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***Rpl27a* mutation in the sooty foot ataxia mouse phenocopies high p53 mouse models**

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Abstract

Ribosomal stress is an important, yet poorly understood, mechanism that results in activation of the p53 tumour suppressor. We present a mutation in the ribosomal protein *Rpl27a* gene (sooty foot ataxia mice), isolated through a sensitized *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis screen for p53 pathway defects, that shares striking phenotypic similarities with high p53 mouse models,

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Author contributions TT performed experiments, analysed data, prepared figures and wrote the paper; MD performed HSC analysis by FACS; CT performed ISH and Nissl staining; LAD contributed to financial support and in FACS analysis assay design; FA performed the histopathology; GL provided mice and contributed to the phenotypic assay; MJJ provided mice and financial support; DRR provided financial support and helpful discussions; NB conceived and performed experiments, analysed data, provided financial support and contributed to writing. All authors had final approval of the submitted and published versions.

SUPPORTING INFORMATION ON THE INTERNET The following supporting information may be found in the online version of this article:

Supplementary materials and methods Table S1. No *SFA/SFA* genotype observed in progeny from the cross *SFA*⁺ × *SFA*⁺. Embryonic lethality of *SFA* homozygous mice.

Table S2. Phenotypes observed in progeny of intercross *SFA*⁺ mice with two BAC transgenics expressing *Rpl27a* from its genomic locus.

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Figure S2. An example of gating for HSCs in a WT mouse. HSCs sorting of cells by lineage (c-Kit/Sca-1) and PI staining of BMs of mice.

Figure S3. *SFA*⁺ mice suffer from an unsteady gait in comparison to WT mice. Record of *SFA*⁺ walking with ink footprints.

Figure S4. *SFA*⁺ mice are sensitive to γ-IR compared to WT mice. Kaplan–Meier survival curve of *SFA*⁺ and WT mice after 6 Gy of IR and IF staining for p53 on irradiated and non-irradiated thymi.

Figure S5. p53 immunopositivity in *SFA*⁺ bone marrow. p53 IHC staining in WT and affected *SFA*⁺ tissues.

No conflicts of interest were declared.

including cerebellar ataxia, pancytopenia and epidermal hyperpigmentation. This phenocopy is rescued in a haploinsufficient *p53* background. A detailed examination of the bone marrow in these mice identified reduced numbers of haematopoietic stem cells and a *p53*-dependent c-Kit down-regulation. These studies suggest that reduced Rpl27a increases *p53* activity *in vivo*, further evident with a delay in tumorigenesis in mutant mice. Taken together, these data demonstrate that Rpl27a plays a crucial role in multiple tissues and that disruption of this ribosomal protein affects both development and transformation.

Keywords

ribosomal stress; *p53*; development; tumorigenesis; stem cells; c-Kit

Introduction

Cellular defects that alter ribosomal protein levels or ‘ribosomal stress’ are often capable of invoking a powerful *p53* response. In 1994, Levine and co-workers first suggested that *p53* protein levels are coupled to ribosomal protein levels, when they showed that a ribosomal protein, RPL5, forms a ternary protein complex with *p53* and its major inhibitor, Mdm2 [1]. Thereafter, other ribosomal proteins have been shown to directly interact with Mdm2 to affect *p53* levels [1–7]. Loss-of-function/expression of some ribosomal proteins decreased *p53* levels [4,5,7–10], while for others *p53* levels increased [6,11–16]. Conversely, over-expression of some ribosomal proteins raised *p53* levels [3,5–10,17]. Both reduction and over-expression of RPL23 induced *p53* [6]. In mice, ribosomal protein mutations or heterozygous deletions affecting their expression have been associated with various *p53*-dependent pathologies, including embryonic lethality during gastrulation [11] (*Rps6*; partial rescue on *p53*-null background), T cell-specific lineage defects [13] (*Rpl22*), hyperpigmentation and mild anaemia [12] (*Rps19* and *Rps20*), hypopigmentation and skeletal and retinal abnormalities [14] (*Rpl24*). The diversity of these phenotypes may be due to differences in basal levels of certain ribosomal proteins in different tissues and may reflect the developmental stage and differentiation-specific expression of these ribosomal proteins. Moreover, the absence of obvious phenotypes that recapitulate *p53* pathway mouse models indicates that *p53* activation in these mutant mice originated in differentiated cells rather than in undifferentiated stem-like cells, which would have resulted in graver and more diverse pathologies.

Through a *p53*-sensitized mutagenesis *N*-ethyl-*N*-nitrosourea (ENU) screen, we isolated a model of ribosomal stress that uniquely phenocopies mice with reduced levels of *p53* inhibitors, such as *Mdm2*^{*purol*Δ7–12} [18], *Mdm4*^{+/-} [19,20] and *Mdm2*^{+/-}:*Mdm4*^{+/-} [20] mice. This model provides evidence of a link between a deficiency in the *Rpl27a* ribosomal protein and *p53*-dependent developmental defects, tumour development and progression.

