

Inactivating *E2f1* reverts apoptosis resistance and cancer sensitivity in *Trp53*-deficient mice

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The E2f1 transcription factor, which regulates genes required for S-phase entry^{1–4}, also induces apoptosis by transcriptional and post-translational mechanisms^{5–8}. As E2f1 is inducible by DNA damage^{9,10} we investigated its importance *in vivo* in ultraviolet (UV)-induced apoptosis, a protective mechanism that prevents the epidermis from accumulating UV-induced mutations^{11,12}. Contrary to expectation, *E2f1*^{−/−} mice demonstrated enhanced keratinocyte apoptosis after UVB exposure, whereas apoptosis was suppressed by epidermis-specific overexpression of human E2F1. Apoptosis induced by γ -radiation was also repressed by E2f1. *E2f1*^{−/−}; *Trp53*^{−/−} double knockout mice exhibited the elevated UVB-induced apoptosis of *E2f1*^{−/−} alone, rather than the profound apoptosis defect seen in *Trp53*^{−/−} mice, indicating that Trp53 (p53) lies functionally upstream of E2f1. Transfecting E2F1 into *E2f1*^{−/−}; *Trp53*^{−/−} primary fibroblasts suppressed UVB-induced apoptosis and this suppression was relieved by *Trp53*. The double knockout also reverted the abnormal sex ratio and early-onset tumours of *Trp53*^{−/−} mice. These results imply that E2f1 functions as a suppressor of an apoptosis pathway that is initiated by DNA photoproducts and perhaps genetic abnormalities; p53 relieves this suppression.

The function of E2f1 in the DNA damage response *in vivo* was analysed by first exposing the dorsal skin of *E2f1*^{−/−} mice¹³ to physiological doses of UVB radiation, the highest dose (1250 J m^{−2}) corresponding to a minimally perceptible sunburn. After 24 h, the skin showed no macroscopically visible damage and was scored for apoptotic epidermal keratinocytes, which arise in mouse or human tissue after overexposure to UV or sunlight (*Trp53*-dependent 'sunburn cells'¹¹). The frequency of apoptosis was threefold greater in *E2f1*^{−/−} keratinocytes than in wild-type and the difference between genotypes increased with dose (Fig. 1a). This difference may be underestimated because at the highest dose, the epidermal structure in the knockout mice was disordered owing to the high number of apoptotic figures. The presence of apoptotic cells was confirmed using a TUNEL assay, which demonstrated greater numbers of TUNEL-positive keratinocytes in *E2f1*-knockout epidermis (data not shown).

Transgenic mice overexpressing moderate levels of the human *E2F1* gene from an epidermis-specific keratin 5 promoter (which is active in the basal cells of squamous epithelia¹⁴) were also UVB-irradiated and scored for apoptotic keratinocytes. Overexpressing E2F1 in the epidermis resulted in a three to fourfold reduction in the number of apoptotic keratinocytes compared with isogenic wild-type siblings (Fig. 1b). This magnitude is biologically significant, as a threefold decrease in epidermal apoptotic keratinocytes through a mutation in *FasL* results in rapid accumulation of surviving mutant keratinocytes after UVB irradiation¹². A similar effect of *E2F1* was obtained using the same *E2F1* transgenic line backcrossed into the SKH-1 hairless background (data not shown).

To explore the apoptosis suppression mechanism of E2f1, we next determined whether E2f1 functions by downregulating the ability of UV light to induce p53, as UV-induced apoptosis requires p53 (ref. 11). However, immunoblotting demonstrated that UV induction of p53 protein was not greater in primary *E2f1*^{−/−} fibroblasts — in fact, induction was reduced and delayed (Fig. 1a inset). Immunostained epidermal sections from *E2f1*^{−/−} and E2F1-overexpressing mice also indicated that E2f1 does not downregulate induction of p53 by UV irradiation (data not shown). The transcriptional activation activity of p53, assayed by induction of the downstream target p21, was also unaltered (Fig. 1a, inset). Although reduced p53 induction in an *E2f1* knockout is the result expected from the E2f1–Arf–Mdm2–p53 stimulatory pathway, the apoptosis-suppressing function of E2f1 remained unexplained. It remained possible that the apoptotic activity suppressed by E2f1 involved a p53 target for which p21 was not a good indicator, or involved the transcriptional inhibition activity of p53. A definitive test for the placement of p53 downstream is that an *E2f1*^{−/−}; *Trp53*^{−/−} double-knockout animal would resemble the *Trp53*^{−/−} single knockout and exhibit low levels of UV-induced apoptosis. By contrast, if p53 was functioning upstream of E2f1, then double-knockout animals would exhibit the same high levels of UV-induced apoptosis as the *E2f1*^{−/−} single knockout. We therefore crossed *E2f1*^{−/−} knockout mice with *Trp53*^{−/−} knockouts, then irradiated *E2f1*^{−/−}; *Trp53*^{−/−} double-knockout animals and matched controls with UVB-treated animals and scored for apoptotic epidermal keratinocytes. At the highest dose, the number of apoptotic keratinocytes in double knockouts was

