***In Vivo* Role of Alternative Splicing and Serine Phosphorylation of the Microphthalmia-Associated Transcription Factor**

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ABSTRACT The microphthalmia-associated transcription factor (MITF) is a basic helix-loop-helix leucine zipper protein that plays major roles in the development and physiology of vertebrate melanocytes and melanoma cells. It is regulated by post-translational modifications, including phosphorylation at serine 73, which based on *in vitro* experiments imparts on MITF an increased transcriptional activity paired with a decreased stability. Serine 73 is encoded by the alternatively spliced exon 2B, which is preferentially skipped in mice carrying a targeted serine-73-to-alanine mutation. Here, we measured the relative abundance of exon 2B⁺ and exon 2B[−] RNAs in freshly isolated and FACS-sorted wild-type melanoblasts and melanocytes and generated a series of knock-in mice allowing forced incorporation of either alanine, aspartate, or wild-type serine at position 73. None of these knock-in alleles, however, creates a striking pigmentation phenotype on its own, but differences between them can be revealed either by a general reduction of *Mitf* transcript levels or in heteroallelic combinations with extant *Mitf* mutations. In fact, compared with straight serine-73 knock-in mice with their relative reduction of 2B⁺ *Mitf*, forced incorporation of alanine 73 leads to greater increases in MITF protein levels, melanoblast and melanocyte numbers, and extent of pigmentation in particular allelic combinations. These results underscore, *in vivo*, the importance of the link between alternative splicing and post-translational modifications and may bear on the recent observation that exon 2B skipping can be found in metastatic melanoma.

ALTERNATIVE splicing and post-translational modifications are among the major mechanisms by which individual genes generate multiple protein products. In fact, modern sequencing technologies and proteomic analyses have shown that most if not all multiexon pre-mRNAs give rise to alternatively spliced mature mRNAs (Nilsen and Graveley 2010) and that post-translational modifications modulate the activities of most eukaryotic proteins (Mann and Jensen 2003). In a simple sense, then, the two mechanisms are linked as post-translational modifications depend

on the presence of the particular exons encoding the modifiable residues or proteolytic cleavage sites. Here, we present a mouse model that allows us to probe this link *in vivo* by separately targeting a splice site and a codon for a biologically relevant phosphoacceptor site in the gene encoding the pigment cell transcription factor, microphthalmia-associated transcription factor (MITF). In fact, pigmentation is particularly suitable for such studies as it provides for an easily visible and highly sensitive readout of gene function.

Mitf plays a crucial role in the development and function of melanin-bearing pigment cells in skin, eye, and inner organs (Hodgkinson *et al.* 1993; Arnheiter *et al.* 2006; Arnheiter 2010). Its protein product is a basic helix-loop-helix leucine zipper transcription factor that regulates target gene promoters by binding specific E box sequences as homodimers or heterodimers with the related proteins TFEB, TFE3, and TFEC (Strub *et al.* 2011). *Mitf* mutations have been found from zebrafish to man and their analyses have established that *Mitf* controls specification, proliferation, death, differentiation, and stress

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responses of normal pigment cells and regulates migration, metastasis, and death of melanoma cells (Arnheiter *et al.* 2006; Hoek and Goding 2010).

In vitro experiments have shown that phosphorylation of MITF serine 73 increases the protein's transcriptional activity about threefold (Hemesath *et al.* 1998) and that serine-73 phosphorylation alone or double serine-73/serine-409 phosphorylation leads to polyubiquitination and degradation of MITF (Wu *et al.* 2000; Xu *et al.* 2000). To address the question of whether serine-73 phosphorylation plays a role *in vivo*, we have previously generated targeted mice in which codon 73 was changed to one encoding alanine 73, which cannot be phosphorylated (Bismuth *et al.* 2008). These mice had a normally pigmented coat and normal eyes, a finding that was later confirmed by using a transgenic bacterial artificial chromosome (BAC) rescue strategy with BACs engineered to encode alanine 73 alone or alanine 73 and alanine 409 together (Bauer *et al.* 2009). A complication of these experiments was the fact that the *Mitf* exon, which encodes serine 73, exon 2, contains a 5' alternative splice site that divides it into an exon 2A of 20 codons and an exon 2B of 56 codons, the latter including codon 73. This arrangement normally leads to a major transcript (at least 90% of total *Mitf* RNA in skin or heart) that contains the entire exon 2, and a minor one in which exon 2B, and hence codon 73, is missing, although the open reading frame is maintained (Bismuth *et al.* 2008; Bauer *et al.* 2009). Interestingly, the codon-73 mutation in both targeted mice and BAC transgenics is associated with a substantial decrease of the transcripts containing exon 2B (representing only ~10% of total *Mitf* mRNA in the targeted mice and ~45% in the BAC rescue mice), and a concomitant increase of the transcripts lacking exon 2B (Bismuth *et al.* 2008; Bauer *et al.* 2009). This splice change, which is likely due to the fact that codon 73 is embedded in a splice enhancer sequence whose affinity for the serine/arginine-rich splice regulator SRSF5 is reduced upon mutation (Wang *et al.* 2009), precluded meaningful conclusions on the specific role of serine 73. To separate the potential effects of mutations at residue 73 from the splice-dependent absence of this residue, we retargeted *Mitf* in a way that renders the alternative 5' splice site nonfunctional and incorporates either a wild-type serine, a phosphomimetic aspartate, or an alanine at position 73. We find that although the new mutations cause no visible coat phenotypes on their own, they sensitize melanocytes to the phenotypic effects of reductions in total *Mitf* levels and to the effects of extant *Mitf* alleles in compound heterozygotes. Intriguingly, when tested under such conditions, the targeted allele containing the wild-type serine behaves as a hypomorph, while that containing the alanine behaves as a hypermorph. The results show that alternative splicing of exon 2B is indeed relevant *in vivo*, although in an unexpected manner and in a way that would be difficult to detect solely on the basis of *in vitro* experiments.

Material and Methods

Melanoblast/melanocyte purification and RNA-Seq data

Melanocytes were purified by FACS sorting from doxycycline-treated bitransgenic mice (iDct-GFP mice, strain FVB/N) that express H2B-GFP fusion proteins specifically in *Dct*-positive melanocytes, using previously published protocols (Zaidi *et al.* 2011a,b,c). To purify melanoblasts from E15.5 and E17.5 C57BL/6 iDct-GFP embryos and P1 pups, the dams were fed doxycycline-fortified chow for the entire duration of gestation until harvest. For P3 and P7 pups, doxycycline was injected intraperitoneally at 80 µg/g body weight 24 hr before harvest. Total RNA was prepared from FACS-sorted melanoblasts/melanocytes according to standard Illumina RNA-Seq paired-end protocol and sequenced on the Illumina GAIIx to 80 bp per read. For analysis of alternative exon 2B splicing, a set of possible splice sites was determined and assigned to the aligner, indicating that a given splice variant might exist. The sequences were aligned using the genomic short-read nucleotide alignment program (Wu and Nacu 2010) to mouse genome assembly mm9. A custom Python script was then used to count introns that had one boundary at position 97,944,374 on chromosome 6 (5' end of exon 3). The analysis revealed that only three splice sites were used, one at position 97,943,348, yielding 2B⁺ *Mitf*, one at position 97,943,180, yielding 2B⁻ *Mitf*, and a single read for one at position 97,879,927, yielding *Mitf* with a junction between exon H and exon 3.

