure 1a), *scratch1b* was most strongly expressed in the hindbrain and diencephalic ventrocaudal cluster¹⁹ (vcc, Figure 1d). At 24 hours post-fertilization (h.p.f.), the expression of both genes was better defined in the same regions (Figures 1b and e). Similarly, *scratch2* was expressed in both the drc and vcc, and in the hindbrain at the same stages, and at 24 h.p.f., it was also strongly expressed in the spinal cord (sc, Figures 1g–i), which was devoid of *scratch1a* and *1b* transcripts (Figure 1c and f). *scratch2* transcripts were detected in the spinal cord from the onset of its expression at the six-somite stage (Figure 1m), when it was also expressed in sensory Rohon-Beard neurons and in the placode of the trigeminal ganglion (tg, Figures 1j and k). However, this spinal cord expression was dynamic and transient, with no transcripts detected after 36 h.p.f. (data not shown). At 24 h.p.f., *scratch2* transcripts were evident in the mantle layer of the spinal cord at different dorsoventral levels (Figure 1l) and not in the ventricular zone, indicating that it is expressed where postmitotic neurons are found. Hence, like the *Drosophila*,⁹ *C. elegans*¹⁰ and mouse *scratch1*^{6,7} and *scratch2*⁷ homologs, the three zebrafish *scratch* genes are restricted to the nervous system, with *scratch2* being expressed in the same territories as the two *scratch1* genes, and in the spinal cord at the time of neuronal differentiation.

***scratch2* knockdown induces cell death in the spinal cord.** To study the function of *scratch* genes, we examined the effects of disrupting *scratch2* activity in the spinal cord, which only expresses this *scratch* gene. Two morpholino oligonucleotides, one that overlapped the start codon (MO1) and another against the 5' UTR (MO2, see Supplementary Figure 3), were injected into one-cell embryos. The survival of the embryos injected with MO1 was 86% (7 ng) and 70% (15 ng), whereas up to 84% (2 ng) and 50% (7 ng) of embryos survived when injected with MO2 ($n > 150$ embryos per condition). Similar survival (84%) was evident after injection of a control morpholino (MOC), which did not produce any detectable phenotype at the concentrations used. In contrast, both MO1 and MO2 induced dose-dependent shortening of the embryos and opacity, characteristic of cell death at 24 h.p.f. (Figure 2a). At concentrations that produced similar embryo survival and opacity, injection of both MO's (7 ng of

MO1 or 2 ng of MO2 per embryo) increased apoptosis in 94% of the embryos, when compared with the control MO ($n = 100$; Figure 2b). Although distributed throughout the embryo (Supplementary Figure 3), the injected morpholinos should only affect regions that express target genes, and, indeed, apoptosis only increased in the nervous system. This cell death did not appear to be an off-target effect of morpholino injection (as described for around 20% of sequences²⁰) because both MOs produced the same effect. Moreover, there was no morphological phenotype or additional apoptosis in injected embryos analyzed at the six-somite stage when *scratch2* expression commences (Figure 2b). Indeed, when we co-injected MO2 (directed against the 5' UTR sequences) with an mRNA containing the coding region of *scratch2*, morpholino-induced apoptosis was fully rescued in up to 85% of embryos ($n = 100$; Figures 3g–i), reflecting the specificity of this effect. Hence, *scratch2* might act as an anti-apoptotic factor in developing zebrafish embryos, although endogenous embryonic apoptosis persisted (Figures 3h and i) and *scratch2* overexpression did not impede the endogenous cell death in the spinal cord (Figures 3j–l) and trigeminal ganglion ($n = 8$ ganglia per condition) in which it is prominent at these stages (Supplementary Figure 4).

***scratch2* interacts with the p53 pathway to protect from cell death.** As cell death augments in the developing spinal cord of *scratch2* morphants, we wanted to determine the molecular pathway by which Scratch2 regulates apoptosis. Members of the p53 family are critical elements in the apoptotic program, p53 being the key family member in the zebrafish nervous system.²¹ An increase in p53 activity is associated with considerable apoptosis in the nervous system and growth arrest in embryos,¹⁴ a phenotype reminiscent to that observed in the *scratch2* morphants. Hence, we assessed whether *scratch2* and p53 might be acting in the same pathway. Injection of *p53* MO alone did not induce any embryonic defects (98%, $n = 100$; Figures 3m–o) as expected, as p53 is not essential for zebrafish (or mouse) embryonic development.¹⁴ Indeed, when MOs against *scratch2* and *p53* were co-injected into embryos, they were indistinguishable from control embryos (91%, $n = 77$; Figures 3p–r). Hence, it appears that Scratch2 protects neurons from apoptosis and that the death induced by Scratch2 downregulation is mediated by p53.

As Snail/Scratch proteins are transcriptional repressors,⁴ we assessed whether Scratch2 could protect cells from apoptosis by repressing *p53* transcription. There were no significant changes in *p53* expression in *scratch2* morphant embryos (Figure 4a), indicating that Scratch2 does not act directly upstream of p53. However, Scratch2 downregulation activates p53, as it influences the expression of its main direct target, *mdm2* (Figure 4a). Conversely, we determined whether activating p53 with camptothecin (CPT), an anti-cancer drug that promotes p53-mediated apoptosis in zebrafish, affects *scratch2*.¹⁴ CPT induced DNA damage and large-scale apoptosis in the spinal cord of 98% of embryos ($n = 100$), which was impaired by injecting *p53* MO (75%; $n = 100$). Hence, the apoptosis induced by CPT was mediated by p53 and it was concomitant with increased

