Published in final edited form as:

Mol Carcinog. 2012 September ; 51(9): 754–760. doi:10.1002/mc.20852.

Loss of *Trp53* Promotes Medulloblastoma Development but not Skin Tumorigenesis in *Sufu* Heterozygous Mutant Mice

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Abstract

Basal cell carcinoma of the skin typically carries genetic alterations in components of the hedgehog (HH) signaling pathway. Previously, we generated a knockout mouse with a loss-of-function mutation in suppressor of fused (*Sufu*), an essential repressor of the pathway downstream of Hh ligand cell surface reception. Mice heterozygous for the mutated *Sufu* allele develop a skin phenotype that includes lesions similar to basaloid follicular hamartomas. The purpose of the current study was to test the possibility that the simultaneous loss of the tumor suppressor gene, *Trp53*, would aggravate the *Sufu* skin phenotype since *Trp53* loss is known to enhance the growth of other Hh-driven tumors. Consistent with previous reports, medulloblastomas and rhabdomyosarcomas developed in *Sufu*^{+/-}; *Trp53*^{-/-} mice. However, the characteristic *Sufu*^{+/-} skin phenotype was not altered in the absence of *Trp53*, and showed no changes in latency, multiplicity, cellular phenotype or proliferative capacity of the basaloid lesions. This finding was both novel and intriguing and demonstrated a differential, tissue-specific sensitivity to *Sufu* and *Trp53* tumor suppressor gene loss, which may be linked to developmental stage and the degree of proliferative activity in specific cell types.

Keywords

Hedgehog; suppressor of fused; basal cell carcinoma; p53

INTRODUCTION

Sporadic basal cell carcinoma (BCC) is the most commonly diagnosed human cancer among Caucasians [1]. These tumors have a high frequency of inactivating mutations in the tumor suppressor gene *patched1* (*PTCH1*) [2,3] or, to a lesser extent, activating mutations in the proto-oncogene *smoothened* (*SMO*) [3–5], both of which are components of the hedgehog (HH) signaling pathway. In fact, the current understanding is that an aberrantly activated HH pathway is a prerequisite for BCC tumorigenesis [1], and the level of hedgehog pathway activation may be linked to the morphological heterogeneity of BCCs and related tumors [1,6,7]. In the HH pathway, *PTCH1* is a cell surface receptor that becomes inactivated upon binding of the HH ligands, leading to activation and recruitment of *SMO* to the primary cilium [8,9]. *SMO*, in turn, signals to a downstream complex consisting of the suppressor of fused (*SUFU*), a negative regulator of the HH pathway, and the *GLI* proteins, transcriptional

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effectors of the pathway [10,11]. This leads to dissociation of the complex and translocation of GLI into the nucleus where it activates the HH transcriptional program.

Loss-of-function mutations in the *PTCH1* gene are also responsible for the nevoid basal cell carcinoma syndrome (NBCCS), known as Gorlin syndrome, a rare autosomal dominant disorder in which patients have a marked susceptibility to develop BCCs [12,13]. Beside BCCs, NBCCS patients also have an increased risk of developing medulloblastoma (MB), the most common malignant brain tumor in children. Interestingly, it was recently reported that a family with features of NBCCS, including MB but not BCC, lacked *PTCH1* mutations and instead had a *SUFU* germline mutation [14].

Mice with engineered heterozygosity for a loss-of-function allele of *Ptch1* [15,16] spontaneously develop MB and microscopic basaloid proliferations in the skin, but irradiation by UV or X-rays is required for BCC development [17,18]. The BCC lesions in irradiated *Ptch1*^{+/-} mice show frequent loss of the remaining wild-type *Ptch1* allele and exhibit up-regulated Hh signaling activity. We have shown previously that non-irradiated mice carrying a heterozygous inactivating mutation of the tumor suppressor, *Sufu*, develop similar basaloid proliferations but with a higher multiplicity than the *Ptch1*^{+/-} mice. However, *Sufu*^{+/-} mice do not spontaneously develop MB [19,20].

