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The transcription factors *Ets1* and *Sox10* interact during murine melanocyte development

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ABSTRACT

Melanocytes, the pigment-producing cells, arise from multipotent neural crest (NC) cells during embryogenesis. Many genes required for melanocyte development were identified using mouse pigmentation mutants. The *variable spotting* mouse pigmentation mutant arose spontaneously at the Jackson Laboratory. We identified a G-to-A nucleotide transition in exon 3 of the *Ets1* gene in *variable spotting*, which results in a missense G102E mutation. Homozygous *variable spotting* mice exhibit sporadic white spotting. Similarly, mice carrying a targeted deletion of *Ets1* exhibit hypopigmentation; nevertheless, the function of *Ets1* in melanocyte development is unknown. The transcription factor *Ets1* is widely expressed in developing organs and tissues, including the NC. In the chick, Ets1 is required for the expression of *Sox10*, a transcription factor critical for the development of various NC derivatives, including melanocytes. We show that Ets1 is required early for murine NC cell and melanocyte precursor survival *in vivo*. Given the importance of *Ets1* for *Sox10* expression in the chick, we investigated a potential genetic interaction between these genes by comparing the hypopigmentation phenotypes of single and double heterozygous mice. The incidence of hypopigmentation in double heterozygotes was significantly greater than in single heterozygotes. The area of hypopigmentation in double heterozygotes was significantly larger than would be expected from the addition of the areas of hypopigmentation of single heterozygotes, suggesting that *Ets1* and *Sox10* interact synergistically in melanocyte development. Since *Sox10* is also essential for enteric ganglia development, we examined the distal colons of *Ets1* null mutants and found a significant decrease in enteric innervation, which was exacerbated by *Sox10* heterozygosity. At the molecular level, Ets1 was found to activate an enhancer critical for *Sox10* expression in NC-derived structures. Furthermore, enhancer activation was significantly inhibited by the *variable spotting* mutation. Together, these results suggest that *Ets1* and *Sox10* interact to promote proper melanocyte and enteric ganglia development from the NC.

Introduction

Melanocytes are derived from neural crest (NC) cells, a transient population of multipotent cells that arises from the dorsal aspect of the neural tube during vertebrate embryogenesis. In addition to melanocytes, other NC-derived lineages include endocrine cells, glial cells, neurons, craniofacial bone and cartilage, and cardiac cells (Le Douarin and Kalcheim, 1999). Defects in NC cell development result in a range of human disorders known as Neurocristopathies (Bolande, 1997). These include craniofacial malformations, cardio- and neurocutaneous syndromes, Waardenburg Syndrome, Hirschsprung's disease, and piebaldism, among others (reviewed in Hou and Pavan (2008) and Pingault et al. (2010)). Neurocristopathies are often characterized by pigmentation defects, hence the study of mouse pigmentation mutants has been instrumental for the identification of genes and pathways involved in the development of various NC cell lineages, including melanocytes (Tassabehji et al., 1992; Hosoda et al., 1994; Puffenberger et al., 1994).

The deletion of the transcription factor *Ets1* was recently shown to cause hypopigmentation in mice (Gao et al., 2010); however, the

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role of *Ets1* in melanocyte development is unknown. Ets1 is a member of a large family of helix-turn-helix transcription factors that are characterized by the presence of the E26 transforming specific sequence (Ets) domain, which recognizes the core nucleotide sequence GGAA/T (reviewed in Dittmer (2003)). Ets1 is required for the survival of various cell lineages, including thymocytes, T cells (Muthusamy et al., 1995), and endothelial cells (Wei et al., 2009). During embryonic development, *Ets1* is expressed in various organs and tissues, including the NC (Vandenbunder et al., 1989; Quéva et al., 1993; Kola et al., 1993; Maroulakou et al., 1994; Fafeur et al., 1997; Théveneau and Mayor, 2007). *Ets1* expression has also been correlated with pathological invasive processes. This transcription factor has been found to be upregulated in various tumor types, including melanoma (Keehn et al., 2003; Rothhammer et al., 2004, 2005; reviewed in Garrett-Sinha (2013)), and its expression in certain tumors has been correlated with the upregulation of matrix metalloproteases (MMPs), particularly MMP1, MMP2 and MMP9 (Rothhammer et al. (2004), and Okuducu et al. (2006); reviewed in Dittmer (2003)).

Several studies have implicated *Ets1* in the proper development of NC cell derivatives. During chick embryonic development, *Ets1* expression is correlated with epithelial-to-mesenchymal transition (EMT) (Fafeur et al., 1997), and although it is not required for EMT, Ets1 promotes cell mobilization, which is necessary for the initiation of cranial NC cell delamination (Théveneau et al., 2007). In the mouse, *Ets1* deletion was found to produce cardiac malformations (Gao et al., 2010; Ye et al., 2010). Some of these cardiac malformations were found to arise as a result of the lack of NC-derived cells in the proximal aspects of the outflow tract endocardial cushions and the presence of an abnormal nodule of NC-derived cartilage within the heart. These defects suggest a role for *Ets1* in proper NC cell migration and differentiation (Gao et al., 2010).