Materials and methods

Positional cloning

The ENU-induced mutation was generated on a C57BL/6J background and affected mice carrying the mutation were backcrossed for two generations to the 129S6SvEv^{TAC} background for meiotic mapping (see Supporting information, Supplementary Materials and Methods).

Mice and colony maintenance

The *SFA*⁺ mouse was isolated from a previously described mutagenesis screen [21] and maintained on a C57BL/6J background (for genotyping and maintenance, see Supporting information, Supplementary materials and methods). *p53*^{+/-} mice [22] were purchased from the Jackson laboratory. *Mdm2*^{+/-Δ7-12} mice [18] were obtained from Mouse models of human cancers consortium, while *Mdm2*^{+/-}, *Mdm4*^{+/-} [19], and *Mdm4*^{+/-Δ2} [23] mice were provided by Dr G. Lozano. *Dopachrome Tautomerase (DCT)-LacZ* transgenic mice [24] were provided by Dr P. Overbeek.

Bacterial artificial chromosome (BAC) transgenics were made using RP23–74E7 and RP23–341B3 clones, obtained from the Children's Hospital, Oakland Research Institute, and containing the entire *Rpl27a* genomic locus and its immediate 5' regulatory sequences.

All animal protocols were approved by the Institutional Animal Care and Use Committee.

Complete blood counts (CBCs)

Peripheral blood was collected by retro-orbital sinus puncture of P21–25 anesthetized mice into Microtainer tubes containing dipotassium-EDTA (Becton-Dickinson). CBCs were performed using the Cell-Dyn 3500R haematology analyser (Abbott Diagnostics).

Histology and immunostaining

Tissues were fixed for 24 h in 4% formalin, processed and then embedded in paraffin. Immunostaining for p53 (1 : 400; Novocastra Laboratories, UK) and cleaved caspase-3 (1 : 200; Cell Signalling) were performed as described [20] and according to the respective manufacturers' recommendations. For proliferation, P3 pups were injected intraperitoneally with BrdU (1 ml/100 g body weight) and tissues were harvested 2 h later and processed for immunofluorescence (IF) [20], using anti-BrdU-FITC antibody (BD Biosciences). Propidium iodide (PI) was used as nuclear stain. Nissl and haematoxylin and eosin (H&E) staining were performed by the Baylor College of Medicine (BCM) *in situ* hybridization (ISH) core facility.

Bone marrow proliferation, apoptosis and HSC counts by flow cytometry

Bone marrows (BM) of postnatal day 21–25 (P21–25), wild-type (WT) and *SFA*⁺ mice were flushed from both femurs and haematopoietic stem cells (HSCs) were isolated as described previously [25]. Proliferation and apoptosis were assessed by BrdU/PI and annexin V staining, respectively, as described [25].

RNA isolation, expression assays and 3' RACE

P3 skin and cerebellums were snap-frozen in liquid nitrogen and whole RNA was extracted for cDNA synthesis and expression assays and as a template for 3' Rapid Amplification of cDNA 3' Ends (3' RACE) of the *Rpl27a* locus to identify any alternatively spliced RNAs (see Supporting information, Supplementary Materials and Methods).

In situ hybridization

ISH for *Mdm2*, *Mdm4*, *p53* and *Cdkn1a* was performed using digoxigenin-labelled probes on 20 μm mid-sagittal brain sections, as described [26], by the BCM ISH core facility.

IR experiment

Five week-old WT and *SFA*⁺ littermates were subjected to a sublethal 6 Gy dose of γ-rays (γ-IR) from a ¹³⁷cesium source. After 5 h thymi were collected for IF. Ten mice of each genotype were also followed for survival.

Statistical and survival analysis

To compare the means of two datasets, we used the unpaired Student's *t*-test. To compare the survival of two cohorts, we performed a Log-rank test and Kaplan–Meier analysis. Differences between observed and expected genotypes in Table S4 (see Supporting information) were assessed by χ^2 analysis. Statistical differences were considered significant if they had a two-sided *p* value of <0.05 (see Supporting information, Supplementary Materials and Methods).

Rotarod assay

The rotarod assay is a performance test based on forced motor activity of an experimental animal, usually rodents. We measured the riding time (s) or endurance of mice running on an accelerating rotating rod (Ugo Basile). Latency to fall was recorded in four successive five minute trials on the first day, followed by a second set of four trials on the second day. The last trial was plotted.

Results

A mouse mutagenesis screen for p53 pathway modifiers

A large-scale random ENU mutagenesis screen that was the first to use a balancer chromosome in a mouse screen was performed in a previous study [21]. As part of the breeding scheme, mutant mice were crossed to mice that inherited an inversion allele of chromosome 11 with breakpoints at the *p53* and *Wnt3* loci [27]. This 23 centiMorgan (cM) chromosomal inversion, *Inv(11)8Brd^{p53-Wnt3}*, is homozygous lethal, due to disruption of the *Wnt3* locus, and is haploinsufficient for *p53* in the heterozygous state [28]. We identified that this mutagenesis screen doubles as a sensitized screen for p53 and Wnt pathway defects, since it included breeding all mutagenized progeny with mice carrying the *Inv(11)8Brd^{p53-Wnt3}* inversion allele. In 230 isolated mutations, we identified the ‘sooty foot ataxia’ phenotype that demonstrated a unique inheritance pattern that suggested genetic interaction with either *p53* or *Wnt3*. This phenotype, mainly characterized by excessive shaking (ataxia) and hyperpigmentation of the footpads, was noticeably absent when inherited together with the *Inv(11)8Brd^{p53-Wnt3}* allele, but strikingly penetrant when the inversion allele was absent.