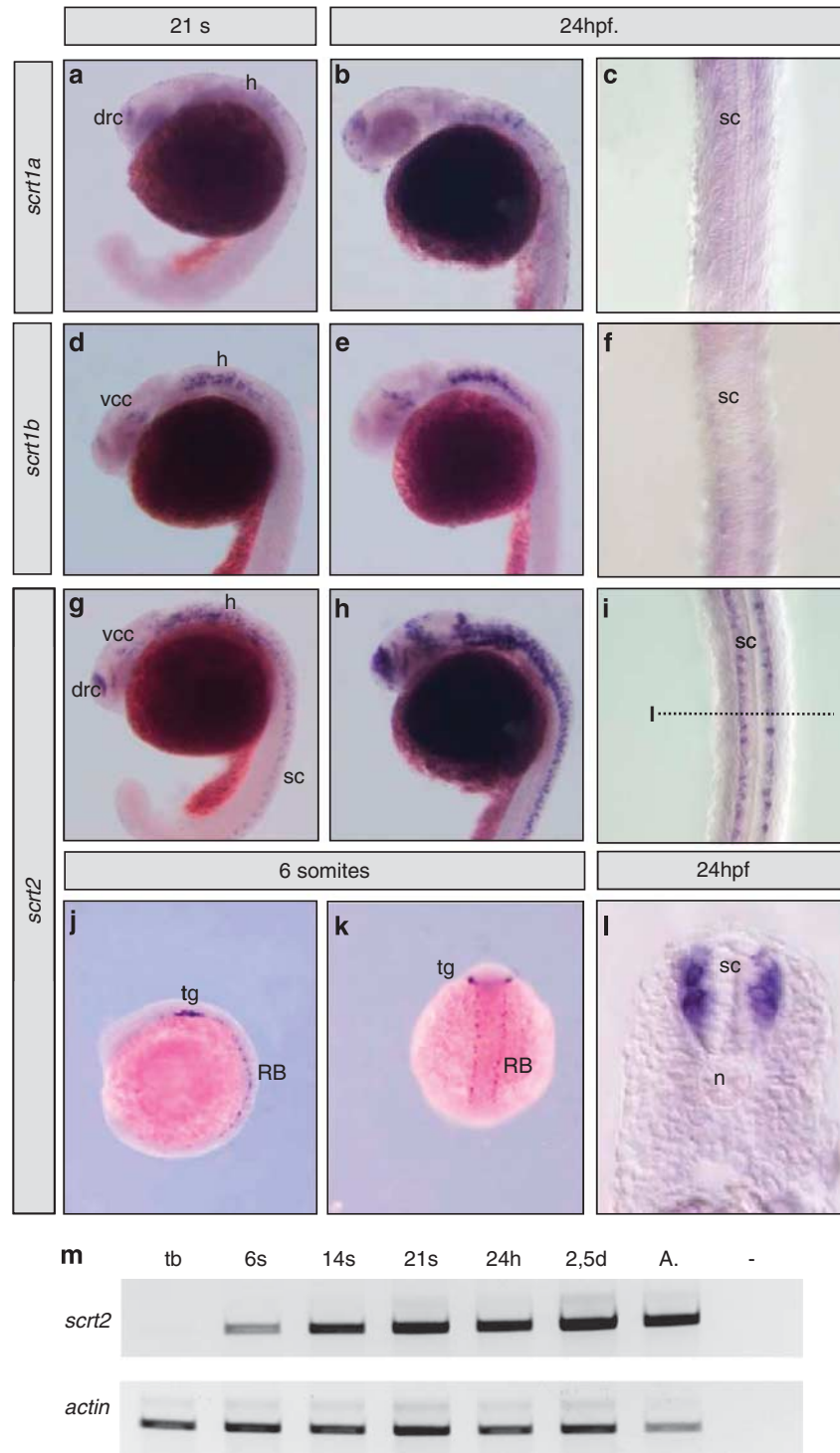


Figure 1 Expression of *scratch* genes in zebrafish embryos. The expression of *scratch1a*, *scratch1b* and *scratch2* was analyzed at different developmental stages. (a–c) *scratch1a* is first expressed in the telencephalon and the hindbrain of 21-somite embryos, and this pattern is better appreciated at 24 h.p.f. (d–f) The onset of *scratch1b* expression is similar to that of *scratch1a*, although the expression in the hindbrain is more prominent and transcripts can also be detected in the diencephalon. (g–i) *scratch2* is prominently expressed in the regions of *scratch1a* and *1b* expression and in the spinal cord. (j and k). *scratch2* is expressed in the precursors of the trigeminal ganglion and in the sensory Rohon-Beard neurons from the six-somite stage (lateral and dorsal views, respectively). (l) *scratch2* expression in the spinal cord at 24 h.p.f. is restricted to the mantle layer at all dorsoventral levels. (m) RT-PCR confirms the onset of *scratch2* expression in six-somite stage embryo. h, hindbrain; n, notochord; drc, telencephalic dorsorostral cluster; RB, Rohon-Beard neurons; sc, spinal cord; tg, precursors of the trigeminal ganglion; vcc, diencephalic ventrocaudal cluster

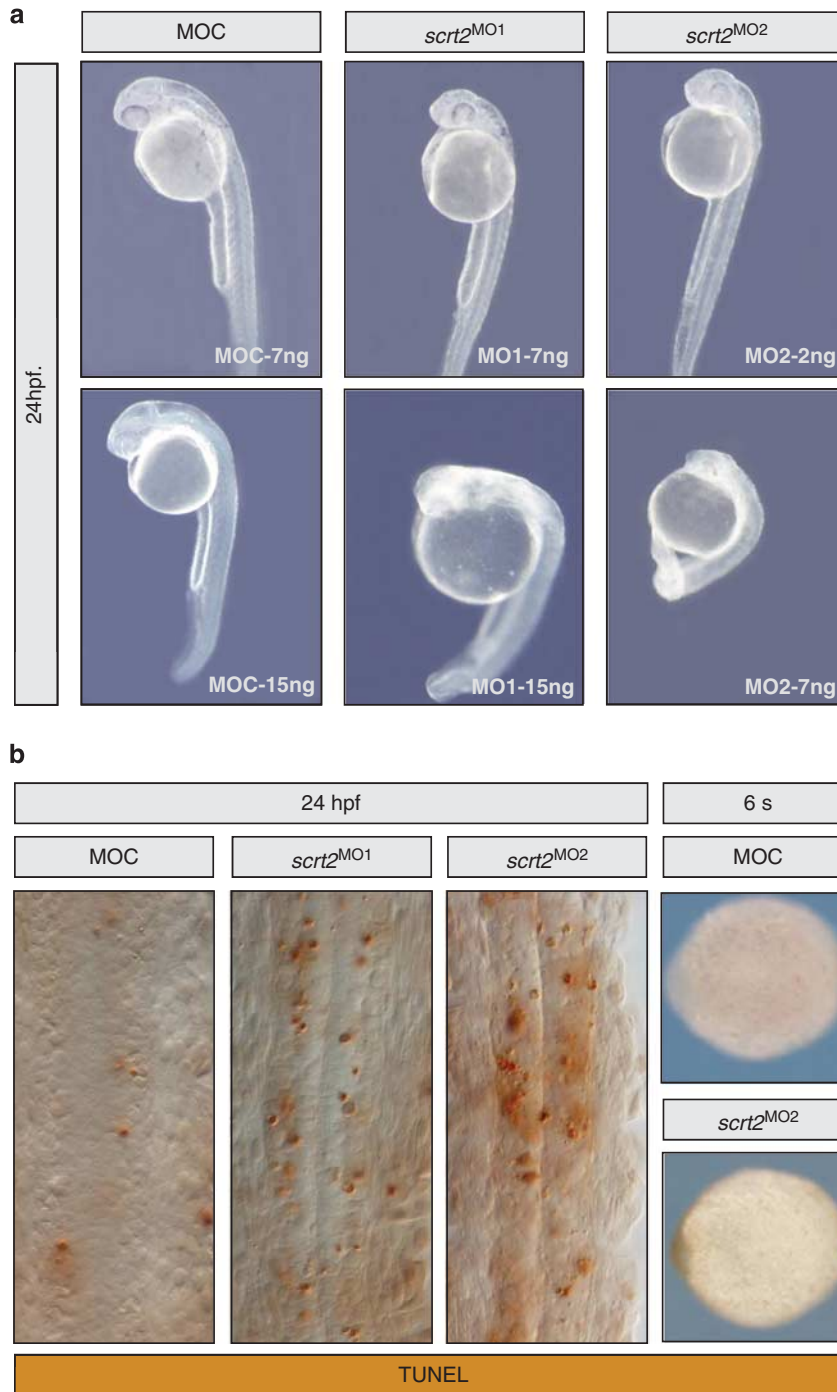


Figure 2 *scratch2* knockdown induced cell death in zebrafish embryos at 24 h.p.f. (a) Two different morpholino antisense oligonucleotides were designed against adjacent sequences in the 5' region of *scratch2*. *scratch2* morpholino injections produced shortening and opacity, as assessed by morphology in embryos at 24 h.p.f. (b) A significant increase in cell death in the spinal cord was detected by TUNEL staining. Pictures show flat-mounted embryos highlighting dorsal views of the spinal cord. No morphological defects or significant cell death could be observed in embryos injected with control morpholino (MOC) or with *scratch2* MOs analyzed as whole embryos at the six-somite stage (6s), when endogenous *scratch2* begins to be expressed (anterior is to the left)

p53 transcription (Figures 4b and c). Upregulation of direct transcriptional targets of *p53*, *mdm2* and the BH3-only *puma* further confirmed the functional activation of *p53* (Figure 4c). Moreover, *scratch2* transcription was also upregulated in CPT-treated embryos (Figure 4c), augmenting the number of

cells expressing *scratch2* in the spinal cord by 55% in a *p53*-dependent manner (from around 11 to 17 per somite in three independent embryos per condition, Figure 4d). The complement of cells expressing *scratch2* was normal in *p53*-morphant embryos, indicating that *p53* is not the