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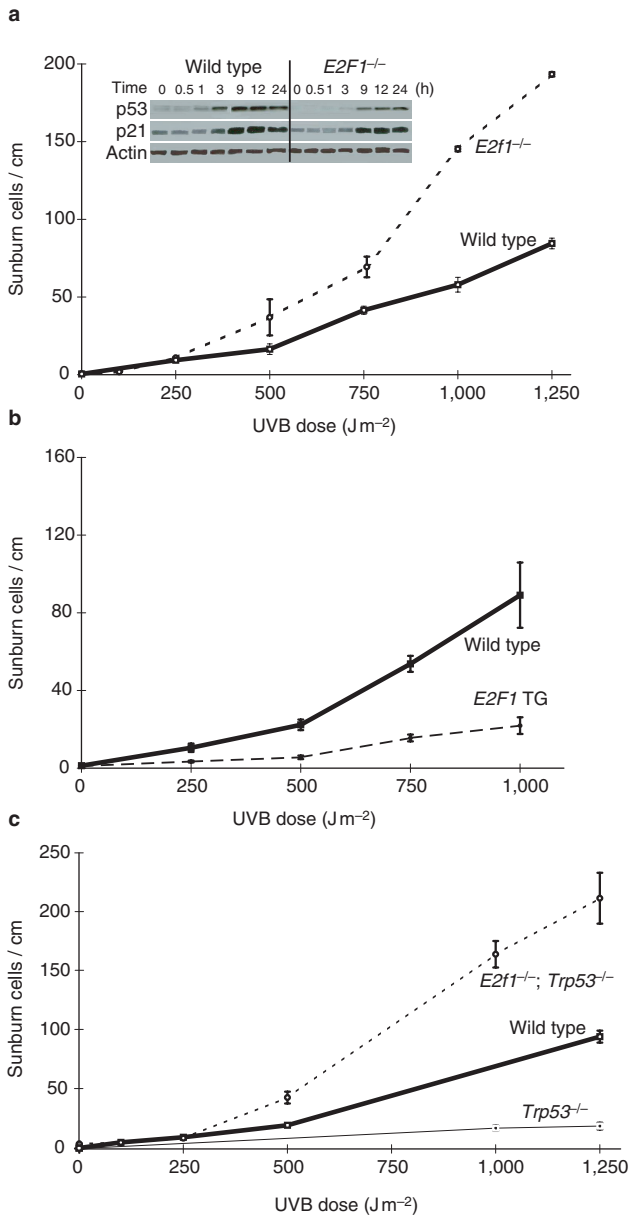


Figure 1 E2f1 inhibits UVB-induced apoptosis *in vivo* and p53 relieves this suppression. **(a)** Increased UVB-induced apoptosis of epidermal keratinocytes (sunburn cells) in the absence of E2f1. Solid line, wild-type; dashed line, *E2f1*^{-/-} (mean ± SEM; *n* = 18; *p* = 0.04 and 0.0001 for the difference between the curves at 1,000 and 1,250 J m⁻², by two-tailed heteroscedastic *t*-test). Inset, time course for inducing p53 protein and its downstream transcriptional activation target, p21, in *E2f1*^{-/-} primary fibroblasts. **(b)** Suppression of UVB-induced apoptosis by epidermis-specific overexpression of human E2F1. Solid line, wild-type SSIN strain; dashed line, *E2F1* transgenic (TG) SSIN (mean ± SEM; *n* = 29; one-tailed *p* = 0.01, 0.002 and 0.03 at 500, 750 and 1,000 J m⁻²). **(c)** Inactivating *E2f1* reverts the UVB-induced apoptosis resistance of *Trp53*-deficient keratinocytes *in vivo*. Solid line, wild-type; thin solid line, *Trp53*^{-/-}; dashed line, *E2f1*^{-/-}; *Trp53*^{-/-} (mean ± SEM; *n* = 17; two-tailed *p* = 0.001–0.04 for comparisons of wild-type to *E2f1*^{-/-}; *Trp53*^{-/-} and *Trp53*^{-/-} or of *E2f1*^{-/-}; *Trp53*^{-/-} to *Trp53*^{-/-}, 500 or 1,250 J m⁻²).