The sooty foot ataxia phenotype and identification of the mutation

The sooty foot ataxia (SFA) phenotype has a semidominant mode of inheritance, with embryonic lethality of homozygotes prior to E7.5 (see Supporting information, Table S1). The *SFA* mutation was localized to a large interval on mouse chromosome 7, using a meiotic mapping approach. Fine mapping in this interval identified a region that included 12 odorant receptor genes which we did not consider as candidates, and 13 additional genes with at least 120 exons that were subjected to sequence analysis in C57BL/6J and mice heterozygous for the *SFA* mutation (*SFA*+/ mice) (Figure 1A). The scan for mutations identified an A > G point mutation at position –15 in the 3' splice acceptor of intron 4 (IVS4-15A > G) within the *Rpl27a* gene, which is a large subunit ribosomal protein of 148 amino acids encoded by five exons [29] (Figure 1B). This mutation was the only one found in 82 kb of sequenced genomic DNA.

To validate the cloning of the putative *SFA* mutation, we used the RP23-74E7 and RP23-341B3 BAC clones to generate transgenic mice that carried an extra copy of the *Rpl27a* gene and its genomic regulatory sequences (see Supporting information, Figure S1A). An intercross between these transgenics and *SFA*+/ mice showed that the extra *Rpl27a* genomic copy was capable of restoring the WT phenotype in these mice (see Supporting information, Table S2). Accordingly, *Rpl27a* expression levels appeared to be restored on

average in the *SFA*^{+/+} BAC footpads compared to *SFA*^{+/+} mice (see Supporting information, Figure S1B).

Analysis of the *SFA* phenotype

Since the *SFA* mutation is situated at the intron–exon junction, we checked for alternatively spliced products of the *Rpl27a* gene by 3′ RACE analysis in E14.5 whole embryos, or in either cerebellums or footpad skins of P3 mice. We could not detect any splice variants in *SFA*^{+/+} : versus WT mice. However, lower levels of *Rpl27a* transcripts were observed by quantitative PCR (qPCR) in P3 mice. *Rpl27a* expression was reduced in *SFA*^{+/+} : cerebellum (59.5%), BM (72.7%) and skin (79.2%) compared to WT tissues (Figure 1C).

Low body weight and anaemia—*SFA*^{+/+} : mice presented with variably penetrant low body weight and anaemia (Figure 1D). Seriously affected *SFA*^{+/+} : mice were unable to undertake the rapid growth spurt that normally occurs 19–30 days after birth, indicating that normal body weight is dependent on a basal expression level of *Rpl27a*. Approximately 20% of affected animals looked anaemic and died in early postnatal development (Figure 1E). The mutant thymi and spleens were also significantly reduced in size in comparison to WT tissues (Figure 2A, B). Blood was drawn for CBCs from a number of severely affected P21–23 *SFA*^{+/+} : mice. These mice had thin, watery blood and suffered from pancytopenia, a condition in which the numbers of all blood cells were reduced. Thus, affected mice had 14% white blood cells, 46% red blood cells and 30% platelets compared to WT littermates (see Supporting information, Table S3), confirming the histopathological examination of affected *SFA*^{+/+} : BM (Figure 2C) and indicating that all types of blood cells were affected by sub-physiological levels of *Rpl27a*.

To determine whether this defect was due to a decrease in the number of HSCs, we performed a count using standard *lin*^{−/lo}/c-Kit/Sca-1 staining for flow cytometry [25] in combination with the Flk-2 antibody to differentiate between short-term (ST) and long-term (LT) HSCs [30]. Severely affected *SFA*^{+/+} : mice had ~40% total HSC counts compared to WT littermates, with a sharp reduction in both ST and LT HSCs (Figure 2D). HSC detection by flow cytometry using the c-Kit antibody (see Supporting information, Figure S2) identified a strong reduction (~42%) of cell surface c-Kit receptors on BM cells in all severely affected mice compared to WT mice (Figure 2E, F), confirming the loss of HSCs. We were able to detect a significant increase in apoptosis in three of eleven *SFA*^{+/+} : whole BMs (~20% versus ~3% in WT mice; Figure 2G). However, a significant increase in HSC proliferation was observed in *SFA*^{+/+} : mice, while whole BM proliferation was not significantly different from WT tissues (Figure 2G). Furthermore, it was notable that *SFA*^{+/+} : mice with the lowest HSC numbers had the highest proportion of proliferating HSCs, as shown by the linear trend in Figure 2H.