Mutations in the human tumor suppressor gene, *TP53*, are frequently found in BCCs [3,21] but it is not clear to what extent the mutant TP53 protein cooperates with aberrant HH pathway activity in BCC tumorigenesis. In mice, it has been shown that *Trp53* loss can enhance Hh-driven tumorigenesis, for example, the incidence of MB in *Ptch1*^{+/-} [22] and *Sufu*^{+/-} [23] mice is dramatically increased on a *Trp53* null background.

To investigate whether loss of the mouse p53 protein has an effect on the *Sufu*^{+/-} skin phenotype, *Sufu*^{+/-} mice were generated on a *Trp53* null background. The results demonstrated that the *Sufu*^{+/-} skin phenotype was not aggravated upon loss of *Trp53* during the four to five month life-span of these mice. At the end of this period, all the *Sufu*^{+/-}; *Trp53*^{-/-} mice had succumbed to non-skin tumors, mostly MB and malignant lymphomas, consistent with previously published data [23]. These findings indicated that the cancer-promoting effects of *Trp53* loss on a *Sufu* heterozygous background is cell type-specific and may be dependent upon the developmental stage and/or degree of proliferative activity of a particular cell type.

MATERIALS AND METHODS

Mouse strains and Genotyping

Sufu^{+/-} mice (strain B6;129X1/SvJ-*Sufu*^{tm1Rto}, [19]) were intercrossed with *Trp53*^{+/-} mice (strain B6;129-*Trp53*^{tm1Brd}, [24]) to generate compound *Sufu*^{+/-}; *Trp53*^{+/-} mice, which in turn were intercrossed to obtain *Sufu*^{+/-}; *Trp53*^{-/-} mice together with the necessary controls. A cohort of 84 animals (wt n=11, *Sufu*^{+/-} n=13, *Trp53*^{+/-} n=9, *Sufu*^{+/-}; *Trp53*^{+/-} n=26, *Trp53*^{-/-} n=4, *Sufu*^{+/-}; *Trp53*^{-/-} n=21) was followed up to 10 months of age or until severe symptoms developed, whereafter the mice were sacrificed and dissected. Genotyping was performed as previously described [19,24,25]. Mice were housed according to local and national regulations, and the study was approved by the Stockholm South Animal Ethics Committee.

Histological analyses

For the histological analyses, adult mouse tissues were fixed in 4% paraformaldehyde (Sigma) in phosphate buffered saline (PBS) overnight or in Bouin's solution (Sigma) for a week. The latter fixation enabling *in situ* visualization of the brain within the skull cavity.

Tissues were subsequently dehydrated and paraffin embedded, sectioned at 4 μm and stained with hematoxylin-eosin. For quantification of skin lesions, plantar skin from the hind paws of three randomly selected two- and six-month-old *Sufu*^{+/-} and *Sufu*^{+/-}; *Trp53*^{-/-} mice each, were processed as above and sagittally sectioned at 10 μm to give between 20 to 40 consecutive tissue sections per paw. Skin lesions were counted for all sections and the average number of lesions per tissue section was calculated. The following primary antibodies were used for immunohistochemistry: anti-K5 (1:5000, PRB-160P, Covance); anti-K6 (1:5000, PRB-169P, Covance); anti-K10 (1:5000, PRB-159P, Covance); anti-Ki67 (1:500, clone SP6, NeoMarkers); anti-p63 (1:500, sc-8431, Santa Cruz Biotechnology) and anti-p53 (1:1000, ab26, Abcam). For all antibodies, antigen retrieval was performed for 30 minutes in DIVA Decloaker (DV2004MX, Biocare Medical) in a 2100-Retriever (Proteogenix). Mouse primary antibodies were used in combination with HistoMouse™ SP (Invitrogen). Biotinylated anti-rabbit IgG secondary antibodies (1:200, BA-1000, Vector Laboratories) and streptavidin-conjugated peroxidase (Invitrogen), with diaminobenzidine (DAB, Zymed Laboratories) as a substrate, were used to visualize the bound primary antibodies, followed by hematoxylin counterstaining and mounting in Pertex® (Histolab).