Targeting constructs, minigenes, and expression plasmids

The 17.9-kb targeting construct described by Bismuth *et al.* (2008) was used as template to PCR amplify a 1.2-kb *EcoRI/HindIII*-flanked fragment encompassing part of intron 1, exon 2A/2B, and part of intron 2/Neo-loxP cassette. The PCR-amplified fragment was cloned into pcDNA 3.1 and used for PCR mutagenesis. In a first round, the exon 2A/2B junction was altered by changing four bases without changing the coding sequence. In a second round, the previously introduced *ApaI* site was changed back to the wild-type sequence and codon A73 was either left intact or was changed into one encoding either aspartate or wild-type serine. Sequence-confirmed clones for each of the three different codons at residue 73 were digested with *AflIII* and *AgeI* and used to replace the corresponding *AflIII/AgeI* fragment in a 7.5-kb *BamHI* fragment representing the 3' arm of the original construct. The resulting constructs were digested with *BamHI* and ligated to a *BamHI*-linearized construct containing the thymidine kinase gene and the 5' arm of the original construct, yielding three final reengineered targeting constructs. The correct orientation of the 7.5-kb *BamHI* fragment was verified by PCR with two primer pairs described in [Supporting Information, Table S1](#). To test for the effect of the 5' splice junction mutation, an *Mitf* minigene comprising exon 1M, intron 1, exon 2A/B, intron 2, and exon 3 was subjected to PCR mutagenesis to alter the 5' splice

junctions and codon 73 as in the targeting constructs, using the Quickchange Site-Directed Mutagenesis kit (Stratagene). For expression analysis, N-terminal estrogen-receptor (ER)-coupled Mitf 6A⁺ pBABE vectors were obtained from Colin Goding (Carreira *et al.* 2005) and subjected to PCR mutagenesis to obtain Mitf^{S73A} (ER S73A) and Mitf^{Δ2B} (ER 2B⁻) expression plasmids, using a primer set described in Table S1. All constructs were sequence verified.

Mice

Mice carrying the allele *Mitf*^{tm1.2Arnh}, containing a S73A mutation with or without a floxed Neo cassette in intron 2, have been described (Bismuth *et al.* 2008). For the purpose of this study, *Mitf*^{mi-S73A} mice from a separate targeting experiment were used and are designated *Mitf*^{tm7Arnh} (containing the neomycin resistance cassette) and *Mitf*^{tm7.1Arnh} (lacking the neomycin resistance cassette) (MGI:5050706). For targeting the exon 2A/2B 5' splice site in conjunction with mutations at serine 73, LC3 embryonic stem (ES) cells [genotype (C57BL/6Nx129S6)F1] were electroporated with 20 μg of the respective NotI linearized targeting constructs and grown under standard G418/FIAU selection protocols. After screening, targeted ES cells were injected into C57BL/6N blastocysts and chimeric mice were bred with C57BL/6J to test for germ line transmission and to establish Neo-cassette-containing targeted lines. To remove the floxed Neo cassette, mice were bred with C57BL/6 • 129S4-Prm1-Cre deleter mice. After removal of the Neo cassette, mice were either intercrossed to obtain homozygous targeted mice or crossed with mice carrying other mutant *Mitf* alleles. The official designation of these targeted mice are: *Mitf*^{tm4Arnh} (MGI:5050700) and *Mitf*^{tm4.1Arnh} (MGI:5050701) for the splice junction/S73 allele; *Mitf*^{tm5Arnh} (MGI:5050702) and *Mitf*^{tm5.1Arnh} (MGI:5050703) for the splice junction/S73A allele; and *Mitf*^{tm6Arnh} (MGI:5050704) and *Mitf*^{tm6.1Arnh} (MGI:5050705) for the splice junction/S73D allele, each with or without the Neo cassette, respectively. C57BL/6 • 129S2 *Kit*^{tm1Alf} (Bernex *et al.* 1996) heterozygous males were bred with *Mitf*^{mi-S73AΔneo} and *Mitf*^{mi-S-S73AΔneo} homozygous females. Double heterozygous offspring were then crossed with the corresponding *Mitf* homozygotes to establish E12.5 and E15.5 embryos and P1 pups homozygous for *Mitf* and heterozygous for *Kit*^{tm1Alf}. X-gal-labeling was done as described (Wen *et al.* 2010). For generation of melanocyte lines, mice were crossed with B6 • *Cdkn2a*^{tm1Rdp} mice to obtain double homozygous *Mitf*/*Cdkn2a* mutants. The bitransgenic iDct-GFP mice (Zaidi *et al.* 2011a) used for RNA-Seq were kept on a FVB/N background.

Reverse transcription, RT-PCR, and real-time (q)RT-PCR, Western blots

Random primed cDNAs were made from 500 ng of total RNA extracted from heart or 50 ng extracted from melanocyte lines using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) and RNase inhibitor cocktail (Invitrogen). PCR and qPCR were performed as described

(Bharti *et al.* 2010), using primers described in Table S1. For Western blots, 600 μg of heart tissue or 120,000 cultured cells were separated by SDS-PAGE and blotted onto nitrocellulose membranes. The blots were exposed to appropriate antibodies and bands were revealed using SuperSignal West Pico chemiluminiscent substrate. Quantitation was done using a Biorad ChemiDoc XRS⁺ system.

Genotyping and Southern analysis

DNA extracted by standard procedures from tissue samples was analyzed by PCR, using primers indicated in Table S1. For Southern blots, liver DNA was prepared, digested as indicated, and probed using PCR-amplified probes as previously published (Bismuth *et al.* 2008).

Photography

Photographs of knock-in mice and their intercrosses with other *Mitf* alleles were taken between 3 weeks and 2 months of age.

Cell lines stably expressing Mitf proteins

pBABE-ER vectors allowing expression of HA-tagged exon 6A⁺ MITF fused with the estrogen-responsive portion of the estrogen receptor were obtained from Colin Goding (Carreira *et al.* 2005). Virus was produced by cotransfection of pBABE vectors and pCL Amphi vectors in 293T cells, and ARPE-19 cells (spontaneously immortalized human retinal pigment epithelium cells) were infected and selected with puromycin (1 μg/ml) without subsequent cloning. Stable cell lines were prepared allowing expression of only ER (ER Ø) or MITF-ER with wild-type S73 (ER WT), S73A (ER S73A), or 2B⁻ MITF (ER 2B⁻). For induction, cells were treated with 300 nM 4-hydroxy-tamoxifen (TM) for 48 hr. Total extracts were probed with 6A5 anti-MITF antibodies (Bharti *et al.* 2008).

Flow cytometry

For assaying MITF expression, ARPE19 cells were washed and fixed with 4% paraformaldehyde, permeabilized, and incubated with mouse monoclonal antibody 6A5 and R-phycoerythrin-goat antimouse IgG1 as second antibody. Flow cytometry was performed using a FACSVantage SE flow cytometer (Becton Dickinson) and the data were processed using CellQuest software. The gating was determined empirically and applied in the same way to all experiments.