twice that of wild-type littermates (Fig. 1c), similar to that of the *E2f1* knockout alone. Thus, although inactivation of p53 reduces UV-induced apoptosis almost tenfold (Fig. 1c; ref. 11), subsequent inactivation of the

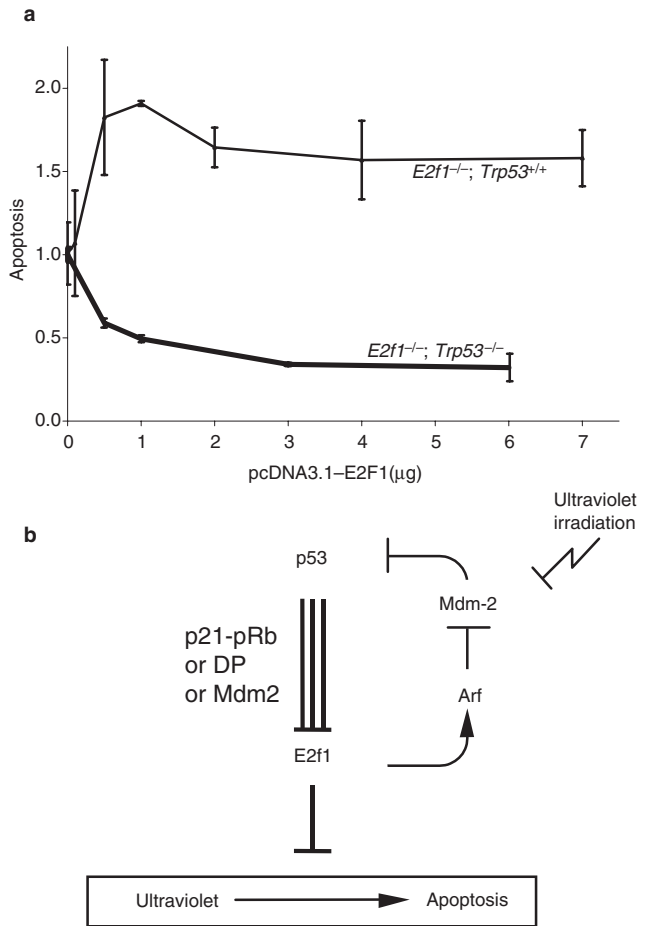


Figure 2 E2F1-mediated suppression of UVB-induced apoptosis is relieved by p53. **(a)** Increasing concentrations of pcDNA3.1-E2F1 under the control of the CMV promoter were transfected into primary *E2f1*^{-/-}; *Trp53*^{-/-} fibroblasts (solid line) or primary *E2f1*^{-/-}; *Trp53*^{+/+} fibroblasts (thin line). Apoptosis at 0 J m⁻² is background-subtracted and maximum UVB-induced apoptosis without vector is normalized between strains. In the absence of irradiation, apoptosis was not appreciably induced by these vector concentrations. Two-tailed *P* = 8 × 10⁻⁴ for the comparison of the two highest vector concentrations. **(b)** Three known pathways enable E2f1, functioning as a suppressor of UVB-induced apoptosis *in vivo*, to control a switch from a direct pathway for UVB-induced apoptosis to one that is p53-dependent. A p53-independent apoptosis pathway inducible by UVB irradiation is suppressed by E2f1, which is in turn suppressed by p53. The classic p53–p21–CyclinD–Cdk4,6–Rb–E2f1 axis regulates the extent to which E2f1 is bound in an inactive complex with Rb^{1,2}. p53 has also been reported to displace DP1 from the E2f1–DP1 complex, resulting in loss of DNA binding^{1,2}. In many cell types p53 upregulates Mdm2, promoting E2f1 degradation⁵. p53 regulates UVB-induced apoptosis by relieving the suppression of E2f1. The stimulatory pathway, E2f1–Arf–Mdm2–p53, cannot itself account for the apoptosis observed in *Trp53*^{-/-}; *E2f1*^{-/-} mice, but its contribution to UVB induction of p53 and p21 is evident in the *E2f1* knockout (Fig. 1a, inset).

E2f1 gene more than reversed this loss. Similar results were obtained using mice bred from a different *Trp53*^{-/-} strain¹⁵ (data not shown). *In vivo*, p53 evidently operates upstream of E2f1 and functions by relieving the suppression of UVB-induced apoptosis by E2f1. UV-induced apoptosis proceeds in the absence of both genes.