Statistical analysis

Statistical analysis of the Kaplan-Meier survival rates (Figure 1A) was performed with the log-rank Mantel-Cox test and the quantification of paw skin lesions (Figure 2A) with unpaired *t*-test using the Prism 5.0 software program (GraphPad Software, Inc., La Jolla, CA).

RESULTS

The *Sufu*^{+/-} Skin Phenotype is Unaffected by *Trp53* Loss, but Medulloblastoma Tumorigenesis is Promoted

In order to investigate the effect of the absence of p53 functional activity on the *Sufu*^{+/-} skin phenotype [19], a cohort of 84 mice was generated, bearing the following genotypes: wild-type, *Sufu*^{+/-}, *Trp53*^{+/-}, *Sufu*^{+/-}; *Trp53*^{+/-}, *Trp53*^{-/-} and *Sufu*^{+/-}; *Trp53*^{-/-} (Figure 1A). In line with a previous study [23], 57% (12/21) of the *Sufu*^{+/-}; *Trp53*^{-/-} mice developed MB (Figure 1B; Table 1) with a peak incidence at three to four months of age (Figure 1C). Eight of the *Sufu*^{+/-}; *Trp53*^{-/-} mice (38%) developed malignant lymphomas, which is a characteristic *Trp53* null background lesion, before six months of age, and two of the 12 *Sufu*^{+/-}; *Trp53*^{-/-} mice with MB also suffered from malignant lymphomas. We also observed one case of rhabdomyosarcoma (5%) in the *Sufu*^{+/-}; *Trp53*^{-/-} mouse cohort (Table 1), a tumor type that was also noted in the study by Lee et al. [23]. Two out of the 21 *Sufu*^{+/-}; *Trp53*^{-/-} mice died of unknown causes, although seemingly not from MB or malignant lymphomas. The median survival time of the *Sufu*^{+/-}; *Trp53*^{-/-} mice was four months (Figure 1A). However, there was no statistical difference in their survival rate compared to that of the *Sufu*^{+/-}; *Trp53*^{+/-} mice (*P* = 0.2463), neither was there any difference in survival rate between *Sufu*^{+/-}; *Trp53*^{+/-} versus *Sufu*^{+/-}; *Trp53*^{-/-} mice (*P* = 0.9835). The sex ratio distortion among *Trp53* deficient mice (Table 1) was likely due to female-specific exencephaly, an embryonic lethal condition affecting a fraction of these mice [26].

The *Sufu*^{+/-} skin proliferations first appear on the non-haired palmo-plantar aspects of the paws, and since very few lesions can be detected elsewhere in the skin before six months of age [19], the current study was focused on this particular tissue. The skin lesions on the paws of *Sufu*^{+/-}; *Trp53*^{-/-} mice were of the same type of basaloid invaginations as we have previously described for the *Sufu*^{+/-} mice (Figure 3II–IV). However, by comparison, there was no statistical difference either at two or six months of age (*P* = 0.17 and 0.97, respectively; *t*-test) in the latency or multiplicity of the lesions (Figure 2A). Also, the