Results

Alternative splicing of exon 2B of Mitf in melanocytes

Previous results have shown that heart and skin of wild-type mice express *Mitf* splice variants that specifically lack exon 2B (Hallsson *et al.* 2000; Bismuth *et al.* 2008; Bauer *et al.* 2009). To confirm these findings in freshly isolated embryonic and postnatal melanocytes, we purified the respective cells by FACS, using the recently described bitransgenic

iDct-GFP mice that allow for tetracyclin-regulated expression of a histone H2B-GFP fusion protein in melanoblasts and melanocytes (Zaidi *et al.* 2011a,b,c). For these experiments, we used wild-type embryos at E15.5 and E17.5 and postnatal mice at P1, P3, and P7. RNA-Seq of sorted cells clearly showed the presence of *Mitf* RNA. As shown in Figure 1A, detailed analyses revealed that although in all samples the majority of reads across the 5' alternative splice junctions of exon 2 corresponded to the exon 2B-exon 3 sequence, a small subset corresponded to the exon 2A-exon 3 sequence. To further confirm these results, we subjected RNA from P1 and P3 FACS-sorted cells to real-time RT-PCR as described (Bharti *et al.* 2008). The results showed a 2B⁻/(2B⁻ + 2B⁺) ratio of between 1.85 and 4.36%. These values were similar to those in passage 10 melanocytes obtained from P2 mice that were wild-type at *Mitf* and homozygous for a *Cdkn2a* targeted null allele used to prevent cell senescence (Sviderskaya *et al.* 2002) (Figure 1B). In contrast, in corresponding melanocytes from P2 skin of *Mitf*-S73A mutated, *Cdkn2a*^{-/-} mice (labeled S73A in Figure 1B), the ratio was 90.75%. These results indicate that freshly isolated embryonic or postnatal melanocytes or cultured melanocytes express a small amount of *Mitf* mRNAs lacking exon 2B and that a serine-73 mutation substantially increases this alternatively spliced transcript.

A targeted mutation in the exon 2A/2B junction of *Mitf* prevents exclusion of exon 2B *in vitro* and *in vivo*

As shown in Figure 2A and Figure S1, the RNA sequence of the alternative 5' splice site, CACCAG'GUAAG, is close to the consensus splice sequence, C/AAG'GUA/GAGU. To ensure efficient inclusion of exon 2B despite codon-73 mutations, we deliberately mutated this splice site in genomic DNA to yield RNAs with the sequence CAUCAA'GUUAAA. The mutation was expected to render the 5' splice junction nonfunctional without, however, changing the encoded amino acid sequence. We first tested whether the mutation would function in the intended way in a minigene construct that comprised exon 1M-exon 3 of *Mitf*. Upon transfection into heterologous cells, this construct normally recapitulates the alternative exon 2B splicing and its dependence on the sequence of codon 73. When both the 5' splice junction and codon 73 were mutated, however, only exon 2B⁺ RNA and no aberrant splice products were produced *in vitro* (not shown). We then prepared three separate targeting constructs that carried the same splice mutation along with a codon for either wild-type serine, aspartate, or alanine (Figure 2A; for details, see *Materials and Methods*). After electroporation into embryonic stem cells, three lines of targeted mice designated *Mitf*^{mi-S-73S}, *Mitf*^{mi-S-73A}, and *Mitf*^{mi-S-73D} (S-S for splice site and serine 73) were obtained and characterized by Southern blot analysis (Figure 2, B and C) and by sequencing (not shown). Together with *Mitf*^{mi-S73A} mice, which do not carry the 5' splice site mutation, we established eight separate lines, four of them containing a floxed neomycin resistance cassette in intron 2 (see Figure 2A),

A RNA-seq analysis of *Mitf* exon 2B alternative splicing profile on GFP-positive sorted melanoblasts and melanocytes

Embryo stage	Ratio $\frac{2B^-}{(2B^- + 2B^+)}$	Total <i>Mitf</i> reads containing exon 3 junctions
E15.5	2.7%	37
E17.5	0.7%	277
Post natal Day 1	2.2%	557
Post natal Day 7	1.9%	206
Total averaged	1.8%	1077

B RT-PCR analysis of *Mitf* exon 2B alternative splicing on both GFP-sorted melanocytes and melanocyte cell lines

Figure 1 Detection of *Mitf* isoforms in melanocytes *in vivo*. (A) GFP⁺ melanoblasts from bitransgenic (iDct-GFP) embryos and postnatal pups of the indicated ages were isolated and subjected to sorting by FACS. Corresponding RNA-seq data were analyzed as described in *Materials and Methods*. The ratios of the reads across exon 2A/exon 3 and exon 2B/exon 3 [$2B^-/(2B^- + 2B^+)$] as well as the total number of reads analyzed are shown. (B) Relative abundance of *Mitf* 2B⁺ and *Mitf* 2B⁻ RNA prepared from *in vivo* isolated P1 and P3 melanocytes, expressing GFP and FACS-sorted as for A, was determined by qRT-PCR using the appropriate primer pairs (Table S1). In addition, RNA from melanocyte cell lines derived from either *Cdkn2a*^{-/-}; *Mitf*^{+/+}/*Mitf*⁺ and *Cdkn2a*^{-/-}; *Mitf*^{mi-S73A}/*Mitf*^{mi-S73A} P1 dorsal skins and cultured for 10 passages served as controls. The calculated percentages correspond to the ratio of 2B⁻/(2B⁻ + 2B⁺).

and four corresponding ones in which this cassette was removed by breeding with *Prm1-Cre* deleter mice.

For RNA analyses, we first used heart tissue because heart expresses relatively high levels of *Mitf* RNA and, unlike melanocytes, is not altered in a major way by *Mitf* mutations. RT-PCR analyses showed the presence of both exon 2B⁺ and exon 2B⁻ RNA in wild-type mice; the predominant lack of exon 2B in mice homozygous for the *Mitf*^{mi-S73AΔneo} allele; and the exclusive presence of exon 2B in hearts of mice homozygous for either of the three 5' splice-mutant alleles [regular RT-PCR in Figure S2A, qRT-PCR (Bharti *et al.* 2010) in Figure S2B]. Total levels of *Mitf* RNAs, averaged from quantitative RT-PCR analyses using primers for exons 6-7 and 8-9 (Table S1), were similar to wild type in the mutants in which the Neo cassette has been removed (Figure S2C). To determine RNA levels in mice in which the Neo cassette was left intact, we used primers specifically covering the mutated exon 2 region to avoid measuring aberrant RNAs potentially starting from the

Figure 2 Generation of four knock-in *Mitf* alleles. (A) Schematic representation of the *Mitf* genomic structure and the mutations generated in *Mitf*^{mi-S73Aneo} [previously described (Bismuth et al. 2008)] and in the three novel splice alleles. Sequence modifications at the exon 2A–2B junction as well as at the S73 codon are highlighted in red, and the position of the neomycin resistance cassette in intron 2 is shown. (B and C) Differential restriction profile of the *Mitf* locus before and after targeting and Southern blot analysis of wild-type and one line each of homozygous targeted mice. DNA was digested as indicated and probed with the respective 5' and 3' probes shown in B.