This circuitry was unexpected, as overexpressed E2f1 is pro-apoptotic in several systems, often being p53-dependent^{5–8}. Therefore, the ability

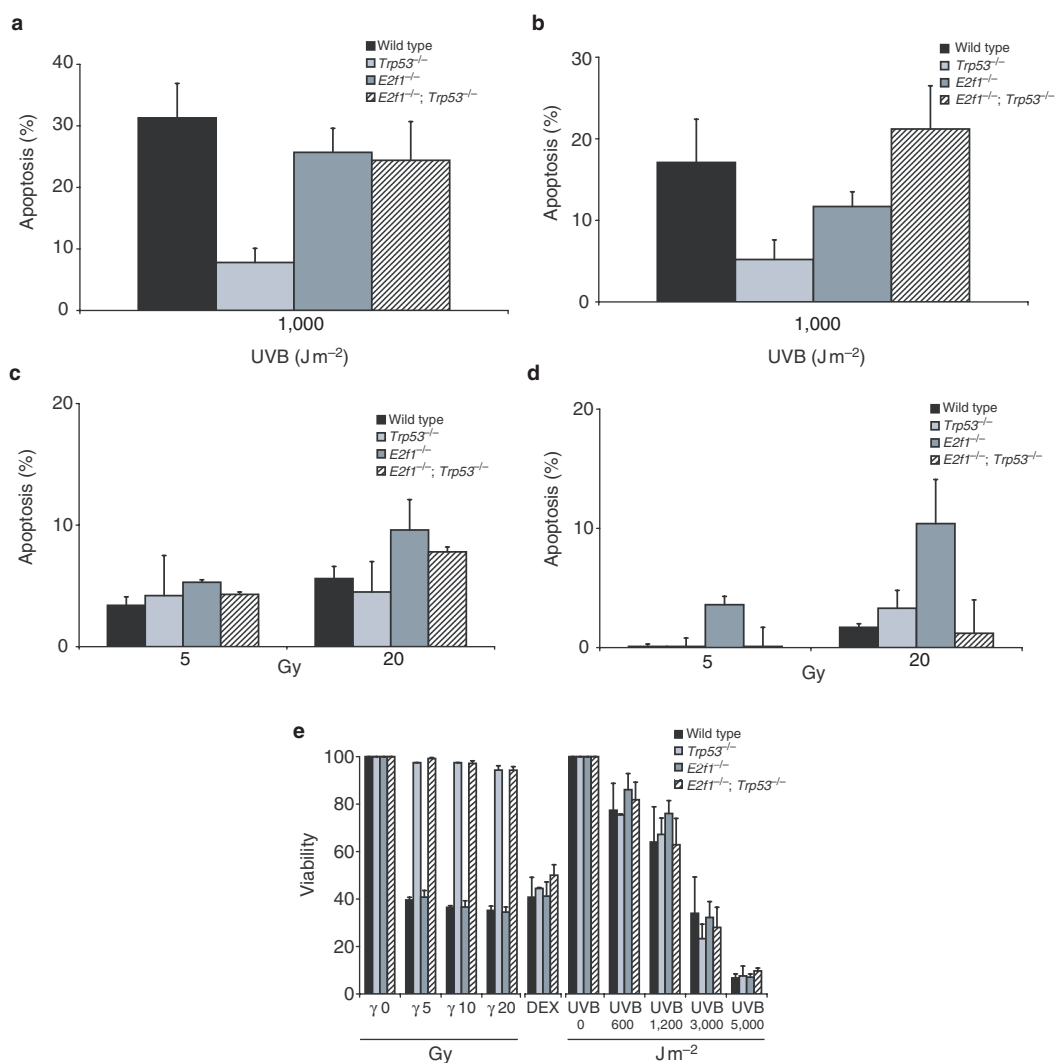


Figure 3 *E2f1* suppresses apoptosis induced by DNA photoproducts or γ -radiation. (a) UVB-irradiated primary fibroblasts and (b) keratinocytes undergo p53-dependent apoptosis which is suppressed by *E2f1* (one-tailed $p = 4 \times 10^{-4}$ and 4×10^{-5} , respectively). Cells were irradiated with 0 or 1000 J m⁻² UVB and assayed for apoptosis 24 h later by annexin. (c) γ -irradiated primary wild-type fibroblasts and (d) keratinocytes are apoptosis-resistant (note scales). *E2f1*^{-/-} keratinocytes are more sensitive, indicating that *E2f1* also represses γ -induced apoptosis. Cells were treated with 0, 5, or 20 Gy γ -radiation and assayed for apoptosis 24 h later by annexin. (e) Primary thymocytes undergo p53-dependent cell