number and average size of the typical *Sufu*^{+/-} lesions increased similarly with age, independent of *Trp53* status (Figure 2A–B). We did not detect any apparent signs of inflammation regardless of genotype. Since all *Sufu*^{+/-}; *Trp53*^{-/-} mice died of MB or malignant lymphoma before six months of age, it was obviously not possible to observe the phenotype at later time points. As expected, no basaloid proliferations could be detected in the skin of wild-type or *Trp53*^{-/-} mice (Figure 3I and 3V). In order to reveal potential cellular differences between the *Sufu*^{+/-} and *Sufu*^{+/-}; *Trp53*^{-/-} skin lesions, paw skin sections were immunostained for both epidermal and proliferation markers. Keratin 5 (K5) is a marker for keratinocytes in the basal layer of the epidermis [27], and its expression in *Sufu*^{+/-}; *Trp53*^{-/-} skin proliferations was relatively homogeneous and similar to that seen in *Sufu*^{+/-} and *Sufu*^{+/-}; *Trp53*^{+/-} skin (Figure 3VI–X), supporting a basal cell origin for the lesions. In the skin, keratin 6 (K6) is normally only present in the palmoplantar epidermis and in the companion cell layer of hair follicles [28]. K6 expression is also associated with hyperproliferations in the interfollicular epidermis (IFE), although expression of K6 in BCCs is normally low or absent [7,29]. In this study, K6 showed a similar expression pattern in the *Sufu*^{+/-}; *Trp53*^{-/-} proliferations when compared with those developing in the *Sufu*^{+/-} or *Sufu*^{+/-}; *Trp53*^{+/-} mice. In contrast to K5, K6 expression was rather heterogeneous and tended to decrease, and even disappear, in the deeper portions of the larger proliferations (Figure 3XI–XV). Interestingly, keratin 10 (K10), which is a marker for suprabasal cells, predominantly stained cells in the inner portions of the proliferations suggesting that there was some differentiation occurring within the lesions, supporting their basaloid follicular hamartoma-like features (Figure 3XVI–XX). Again, the presence, or absence of p53 had no apparent effect.

To investigate the degree of proliferative activity within the lesions, tissue sections were stained for the proliferation marker Ki67. Similar to the observations in *Sufu*^{+/-} and *Sufu*^{+/-}; *Trp53*^{+/-} mice, the proliferative activity in *Sufu*^{+/-}; *Trp53*^{-/-} lesions was relatively low as indicated by the small number of cells staining positive for Ki67 (Figure 3XXI–XXV). This observation was consistent with the slow growth of the lesions and their basaloid follicular hamartoma-like characteristics, and also indicated that loss of *Trp53* does not increase the proliferative capacity of these early lesions.

In adult epidermis, the transcription factor, p63, is expressed in the basal cell layer of the IFE and in hair follicles where it plays a role in transit amplifying cells and stem cell maintenance [30]. In this study, p63 immunostaining was seen in most of the cells in the epithelial proliferations regardless of genotype, again supporting the basal cell origin of these lesions (Figure 3XXVI–XXX).

In normal skin, p53 is usually undetectable due to its rapid turnover. However, in situations of cellular stress, such as that caused by irradiation or oncogenic insult, p53 becomes stabilized. To explore whether the cells in the *Sufu*^{+/-} proliferations were under cellular stress, the tissue sections were stained for p53, but we were unable to detect an increase of p53 expression in *Sufu*^{+/-} or *Sufu*^{+/-}; *Trp53*^{+/-} lesions (data not shown).

DISCUSSION

In view of the fact that *TP53* mutations often coexist with mutations in the HH signaling pathway in human BCCs [3,21], and that *Sufu*^{+/-} mice develop basaloid follicular hamartomas [19], *Sufu*^{+/-}; *Trp53*^{-/-} mice were generated in order to study possible genetic cooperativity between *Sufu* and *Trp53* in skin tumorigenesis. Since the loss of *Trp53* in *Ptch1*^{+/-} [22] and *Sufu*^{+/-} [23] mice leads to enhanced MB tumorigenesis, we hypothesized that the loss of *Trp53* might also aggravate the skin phenotype in *Sufu*^{+/-} mice. Interestingly, however, no such enhancement of the *Sufu*^{+/-} skin phenotype was observed in

contrast to the striking effect that the loss of *Trp53* had on the developing cerebellum, leading to MB development in 57% of the *Sufu*^{+/-}; *Trp53*^{-/-} mice.