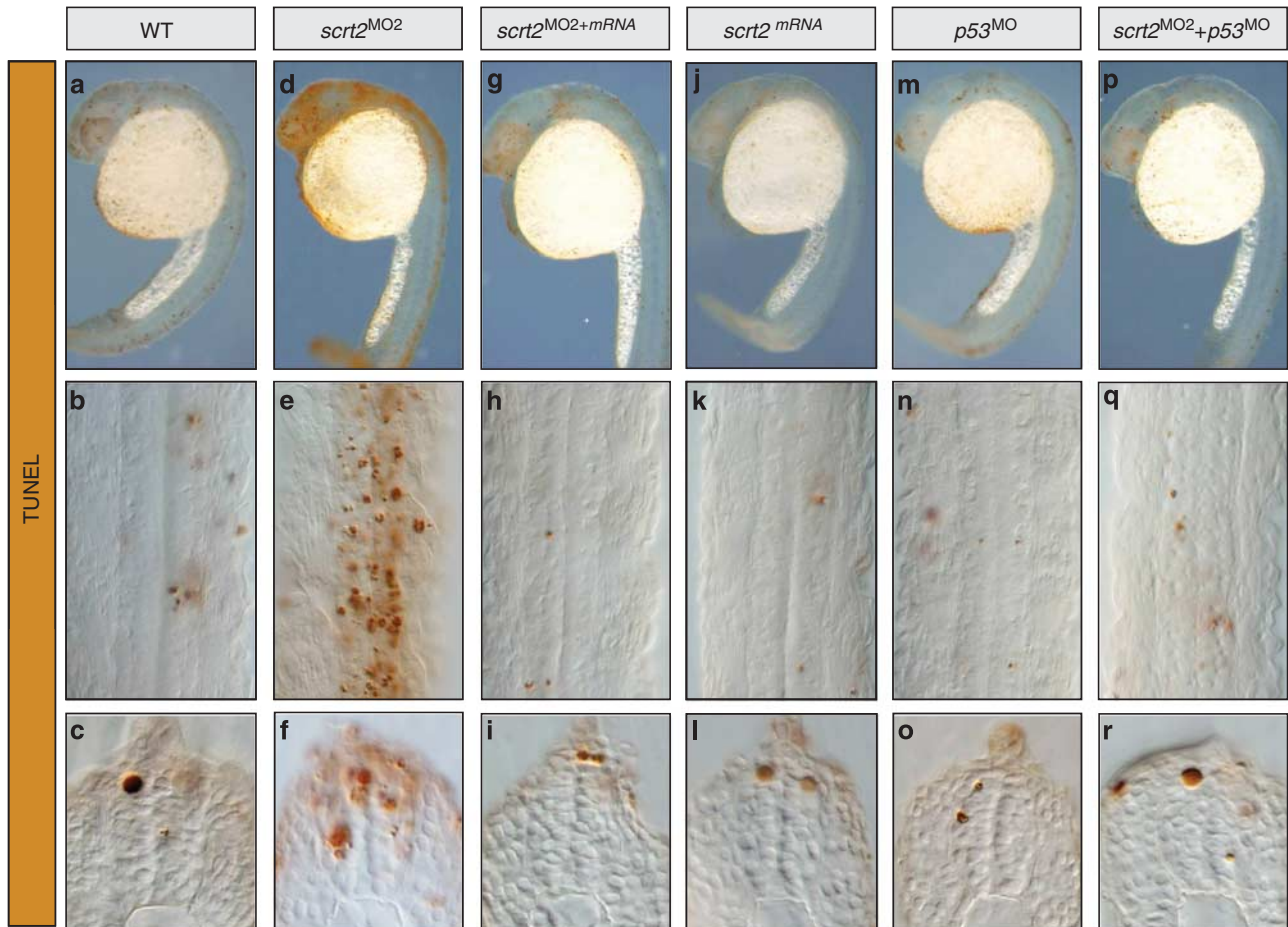


Figure 3 *scratch2* protects from cell death by antagonizing the p53 pathway. Embryos at 24 h.p.f. were subjected to TUNEL staining and shown as whole embryos, flat mounts or in transverse sections through the spinal cord. (a–l). The cell death phenotype observed after *scratch2* MO injection could be rescued by co-injection of *scratch2* mRNA. (j–l) Injection of *scratch2* mRNA alone does not protect from endogenous cell death. (m–o) *p53* morphant embryos are indistinguishable from control embryos. (p–r) The co-injection of *p53* MO prevents the death induced by *scratch2* MO

endogenous inducer of *scratch2*. These results demonstrate that *scratch2* is a novel p53 target activated after DNA damage.

***scratch2*, a new direct target of p53.** To determine whether p53 directly activates *scratch2* transcription, we looked for putative p53-responsive elements in the *scratch2* gene with the p53MH algorithm.²² We identified two putative p53-binding sites for which luciferase reporter constructs were generated, one in the 5' UTR and another in the *scratch2* intronic sequences (Figure 5a). A similar construct containing the p53 RE in *puma* was used as a positive control.¹² The constructs were independently injected into one-cell zebrafish embryos and p53 was activated by treating the zebrafish embryos with CPT at the 21-somite stage. This activation of p53 transactivated each *scratch2* reporter, and as this transactivation was prevented when *p53* was knocked down, p53 appears to activate *scratch2* transcription through the putative binding sites identified (Figure 5b). Moreover, in chromatin immunoprecipitation assays (ChIP) performed on embryos treated with CPT, p53 was specifically recruited to fragments containing the binding site in the *scratch2*

promoter (Figure 5c) and, hence, we consider *scratch2* to be a novel direct target of p53.

Scratch2 directly represses *puma* transcription. As *scratch2* knockdown did not affect *p53* transcription (Figure 4) and Snail/Scratch factors act as transcriptional repressors, Scratch2 could repress the transcription of pro-apoptotic genes downstream of *p53*. Hence, we assessed *puma* expression when Scratch2 function was compromised, one of the main mediators of apoptosis in the nervous system. Injection of MO2 strongly upregulated *puma* expression in zebrafish embryos at 24 h.p.f., an effect that was prevented by co-injection with *p53* MO (Figures 6a and b). Thus, this increase in *puma* transcription appears to be mediated by p53, explaining the rescue of cell death observed when *p53* MO was co-injected with *scratch2* MO (Figures 3p–r). These data also support that Scratch2 could repress *puma* transcription. Similar to Snail, Scratch binds to E-boxes, and in murine hematopoietic cells, Snail2 has already been shown to bind to a consensus E-box in an intron of *Puma*.¹² Indeed, we found two putative Scratch-binding sites in the first intron of zebrafish *puma*. As antibodies against Scratch are not available, we