The hyperpigmentation phenotype—*SFA*^{+/+} : mice exhibited fully penetrant hyperpigmentation of the tail, feet, ears and genital areas (Figure 3A, B and data not shown). To further examine this phenotype, we crossed the *SFA*^{+/+} mouse to the *DCT–LacZ* transgenic mouse, which expresses the β -galactosidase reporter gene under the melanocyte-specific tyrosinase-related protein-2 promoter [24,31], permitting us to easily visualize and count the melanocytes. A dramatic increase in epidermal melanocyte numbers with increased deposition of melanin granules was noted in *DCT–LacZ* : *SFA*^{+/+} footpads and tails compared to *DCT–LacZ* mice [32] (Figure 3C, D).

Ataxia—Cerebellar ataxia is the third major defect observed in *SFA*^{+/+} mice that suffered from an unsteady gait and consistent falling (see Supporting information, Figure S3). Nissl staining for neurons in 12–14 week-old adult mice revealed a pronounced reduction in cell

numbers in the cerebellar granule cell layer (Figure 4A). Furthermore, Calbindin-1^{D28K} IF staining revealed a severe disruption of the layers of the cerebellum (the molecular, Purkinje and granule layers), where Purkinje neurons were disorganized and found ectopically located throughout the cerebellum (Figure 4B). We also examined P0 and P3 cerebellums because much of normal development occurs in the first postnatal week, when granule neurons rapidly proliferate in a transient structure called the external granule layer (EGL). An abnormally small EGL was observed in *SFA*⁺ mice, starting at P2. Quantification of BrdU⁺ staining of proliferating cells at P3 showed that 51% of WT EGL was proliferating versus 21% in *SFA*⁺ mice (Figure 4C, D). We also detected increased levels of apoptosis by caspase-3 IHC staining in the *SFA*⁺ EGL (Figure 4C). In conclusion, these data suggest that abnormally low proliferation and high apoptosis levels in developing granule neurons significantly contribute to the *SFA*⁺ defect, resulting in ataxia.

***SFA*⁺ mouse phenocopies and other high *p53* mouse models**

Based on the observation that the *SFA* phenotypes were rescued by the *Inv(11)8Brd^{p53}-Wnt3* balancer chromosome used in the original mutagenesis screen [21], we hypothesized that these phenotypes were *p53*-dependent. In support of this, we observed that the BM depletion and cerebellar granule neuron deficiency found in affected *SFA*⁺ mice bore a striking histological similarity (Figure 5A, B) to the *Mdm2*^{+/-}:*Mdm4*^{+/-} high *p53* mouse model [20] and to *Mdm2*-null mice carrying the *p53* R172P mutation (*Mdm2*^{-/-}:*p53*^{R172P/P} mice [33]). Histological sections of BMs show that *SFA*⁺, *Mdm2*^{+/-}:*Mdm4*^{+/-} and *Mdm2*^{-/-}:*p53*^{R172P/P} mice have a hypocellularity indicative of aplastic anaemia (Figure 2C, 5A), as in the *Mdm2* hypomorphic mouse (*Mdm2*^{puro/Δ7-12}), another high *p53* mouse model [18]. Also, all three *SFA*⁺ *Mdm2*^{+/-}:*Mdm4*^{+/-} and *Mdm2*^{-/-}:*p53*^{R172P/P} cerebellums were small compared to WT, with considerably reduced numbers of granule neurons and disorganized distribution of Purkinje neurons (Figure 4B, 5B). Interestingly, *Mdm2*^{+/-}:*Mdm4*^{+/-} mice had heavily pigmented footpads, detectable first at P4, identical to those observed in *SFA*⁺ mice (Figure 5C). We carried out a closer examination of other *p53* pathway mouse models, such as *Mdm2*^{+/-} [34], two different loss-of-function alleles of *Mdm4*: *Mdm4*^{+/-} (deletion of part of introns 2–5) [19], *Mdm4*^{+/-Δ2} (deletion of exon 2) [35], and *Mdm2*^{+/-}:*Mdm4*^{+/-Δ2} mice. We identified a mild footpad hyperpigmentation beginning at around 4–6 months of age in *Mdm2*^{+/-} and *Mdm4*^{+/-Δ2} mice, and a stronger hyperpigmentation at around 3–4 weeks in *Mdm4*^{+/-} mice and in early postnatal development in *Mdm2*^{+/-}:*Mdm4*^{+/-Δ2} mice (Figure 5C and not shown). In conclusion, all high *p53* mouse models that we examined had hyperpigmented extremities.

Since it has been shown that hypomorphic *Mdm2* (*Mdm2*^{puro/Δ7-12}), *Mdm2*^{+/-Δ7-12}, *Mdm2*^{+/-} and *Mdm4*^{+/-} mice are highly sensitive to γ-IR [18,20], we hypothesized that *SFA*⁺ mice would be similarly radiosensitive. A cohort of WT and least affected *SFA*⁺ littermates (near-normal body weight and minimal signs of ataxia) were subjected to a sub-lethal dose of γ-IR (6 Gy). *SFA*⁺ mice were highly sensitive to irradiation and died <20 days post-treatment, whereas WT littermates survived (see Supporting information, Figure S4A). A more than two-fold increase in *p53*-positive cells was seen in the *SFA*⁺ versus WT thymi after irradiation (see Supporting information, Figure S4B, C), indicating that the *SFA* mutation may synergize with DNA damage to stabilize *p53*, contributing to this radiosensitivity.

