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Temperature-sensitive splicing of *mitfa* by an intron mutation in zebrafish

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Dear *Editor*

Temperature-sensitive (*ts*) mutations have provided fundamental insight into our understanding of gene function *in vivo*. Although they are rare in higher organisms, temperature sensitive mutations in the pigmentation pathways include *tyrosinase ts* mutations found in oculocutaneous albinism (OMIM: 606952), in Siamese cats and the Himalayan mouse (Giebel et al., 1991), and in *kit* in zebrafish (Rawls and Johnson, 2001). Microphthalmia-associated transcription factor (*Mitf*) is the master melanocyte transcription factor that has critical functions in melanocyte development, melanocyte stem cell renewal and the tanning response, and is an important drug target (Hsiao and Fisher, 2014). Given the importance of *MITF*, it is critical to develop laboratory animals that enable conditional control of *MITF* activity in different temporal, cellular, developmental and disease contexts. Conditional or *ts* alleles in *MITF* have not been described in mammals. We (SLJ, JAL) have previously identified the first conditional *MITF* mutation (*mitfa^{vc7}*) in an animal in an ENU-based genetic screen in zebrafish, but the molecular mechanism underlying this mutation was unknown (Johnson et al., 2011). Here, we explain the molecular mechanism that enables the *mitfa^{vc7}* mutation to confer conditional temperature dependent control of *MITF* activity in zebrafish, and describe an unusual intron mutation that leads to aberrant splicing of wild type and dominant negative splice variants at the restrictive temperatures.

Most temperature-sensitive mutations affect exon sequences, but the *mitfa^{vc7}* mutation is unusual because it is in an intron splice donor (t>a at position 39638 in *mitfa* genomic locus or position 2 in intron 6) (Johnson et al., 2011). We collected wild type and *mitfa^{vc7}* mutant zebrafish embryos, and grew the animals at 24°C, 26°C, 28.5°C and 32°C. At higher temperatures zebrafish embryos grow more rapidly, and to compensate for this, for all experiments, zebrafish embryos were carefully stage-matched. At 32°C *mitfa^{vc7}* zebrafish embryos lack all neural crest derived melanocytes due to loss of *mitfa* activity (**Figure 1A**). At 24°C, wild type and *mitfa^{vc7}* zebrafish embryos develop similar numbers of clearly

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visible and pigmented melanocytes (Johnson et al., 2011), albeit with a delay in differentiation and/or cell size in the *mitfa^{vc7}* mutant embryos at two days post-fertilization (**Figure 1A**). We examined the *mitfa* RNA encompassing exons 4-7, and found correct splicing occurs at low (permissive) temperatures along with aberrant splicing, while aberrant splice forms predominate with increasing temperature (**Figure 1B**; as described in the Supplementary Methods). Temperature-sensitive splicing at this locus had not previously been detected (Johnson et al., 2011), possibly due to PCR conditions. We cloned each of the splice forms (labeled **a-c**), and found that the aberrant splice forms include those that skip exons or retain introns (**Figure 1C**). Splice form **a** includes intron 5 and 6 (*mitfa^{+in5,6}*), form **b** includes an in-frame intron 6 (*mitfa^{+intron6}*), and splice form **c** leads to an in-frame deletion of exon 6 (*mitfa^{ex6}*). At the highest temperature (32°C), almost no wild type *mitfa* or *mitfa⁺ⁱⁿ⁶* was detectable, while there was strong expression of *mitfa^{ex6}*. These results demonstrate that aberrant of the *mitfa* RNA correlates with the temperature sensitivity of the *mitfa^{vc7}* mutation.

Aberrant splice variants can have partial or neomorphic function. To establish if the splice variants had activity, we cloned the splice variants under the control of the *mitfa* promoter, and microinjected them into zebrafish null *mitfa* (the *mitfa^{vc7}* (*nacre*) mutation, a premature stop in exon 3, in which there are no neural crest derived melanocytes; Lister et al. 1999) (**Figure 1C**). Expression of wild type *mitfa* rescued the *nacre* mutation, and melanocytes were clearly visible at 5 dpf. In contrast, the *mitfa^{ex6}* isoform was unable to stimulate melanocyte development in *nacre* mutants. The *mitfa⁺ⁱⁿ⁶* splice variant was functional in this assay, albeit with significantly reduced activity compared to wild type *mitfa*. In addition, the *mitfa^{+in5,6}* isoform demonstrated significantly reduced activity compared to *mitfa* and compared to *mitfa⁺ⁱⁿ⁶*. At 32°C, *mitfa⁺ⁱⁿ⁶* was significantly reduced in its activity and *mitfa^{+in5,6}* had no function at all, demonstrating the temperature sensitivity of the splice products.