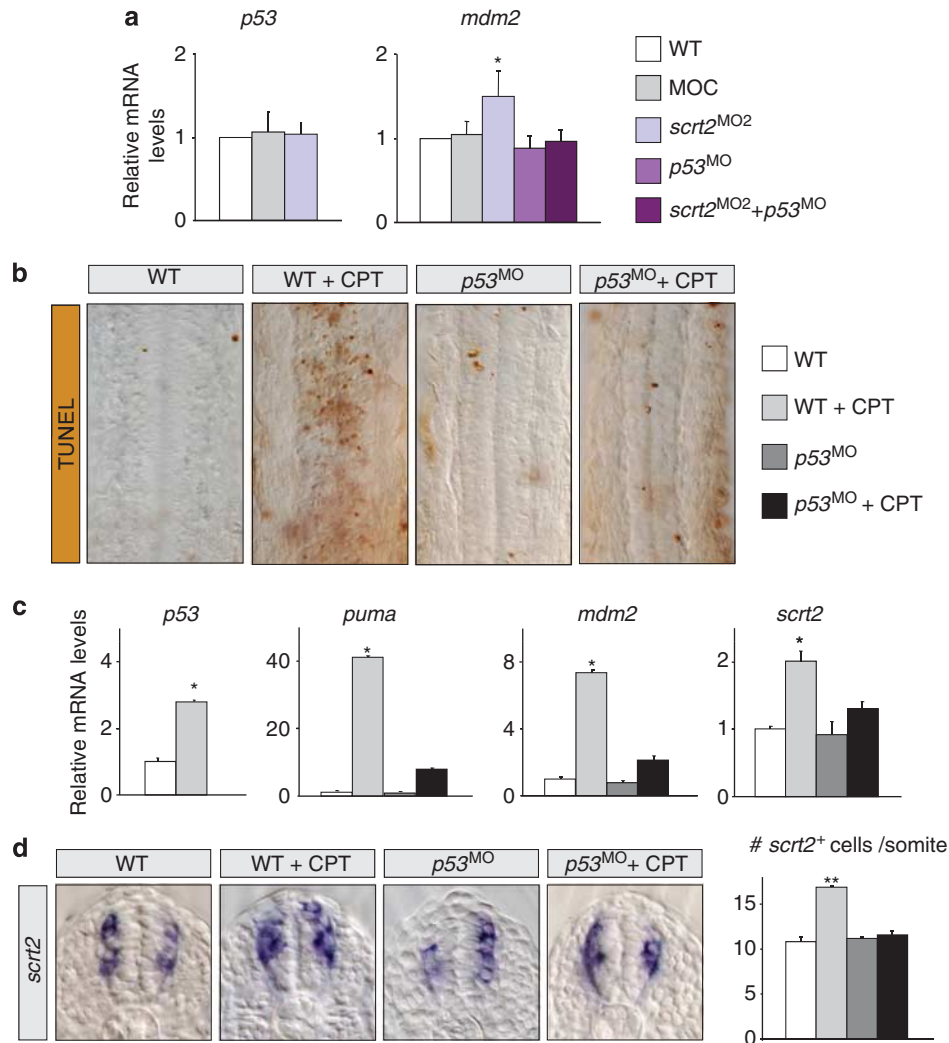


Figure 4 p53 activation induces *scratch2* expression in the spinal cord. (a) Scratch2 does not regulate *p53* transcription. Real-time RT-PCR from embryos injected with control (MOC), *scratch2*, *p53* or *scratch2* plus *p53* MOs. The transcription of *p53* is not affected by *scratch2* knockdown, but its transcriptional activity, measured by the levels of its readout *mdm2*, is increased. (b) Flat-mounted spinal cords from 24 h.p.f. embryos showing the apoptosis induced by camptothecin (CPT)-mediated p53 activation.¹⁴ (c) Real-time RT-PCR to assess the transcription of *p53*, its targets *puma* and *mdm2* and of *scratch2* in embryos subjected to the treatments shown in b. *scratch2* transcription is activated in response to CPT treatment. (d) *scratch2* is activated in ectopic spinal cord cells on CPT treatment in a p53-dependent manner. Real-time RT-PCR data in a are means \pm S.E.M. of five independent experiments and c shows a representative experiment ($n = 3$), with each point examined in triplicate in all cases. * $P < 0.05$; ** $P < 0.01$

generated a myc-tagged Scratch2 fusion protein to perform ChIP assays. Moreover, as *scratch2* overexpression is insufficient to protect against endogenous apoptosis (Figure 3), the ChIP experiment was performed in a *scratch2*-morphant background that can be rescued by injection of *scratch2* mRNA (Figure 3). As expected, co-injection of *scratch2* MO and *myc-scratch2* mRNA prevented the apoptosis induced by *scratch2* knockdown (75%, $n = 40$; Figure 6c). Under these conditions, Scratch2 can bind to a fragment containing the first E-box in the *puma* intron (Figure 6d), demonstrating that Scratch2 can directly repress *puma* transcription, preventing embryonic cell death in the absence of damage.

***scratch2* downregulation activates intrinsic and extrinsic apoptotic pathways.** In the *scratch2*-morphant embryos studied here, apoptotic cells appeared in regions

of endogenous *scratch2* expression and in adjacent cells extending two or three cell layers, indicative of a non-cell-autonomous phenotype (Figures 3f and 7c). These apoptotic cells appear to account for about one-third of the total terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL)-positive cells. As this ectopic cell death was prevented by co-injection of *scratch2* mRNA, it was considered a specific consequence of *scratch2* knockdown. As a transcription factor, Scratch2 should act in a cell-autonomous manner and, thus, *scratch2* MO should only produce effects in cells that normally express this factor. Nevertheless, these cells could produce non-cell-autonomous effects by activating an extrinsic apoptotic pathway. Accordingly, overexpression of a specific inhibitor of the extrinsic pathway (FLICE-inhibitory protein (FLIP))²³ should block the apoptosis observed outside the endogenous domain of *scratch2* expression. Injection of *flip* mRNA

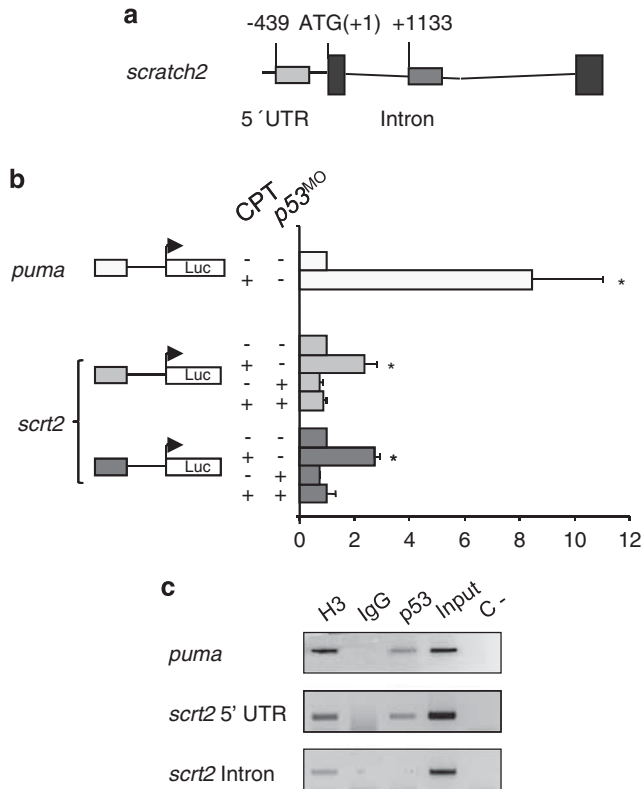


Figure 5 *scratch2* transcription is directly activated by p53. (a) p53-binding sites in *scratch2*. Putative p53 response elements are found in the promoter and within the intron of *scratch2* sequences (gray boxes, positions relative to the ATG). (b) Luciferase assays carried out in whole zebrafish embryos. CPT-mediated p53 activation correlates with reporter activity for *puma*-positive control constructs and *scratch2* constructs containing p53-binding sites found either in the promoter or in the intron. Transactivation is prevented by the co-injection of p53 MO. * $P < 0.05$ (c) Chromatin immunoprecipitation assays (ChIP) in embryos treated with CPT (see Materials and Methods). Input material was tested for each primer set. ChIP analyses were carried out with anti-p53 antibodies on 24 h.p.f. zebrafish embryos amplifying the p53 response element in *puma* intron as a positive control for immunoprecipitation. H3 and IgG are the positive and negative controls of the immunoprecipitate, respectively; C⁻ is a PCR negative control. The data presented are representative of three independent experiments