death 8 h after γ -radiation induction of DNA double-strand breaks, as reported (left; doses in Gy). Cell death is not suppressed by *E2f1*, perhaps leading to the γ -radiation sensitivity of thymocytes. Dexmethasone-induced cell death (1 μ M) is independent of *Trp53* and *E2f1* (centre). Thymocytes require high doses of UVB-induced DNA photoproducts (right; doses in J m⁻²) and this cell death is independent of both *Trp53* and *E2f1*. Viability was assayed as 7-amino-actinomycin D uptake (similar results were obtained with propidium iodide, data not shown). Annexin assay indicated that UVB-induced thymocyte death was not apoptotic (data not shown).

of *E2f1* to repress UV-induced apoptosis was confirmed by *in vitro* transfection. Transfecting primary *E2f1*^{-/-}; *Trp53*^{-/-} fibroblasts with increasing amounts of human *E2F1* under control of the CMV promoter progressively suppressed subsequent UVB-induced apoptosis (Fig. 2a). The experiment was then repeated in the presence of p53 to determine whether p53 relieves suppression of apoptosis by *E2f1*. To avoid artefacts from double transfections, p53 was introduced chromosomally by using primary fibroblasts derived from matched *E2f1*^{-/-}; *Trp53*^{+/+} animals. Addition of *E2F1* no longer suppressed UVB-induced apoptosis (Fig. 2a). These results indicate that *E2F1* suppresses UVB-induced apoptosis and this suppression is relieved by p53 (Fig. 2b). The internal operation of this genetic circuit, by double suppression, is not apparent from its overall behaviour in wild-type cells.

The general importance of *E2f1* in regulating apoptosis induced by DNA damage was tested in primary cultures derived from all four genotypes. In both fibroblasts (Fig. 3a) and keratinocytes (Fig. 3b), UVB-induced apoptosis was p53-dependent and the *Trp53*^{-/-} defect was reverted by also inactivating *E2f1*^{-/-}. Levels of apoptosis in *E2f1*^{-/-} cells were closer to that of wild-type in these experiments, possibly because the annexin assay used *in vitro* measured earlier events than those measured by the sunburn cells. The DNA damage specificity for the regulation by *E2f1* was investigated by γ -irradiation. Fibroblasts and keratinocytes were resistant to γ -irradiation, but became sensitized in the *E2f1* knockout, indicating that *E2f1* also represses γ -induced apoptosis (Fig. 3c, d). The low levels of γ -irradiation-induced apoptosis make it difficult to draw conclusions about the importance

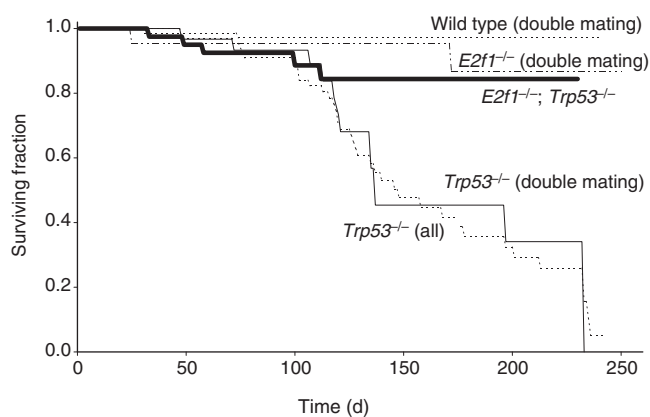


Figure 4 Inactivating *E2f1* restores cancer resistance to *Trp53*^{-/-} mice. The Kaplan–Meier survival plot for *Trp53*^{-/-};*E2f1*^{-/-} mice (thick solid line; *n* = 40) is increased compared with *Trp53*^{-/-} animals (dotted line, mice from single matings plus the double matings which produced *Trp53*^{-/-};*E2f1*^{-/-}, *p* = 0.0086 by logrank test, *n* = 66; thin solid line, sub-population derived from the double mating, *n* = 30) and is indistinguishable from that of *E2f1*^{-/-} derived from the double mating (dashed line, *p* = 0.85, *n* = 22). The *p* values for the comparisons of wildtype to *Trp53*^{-/-} from all matings or matched double mating are *p* = 0.0016 and 0.0054, respectively. Wildtype, upper dotted line, *n* = 69.

of p53. Wild-type thymocytes were sensitive to γ -irradiation but UVB-resistant (Fig. 3e); thymocytes of all four strains were >95% CD4⁺CD8⁺. Apoptosis induced by γ -irradiation, but not by dexamethasone, was *Trp53*-dependent. The *Trp53*^{-/-} defect in γ -irradiated thymocytes was not reverted by inactivating *E2f1* (Fig. 3e). These results indicate that *E2f1* can suppress apoptosis after DNA damage *in vivo* or in primary cells. In tissues that are sensitive to DNA photoproducts (keratinocytes and fibroblasts), this suppression was relieved by UV-induced p53; in cells unusually sensitive to DNA double-strand breaks (thymocytes), γ -apoptosis has not been suppressed by *E2f1* and other functions of p53 are significant.