Given the activity of *mitfa⁺ⁱⁿ⁶*, and the very weak activity of *mitfa^{+in5,6}*, we hypothesized that some *mitfa* pre-RNA species can be correctly spliced in these transcripts to wild type *mitfa* and promote melanocyte development. To explore this idea, we examined the *mitfa* transcripts in the *mitfa⁺ⁱⁿ⁶* embryos at 24°C and 28°C and found transcripts that were the same size as wild type *mitfa*. However, the interpretation of this was complicated by the presence of *mitfa^{nacre}* transcripts that are also the same size as wild type *mitfa* (502 bp) (**Figure 2A**). Sequencing the 502 bp species in the *mitfa⁺ⁱⁿ⁶*-expressing *nacre* mutant embryos revealed both the *nacre* and wild type *mitfa* transcripts indicating that *mitfa⁺ⁱⁿ⁶* splice variants could be correctly spliced to the wild type form in the *nacre* embryos (**Figure 2B**). While not tested, we anticipate a similar mechanism explains the few melanocytes that develop in the *mitfa^{+in5,6}*-expressing *nacre* animals, rather than functional Mitfa activity of the protein product of the splice variant, because the encoded protein for the *mitfa^{ex6-9}* lacks the DNA binding and dimerization domains (**Figure 1C, D**). Subsequent PCR analysis suggests that the reduction of the *mitfa^{nacre}* transcript in the *mitfa⁺ⁱⁿ⁶* injected embryos (**Figure 2A**) is due to PCR template competition in favour of the ectopically expressed *mitfa⁺ⁱⁿ⁶*, rather than regulation of *mitfa^{nacre}* expression by Mitfa^{+In6} (*data not shown*).

Next, we altered the splice acceptor and donor sites to test if the *mitfa*⁺ⁱⁿ⁶ splice variants can have activity independently of splicing to the wild type form (**Figure 2C-E**). Restoration of the splice donor site (ga>gt) in the *mitfa*⁺ⁱⁿ⁶ cDNA was sufficient to restore Mitf activity in the transgene, while change to another nucleotide (ga>gg) resulted in further reduced activity (**Figure 2D**). In contrast, when we maintained the *mitfa*⁺ⁱⁿ⁶ mutation, but destroyed the 3' splice acceptor site (ag>tg) it was non-functional in the *nacre* mutants (**Figure 2E**). Taken together, these experiments indicate that the *mitfa*⁺ⁱⁿ⁶ can be spliced to the wild type *mitfa* form, and that the activity of *mitfa*⁺ⁱⁿ⁶ is due to the minor accumulation of the wild type species. The particular mutation (t>a) appears to be crucial to the unique temperature sensitive splicing.

MITF binds to DNA as a homodimer, and the aberrant splice variants could interfere with wild type MITF activity. Deletion of exon 6 is predicted to truncate the basic region (**Figure 1C**). The dominant negative alleles in mouse cluster in the basic region, which is necessary for DNA binding, while retaining dimerization capability (reviewed in Steingrimsson et al. 2004). Heterozygous *mitfa*^{vc7} mutants appear similar to wild type zebrafish at 5 dpf (Johnson et al., 2011), suggesting that the dominant negative activity of the *vc7* mutation is not sufficient to produce a robust phenotype by interfering with wild type protein. However, when combined with the weakly active *mitfa*^{z25} mutation (a substitution, 1219F, in the first helix of the HLH domain)(Johnson et al., 2011), the *mitfa*^{z25/vc7} mutant embryos failed to develop melanocytes at 32°C, while the *mitfa*^{z25/w2} mutant embryos were able to develop a few and weakly pigmented melanocytes (**Figure 2F**). These results indicate that the *mitfa*^{vc7} splice variants have some weak dominant interfering activity that may contribute to complete loss of *mitfa* activity at 32°C.