Neo cassette. The results showed that when the Neo cassette was present, *Mitf* RNAs containing part or all of exon 2 were reduced by ~50–75% (Figure S2D). Immunoprecipitation/immunoblotting assays of hearts of the different mutants indicated that *Mitf*^{mi-S73Aneo} mice produced MITF protein with a faster electrophoretic mobility, consistent with the predominant expression of MITF lacking exon 2B in such mice (Figure S2E). In contrast, the different S-S73 mutants all produced predominantly full-length MITF protein, but their relative expression levels differed. Compared to *Mitf*^{mi-S-S73Sneo} mice, the levels were ~1.3 fold higher in

Mitf^{mi-S-S73Dneo} and ~1.6 fold higher in *Mitf*^{mi-S-S73Aneo} mice, suggesting that the particular residue at position 73 influences protein stability (Figure S2E).

To determine RNA and protein expression specifically in melanocytes, we then tested above described *Mitf*^{+/+}/*Mitf*⁺; *Cdkn2a*^{-/-} and *Mitf*^{mi-S73Aneo}/*Mitf*^{mi-S73Aneo}; *Cdkn2a*^{-/-} melanocyte lines as well as a corresponding line derived from P2 skin of *Mitf*^{mi-S-S73Aneo}/*Mitf*^{mi-S-S73Aneo}; *Cdkn2a*^{-/-} mice. RT-PCR assays showed a predominant band representing the *Mitf* 2B⁺ transcript in wild type, a double band representing the *Mitf* 2B⁺ and *Mitf* 2B⁻ transcripts in *Mitf*^{mi-S73Aneo}, and a single band representing the *Mitf* 2B⁺ transcript in *Mitf*^{mi-S-S73Aneo} (Figure 3A). As schematically depicted in Figure 3B, MITF protein can undergo numerous post-translational modifications in melanocytes and other cell types, including phosphorylations, sumoylations, and caspase cleavage. These modifications may lead to proteins with distinct electrophoretic mobilities on Western blots. In fact, as shown in Figure 3C, wild-type melanocytes showed the characteristic double band of MITF protein representing S73-phosphorylated and S73-nonphosphorylated exon 2B⁺ protein. *Mitf*^{mi-S73Aneo} lines, however, showed a band with an electrophoretic mobility corresponding to nonphosphorylated MITF 2B⁺ and one corresponding to MITF 2B⁻. In contrast to the relative amounts of the respective transcripts, however, the MITF 2B⁺ protein band was more prominent than the MITF 2B⁻ protein band. This observation suggests that compared to the wild-type full-length protein, either nonphosphorylated full-length MITF is more stable or MITF lacking exon 2B less stable, provided exon 2B⁺ and exon 2B⁻ mRNA share equal translational efficiency. In addition, *Mitf*^{mi-S73Aneo} lines showed one band of lower and one of higher electrophoretic mobility (marked with arrows in Figure 3C) that may represent differently modified or cleaved products. *Mitf*^{mi-S-S73Aneo} lines showed a single RT-PCR product corresponding to the *Mitf* 2B⁺ transcript and a simpler Western blot signal with a prominent band corresponding to nonphosphorylated MITF 2B⁺ protein and one band each of higher and lower electrophoretic mobility (arrows), again potentially representing differently modified or cleaved products. Quantitation of the Western blot bands revealed that *Mitf*^{mi-S73Aneo} and *Mitf*^{mi-S-S73Aneo} cells had higher MITF levels than the corresponding wild-type cells (Figure 3C), consistent with the notion that lack of phosphorylation at residue 73 may increase accumulation of full-length MITF because of increased protein stability.

Genetic dissection of pigmentation phenotypes associated with the targeted mutations

To determine the phenotypic consequences of the above mutations, we performed extensive breeding tests. First, we generated mice containing the neomycin resistance cassette that were either homozygous for the four targeted *Mitf* alleles or were compound heterozygotes with any of three extant *Mitf* alleles: *Mitf*^{mi-vga9}, which is a null allele due to insertion of a transgene array in the M-Mitf promoter

Figure 3 Mitf exon 2B alternative splicing profile at the RNA and protein level in melanocyte lines. (A) RNA extracts were prepared from passage 10 *Cdkn2a*^{-/-} melanocyte lines from wild-type B6, *Mitf*^{mi-S73AΔNeo} (S73A), and *Mitf*^{mi-S-S73AΔNeo} (S-S73A) mice and subjected to RT-PCR. (B) Schematic diagram of select post-translational modifications of MITF seen in melanocytes and other cell types. Indicated are phosphorylation, sumoylation, and caspase cleavage sites, while sites for acetylation, ubiquitination, and other modifications are not shown. (C) Western blots of protein extracts of the cell lines used in A, probed with 6A5 anti-MITF antibodies. The double band seen in the wild-type sample corresponds to phosphorylated and nonphosphorylated exon 2B⁺ MITF. *Mitf*^{mi-S73A} cells show both a minor band corresponding to 2B⁻ MITF and a major band corresponding to 2B⁺ MITF, in addition to a band of higher molecular weight and one of lower molecular weight, likely representing another post-translationally modified form and a degradation product (arrows). *Mitf*^{mi-S-S73A} cells show a predominant 2B⁺ MITF band and no 2B⁻ MITF band. Also, there is a band of higher molecular weight and one of lower molecular weight (arrows). MITF quantification relative to the endogenous β-tubulin is indicated below the gel.

(Hodgkinson *et al.* 1993); *Mitf*^{mi-wh}, a semidominant allele characterized by an Ile212-to-Asn mutation in the DNA-binding basic domain (Steingrímsson *et al.* 1994); and *Mitf*^{mi}, a semidominant allele characterized by the lack of an Arg in a row of four Arg in the basic domain (Steingrímsson *et al.* 1994). The targeted homozygotes showed normal eyes and normal pigmentation in coat, ears, and feet except for a white belly spot, which on average was largest in *Mitf*^{mi-S-S73SNeo} but almost always absent in *Mitf*^{mi-S-S73ANeo} mice (Figure 4, A–D). Similar phenotypic differences between the targeted alleles were seen in compound heterozygotes with *Mitf*^{mi-vga9}. While *Mitf*^{mi-vga9}/*Mitf*⁺ mice are normally pigmented (Figure 4E) and *Mitf*^{mi-vga9}/*Mitf*^{mi-vga9} mice totally white and microphthalmic (Figure 4H), the compound heterozygotes showed various extents of white spotting whereby *Mitf*^{mi-S-S73ANeo}/*Mitf*^{mi-vga9} mice had the smallest areas of white coats; *Mitf*^{mi-S-S73SNeo}/*Mitf*^{mi-vga9} mice were almost completely white; and *Mitf*^{mi-S73ANeo}/*Mitf*^{mi-vga9} and *Mitf*^{mi-S-S73DNeo}/*Mitf*^{mi-vga9} mice were intermediate (Figure 4, F, G, I, and J). In contrast, no such differences between the targeted alleles were seen in combination

with *Mitf*^{mi-wh} as all mice had a fawn coat with white spots of similar sizes (Figure S3A). Only rarely did we observe small black spots in these mice, which may reflect somatic loss-of-function mutations in the dominant-negative *Mitf*^{mi-wh} allele (an example shown in Figure S3A for *Mitf*^{mi-S-S73ANeo}/*Mitf*^{mi-wh}). We also saw no major codon-73-dependent differences in combinations with *Mitf*^{mi} as all mice were totally white except for small black spots that appeared in highest frequency and sizes in *Mitf*^{mi-S-S73ANeo}/*Mitf*^{mi} mice but were always absent in *Mitf*^{mi-S-S73SNeo}/*Mitf*^{mi} mice (Figure S3B). These results indicate that in alleles containing the neomycin resistance cassette, the presence or absence of exon 2B and the phosphorylatability or permanent negative charge of residue 73 are associated with phenotypic differences that are, however, only seen in particular allelic combinations.