alone did not affect embryos, which were indistinguishable from controls (96%, $n = 30$; Figures 7a, b and e), whereas there were fewer apoptotic cells in embryos co-injected with *flip* mRNA and *scratch2* MO than when *scratch2* was knocked down (89% $n = 37$; compare Figure 7c with f). Interestingly, the remaining apoptotic cells were only found in the mantle layer of these embryos, within the limits of the endogenous *scratch2* expression domain (compare Figures 7d with f). Hence, *scratch2* knockdown leads to cell-autonomous activation of the intrinsic apoptotic pathway in cells that normally express *scratch2*, subsequently inducing cell death in adjacent cells through an extrinsic apoptotic pathway.¹⁵ Indeed, B-cell lymphoma 2 (*bcl2*) over-expression completely abolished the apoptotic phenotype in 75% of embryos co-injected with *scratch2* MO ($n = 65$; Figure 7h), both in endogenous territories of *scratch2* expression and in adjacent cells.

The apoptosis in the *scratch2* morphants did not significantly reduce the size of the spinal cord, perhaps because compensatory proliferation can be triggered in adjacent cells, as in *Drosophila* and mice.^{24–26} Indeed, the apoptosis observed in *scratch2* morphants was followed by an increase in mitosis in the ventricular zone, assessed by phosphohistone 3 (pH3) staining (Figures 7i–l). This increased proliferation was specifically related to *scratch2* knockdown, as the normal number of mitotic cells were observed when the morpholino was co-injected with *scratch2* mRNA (Figures 7k and l). Hence, the apoptosis induced by loss of Scratch2 is associated with compensatory proliferation that attempts to heal the tissue.

Discussion

By characterizing the zebrafish *scratch* genes, we show that, similar to their homologs in other species, they are specifically expressed in the nervous system during early development.^{6,7,9,10} The three *scratch* genes are expressed in overlapping and specific patterns in the developing brain and *scratch2* alone is expressed in the embryonic spinal cord. This specific expression of *scratch2* allowed us to analyze vertebrate Scratch function *in vivo* for the first time, avoiding the problems of compensation due to co-expression of multiple family members. Accordingly, we pinpoint a role for Scratch in neuron survival during normal embryonic development, repressing Puma expression in the absence of DNA damage.

Scratch2 promotes neuronal survival by suppressing Puma expression during normal development. The induction of Puma expression by stress can provoke indiscriminate death of mature cells, whereas in deregulated systems (e.g., tumors), the loss of Puma may be sufficient to overcome apoptosis.²⁷ Thus, maintaining Puma levels low is essential for cell survival. Snail2 can protect hematopoietic progenitor cells from cell death induced by DNA damage by repressing *Puma* transcription.¹² In the nervous system, *scratch2* knockdown de-represses *puma* and provokes neuronal death, even in the absence of DNA damage. This indicates that Scratch2 is required for neuron survival during normal development, and that Puma must be actively repressed, even in the absence of stress signals. Newly differentiated spinal cord neurons need an effective way to repress Puma activity to survive, and this can be achieved during embryonic development through Scratch2 (Figure 8a).

The knockdown of *scratch2* augments *puma* expression and neuronal death in a p53-dependent manner (Figure 8b), even though *p53* expression remains unaffected. As p53 is constitutively transcribed during development, its activity is tightly regulated through proteasomal degradation triggered by its own target, Mdm2,²⁸ and by its sequestration by anti-apoptotic members of the Bcl-2 family.²⁹ *Puma* is a common target of p53 and Scratch2 for activation or repression, respectively. Indeed, Scratch2 binds to an E-box located very close to the p53 consensus RE in a *puma* intron, suggesting that they might compete for binding, as previously postulated for Snail2.³⁰ In the absence of damage, low levels of p53 enable Scratch2 to bind to the intron of *puma* and prevent its