Temperature sensitive mutations are classic genetic tools that enable functional and temporal control of gene action. Our zebrafish *mitfa*^{vc7} temperature sensitive mutant is the only conditional MITF mutation in vertebrates, and has already provided insight into the function of MITF in melanocyte stem cells (Johnson et al., 2011), melanocyte development and differentiation (Johnson et al., 2011; Taylor et al., 2011), and in melanoma (Lister et al., 2014). Unusually, rather than increasing temperature affecting the protein function directly as is the case with most temperature sensitive mutations, *mitfa*^{vc7} is an intron mutation that leads to aberrant splice forms. Few examples of temperature sensitive splicing due to intron mutations are found in the literature. In human disease, temperature-dependent splicing in β -globin pre-mRNA of thalassemic patients is caused by a mutation in intron 2 (Gemignani et al., 2002), and temperature-sensitive aberrant splicing of Type 111 Procollagen transcripts is caused by mutations in splice-donor sites within introns in patients with Ehler-Danlos Syndrome Type IV (Lee et al., 1991; and references therein). In *Arabidopsis*, an exon mutation close to a 5'-splice site confers temperature-sensitivity of RNA splicing in the floral homeotic gene APETALA3 (called the *ap3-1* mutant) that controls stamen and petal development(Sablowski and Meyerowitz, 1998). Conditional mutants have been cleverly engineered by the addition of temperature sensitive DEGRON (Dohmen et al., 1994) and self-excising intein (excising protein) sequences (Tan et al., 2009; Zeidler et al., 2004). We have tested if the intron 6 can confer temperature sensitivity to GFP in zebrafish but have

thus far been unsuccessful: additional exonic sequences may be required to enable temperature-sensitive splicing to be engineered into other genes.

To conclude, our work explains the temperature sensitivity of the *mitfa*^{vc7} mutation to be due to an unusual intron 6 mutation that leads to reduced levels of wild type *mitfa* RNA. The production of interfering variants may also ensure that no melanocytes develop at 32°C. We suggest that the *mitfa*^{vc7} mutation compromises base-pairing with the small nuclear RNAs of the spliceosome, and that this interaction becomes destabilized at the restrictive temperatures. Alternative splicing is an integral feature of *MITF* pre-RNA processing, and gives rise to multiple MITF splice variants that are both melanocyte specific and relevant in melanocyte development and melanoma (Bharti et al., 2010; Cronin et al., 2009; Debbache et al., 2012; Simmons et al., 2014). Given the importance of MITF in melanocytes, the *mitfa*^{vc7} allele enables careful examination of MITF activity at multiple stages of melanocyte development, stem cells and melanoma, and is a unique means to explore the function of aberrant pre-RNA splicing in zebrafish.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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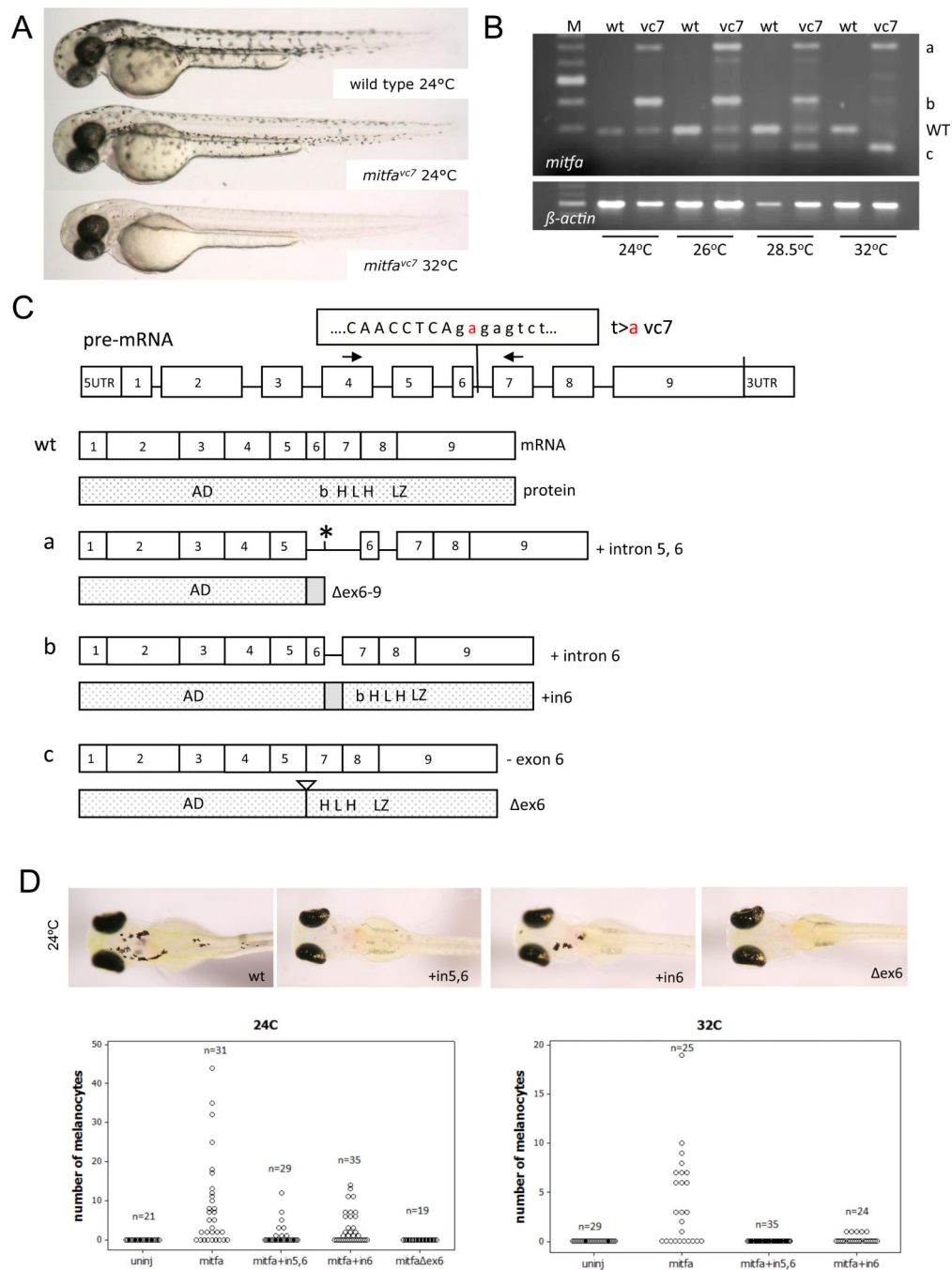