The *SFA* phenotype is rescued by haploinsufficient *p53*

To directly test the contribution of *p53* to the *SFA* phenotype, we crossed *SFA*⁺ mice to *p53*^{+/-} mice. We compared the degree of ataxia in 10 week-old *SFA*⁺, *p53*^{+/-} and *p53*^{+/-}:*SFA*⁺ littermates, using the rotarod assay (Figure 6A), which tests for balance and motor coordination. We noticed that the *SFA*⁺ mice lacked motor coordination and were

generally underweight, whereas $p53^{+/-};SFA/+$ mice performed almost identically to the $p53^{+/-}$ group (Figure 6A). In addition, we observed that the smaller the animal, the stronger the ataxia and the more likely it was to have anaemia and early death.

Histological examination of $p53^{+/-};SFA/+$ mice showed no deficiencies in either the BM or cerebellar compartments. However, the hyperpigmentation phenotype was not fully rescued and a complete rescue was only observed when both $p53$ alleles were lost (Figure 6B). Thus, pigmentation is strongly influenced by sub-physiological levels of *Rpl27a* and is a p53-dependent phenotype, as seen in *Rps19* and *Rps20* mice [12].

Given that all major phenotypes of the *SFA/+* mouse are p53-dependent, we investigated whether deletion of both $p53$ alleles could rescue the embryonic lethality of *SFA/SFA* mice. We intercrossed $p53^{+/-};SFA/+$ mice, and no $p53^{-/-};SFA/SFA$ mice were observed+out of over 100 offspring (see Supporting information, Table S4), indicating that a homozygous reduction in levels of *Rpl27a* resulted in p53-independent lethality.

Low *Rpl27a* increases survival of $p53^{+/-}$ mice, yet promotes metastasis

Given that *SFA/+* mice have increased p53 activity compared to WT mice, we examined the role of low *Rpl27a* in tumorigenesis by monitoring $p53^{+/-};SFA/+$ and $p53^{+/-}$ mice. Reduced *Rpl27a* levels significantly delayed the tumour onset and increased survival in the $p53^{+/-}$ background (Figure 6C). Low *Rpl27a* increased the median survival of $p53^{+/-}$ mice (538 days) compared to $p53^{+/-}$ mice (487 days). Moreover, we observed tumours starting at ~13 months in the $p53^{+/-};SFA/+$ cohort, while $p53^{+/-}$ tumours started at ~9 months of age. Examining the tumour spectrum, we noticed that the percentage of sarcomas and carcinomas were similar in both cohorts, while lymphomas slightly decreased (8.5%) and tumour type multiplicity increased (9.9%; Table 1) in $p53^{+/-};SFA/+$ mice. Further, metastasis greatly increased from 3.7% ($p53^{+/-}$) to 22% in $p53^{+/-};SFA/+$ tumours. If we only considered osteosarcoma, this increase was further elevated (33.3%; Table 1).

p53 pathway signalling in *SFA/+* mice

We checked p53 protein levels by IHC staining in *SFA/+* P14 BM ($n = 2$) and P3 cerebellums ($n = 3$). We observed a widespread but variable stabilization of p53 in the BM (see Supporting information, Figure S5) and a more localized increase in p53 protein in the EGL (Figure 7A). ISH on P3 brain sections of the p53 pathway members *Mdm2*, *Mdm4*, *p53* and *Cdkn1a* were performed, and we found that *Mdm2* and *Mdm4* were broadly expressed in the cerebellum, hampering reliable detection of expression differences (Figure 7B). Interestingly, the EGL showed the highest levels of *p53* in both WT and *SFA/+* brains, suggesting that SFA phenotypic defects involve sites of elevated *p53* expression during development (Figure 7B). Moreover, *Cdkn1a* was elevated in the *SFA/+* EGL (Figure 7B). qPCR quantification revealed a significant increased expression of *p53* by 1.4-fold, *Mdm2* by 3.5-fold, *Cdkn1a* by ~2.1-fold and *Puma* by 2.5-fold in *SFA/+* compared to WT mice (Figure 7C). Since *Mdm2* was elevated concurrently with p53 in the *SFA/+* cerebellum, it appears that, in the context of low *Rpl27a*, Mdm2 is less efficient in degrading p53.