Why *Trp53* loss in *Sufu*^{+/-} mice had a completely different outcome in the cerebellum compared to skin is an intriguing question. The granule precursor cells (GPCs) of the cerebellum are believed to be the cell of origin for Hh pathway-dependent MB [31]. These cells undergo rapid expansion in the external germinal layer during the first two weeks of postnatal life. They then undergo terminal differentiation, and lose their proliferative capacity. Consequently, there is only a very limited time period during which the presumed tumor precursor cells are able to develop. We did not find any evidence of MB in our *Sufu*^{+/-} or *Sufu*^{+/-}; *Trp53*^{+/-} mice, indicating that genomic instability, such as complete loss of *Trp53* function, is necessary to promote this tumor type. Loss of *Trp53* leads to the accumulation of cytogenetic abnormalities [32,33] that may act synergistically with *Sufu* haploinsufficiency to produce MB, or cause the second hit leading to loss of heterozygosity of this gene.

Unlike cerebellar GPCs, epidermal cells exhibit unlimited proliferative activity and are under constant environmental stress. Therefore, these cells may need better protection against genomic aberrations, perhaps provided by mechanisms additional to p53, which do not act in GPCs. This may be one explanation for the fact that the loss of *Trp53* does not have the same drastic effect on early tumor induction and growth in the epidermis as it does in the cerebellum of *Sufu*^{+/-} mice.

Another, more direct example of Hh and p53 pathway cooperativity was provided recently in a report describing the GLI1-p53 inhibitory loop [34]. In neural stem cells p53 inhibits the activity, and reduces the level, of GLI1, and in turn GLI1 represses p53. However, if this GLI1-p53 inhibitory loop also functions in the epidermis, disruption of the loop due to *Trp53* loss is apparently not sufficient to cause any significant alteration of the early *Sufu*^{+/-} skin phenotype.

Recently, it was shown that X-ray induced BCC tumorigenesis in *Ptch1*^{+/-} mice, could be enhanced by conditional loss of *Trp53* [35]. In the current study *Trp53* was deleted in all tissues, but its loss of expression did not promote BCC development in *Sufu*^{+/-} skin. The discrepancy may be explained by the use of ionizing radiation to induce tumorigenesis in the study by Wang *et al.* [35], who also suggested that increased Smo expression could promote the tumorigenic effects caused by the loss of *Trp53*. The possible increase in Smo expression is likely irrelevant in the current study since *Sufu* is downstream of Smo. Studying *Sufu*^{+/-} mice in combination with irradiation and/or a skin-specific *Trp53* deletion should help to shed more light on this issue. Nevertheless, since the penetrance of the basaloid lesions is 100%, the *Sufu*^{+/-} mouse model may be a useful screening tool to find additional genes necessary for mediating the possible conversion of basaloid hamartomas to full-blown BCCs.

In summary, this study demonstrates a differential tissue-specific sensitivity to tumor development in mice upon *Trp53* loss in a *Sufu*^{+/-} background, suggesting that Hh and p53 pathway cooperativity is intimately linked to developmental stage and the degree of proliferative activity in specific cell types.

Acknowledgments

We would like to thank M. Nistér for the *Trp53* mice and E. Tüksammel for technical assistance. We are grateful to V. Jaks, M. Kasper, M. A. Hoelzl, A. Are and A. Schwäger for helpful discussions, and to S. Lang for language editing.

GRANT SUPPORT This work was supported by the Swedish Research Council (S.T.), the Swedish Cancer Society and NIH/NCI MMHCC (R.T.), the Wallenberg Consortium North (B.R.) and the Robert Lundberg Memorial Foundation (K.H.-H.).

ABBREVIATIONS

BCC	basal cell carcinoma
Gli	glioma associated protein
Hh	hedgehog
IFE	interfollicular epidermis
MB	medulloblastoma
NBCCS	nevoid basal cell carcinoma syndrome
Ptch1	patched1
Shh	sonic hedgehog
Smo	smoothened
Sufu	suppressor of fused
Trp53	transformation related protein 53

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Figure 1.