Figure 1. The *mitfa^{vc7}* intron 6 mutation causes defective temperature-dependent splicing of *mitfa*

A. Images of *mitfa^{vc7}* zebrafish embryos (2 days post fertilization) at 24°C (permissive temperature) and 32 °C (restrictive temperature). Pigmented melanocytes are clearly visible on the body of the zebrafish.

B. RT-PCR *mitfa* RNA expression at 24°C, 26°C, 28.5°C, and 32OC in wild type and *mitfa^{vc7}* mutant embryos. Four types of *mitfa* transcripts (wt, a, b, c) were consistently

observed in *mitfa^{vc7}* embryo across all temperatures while only one transcript (wt) was observed in wild type embryo.

C. Schematic overview of *mitfa^{vc7}* splice variants transcripts and their predicated MITF protein products.

D. Images and quantitation of zebrafish embryos following injection with transgenes expressing the *mitfa^{vc7}* splice variants from the *mitfa* promoter at 24°C and 32°C. At 24OC, the number of melanocytes promoted by expression of the *mitfa⁺ⁱⁿ⁶* splice variant (2.97 (2.27 to 3.67) [mean (95% CI)]) was significantly less active compared to wild type *mitfa* (8.71 (6.66 to 10.76) [mean (95% CI)]) (p=0.007; ANOVA). In addition, the number of melanocytes promoted by expression of *mitfa^{+in5,6}* (1.138 (0.64 to 1.636) [mean (95% CI)]) was significantly less than *mitfa* (p=0.001; ANOVA) and compared to *mitfa⁺ⁱⁿ⁶* (p=0.045; ANOVA).