To examine the interplay of *Rpl27a* with the p53 pathway, we checked whether haploinsufficiency of either *Mdm2* or *Mdm4* may increase the severity of the *SFA/+* phenotype. We crossed the *SFA/+* mice with *Mdm2*^{+/Δ7-12} [18] or *Mdm4*^{+/-} mice [19]. Of 40 progeny from each cross, no *Mdm2*^{+/Δ7-12};*SFA/+* (see Supporting information, Table S5) or *Mdm4*^{+/-};*SFA/+* progeny were born (see Supporting information, Table S6), showing the genetic interaction between *Rpl27a* and *Mdm2* and *Mdm4* during development.

Discussion

This study identifies a new high *p53* mouse model characterized by low *Rpl27a* expression and bearing the four known *p53*-dependent phenotypes: hyperpigmentation [12], anaemia [12,18,20,33], cerebellar ataxia [20,33] and radiosensitivity [18,20]. In this mouse, the reduction in *Rpl27a* expression is due to a mutation located in the final splice acceptor of the gene. The mode of action of this mutation was comparable to other ribosomal nonsense destabilizing mutations [36]. Low *Rpl27a* resulted in raised *p53* levels, driving the system closer to a survival threshold that is crossed with HSC depletion in severely affected mice. While this resulted in pancytopenia, the *p53*-dependent red blood cell anaemia in mutant mice of *Rps19* and *Rps20* (dominant loss-of-function) was reported as an erythrocyte specific lineage defect [12], rather than a stem cell shortage.

In addition to HSC depletion, significant down-regulation of *c-Kit* expression was noted in residual *SFA*⁺ HSCs, indicating that *c-Kit* may be a transcriptional target of *p53* in the BM, as previously been observed for melanocytes [37]. Interestingly, a *c-Kit* loss-of-function mutant mouse had significantly reduced HSCs, causing early postnatal lethality [38], suggesting that *p53*-dependent down-regulation of *c-Kit* may be the mechanism causing early postnatal lethality in severely affected *SFA*⁺ mice. We also observed an increase in HSC proliferation with a significant increase in apoptosis in severely affected mice. Interestingly, as HSC numbers decreased, HSC proliferation increased, suggesting a compensatory mechanism as seen in mice lacking *Mdm2* or *Mdm4* in the intestinal epithelium with elevated *p53* levels [39,40]. Nevertheless, we have not directly examined the cell autonomous nature of this effect in our mouse model. It is of interest to note that a *p53*-dependent effect on stem cells or proliferating progenitor cells is an emerging paradigm, explaining developmental defects associated with high *p53* mouse models [12,18,20,33], and is concordant with increased HSC numbers observed in *p53*-null mouse BM [25,41,42].

In contrast to the low penetrant defect of the BM, the hyperpigmentation is fully penetrant and is attributed to the stabilization of keratinocyte *p53*, resulting in the release of Kitlg and other paracrine factors, inducing an increase in melanocyte numbers ([12] and unpublished observations). Thus, *p53* may regulate members of the c-Kit signalling pathway differently, depending on the tissue context. On the other hand, in the developing *SFA*⁺ cerebellum, stabilized *p53* in progenitor EGL neurons induced *Puma* and *Cdkn1a* expression, resulting in apoptosis and cell cycle arrest. A *p53*-dependent involvement of c-Kit or Kitlg in the ataxia is less likely, especially since *c-Kit* appears to be primarily expressed in the molecular layer [43].

Together, these phenotypes are due to a developmental interruption of the negative regulation of *p53*, suggesting that normal *Rpl27a* levels are required to efficiently maintain viable *p53* levels during development. Moreover, the variable degree of severity of *SFA*⁺ defects is likely due to different expression levels of *Rpl27a*, depending on the affected tissue type and developmental stage. This also suggests that a number of cellular and biochemical mechanisms operate to couple the level of this ribosomal protein with *p53* levels. We observed no effect on ribosome biogenesis, since low *Rpl27a* phenotypes are completely rescued by homozygous *p53* deletion.

The strong phenocopy of low *Rpl27a* mice with *p53* pathway models [18,20,33] and its synthetic lethality with haploinsufficient *Mdm2/Mdm4* suggests that *Rpl27a* might well be acting through *Mdm2/Mdm4* to influence *p53* protein levels, as is the case of ribosomal proteins RPL5, RPS7, RPL11 and RPL23 [1,3,6,7,9].

Since alterations in ribosomal protein levels (RNA/protein) or stoichiometry are an obligate event in cellular transformation, where many ribosomal proteins are over-expressed to

facilitate rapid cell cycle progression, p53 may be recruited in mammalian ribosomal surveillance to suppress transformation. Thus, we sought to test the *in vivo* impact of reduced *Rpl27a* levels on transformation. In *p53^{+/-}:SFA/+* mice, we observed a statistically significant delay in tumour onset, and an extended survival compared to *p53^{+/-}* mice. This improvement in survival constitutes another functional evidence of high p53 levels in *SFA/+* mice, as found in Eμ-*myc*-driven *Mdm2* or *Mdm4* haploin-sufficient mice [20,44]. In contrast, *p53^{+/-}:Mdm4^{+/-}* mice developed tumours sooner than *p53^{+/-}* mice, which is believed to be through a p53-independent role of Mdm4 [45]. Surprisingly, reduced *Rpl27a* levels in *p53^{+/-}:SFA/+* mice also inherently conferred a higher risk for metastatic conversion, concomitant with the extended survival (~18% increase in metastasis from overall tumours and ~25% from osteosarcomas). The fact that no difference in tumour onset, spectrum or metastasis was reported for *Rpl24*-deficient mice on a *p53^{+/-}* background suggests that the role of Rpl27a in transformation and prevention of malignant conversion may not be a generalized feature of ribosomal proteins [46]. This metastasis promoter effect of Rpl27a underlines a p53-independent role of this protein that is more complex and will require further investigation, especially in light of RPL27A being over-expressed/amplified in a number of tumour types [47–49] and cancer cell lines [50].