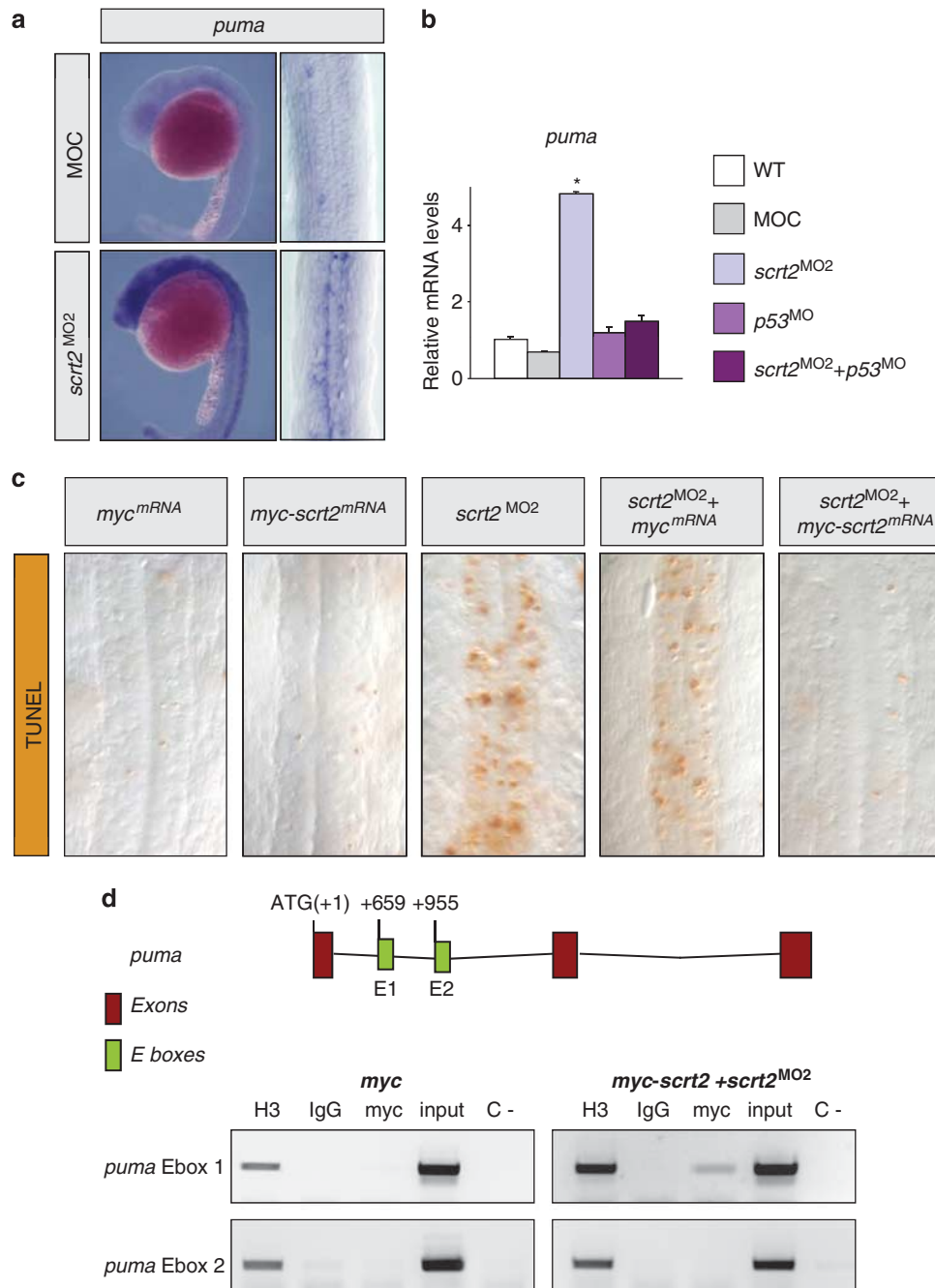


Figure 6 *scratch2* antagonizes p53-mediated apoptosis by directly repressing *puma* transcription. (a) Increased *puma* transcription is observed by *in situ* hybridization in *scratch2* morphants. (b) Real-time RT-PCR showing the increase in *puma* transcripts in *scratch2*-morphant embryos, which can be prevented by co-injection with p53 MO. **P* < 0.05. (c) A *myc-scratch2* fusion mRNA is, similar to *scratch2* mRNA, able to rescue the cell death induced by *scratch2* MO. (d) Zebrafish Scratch2 is recruited to the E1-box in the first *puma* intron. Schematic representation of *puma* with E-boxes in green and positions relative to the ATG. Chromatin immunoprecipitation assays (ChIP) in embryos treated as in c. Input material was tested for each primer set and ChIP analyses were carried out with anti-myc antibody on 24 h.p.f. zebrafish embryos. An irrelevant sequence in *scratch2* intron was amplified as a negative control of the immunoprecipitation with the anti-myc antibody (data not shown). H3 and IgG are the positive and negative controls of the immunoprecipitate, respectively. C- is a PCR negative control. The data presented are representative of three independent experiments

transcription (Figure 8a). Conversely, p53 can activate *puma* transcription in the absence of Scratch2 (Figure 8b). Interestingly, the p53/Bcl-XL equilibrium is disrupted by Puma, releasing p53 into the cytoplasm.³¹ Thus, newly expressed Puma may augment the p53 cytoplasmic pool, reinforcing Puma expression and cell death.

After DNA damage, p53 activation directly increases *scratch2* transcription in the zebrafish, establishing a negative feedback loop similar to that described for Mdm2, but through direct repression of *Puma* rather than p53 degradation (Figure 8c). Similarly, p53 upregulates the transcription of other prosurvival genes, including *Snail* family members.¹²

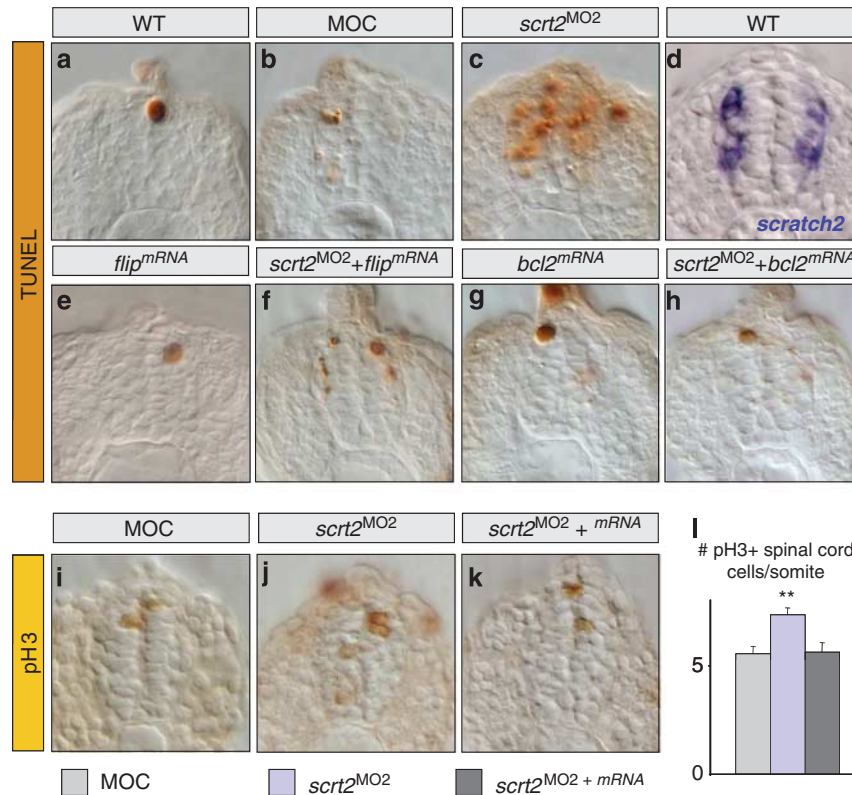


Figure 7 *scratch2* downregulation activates both the intrinsic and extrinsic apoptotic pathways in the spinal cord, concomitant with compensatory proliferation. Embryos at 24 h.p.f. were TUNEL stained (a–c and e–h) or subjected to *in situ* hybridization for *scratch2* (d), and transverse sections through the spinal cord are shown. (a–d) After *scratch2* MO injection cell death is observed in regions of endogenous *scratch2* expression and in adjacent cells. Compare c with d. (e–f) Co-injection of *scratch2* MO and mRNA for the specific inhibitor of extrinsic apoptotic pathway *flip* prevents the death induced outside the *scratch2* expression domain, confirming the activation of this pathway after *scratch2* downregulation. (g and h) The co-injection of *scratch2* MO and *bcl2* mRNA prevents the activation of both the intrinsic and the extrinsic death pathways induced by *scratch2* MO. (i–l) pH3 immunoreactivity and cell quantification reveals that cell death in *scratch2* morphants induce compensatory proliferation in the ventricular zone (compare j with i, and l). Co-injection of *scratch2* MO and mRNA rescues this phenotype (k and l). ** $P < 0.01$

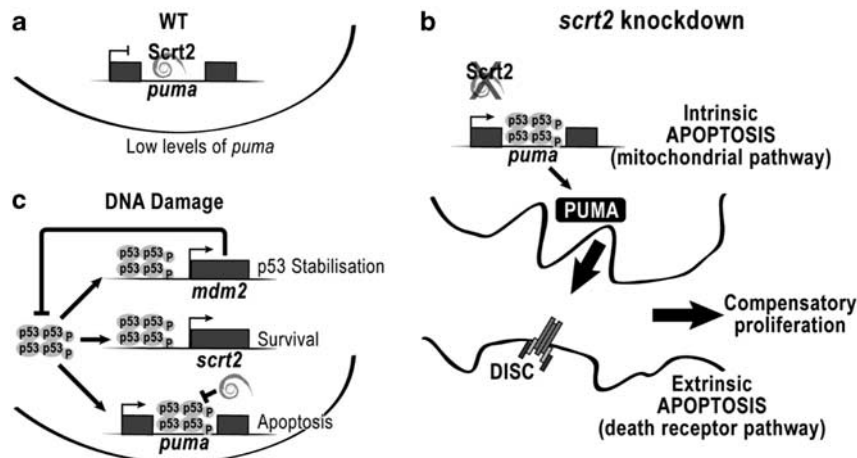


Figure 8 Role of Scratch2 in neuron survival during development. (a) In the wild-type embryo, Scratch2 maintains *puma* transcription repressed, allowing neuronal differentiation and survival. (b) Scratch2 downregulation de-represses *puma*, permitting p53 binding to its response element in the intron and resulting in neuronal cell death. Apoptosis mediated by the intrinsic pathway in these cells induces the extrinsic apoptotic program in the neighboring cells. Dying cells induce progenitor cells in the ventricular zone to increase proliferation in an attempt to compensate for cell loss. (c) On DNA damage, p53 induces the transcription of several target genes, including *mdm2*, *puma* and *scratch2*. p53 protein levels are controlled by degradation induced by Mdm2. Cells expressing high levels of Scratch2 can interfere with the p53 apoptotic program through the repression of *puma*, leading to cell survival. A long-lasting or strong damage induces apoptosis as the final outcome of persistent p53 activity

Weak Scratch expression permits *Puma* transcription, whereas strong Scratch expression would prevent p53 from acting on *Puma*, probably by interfering with the assembly of the transcriptional complex.