In a second round of breedings, we used mice lacking the neomycin cassette to generate targeted homozygotes or compound heterozygotes as above. As shown in Figure 4, K–N, homozygotes were all normally pigmented regardless of the splice and serine-73 mutations. In contrast, phenotypic differences between the different targeted *Mitf*

Figure 4 Phenotypes associated with the four knock-in alleles alone and in combination with extant *Mitf* alleles. (A–J) Controls and Neo-cassette-containing lines. (A–D) White belly spots found in homozygotes of the indicated genotypes. Note that *Mitf*^{mi-S73A} homozygotes show no belly spots, in contrast to the other lines. (E) *Mitf*^{mi-vga9} heterozygotes show no pigmentary phenotype while *Mitf*^{mi-vga9} homozygotes are completely white and microphthalmic (H). (F, G, I, and J) Heteroallelic combinations with *Mitf*^{mi-vga9}. Note different degrees of white spotting with the different targeted alleles. (K–R) Lines lacking the Neo cassette (Δ neo). (K–N) Regardless of the genotype, all homozygotes are normally pigmented and indistinguishable by visual inspection from wild-type B6 mice, except that *Mitf*^{mi-S73A Δ neo}/*Mitf*^{mi-S73A Δ neo} mice have a tail with generally lighter pigmentation compared to the other genotypes. (O–R) Heteroallelic combinations with *Mitf*^{mi-wh}. Note the darker pigmentation seen in combinations with either *Mitf*^{mi-S73A Δ neo} or *Mitf*^{mi-S73S Δ neo} compared to combinations with *Mitf*^{mi-S73D Δ neo}, which yield mice with a coat that was only slightly darker, and combinations with *Mitf*^{mi-S73S Δ neo}, which yield mice that are indistinguishable from *Mitf*^{mi-wh}/*Mitf*⁺ heterozygotes. Each photograph in O–R represents littermates.

alleles were seen in combination with *Mitf*^{mi-wh} (Figure 4, O–R). Compared with *Mitf*^{mi-wh}/*Mitf*⁺ littermates, both *Mitf*^{mi-S73A Δ neo}/*Mitf*^{mi-wh} and *Mitf*^{mi-S73S Δ neo}/*Mitf*^{mi-wh} mice had a considerably darker coat, yet they still carried white belly spots typical of the presence of the *Mitf*^{mi-wh} allele. However, the coat was only slightly darker in *Mitf*^{mi-S73D Δ neo}/*Mitf*^{mi-wh} mice, and *Mitf*^{mi-S73S Δ neo}/*Mitf*^{mi-wh} mice were phenotypically indistinguishable from *Mitf*^{mi-wh}/*Mitf*⁺ mice (Figure 4, O–R). Because it was theoretically possible that the presence of MITF protein that cannot be phosphorylated at residue 73 might influence the phosphorylation status of the MITF^{mi-wh} protein and so influence the stability and dominant-negative activity of the latter, we also performed immunoprecipitation/immunoblot assays of heart tissues of the different allelic combinations. As shown in Figure S2F, however, there was no clear indication for a change in phospho-MITF^{mi-wh} as the bands in *Mitf*^{mi-S73A Δ neo}/*Mitf*^{mi-wh} hearts (lane 5) appeared as the sum of those seen in *Mitf*^{mi-S73A Δ neo}/*Mitf*^{mi-S73A Δ neo} (lane 3) and *Mitf*^{mi-wh}/*Mitf*^{mi-wh} (lane 8) hearts. Furthermore, none of the targeted alleles showed any differences in combinations with *Mitf*^{mi-vga9} or *Mitf*^{mi} (Figure S3, C and D). These results again indicate that phenotypic differences between the different targeted alleles can be revealed only in particular allelic combinations.

Melanoblast and melanocyte accumulation is differently affected by *Mitf*^{mi-S73A} and *Mitf*^{mi-S73A}

The darker coat associated with the presence of the serine-73-to-alanine mutated MITF protein in *Mitf*^{mi-wh} compound heterozygotes could be explained by the accumulation of a greater number of melanoblasts and melanocytes in the corresponding mice. The availability of *Kit*^{tm1Alf}, a *Kit* null allele characterized by the insertion of a bacterial lacZ gene in exon 1 of *Kit*, allowed us to specifically and reliably label melanoblasts during development. In fact, previous results have shown that the nuclear β -GAL produced by *Kit*^{tm1Alf} is not affected by *Mitf* mutations, at least not in melanoblasts (Hou *et al.* 2000). It needs to be kept in mind, however, that *Kit*^{tm1Alf}/*Kit*⁺ mice have white feet, tail tips, and belly spots as only two fully functional *Kit* alleles allow for completely normal melanoblast development. Hence, the use of *Kit*^{tm1Alf} gave us the added opportunity to compare the phenotypic effects of the different targeted *Mitf* alleles in conjunction with a separate mutation affecting melanogenesis. Inter-crosses showed, however, that homozygosity for *Mitf*^{mi-S73A} and *Mitf*^{mi-S73A} did not grossly change the *Kit*^{tm1Alf}/*Kit*⁺ phenotype except that on average, the belly spots seemed slightly larger with both *Mitf* alleles. We then harvested corresponding E12.5 and E15.5 embryos as well as P1 skin

Figure 5 Melanocyte numbers in developing embryos. Embryos homozygous for either *Mitf*^{mi-S73AΔNeo} or *Mitf*^{mi-S73AΔNeo} and heterozygous for *Kit*^{tm1Alf} were X-gal-labeled and blue cells counted in selected regions at E12.5 and E15.5 and in P1 skin and P1 pigmented hair follicles. Numbers from 7 to 10 fields from at least three embryos are shown relative to those observed in *Kit*^{tm1Alf}/*Kit*⁺ control samples. Statistical significance (two-tailed unpaired *t* test) is indicated (**P* < 0.1; ***P* < 0.01; ****P* < 0.001).

and subjected the specimens to X-gal labeling (Figure 5). At E12.5, in a representative area around the developing eye, *Kit*^{tm1Alf}/*Kit*⁺; *Mitf*^{mi-S73A}/*Mitf*^{mi-S73A} embryos showed similar numbers of X-gal-labeled cells as *Kit*^{tm1Alf}/*Kit*⁺; *Mitf*^{mi-S73A}/*Mitf*^{mi-S73A} embryos. At E15.5 and P1, however, representative areas showed slightly, but significantly higher

numbers of labeled cells in *Kit*^{tm1Alf}/*Kit*⁺; *Mitf*^{mi-S73A}/*Mitf*^{mi-S73A} compared to *Kit*^{tm1Alf}/*Kit*⁺; *Mitf*^{mi-S73A}/*Mitf*^{mi-S73A} mice. This was also reflected in the number of labeled cells in pigmented hair follicles in a representative dorsal area between the forelimbs. The results suggest that the absence of the entire exon 2B, and the selective absence of