In conclusion, the *SFA/+* mouse offers a unique opportunity to study the effects + of the p53 gradient in many tissue systems, with a particular interest in the role of Rpl27a in ageing, stem cell function and tumour development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BM	bone marrow
BrdU	bromodeoxyuridine
ENU	<i>N</i> -ethyl- <i>N</i> -nitrosourea
HSC	haematopoietic stem cells

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Figure 1.

Meiotic mapping and identification of the mutation responsible for the *SFA* phenotype. (A) Meiotic mapping of the *SFA* locus performed after a two-generation backcross onto the 129S6/SvEv background from the C57BL/6J parent strain. C57BL/6J genotypes are represented by black boxes and 129S6/SvEv by white boxes. Polymorphic microsatellite marker designation is shown on the left, while physical location is shown on the right of the box genotype diagram. Numbers of both *SFA*+/+ and WT mice of each genotype combination for the nine microsatellite markers are shown underneath the box genotype diagram. (B) The first two panels are sequence chromatograms of mice of indicated genotypes. The bottom panel represents the intron–exon structure of the *Rpl27a* gene. The white boxes illustrate the untranslated exons and the red boxes illustrate the coding exons. The ENU-induced point mutation (IVS4 -15A > G) within the intron four-splice acceptor is represented by a red star. (C) Expression levels of *Rpl27a* (mean + SEM) in *SFA*+/+ P3 cerebellum ($n = 8$, $p = 0.0388$), P23-25 BM ($n = 6$, $p = 0.0426$) and P3 footpad skin ($n = 9$, $p = 0.0283$) in comparison to levels in WT tissue indicated by the dotted line (set at 1; $n = 6$ for cerebellum, $n = 7$ for BM and $n = 5$ for skin). (d) Variably penetrant low body weight and size in P35 *SFA*+/+ mice. (E) Postnatal growth and survival curves of WT and *SFA*+/+ mice.

Figure 2.

Blood phenotype in *SFA/+* mice. (A). A representative picture of thymi and spleens from a severely affected *SFA/+* and WT littermate (B) Histogram (mean + SEM, $n = 3$) of thymus and spleen weights relative to whole body weights in WT and *SFA/+* littermates. $p = *0.0079$ and $p = **0.001$. (C) H&E stain of sections from upper femurs of P21-P23 WT and *SFA/+* littermates, showing BM with all subtypes of blood cells present, such as megakaryocyte (MK), macrophage (MO), myeloid (M) and erythroid (E) cells. (D) Histogram presenting flow cytometry analysis of short-term (ST) and long-term (LT) HSC numbers (mean + SEM) in flushed BM of P21-P23 mice ($n = 6$ for WT; $n = 10$ for severely affected *SFA/+*), measured by Lin^{-lo} c-Kit⁺ Sca-1⁺ gated cells, which are sorted into Flk-2⁺ (LT) and FLK-2⁻ (ST). $p = 0.0114$ for LT and $p = 0.001$ for ST. (E) Representative flow cytometric analysis of HSCs from flushed BM of P21-P23 WT and severely affected *SFA/+* mice. Lin^{-lo} cells were gated on c-Kit and Sca-1. Small quadrant (R3) represents Sca-1⁺ c-Kit⁺ cells which contain the stem cells population. The second panel is representative of staining from most of the affected *SFA/+* mice. The third panel shows staining from one of the most affected mice. (F) Quantitation of fluorescence intensity of c-Kit⁺ cells (mean + SEM) in the R3 HSC quadrant of (E) ($n = 6$ mice/genotype). *** $p < 0.0001$. (G) Total BM and HSCs (%) undergoing proliferation (BrdU⁺ cells) [$n = 6$ /genotype; $p = 0.3130$ (ns) and *0.0136], and total BM cells (%) undergoing apoptosis (annexin V staining, $n = 3$ /genotype; *** $p = 0.0001$) measured by flow cytometry (mean + SEM) in WT and severely affected *SFA/+* mice. (H) The relationship between HSC numbers in whole BM and percentage proliferating HSCs (BrdU⁺ cells) is plotted. A 'best-fit' straight dotted line is drawn through the data (linear regression). R^2 = square of correlation coefficient.

Figure 3.