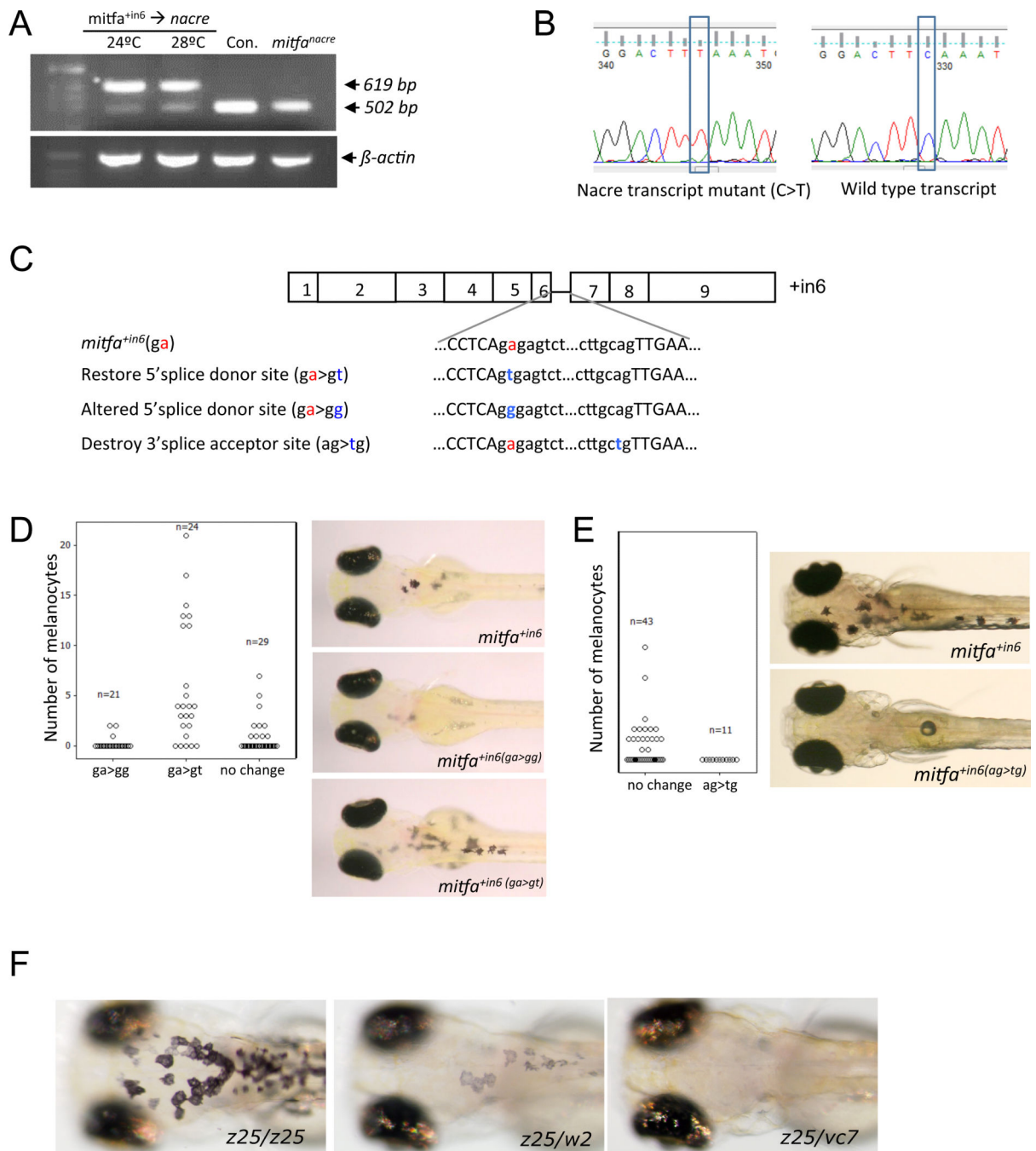


Figure 2. $mitfa^{vc7}$ temperature-sensitivity is determined by reduction of wild type $mitfa$ mRNA coupled with dominant negative activity of novel splice variants

A. RT-PCR analysis of $mitfa$ in $nacre$ mutant zebrafish expressing the $mitfa^{+in6}$ transgene, and in control wild type and uninjected $nacre$ mutant zebrafish. The correctly spliced $mitfa$ (502 bp) and the $mitfa^{+in6}$ variant (619 bp) are indicated.

B. Sequencing traces of the correctly spliced $mitfa$ PCR product identified in **A**. Cloned transcripts contained the $nacre$ transcript with a mutation C>T and wild type $mitfa$ correctly spliced from the $mitfa^{+in6}$ transcript.

C. Illustration of the *mitfa*⁺ⁱⁿ⁶ 5' splice donor site and the 3' splice acceptor site, and engineered mutations.

D. E. Quantitation and images of *nacre* zebrafish embryos: D. expressing the *mitfa*⁺ⁱⁿ⁶ transgene with the restored 5' donor site (A>T), the altered 5' donor site (A>G), E. and when the 3' splice acceptor site is destroyed.

F. Images of *mitfa* mutant zebrafish embryos at 32°C. *mitfa*^{z25} is a hypomorphic allele that results in fewer and pale melanocytes as *mitfa*^{z25/w2}, while no body melanocytes are present in *mitfa*^{vc7/z25}.