Scratch2 is required for newly differentiated neurons to survive: evolutionary considerations. Evolution has favored the need for Scratch to promote neuron survival. Increased scratch (*ces-1*) expression in *C. elegans* leads to the appearance of supernumerary neurons, although it is not, in principle, required for neuron survival.³² However, *scratch2* knockdown in zebrafish is sufficient to promote neuronal death, while its overexpression does not promote the survival of additional neurons, which also seems to be true in the chick embryo (our unpublished results). Interestingly, vertebrate evolution has implemented robust mechanisms to regulate cell number and to eliminate supernumerary neurons,³³ which suggests the need for a system ensuring controlled neuronal survival. Although in tissues with little proliferation, mature cells need to be protected, mature cells are dispensable in those with high proliferation rates, as long as there are sufficient progenitors.¹² For example, the progenitors in the hematopoietic system express Snail2 and, hence, they are protected from p53-induced cell death after DNA damage.¹² Other adult tissues have poor proliferative capacity and they must protect mature cells, as also seems to be the case in the developing nervous system in which differentiated neurons express *scratch* to protect them from cell death.

The role of Scratch2 in the survival of zebrafish and *C. elegans* neurons (refs. 10, 13 and data herein) indicates that resistance to cell death is an ancestral function associated with the Scratch superfamily. Snail proteins have also been implicated in promoting cell survival on different apoptotic stimuli,^{8,11} indicating that cell survival is an ancestral function associated with both families before diploblasts and bilateria diverged.⁴ Accordingly, the better-known role of Snail factors in regulating cell adhesion and movement was probably co-opted later by *Snail* genes alone. It is intriguing that evolutionary patterns also show that *p53* genes existed before the appearance of cancer.³⁴ Therefore, the crucial tumor suppressor functions associated with this family were not ancestral but rather, similar to Snail and Scratch, the ancestral function appears to be the regulation of cell death, which existed even before the regulation of the cell cycle.³⁵ Whether the Snail, Scratch and p53 families evolved hand-in-hand to establish the p53 regulatory networks during evolution might be an interesting topic to explore in the future.

Scratch2 downregulation, activation of intrinsic and extrinsic apoptotic pathways and compensatory proliferation. During embryonic development, *puma* is activated after *scratch2* knockdown to stimulate the mitochondrial apoptotic pathway in the zebrafish neural tube. Scratch2 deficiency provokes cell-autonomous death of differentiated neurons that otherwise express *scratch2*. Similarly, it activates non-autonomous cell death through an extrinsic apoptotic pathway in progenitor cells that do not normally express *scratch2*, revealing a role for the extrinsic apoptotic pathway in the zebrafish nervous

system¹⁵ (Figure 8b). Similar cell death of both proliferative and differentiated cells occurs in the intestine of young mice deficient for Mdm2.²⁶ Interestingly, fish and mice respond similarly to the loss of a p53-induced negative regulator, Scratch2 or Mdm2, not only in terms of cell death but also by inducing compensatory proliferation (ref. 26 and data herein). Indeed, although the apoptosis induced by Scratch2 downregulation could reduce the size of the spinal cord, increased proliferation of neural progenitors in the ventricular zone appears to prevent this from occurring. Hence, this tissue seems to activate mechanisms to overcome neuronal loss and maintain tissue homeostasis, similar to the compensatory mechanisms described in *Drosophila*.^{24,25}

The apoptosis and compensatory proliferation observed in our *scratch2* morphants, is reminiscent of the massive leukocyte cell death and compensatory proliferation, which repopulates damaged tissue following irradiation of wild-type mice and which leads to secondary malignancies appearing because of the damage to progenitor/stem cell pools.^{36,37} Conversely, the lack of cell death and of subsequent compensatory proliferation in *Puma*-deficient mice protects them from developing secondary tumors.^{36,37}

In summary, although both p53 and *Puma* seem to be dispensable for normal embryonic development, they can respond rapidly to damage; thus, inhibitors must be available to tightly control their activity. Here, we describe one such inhibitor, Scratch2, which acts directly on the main readout of p53 signaling, *puma*. When *scratch2* is downregulated, *puma* levels increase, promoting neuronal cell death by activating both intrinsic and extrinsic apoptotic pathways even in the absence of DNA damage. Neuronal loss activates compensatory progenitor cell proliferation, a physiological mechanism at play during development to maintain tissue homeostasis, which may be corrupted in circumstances of previous DNA damage, to provoke the appearance of cancer.

Materials and Methods

Fish maintenance. AB and Tup-Lof wild-type Zebrafish strains maintained at 28°C under standard conditions were used in all experiments. The embryos were staged, as indicated elsewhere.³⁸

Isolation of zebrafish cDNAs. Total RNA was extracted with Trizol (Invitrogen, Paisley, UK) from 24-h.p.f. zebrafish embryos and used to synthesize cDNA using SuperScript III reverse transcriptase (Invitrogen) and oligodT as a primer. Subsequent PCR amplification was carried out using primers directed to the 5'- and 3' UTR sequences as follows: *scratch1a*: 5'-ATGGATCCGGATAACGTGCTGGAAGAGG-3' and 3'-AATCTAGAGAAGTCACGCCTCAATGGAC-5'; *scratch1b*: 5'-CGGGATCCCGGACGAGTGTTCCTCTT-3' and 3'-GCTCTAGATGCCATTAATCCTCGGTGT-5'; *scratch2*: 5'-GGATCCTCTGTGCGACCGTGAACC-3' and 3'-GCCTCGAGGGATTTTTCGAGCATTA-5'. Amplified fragments were subcloned into pTOPO and pCS2+. Zebrafish *actin* was amplified as a control for *scratch2* expression during developmental stages with the following primers: 5'-CTGGTTGTTGACAACGGATCCG-3' and 5'-CAGACTCATCGTACTCCTGCTTGC-3'. Zebrafish *puma* (DQ860151), *flip* (NM_194399) and *bcl2* (ENS DART00000105690) coding sequences were amplified with the following sets of oligonucleotide primers: *puma*, 5'-CACGATTCGGGATAACATCAA-3' and 5'-ACCGTGTGTTTCATCTGAGG-3'; *flip*, 5'-GGATCCGGTGAATGGCAGATGGATT-3' and 5'-GGATCCGGAGTGTGTTGTGTTG-3'; *bcl2*, 5'-GGATCCGCGCTTTCTATCGTATTT-3' and 5'-TCTAGAGAGGCTGTCACTTCTGAGCA-3'. In all cases, sequences represent forward and reverse primers, respectively. The *puma* DNA fragments were subcloned into the pGEM.TE plasmid and those from *flip* and *bcl2* into the pCS2+ plasmid. Myc-tagged *scratch2* was subcloned in pCS2+ -NLS-MT (kindly provided by JL Gómez-Skarmeta) after amplification with

the following primers: Forward 5'-GAATCCATGCCTCGCTCGTTTCT-3' and reverse, 5'-CTCGAGTTAATTCTCTGAGCCAGATTCG-3'.