Sufu^{+/-};*Trp53*^{-/-} mice succumb to medulloblastoma and malignant lymphomas at four to six months of age. (A) Kaplan-Meier survival plot of wild-type, *Sufu*^{+/-}, *Trp53*^{+/-}, *Sufu*^{+/-};*Trp53*^{+/-}, *Trp53*^{-/-} and *Sufu*^{+/-};*Trp53*^{-/-} mice, followed until 10 months of age. (B) Hematoxylin-eosin stained sections of the cerebellum from four-month-old mice: wild-type (left panels); *Sufu*^{+/-};*Trp53*^{-/-} with medulloblastoma (right panels). The boxed regions are shown at higher magnification in the lower panels. Scale bar = 500 μ m, upper panels and 100 μ m, lower panels. (C) Age-related distribution of MB (medulloblastoma), L (malignant lymphoma) and RMS (rhabdomyosarcoma) incidence in *Sufu*^{+/-};*Trp53*^{-/-} mice. The number of mice is indicated above each bar.

Figure 2.

The latency and multiplicity of characteristic early *Sufu*^{+/-} skin lesions is not significantly changed upon *Trp53* loss. **(A)** The average number of basaloid invaginations per sagittal hind paw plantar skin section from two- and six-month-old *Sufu*^{+/-} and *Sufu*^{+/-}; *Trp53*^{-/-} mice. Each bar represents the mean $\pm$ SD of three mice and from each hind paw, 20–40 sections were analyzed. **(B)** Merged panoramic pictures of representative hematoxylin-eosin stained 10- μ m sections used for the quantification shown in (A).

Figure 3.

Immunohistochemical analyses of typical *Sufu*^{+/-} epidermal proliferations show no significant phenotypic differences regardless of *Trp53* status. (I–V) Hematoxylin-eosin (H&E)-stained sections from the palmo-plantar skin of four- to six-month-old mice. (VI–X) Keratin 5 immunostaining showing strong, uniform reactivity in the proliferations. (XI–XV) Keratin 6 immunostaining showing strong but heterogeneous reactivity in the proliferations. (XVI–XX) Keratin 10 immunostaining showing strong reactivity predominantly in the inner portion of the proliferations. (XXI–XXV) Ki67 immunostaining indicating very low proliferative activity in the lesions. (XXVI–XXX) Immunostaining for p63 showing reactivity in most of the cells in the proliferations. Insets show the proliferations at higher magnification. Scale bars correspond to 50 μ m in main figures and 20 μ m in insets.

Table 1

Accumulated 10-month incidence of MB, L, and RMS in female and male mice within the study cohort.

	Wild-type	Sufu ^{+/-}	Trp53 ^{+/-}	Sufu ^{+/-} ;Trp53 ^{+/-}	Trp53 ^{-/-}	Sufu ^{+/-} ;Trp53 ^{-/-}
MB males	0% (0/6)	0% (0/8)	0% (0/7)	0% (0/13)	0% (0/4)	59% (10/17)
MB females	0% (0/5)	0% (0/5)	0% (0/2)	0% (0/13)	0% (0/0)	50% (2/4)
MB total	0% (0/11)	0% (0/13)	0% (0/9)	0% (0/26)	0% (0/4)	57% (12/21)*
L males	0% (0/6)	0% (0/8)	0% (0/7)	0% (0/13)	50% (2/4)	35% (6/17)
L females	0% (0/5)	20% (1/5)	0% (0/2)	0% (0/13)	0% (0/0)	50% (2/4)
L total	0% (0/11)	8% (1/13)	0% (0/9)	0% (0/26)	50% (2/4)	38% (8/21)*
RMS males	0% (0/6)	0% (0/8)	0% (0/7)	8% (1/13)	0% (0/4)	6% (1/17)
RMS females	0% (0/5)	0% (0/5)	50% (1/2)	0% (0/13)	0% (0/0)	0% (0/4)
RMS total	0% (0/11)	0% (0/13)	11% (1/9)	4% (1/26)	0% (0/4)	5% (1/21)

Note: MB, medulloblastoma; L, malignant lymphoma; RMS, rhabdomyosarcoma.
* 2/21 mice (males) were diagnosed with both MB and L. 2/21 mice (males) were not diagnosed with any of the above tumors.