The importance of *E2f1* in mediating *Trp53*-related phenotypes was not confined to apoptosis initiated by DNA damage. Female *Trp53*^{-/-} mice are heavily under-represented in the progeny of *Trp53* heterozygote or homozygote matings, because of developmental abnormalities, such as failed neural tube closure^{16,17}. In contrast, viable female *E2f1*^{-/-};*Trp53*^{-/-} progeny were generated from mating combinations of *Trp53*^{-/-};*E2f1*^{-/-} or *Trp53*^{+/-};*E2f1*^{-/-} parents at nearly the calculated Mendelian rate (Table 1). p53-dependent apoptosis has previously been linked to prevention of birth defects¹⁸. Double-knockout mice exhibited no consistent gross or developmental abnormalities and were fertile. Histologic examination revealed progressive degeneration of spermatogenic precursors and abnormalities of the salivary glands and pancreatic exocrine cells, as observed in *E2f1* single-knockout mice^{13,19}.

Tumour incidence also reverted in the double knockouts. Over 80% of *Trp53*^{-/-} mice develop tumours, predominantly thymic lymphomas, with > 90% mortality by 8 months^{15,20}. In contrast, few spontaneous deaths occurred in *E2f1*^{-/-};*Trp53*^{-/-} double-homozygous mice by the age of eight months (Fig. 4) and two mice sacrificed at 11 months showed no evidence of tumours or abnormal thymus. Eight mice that died spontaneously were autopsied: four had no evident tumours, one had a lymphoma and the remainder had tumours not typical of *Trp53*^{-/-} mice (peripheral nerve sheath tumours, Sertoli cell tumours and lymphosarcoma). The survival curve for *E2f1*^{-/-};*Trp53*^{-/-} double

knockout animals resembled that of the single *E2f1*^{-/-} knockout, in which tumours appear after 15–20 months¹⁹. These genotypic differences were statistically significant. Similar results were obtained with mice bred from a different *Trp53*^{-/-} strain¹⁵ (data not shown). Thus, functional *E2f1* is required for full tumour development in *Trp53*^{-/-} mice. This requirement might be for the ability of *E2f1* to suppress apoptosis or promote proliferation. Loss of *E2f1* reduces the incidence of pituitary and thyroid tumours in *Rb*-defective mice²¹, a result that has been attributed to reduced cell cycling in the absence of *E2f1*. In *E2f1*^{-/-} primary fibroblasts, however, cell proliferation and cell-cycle distribution are unaltered¹³; moreover, this mechanism would not readily account for normalization of the sex ratio. In the primary fibroblasts used here, no statistically significant differences between genotypes were observed in the fraction of cells in S phase (data not shown). *Trp53*^{-/-} cells demonstrated the known increase in S and G2 phases, which was reverted in cells that were also *E2f1*^{-/-}. After irradiation with 500 J m⁻² UVB, almost all of *E2f1*^{-/-} cells, but only approximately 40% of wild-type, *Trp53*^{-/-} or double-knockout cells, exhibited a reduction in the rate of BrdU incorporation (S-phase delay; data not shown). Thus, the hypersensitivity of *E2f1*^{-/-} cells was reverted by loss of *Trp53*.