A MITF expression in stably transfected ARPE-19 cell lines

B FACS Analysis of MITF versus DAPI labeling of stably transfected ARPE-19 cell lines with an inducible ER-MITF vector with or without tamoxifen

C DAPI-based cell cycle distribution of MITF-gated ARPE-19 cell lines with or without tamoxifen

	Cell cycle position	ER Ø	ER WT		ER S73A		ER 2B-	
		Mitf Negative	Mitf Low	Mitf High	Mitf Low	Mitf High	Mitf Low	Mitf High
Tm -	% G0/G1	69.35	68.15	67.82	63.4	63.48	63.73	63.62
	% S	18.21	18.62	17.95	18.5	18.82	17.74	18.68
	% G2/M	12.67	13.41	14.42	18.24	17.99	18.78	17.98
Tm +	% G0/G1	71.39	70.98	77.44	62.69	61.28	70.33	66.06
	% S	19.17	19.98	14.64	21.6	27.62	19.85	23.49
	% G2/M	9.55	9.19	8.06	16.01	11.53	10.12	10.86

a phosphorylatable residue at position 73, affect the accumulation of melanoblasts and melanocytes differentially. The findings are consistent with the facts that the presence of the S-S73A allele in some allelic combinations leads to decreased sizes of white spots (or increased numbers of pigmented spots) compared to mice carrying the S73A allele (see Figure 4, A, B, F, and G; Figure S3B).

Because *Mitf^{mi-S73A}* is a complex allele, giving rise to both exon 2B⁻ as well as exon 2B⁺ protein (see Figure 3C), we finally assessed what the separate effects of each of these two isoforms might be in cells in culture. For this, we used ARPE19 cells, which are spontaneously immortalized human retinal pigment epithelial cells that are low in endogenous MITF and yet are derived from a pigmented cell type, and infected them with retroviral vectors expressing either (wild type) MITF 2B⁺ (corresponding to MITF derived from the *Mitf^{mi-S73S}* allele), MITF^{S73A}, or MITF^{Δ2B} proteins, each fused with the estrogen-responsive portion of the ER (termed ER WT, ER S73A, and ER 2B⁻ in Figure 6). In such cells, MITF proteins normally are found in the cytoplasm at relatively low levels but accumulate at high levels in nuclei after tamoxifen induction. In fact, Western blots revealed the expected MITF bands and electrophoretic mobilities after tamoxifen induction (Figure 6A). The cells were then subjected to flow cytometry, using two-color fluorescence labeling for MITF protein expression and total DNA content (DAPI) for cell cycle analysis. As shown in Figure 6B, 48 hr of tamoxifen induction increased the percentage of gated cells showing above-threshold levels of MITF protein. Intriguingly, wild-type MITF-expressing cells showed distinct MITF^{low} and MITF^{high} populations, whereas MITF^{S73A}- and MITF^{Δ2B}-expressing cells had a distinctly lower MITF^{low} population. This suggested that the MITF^{low} population was generated by reduction of MITF by degradation of the S73-phosphorylated form of MITF. As shown in Figure 6C, tamoxifen induction of the different MITF isoforms had differential effects on DNA content of cells. While there was little change when the tag alone and no ectopic MITF was present, wild-type MITF^{high} cells showed a decrease in the percentage of cells in S phase and an increase in the percentage of cells in G0/G1 48 hr after tamoxifen induction. In contrast, the percentage of MITF^{S73A-high} and MITF^{Δ2B-high} cells in S phase was increased, rather than decreased, after tamoxifen induction, though less prominently with MITF^{Δ2B} compared to MITF^{S73A}. This *in vitro* analysis with heterologous cells and overexpressed, cDNA-derived inducible proteins suggested that wild-type MITF has antiproliferative

activities but that the lack of exon 2B, or the absence of phosphorylation at residue 73, yield MITF proteins lacking antiproliferative activity. The results confirm earlier findings with an S73A mutated protein analyzed in different cells (Bismuth *et al.* 2005) and are consistent with the *in vivo* genetic observations that the relative increased accumulation of nonphosphorylatable MITF protein leads to increased melanoblast/melanocyte numbers, increases in the size and extent of pigmentation in certain heteroallelic combinations, and increased ability to compensate a strong dominant-negative allele, *Mitf^{mi-wh}*.

Discussion

Numerous *in vitro* assays have shown that MITF is regulated by post-translational modifications, which in turn depend on the presence of the exons carrying the modifiable residues. Nevertheless, among the many spontaneous and induced *Mitf* mutations from zebrafish to man, few have been found so far to affect splicing or a specific post-translationally modifiable residue. This is not surprising given the fact that, for instance, targeted mutations in the serine-73 phosphoacceptor site as described here, along with those used in BAC transgenic rescue strategies (Bauer *et al.* 2009), produce few if any phenotypes on their own. It is only in conjunction with additional perturbations, such as the reduction in overall *Mitf* RNA levels or the combination with other *Mitf* alleles, that we start to see the effects of mutations in serine 73, or, in addition, the subtle differences between a serine-73 mutation and the lack of the entire exon carrying serine 73.

Our analysis of the phenotypes produced by the targeted alleles benefited from the wide dynamic range of pigmentation and from the fact that the targeted mutations were produced in the endogenous gene rather than in BACs. Targeting the endogenous gene allows for a direct comparison between the different lines as their transcript levels are all similar to wild type. In contrast, *Mitf* BAC transgenic lines show variable RNA expression levels (Bauer *et al.* 2009) likely due to the fact that the available BACs do not contain the entire 214-kb *Mitf* gene and so may lack the boundary elements necessary for sufficient insulation from neighboring sequences encountered at random transgene insertion sites. Comparable RNA expression levels were all the more important because the task was to evaluate the effects of mutations that may affect the stability of the mutated proteins. It has been shown *in vitro* that serine-73-mutated

Figure 6 Effects of the S73A mutation and exon 2B deletion on protein levels and DNA content in stable cell lines inducibly expressing MITF. ARPE-19 cells expressing either pABE-ER (ER Ø), MITF wild-type S73 (ER WT), MITF S73A (ER S73A), or MITF 2B⁻ (ER 2B⁻) proteins were treated with 4-OH-tamoxifen (TM) for 48 hr before collection, and total extracts were probed with 6A5 anti-MITF antibodies. (A) TM-treated, wild-type MITF expressing cells show the characteristic double band of S73-phosphorylated and nonphosphorylated MITF protein, S73A-MITF expressing cells only one band corresponding to nonphosphorylated MITF, and 2B⁻ MITF-expressing cells a single band corresponding to the 2B⁻ isoform. (B and C) ER MITF and ER only (ER Ø) expressing ARPE-19 cell lines were incubated with or without TM, harvested after 48 hr, and labeled for MITF and DNA content, using DAPI. (B) Flow cytometric analysis displays the percentage of cells expressing relatively low (L) or high (H) MITF intensity and (C) their relative cell cycle stages based on DNA content. Gating was determined empirically and applied equally for all samples.