Morpholino oligonucleotides and mRNA injections. All morpholino antisense oligonucleotides were obtained from Gene Tools LLC (Philomath, OR, USA) and used as described by Nasevicius and Ekker.³⁹ Two antisense morpholinos (MOs) were used. MO1, 5'-GAGGCATGGTTCACGGTCGCACAGA-3', overlapping the start codon and injected from 2 to 15 ng per embryo, and MO2, 5'-CGCACCAGAAAATCCTTCACATCGG-3', located at the 5' UTR and injected from 1 to 7 ng per embryo (Supplementary Figure 3). The sequence of p53 MO was described previously.²⁰ The standard control morpholino from Gene Tools was used for control injections. All oligonucleotides were injected into the yolk of one- or two-cell embryos using a pressure injector (PicoSpritzer, Parker, Fairfield, NJ, USA). MO1 was fluoresceinated to confirm that the morpholino was distributed to all cells after injection and during embryo development (Supplementary Figure 4).

For rescue and overexpression experiments, mRNA was synthesized and capped using the mMessage mMachine Kit (Ambion, Austin, TX, USA). Rescue analyses were performed with 75 pg per embryo of *scratch2* mRNA lacking the 5' UTR region to which MO2 binds. For overexpression analyses, 75–100 pg per embryo of *myc-scratch2*, *flip* and control RNAs or 200 pg per embryo of *bcl2* mRNA were used.

In situ hybridization and immunohistochemistry. *In situ* hybridization and immunohistochemistry of whole-mount zebrafish embryos for *scratch2*, *puma* and pH3 (Rabbit anti-pH3 antibody, 1:1000, Upstate Biotechnology, Lake Placid, NY, USA) were performed as described previously.⁴⁰ Embryos were then fixed and embedded in gelatin to obtain vibratome sections (30 μ m). Embryos were photographed using an Olympus DP70 digital camera (Olympus, Hamburg, Germany) on either a Leica M10 dissecting microscope or a Leica DMR microscope (Leica, Wetzlar, Germany). Flat-mounted 24-h.p.f. embryo spinal cords are shown anterior-up, unless otherwise indicated.

Quantitative reverse transcription PCR. Quantitative reverse transcription PCR was performed using the Step One Plus (Applied Biosystems, Carlsbad, CA, USA) sequence detection system and the SYBR Green method (Applied Biosystems). The data show one representative experiment. Statistics correspond to $n = 3$ unless otherwise indicated, with each point examined in triplicate.

Transcript levels were calculated using the comparative Ct method normalized to GAPDH. The final results were expressed relative to the control condition using the $2^{-(\Delta\Delta C_t)} \pm$ S.D. formula. The primers used are listed below:

Gapdh (AY818346), 5'-GCAGAAAAGCCAGACCATTCC-3' and 5'-CTCGTAGGTGGGAACAGGAA-3'; *scratch2*, 5'-ACTACGAGTCGGCCTGCTT-3' and 5'-GGGATTTTGGCAGCATTAAA-3'; *puma* (DQ860151), 5'-ACGCTGTCTTCCTTCAGAGG-3' and 5'-GGTAGAGGGCATTGATGGTG-3'; *p53* (DRU60804), 5'-CAGTCTGGCACA GCAAATC-3' and 5'-ATTTGAACGGGCAAGTTTT-3'; and *mdm2* (AF356346), 5'-ATTGTTACGGAAGGACTGG-3' and 5'-CCACTGACTGAATGGGCTCT-3'.

CPT treatment and TUNEL assay. CPT (Calbiochem) treatment was performed as described.¹⁴ A 50 μ M stock of CPT in DMSO was diluted in Danieau's buffer to obtain a 500 nM working solution that was applied to 21-somite stage embryos at 28°C until the stage of analysis (24 h.p.f.). Embryos used for luciferase assays and ChIP experiments were treated with CPT for the first 2 h and kept in embryo medium until they reached 24 h.p.f. Control embryos were mock treated with Danieau's buffer containing the same concentration of DMSO. Apoptosis was analyzed with the TUNEL kit (Roche, Basel, Switzerland) according to manufacturer's instructions.

Cell quantification. For the analyses of *scratch2*- and pH3-positive cells, 30 μ m consecutive sections corresponding to five consecutive somites (s5–s9) were analyzed in embryos at 24 h.p.f. Quantification of TUNEL-positive cells in trigeminal ganglia was carried out in flat-mounted 24 h.p.f. embryos. At least three embryos were counted per condition and marker ($n = 3$ for *scratch2*, $n = 6$ for pH3 and $n = 8$ for trigeminal ganglia).

Luciferase assays. The p53MH algorithm²² was used to identify putative p53 REs present in *scratch2* and *puma* genomic sequences. To generate the luciferase reporter plasmids, various fragments containing the p53 putative REs were subcloned in pGL3Basic (Promega, Madison, WI, USA). PCR amplification was

performed on adult zebrafish genomic DNA as template with the following sets of oligonucleotide primers:

scratch2 5' UTR, 5'-CATCATGGTACCTGATCTCACTCCCACCGT-3' and 5'-CATC ATGCTAGCTCAGCACTGGATAGCTCC-3'; *scratch2* intron, 5'-CATCATGCTAGCTC CCACCTGTTACGAAAA-3' and 5'-CATCATCTCGAGTGATGCTTAAACCATTTCAT-3'; and *puma* intron, 5'-CATCATACGCGTTGCATTAATTTCTGACAAGT-3' and 5'-CATC ATCCCGGGACAGCGACAGGCTAT-3'.

Luciferase assays were performed as previously described.⁴¹ Briefly, the reporter plasmid of the firefly *luciferase* gene, under the control of the different p53 REs obtained for *scratch2* (12.5 pg per embryo) and *puma* (2.5 pg per embryo), was injected into one-cell stage zebrafish embryos, together with 1.25 pg per embryo of a plasmid containing the *Renilla luciferase* gene under the control of the Tk promoter. The 21-somite stage embryos were treated with 500 nM CPT for 2 h to promote p53 activation and the embryos were then kept in embryo medium for 2 more hours. Subsequently, 20 embryos of each experimental condition were lysed in 100 μ l lysis buffer (Passive lysis buffer, Promega) and assays were carried out on 10 μ l of the embryo lysate with a Dual-Luciferase Reporter Assay System (Promega). The data are represented as the mean \pm S.D. of three different experiments.

Chromatin immunoprecipitation. ChIP experiments were carried out following the Upstate cell signaling solutions protocol (Upstate cell signaling solutions, Billerica, MA, USA). Briefly, embryos were treated with CPT (see above) and then 30 embryos were sonicated in 300 μ l lysis buffer per condition in each experiment. The chromatin was immunoprecipitated with the anti-p53 antibodies, kindly provided by Professor G Del Sal (University of Trieste, Trieste, Italy) and goat anti-myc (Abcam, Cambridge, UK). The antibodies used as negative and positive controls were Rabbit anti-IgG (Diagenode, Liège, Belgium) and Rabbit anti-H3 (Abcam), respectively. The oligonucleotide primers used for PCR detection were: *scratch2* 5' UTR, 5'-TGATCTCACTCCCACCGT-3' and 5'-CTCAGCACTGGATA GCTCC-3'; *scratch2* intron, 5'-TCCCACCTGTTACGAAAA-3' and 5'-TGATGC TTAAACCATTTCAT-3'; *puma* p53 RE and E-box1, 5'-TGCATTAATTTCTGA CAAGT-3' and 5'-ACCAGCGACAGGCTATCAA-3'; *puma* E-box2, 5'-TCTG CTGACTCTCTCTCA-3' and 5'-GCAACAGCCTGAGCTGAAAAT-3'. The data presented are representative of three independent experiments.

Database search. NCBI and EMBL-EBI/WellcomeTrust Sanger Institute databases were searched through the BLAST family of programs (<http://www.ncbi.nlm.nih.gov/BLAST>) and <http://www.ensembl.org>, respectively. Sequence alignments were carried out using Clustal W (<http://www.ebi.ac.uk/clustalw>; ref. 42) and corrected by visual inspection.

Conflict of interest

The authors declare no conflict of interest.

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