The behaviour of the *E2f1*–p53 regulatory apparatus illustrates the general problem of loops in gene networks. When a protein is linked to another by ‘feedback’ loops, it is difficult to determine whether it functions upstream or downstream and which path is providing the feedback. The familiar *E2f1*–Arf–Mdm2–p53 path (Fig. 2b, thin line) embodies the conventional model where elements such as *E2f1* lie upstream of p53 (refs 5, 6), which then activates pro-apoptotic genes or inhibits anti-apoptotic genes. This positive regulation cannot account for the double-knockout data. In contrast, direct suppression of the apoptosis pathway by *E2f1* and release by p53 can result from three known pathways, in which p53 lies upstream of *E2f1* and negatively regulates it (Fig. 2b, thick line). Upstream p53 will be ineffective whenever *E2f1* is inactivated. Typically, these paths are considered as the p53–p21 pathway for cell-cycle arrest, with only a ‘feedback’ role for apoptosis. An alternative parallel pathway model can also be considered, in which p53-induced apoptosis genes (not shown in Fig. 2b) and *E2f1* suppression are independent and have opposite effects on apoptosis. The various knockout strains would reflect different net activities. But if so, apoptosis in *E2f1*^{-/-} knockouts would have been reduced by the subsequent *Trp53* knockout (unlike Fig. 1c). The parallel-pathways model can be rescued with the constraint that deletion of *E2f1* has a second effect of switching off the apoptosis function of p53. The double knockout would then have no further effect. The Arf–Mdm2 loop does supply precisely this cross-stimulation by *E2f1*, but deleting *E2f1* does not switch off induction of p53 (Fig. 1a). Overexpression of *E2f1* causes apoptosis in the absence of DNA damage, albeit under conditions of growth-factor withdrawal. This apoptosis proceeds through a non-Arf path that can include p73 (refs 5, 6 and 22). Downregulation of *E2f1* can also induce spontaneous apoptosis²³.

We conclude that apoptosis induced by DNA photoproducts can be dissected into two components: an underlying direct apoptosis pathway, which may require JNK1,2 (ref. 24) and a regulatory apparatus that includes p53 and *E2f1* (Fig. 2b). UVB irradiation regulates each component. As *E2f1* is released from Rb during early S phase of the cell cycle^{1,2}, *E2f1* would mediate a cell-cycle-dependent switch between direct and p53-sensitive apoptosis. It may be important, for example, to suppress single-stranded DNA breaks as an initiating signal for apoptosis during times that Okazaki fragments are present. This behaviour is consistent with apoptosis requiring replication through active genes. p53 induction and apoptosis after DNA damage require

Table 1 E2f1 inactivation alters the sex ratio of *Trp53*^{-/-} progeny

Progeny Genotype		Total	Male	Female	Male/Female
<i>Trp53</i>	<i>E2f1</i>				
+/+	+/+	80	44	36	1.22
-/-	+/+	122	100	22	4.55
-/-	-/-	53	27	26	1.04

Trp53^{-/-}; *E2f1*^{-/-} or *Trp53*^{+/-}; *E2f1*^{-/-} animals were mated and the sex of *Trp53*-homozygous progeny was tabulated. Using the two-sided Fisher's exact test, *p* values for the intergenotype comparisons are: WT/WT vs KO/WT, 0.0001; KO/WT vs KO/KO, 0.0001; WT/WT vs KO/KO, 0.72.

DNA replication^{25,26}, UVB-induced p53 induction and apoptosis *in vivo* require DNA photoproducts in actively transcribing genes²⁷ and active genes are replicated in the first half of S phase. Pharmacological inhibitors of E2f1 may sensitize human solid tumours to γ -irradiation therapy. For tumours carrying *Trp53* mutations, E2f1 inhibitors might alleviate resistance to drugs, such as *cis*-platinum, mitomycin C, cyclophosphamide and bischloroethylnitrosourea, which, similarly to UVB, result in bulky DNA lesions. The E2F1 transcription factor may be a target with few side effects, as *E2f1*-knockout mice develop almost normally. □

METHODS

Transgenics. *E2f1*^{-/-} mice were generated by disrupting exon 3 with a *neo* cassette and deleting exon 4 (ref. 13). 129 \times C57Bl/6 animals backcrossed into C57Bl/6 for two generations were obtained courtesy of S. Field (Division of Neuroscience, Children's Hospital, Boston, MA). The progeny genotypes were determined in two independent polymerase chain reaction (PCR) amplifications by targeting the *E2f1* gene or the *neo* cassette. The K5 *E2F1* moderate-overexpression line 1.1 was constructed in a SENCAR inbred strain (SSIN) using the bovine keratin 5 promoter, rabbit β -globin intron 2, human *E2F1* cDNA and SV40 polyadenylation signal¹⁴. Similar results were obtained in an SKH-1 hairless background. *Trp53*^{-/-} mice, constructed by disrupting exon 5 (ref. 20) and backcrossed into C57Bl/6 for five generations, were obtained commercially (Taconic Farms, Germantown, NY). *E2f1*; *Trp53* double-knockout mice and their matched doubly wild-type and single *Trp53*^{-/-} controls, were generated by mating. Offspring from the F1 generation were interbred and the genotype of F2 animals was determined by two independent PCRs that detected the presence of products with flanking primers amplifying *Trp53*, *E2f1* or *neo*. Absence of *Trp53* in sunburn cell experiments was confirmed as the absence of constitutive or UV-induced p53 by immunohistochemistry; in primary fibroblasts, western blotting for constitutive or induced p53 and p21 was used. Single-knockout *E2f1*^{-/-} controls were descended directly from the single-knockout parents. A smaller number of double knockouts and controls were derived using a *Trp53*^{-/-} construct having exons 2–6 disrupted¹⁵ (Jackson Laboratory, Bar Harbor, ME), with similar apoptosis and lifespan results; these mice are not included in the data presented.