MITF has a similar activity on a test target gene promoter as nonphosphorylated wild-type MITF, but a lower activity when compared to phosphorylated wild-type MITF (Hemesath *et al.* 1998). In subsequent *in vitro* experiments, it was reported that S73A-singly mutated MITF or S73A/S409-doubly mutated MITF have an increased stability. Hence, it became important to determine whether there might be similar increases in the stability of mutated MITF protein *in vivo* and whether a possible reduction in transcriptional activity was compensated for by increased protein stability.

The simplest explanation for all genetic results presented in this article is indeed an increased stability of the mutated proteins. First, we find that both in an unrelated tissue, the heart, and in melanocytes, S73A-mutated MITF accumulates to higher levels than corresponding wild-type MITF. Interestingly, in heart, S73D-mutated MITF accumulates to levels intermediate between those in S73A- and S73S-mutated MITF, reflecting the intermediate phenotype that the *Mitf^{mi-S-S73D}* allele produces in some allelic combinations. This suggests that a permanent negative charge at residue 73 neither reflects fully the properties of serine-73-phosphorylated MITF nor those of residue-73-nonphosphorylated MITF. Second, homozygotes for the mutations with wild-type transcript levels are all fully pigmented. Third, the two mutations resulting in MITF that cannot be phosphorylated at residue 73 (*Mitf^{mi-S-S73AΔNeo}* and *Mitf^{mi-S73AΔNeo}*) are capable of partially compensating the effects of the strongly dominant-negative *Mitf^{mi-wh}* allele. Fourth, when the *Mitf* RNA expression levels of the Neo-cassette-containing alleles were further reduced in compound heterozygotes with the null mutant *Mitf^{mi-vga-9}*, *MITF^{S73A}*-expressing mice, with the lowest RNA levels, produced the most extensive pigmentation, and *MITF^{S73S}*-expressing mice, with higher RNA levels, the least amount of pigmentation. Fifth, *in vitro*, both *MITF^{S73A}* and *MITF^{Δ2B}* have milder antiproliferative effects than corresponding wild-type MITF, or they may even stimulate cell proliferation given the nonlimiting amounts of growth factors to which cultured cells are normally exposed. Nevertheless, despite this *in vitro* finding, the number of melanocytes found postnatally *in vivo* is not substantially different in mutants compared to wild type. This is likely due to the fact that only excess amounts of growth factors such as KIT ligand or endothelin 3 would allow for prolonged survival of supernumerary melanocytes *in vivo*.

The above observations imply that increased accumulation of the mutated MITF proteins may not only compensate for the reduction of their activities observed *in vitro*, but may even overcompensate such activity reductions. This underscores the *in vivo* importance of post-translational modifications that would seem to allow for precise regulation of the activities of MITF. The results presented in this article also imply that *MITF^{S73A}* and *MITF^{Δ2B}* have slightly different effects *in vivo*. Although *Mitf^{mi-S73A}* can produce both full-length and exon 2B-deleted MITF, and although the full-length *MITF^{S73A}* protein may be relatively more stable than the internally truncated one, *Mitf^{mi-S73A}* and *Mitf^{mi-S-S73A}*

mice still differ slightly in melanoblast/melanocyte numbers when they are counted during development and postnatally, or in extent of pigmentation in the adult if RNA levels are reduced below a threshold amount by presence of a Neo cassette in combination with the *Mitf* null allele *Mitf^{mi-vga9}*. Although subtle differences in the total levels of MITF may explain these variations, it is equally plausible that exon 2B has additional functions besides simply providing the substrate for serine-73 phosphorylation. In fact, it is conceivable that wild-type MITF, *MITF^{S73A}*, and *MITF^{Δ2B}* differentially stimulate their target genes. This may be important when considering allele-specific genetic interactions of *Mitf^{mi-bws}*, an *Mitf* allele with an exon 2B splice bias but low RNA levels and a wild-type serine 73 (Wen *et al.* 2010). It may also be important to explain the recent finding that human *MITF^{Δ2B}*, when used to rescue pigmentation in zebrafish with a temperature-sensitive mutation in *mitfa*, leads to increased divisions of differentiated, pigmented cells (Taylor *et al.* 2011). Moreover, it may become important to explain the recent finding that a melanoma metastasis carries a somatic mutation at the downstream exon 2B splice junction that leads to elimination of exon 2B (Cronin *et al.* 2009). Although alterations in *MITF* may be only one among many pathways whose changes allow for differentiated cells or melanoma cells to escape normal control of cell proliferation, it may well be that MITF splice alterations, genomically fixed or dynamically regulated, provide for both efficient and precise modulation of melanocyte or melanoma physiology.

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Supporting Information

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***In Vivo* Role of Alternative Splicing and Serine Phosphorylation of the Microphthalmia-Associated Transcription Factor**

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	Exon	Intron		Ex2A	Ex2B	<i>Apa</i> L1	S73
Consensus :	A/CAG	GURAGU					
	Ex2A	Ex2B					
Wild-type sequence:	CACCAG	GUAAAG					
	***	*****					
	H Q	V K					
Mutated sequence:	CAUCAA	GUUAAA					
	**	** *					
	H Q	V K					

	Ex2A	Ex2B	<i>Apa</i> L1	S73
Wild Type :	CACCAG	GTAAAG-----	AGCGCACCCAACAGCCCT	
S73A :	CACCAG	GTAAAG-----	AGTGCACCCAACGCCCT	
S-S73A :	CATCAA	GTATAA-----	AGCGCACCCAACGCCCT	
S-S73D :	CATCAA	GTATAA-----	AGCGCACCCAACGACCCT	
S-S73S :	CATCAA	GTATAA-----	AGCGCACCCAACAGCCCT	

Figure S1 Sequence changes at the 5' alternative splice site and codon-73. The exon 2A-2B junction closely matches the exon-intron consensus motif required for recognition by the spliceosome. Multiple nucleotide changes were made in order to alter this conserved motif while keeping the encoded protein sequence unchanged. In parallel, codon-73 was changed as indicated and the *Apa*L1 site present in *Mitf*^{mi-}_{S73A} was reverted to the wild-type sequence in the novel splice-mutant alleles.

A PCR of heart cDNA
(Mitf exon 2A to exon 3)

B qRT-PCR of Mitf 2B+ and Mitf 2B- cDNA from mutant mouse heart

C Relative Mitf expression
in heart tissue of the different
mutants

D Relative Mitf expression in hearts of lines
with or without the neo-cassette

E MITF expression in heart tissue of wild-type
C57BL/6 and homozygous mutant mice