Hyperpigmentation in *SFA/+* mice. (A) Hyperpigmentation of adult WT footpads in comparison to age-matched *SFA/+* mice. (B) Hyperpigmentation of WT and *SFA/+* tails. (C) *DCT-LacZ* transgenic mouse enables specific identification of melanocytes by β -galactosidase staining (stained in blue). Top panels represent footpads of indicated genotypes. Bottom panels represent β -galactosidase staining of the above corresponding panel. Blue melanocytes are indicated by yellow arrows. The red arrow points to representative melanin granules in *SFA/+* skin. (D) Melanocyte counts/10 mm of linear epidermis [visualized by the blue LacZ-stained cells in (C)] in WT and *SFA/+* footpads and tails ($n = 3$ /genotype/tissue). $p = **0.0024$ and $***p < 0.0001$.

Figure 4.

Cerebellar ataxia in *SFA/+* mice. (A) Nissl stains of mid-sagittal sections of WT and *SFA/+* adult (P56) cerebellum. (B) Calbindin-1^{D28K} IF staining of the Purkinje cells.

Disorganization of Purkinje neurons found ectopically located throughout the cerebellum of P56 *SFA/+* mouse. M, molecular layer showing staining of Purkinje dendrites; P, Purkinje cell bodies; G, granule layer. (C) Top panel, proliferation measured by BrdU (green)/PI (red) IF on mid-sagittal sections of P3 cerebellums; white line delineates the granule layer; bottom panel, apoptosis assayed by caspase-3 IHC staining; apoptotic cells are indicated by black arrows. (D) Quantitation of % BrdU⁺ cells/field of P3 WT and *SFA/+* cerebellums ($n = 3/\text{genotype}$). * $p = 0.0315$.

Figure 5.

Low *Rpl27a* mouse phenocopies high *p53* mouse models. (A) H&E of femur showing the BM in P15 WT, severely affected *SFA/+* and *Mdm2*^{+/-};*Mdm4*^{+/-} mouse (high p53). (B) H&E of cerebellum in WT, severely affected *SFA/+* and *Mdm2*^{+/-};*Mdm4*^{+/-} mice. (C) Footpads from adult mice of p53 pathway models. **Mdm4*-null allele with deletion of exon 2. [§]*Mdm4*-null allele with deletion of part of introns 2–5.

Figure 6.

Genetic interaction between *Rpl27a* and *p53* and impact of low *Rpl27a* on survival. (A) Motor coordination ranges of mice of designated genotypes, measured by the rotarod assay. Latency to fall on the final rotarod trial for each 10 week-old mouse is plotted versus the weight of the mouse. Boxes designate the ranges for weight and scores/genotype. (B) The impact of different *p53* genotypes on pigmentation of *SFA/+* footpads. (C) Kaplan–Meier survival curve (Log-rank test, $p = 0.001$) between $p53^{+/-}; SFA/+$ and $p53^{+/-}$ curves.

Figure 7.

p53 pathway signalling in P3 *SFA*^{+/+} cerebellums. (A) Detection of p53 immunopositivity in mid-sagittal *SFA*^{+/+} cerebellum by IHC. (B) ISH of *p53*, its negative regulators *Mdm2* and *Mdm4*, and target gene *Cdkn1a* on WT and *SFA*^{+/+} cerebellum. (C) Histogram expression of fold expression of indicated genes (mean + SEM, *n* = 6/genotype) in *SFA*^{+/+} cerebellums, measured in triplicate in comparison to levels in WT cerebellums (indicated by the dotted line that is set at 1). *p* = 0.017 (*p53*); *p* = 0.0240 (*Mdm2*); *p* = 0.0002 (*Cdkn1a*); and *p* = 0.0168 (*Puma*).

Table 1Tumour spectrum in $p53^{+/-}$ and $p53^{+/-};SFA/+$ mice

	$p53^{+/-}$ (n = 29)	$p53^{+/-};SFA/+$ (n = 36)
Lymphoma	9 (23.7%)	9 (15.2%)
Sarcoma	21 (55.3%)	30 (50.8%)
Unclassified	8 (21.08%)	8 ^{*1} (13.5%)
Osteosarcoma	12 ^{*1} (31.6%)	21 ^{*7} (35.6%)
Rhabdomyosarcoma	1 (2.6%)	1 (1.7%)
Carcinoma	6 (15.8%)	11 (18.6%)
Unclassified	0	1 (1.7%)
Squamous cell	5 (13.2%)	7 (11.8%)
Adenocarcinoma	1 (2.6%)	3 ^{*1} (5.1%)
Other	2 (5.3%)	9 (15.2%)
Adenoma	2 (5.3%)	3 (5.1%)
Lipoma	0	2 (3.4%)
Leiomyoma	0	1 (1.7%)
Plasmacytoma	0	2 (3.4%)
Haemangioma	0	1 (1.7%)
Tumours total	38	59
Tumours/mouse	1.31	1.64
Mice with multiple primaries	8 (27%)	12 (33%)
Metastasis	1 (3.7%)	9 (22%)
Metastasis from osteosarcoma	1 (8.3%)	8 (33.3%)

* Number of tumours that metastasized.