UVB irradiation and apoptosis. Shaved animals were exposed to UVB (270–420 nm, peak emission 313 nm; UVB, 84.3%; UVA, 15.7%; UVC, 0.1%)^{11,27}. Murine melanocytes do not interfere because they are subepidermal. After 24 h, skin samples were stained and scored for sunburn cells as before, with inclusion of late-stage apoptotic cells with eosinophilic cytoplasm and no nuclei. Apoptosis was confirmed using a TUNEL assay. The animal protocol was reviewed and approved by the Yale Institutional Animal Care and Use Committee. Primary cells were prepared as described²⁸, UVB-irradiated in PBS and scored for apoptosis using Annexin V/propidium iodide flow cytometry (Molecular Probes, Eugene, OR) or 7-AAD uptake (Via-Probe, BD PharMingen, San Diego, CA).

Transfection. Primary fibroblasts were transfected with pcDNA3.1-E2F1 controlled by the CMV promoter²⁹ using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Twenty-four hours after transfection, cells were exposed to the indicated dose of UVB. After a further 24 h, they were harvested for apoptosis

analysis by Annexin V/propidium iodide flow cytometry.

Modifier gene analysis. The *Trp53*^{-/-} parental strain was obtained as a five generation backcross from the hybrid 129–C57Bl/6 background into C57Bl/6, resulting in $\geq 97\%$ homozygous C57Bl/6 genotype. In contrast, the *E2f1*^{-/-} parental strain had been backcrossed two generations and so would carry 25% heterozygous 129 alleles. Thus, it was conceivable that the novel phenotypes observed in *E2f1*^{-/-} and *E2f1*^{-/-}; *Trp53*^{-/-} mice were not caused by the inactivated *E2f1* gene but reflected fortuitous segregation of an unlinked 129-specific modifier gene in *E2f1*^{-/-}-containing mice (but with no segregation to wild-type or *Trp53*^{-/-} controls). A pure 129 background does shorten the mean lifespan of *Trp53*^{-/-} or *Rb*^{+/-} mice by 20–30% compared with a 129 \times C57Bl/6 hybrid^{21,30}. Although the effect of the *E2f1* knockout in the present experiments was to lengthen — not shorten — lifespan, we nevertheless calculated the likelihood that a modifier gene was responsible.

A simulation program for the inheritance of the putative modifier allele was written in C, incorporating the initial allele frequencies of the parental mice and the breeding parentage of the mice used in each experiment. The phenotype probabilities were estimated through one million simulations for each comparison and both dominant and recessive models were considered. For example, let '1' denote the C57Bl/6 allele and '2' denote the 129 allele, then if 2 is fully dominant to 1, all mice with the normal phenotype would have the 11 genotype and mice with the novel phenotype appearing in *E2f1*^{-/-}-knockout mice would have the 12 or 22 phenotype. In addition to considering fully penetrant models, we also allowed some degree of admixture of mice within a phenotype group because the presence of a small genetic sub-population might have been hidden by partial penetrance or experimental variation. For example, for eight mice with a particular phenotype, we imposed that the majority of the mice have genotype 11, but we allowed up to two mice (25%) to have genotype 12 or 22. The simulation program, 'siliColony', can be downloaded at <http://bioinformatics.med.yale.edu/softwarelist.html>.

The probability that a 129-strain dominant modifier allele accounts for the difference in UV-induced apoptosis between *E2f1*^{-/-}; *Trp53*^{-/-} and *Trp53*^{-/-} mice (Fig. 1c) was 2×10^{-3} if that allele was fully penetrant and had assorted only to the double knockout mice and 5×10^{-2} if each experimental group contained up to a 30% sub-population of mice carrying the opposite allele. For a recessive modifier allele, these probabilities were $< 1 \times 10^{-6}$ and 4×10^{-5} . The probabilities for the same strain comparisons in the lifespan experiments were: $< 1 \times 10^{-6}$, 5×10^{-4} , $< 1 \times 10^{-6}$ and 2×10^{-6} (Fig. 4). Modifier genes are thus unlikely to be responsible for the results reported here.

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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