F MITF expression in heart tissue of different
mutant combinations

Figure S2 (A) cDNAs generated from hearts of wild-type and homozygous knock-in mice were amplified using a primer pair across exons 2A and 3. Wild-type B6 cDNA (first lane) shows predominantly the 2B+ isoform while *Mitf*^{mi-S73A} cDNA (second lane) shows predominantly the 2B- isoform. cDNAs of each of the splice mutant lines show only the 2B+ isoform. Quantification by qRT-PCR, using appropriate mutant-specific primer pairs (see Suppl. Table 1) is shown in (B). (C) qRT-PCR of heart *Mitf* RNA from

homozygotes carrying the indicated alleles after removal of the neo cassette. The results were averaged from using primers spanning exon 6B-7 and 7-8 junctions and compared to those obtained from wild-type B6 and *Mitf*^{mi-S73AΔneo}/*Mitf*^{mi-vga9} mice, the latter known to express no *Mitf* RNA from the *Mitf*^{mi-vga9} null allele. The measurements showed no statistically significant changes in *Mitf* expression levels in the knock-in mice compared to wild type. (D) Effect of the neo cassette on *Mitf* expression in heart. The expression levels of *Mitf* 2B- in *Mitf*^{mi-S73ANeo} mice and *Mitf* 2B+ in *Mitf*^{mi-S-S73ANeo}, *Mitf*^{mi-S-S73DNeo} and *Mitf*^{mi-S-S73SNeo} mice (Neo) was compared to those of the corresponding mice lacking the neo cassette (ΔNeo). The results show a reduction of 50% to 75% of *Mitf* expression in hearts of mice carrying Neo-containing alleles. (E) MITF protein expression in heart tissue. Whole protein extracts were immunoprecipitated with a rabbit-anti-MITF serum from wild-type, *Mitf*^{mi-vga9}/*Mitf*^{mi-vga9} and ΔNeo knock-in mice, subjected to SDS-polyacrylamide gel electrophoresis, and probed with 6A5 antibodies, which recognize the C-terminal part of MITF protein. *Mitf*^{mi-S73A} hearts show a band corresponding to exon 2B- MITF in addition to possible degradation products and a background band representing IgG (arrows). Heart extracts from mice homozygous for the 3' splice mutation each show a major band corresponding to exon 2B+ MITF. Their expression levels relative to the internal β-Tubulin control and S-S73S as reference are shown below the gel. The S73A sample was not quantified because of the overlap of some MITF isoforms with the background IgG band. (F) Western blots of heart extracts of various mutants and their combinations. Protein extracts were prepared and processed as for (E). Alleles and allelic combinations are as indicated. Note MITF 2B+ phospho and non-phospho form in wild type (lane 1), absence of MITF in *Mitf*^{mi-vga-9} homozygotes (lane 2), and absence of MITF 2B+ phospho form in *Mitf*^{mi-S73AΔneo} homozygotes. Arrows indicate a band corresponding to IgG heavy chain.

Figure S3 Additional phenotypes associated with heteroallelic combinations between the targeted and extant *Mitf* alleles. The genotypes of the depicted mice are indicated. Note that in combinations with *Mitf*^{Mi-wh}, all four targeted alleles containing the Neo cassette produce a similar lightly tanned coat and variably sized white belly spots (A). In combination with *Mitf*^{mi}, the mice become largely white with small black spots that are most frequent with *Mitf*^{mi-S-S73Aneo} but totally absent in *Mitf*^{mi-S-S73Sneo} (B). (C,D) No phenotypic distinctions are seen in heteroallelic combinations with *Mitf*^{mi} or *Mitf*^{mi-vga9} once the neo cassette has been removed, except for *Mitf*^{mi-S73AΔneo}/*Mitf*^{mi}, *Mitf*^{mi-S-S73SΔneo}/*Mitf*^{mi}, and *Mitf*^{mi-S-S73SΔneo}/*Mitf*^{mi-vga9} mice, which displayed variable, though more extensive depigmentation of the tail tip compared to normal C57BL/6 mice.

Table S1 List of Primers Used

Primers for mutagenesis of <i>Mitf</i>^{mi-S-S73} alleles and pBABE constructs		Specific purpose
5' -CAGCAAGCTCAGAGGCATCAAGTTAAACAGTACCTTTCTACC-3' F 5' -GGTAGAAAGGTACTGTTTAACTTGATGCCTCTGAGCTTGCTG-3' R		Exon 2A-2B junction mutation
5' -CCAGTGCCGGGGAGCAGCGCACCCAACAGCCCTATGGCTATGC-3' F 5' -GCATAGCCATAGGGCTGTGGGTGCGCTGCTCCCCGGCACTGG-3' R		S73S codon mutation
5' -CCAGTGCCGGGGAGCAGCGCACCCAACGCCCTATGGCTATGC-3' F 5' -GCATAGCCATAGGGGCTGTGGGTGCGCTGCTCCCCGGCACTGG-3' R		S73A codon mutation
5' -CCAGTGCCGGGGAGCAGCGCACCCAACGACCTATGGCTATGC-3' F 5' -GCATAGCCATAGGGTCTGTGGGTGCGCTGCTCCCCGGCACTGG-3' R		S73D codon mutation
5' -CAGCAAGCTCAGAGGCACCAGGCATTTTATAAGTTTGAG-3' F 5' -CTCAAACTTATAAAATGCCTGGTGCCTCTGAGCTTGCTG-3' R		Δ2B mutation
Primers for <i>Mitf</i> sequence verification and cloning		
5' -AGCACAATGGCCAGTACCTT-3' F 5' -TTGGTGACAATGACCAGGTG-3' R		Genomic sequencing from M-Mitf Intron 1 to Intron 2
5' -TGCTGGAATGCTAGAATACAGTC-3' F 5' -TAGCTCCTTAATGCGGTCGT-3' R		cDNA sequencing from M-Mitf exons 1M to 7
5' -CATTTGGCTAAAGAGAGGCAGA-3' F 5' -CTAGCCTGCATCTCCAGCTC-3' R		cDNA sequencing from Mitf exons 6B to 9
5' -GGGCGGCAAATTGTTTTATAAAGC-3' F 5' -GAAGTCAGCAAATGGTGGTGG-3' R		5' <i>Bam</i> HI fragment
5' -TGCTTTAGGTAAGAAAGGACCAAG-3' F 5' -CGGCTCGTATGTTGTGTGGAA-3' R		3' <i>Bam</i> HI fragment
5' -ACGTGAATTCGTGATTTAAACATGGCAATATTTCTG-3' F 5' -ACGTAAAGCTTGGGGAACTTCCTGACTAGG-3' R		<i>Eco</i> RI- <i>Hind</i> III Mitf PCR cloning
Primers for qRT-PCR		
5' -AAGCTCAGAGGCACCAGGTAAAG-3' F 5' -ATGGTGAGCTCAGGACTTGG-3' R		qPCR Mitf 2B+
5' -GCTCAGAGGCACCAGGCATTTT-3' F 5' -GACGCTCGAGAGTGCGTGTT-3' R		qPCR Mitf 2B-
5' -GCAAGCTCAGAGGCATCAAGTTAAA-3' F 5' -ATGGTGAGCTCAGGACTTGG-3' R		qPCR Mitf 2B+ on mutated Exon2A-2B junction
5' -CAAGCTCAGAGGCATCAAGCATTTT-3' F 5' -GACGCTCGAGAGTGCGTGTT-3' R		qPCR Mitf 2B- on mutated Exon2A-2B junction
5' -CATTTGGCTAAAGAGAGGCAGA-3' F 5' -TAGCTCCTTAATGCGGTCGT-3' R		qPCR Mitf 6B - 7
5' -CCCCAAGTCAAATGATCCAG-3' F 5' -GCAACTTCCGGATGTAGTCC-3' R		qPCR Mitf 7 - 8
5' -GGACCTTGAAAACCGACAGAAG-3' F 5' -CTAGCCTGCATCTCCAGCTC-3' R		qPCR Mitf 8 - 9
5' -CAGGGCTCAGAGGCACTACT-3' F 5' -GCTCCCTCCCTGCAATACTT-3' R		qPCR USF1