The exact mechanism by which *Ets1* deficiency during embryonic development results in hypopigmentation has not been elucidated. In the chick cranial NC, Ets1 is required for the activation of the transcription factor *sex determining region Y (SRY)-box 10* (*Sox10*) (Betancur et al., 2010), a gene required for the development of various NC cell derivatives, including melanocytes and enteric ganglia (Herbarth et al., 1998; Southard-Smith et al., 1998; Kapur, 1999; Potterf et al., 2001). In humans, mutations in *Sox10* are responsible for Waardenburg syndrome type IV, which is characterized by pigmentation defects and aganglionosis of the distal colon (Hirschsprung's disease) (Pingault et al., 1998). The requirement of Ets1 for *Sox10* expression in the chick embryo and the hypopigmentation phenotype of *Ets1* null mutants, suggest that *Ets1* may play an important role in the regulation of this and other melanocyte-specific genes in the mouse embryo.

In the present study, we report the identification of *Ets1* as the mutated gene in the spontaneous *variable spotting* mouse pigmentation mutant. This finding, along with the observation that mice carrying a targeted deletion of *Ets1* have belly spots (Gao et al., 2010), suggested a role for *Ets1* in melanocyte development. We characterized the temporal requirement of *Ets1* for melanocyte development and established a role for this transcription factor in NC and melanocyte precursor (melanoblast) survival. In light of the functional relationship that exists between *Ets1* and *Sox10* in the chick cranial NC, we hypothesized that, in the mouse, *Ets1* may interact with *Sox10* to promote melanocyte development. Analysis of the pigmentation phenotype of single and double heterozygous mutants for *Ets1* and *Sox10* revealed a synergistic genetic interaction between these transcription factors. Given the importance of *Sox10* for enteric ganglia development, we also investigated the effect of *Ets1* deficiency on enteric innervation. Analysis of the distal colons of *Ets1* null mutant mice revealed defects in enteric ganglia patterning, which were further exacerbated by *Sox10* heterozygosity, hinting to a potential interaction between *Ets1* and *Sox10* in the development of enteric ganglia. Using *in vitro* assays, we show that Ets1 is able to activate an enhancer critical for *Sox10* expression in NC-derived lineages. Additionally, mutating *Ets1*, at the site we characterized in the *variable spotting* mutant, reduces the ability of Ets1 to activate this *Sox10* enhancer. Together, our results indicate that *Ets1* is required early for NC cell and melanoblast survival. Furthermore, the presence of a synergistic genetic interaction between *Ets1* and *Sox10* in melanocyte development, and the ability of Ets1 to activate a *Sox10* regulatory region, suggests that at least some of the functions of Ets1 in melanocyte development may be mediated by Sox10.

Materials and methods

Ethics statement

All animal work was approved by the Florida International University Institutional Animal Care and Use Committee (Protocol no. 13-064) and performed according to institutional guidelines established by the National Institutes of Health (NIH) (Guide for the Care and Use of Laboratory Animals, 2011).

Exome and direct sequencing of the variable spotting mutation

A single heterozygous *variable spotting* sample was processed for exome sequencing. Whole genome libraries with ~170 base inserts and paired-end index adapters were prepared from 3 μg DNA using a SPRI-TE robot and reagents (Beckman-Colter). Libraries were enriched using the SureSelect Mouse All Exon Kit (Agilent) and pooled for sequencing in 2 lanes on a HiSeq2000 (Illumina) using version 3 chemistry. At least 50 million paired-end 100 base reads were obtained for each sample. Data was processed using RTA version 1.12.4.2 and CASAVA 1.7.0. Sequencing reads were aligned with ELAND, and then realigned with cross_match (http://www.phrap.org) to the UCSC mm9 mouse reference sequence. After removal of PCR duplicate reads, diploid genotypes were called using bam2mpg (Teer et al., 2010). Variants were annotated against the UCSC "known genes" gene set (Hsu et al., 2006) using ANNOVAR (http://www.openbioinformatics.org/annovar), and genotypes, gene annotations, and dbSNP (build 128) identifiers were reported in VarSifter format for viewing and filtering (Teer et al., 2012).

Filtering variants to those in coding exons or splice sites within the *variable spotting* interval (Chr 9 proximal to *Apoa1*; Spencer and Davisson, 1988) identified 2 variants with heterozygote genotype calls not present in dbSNP or 4 other unrelated mouse mutant exomes completed as part of the same project. The 2 variants were within *Mtmr2* (c.1757G > A:p.R586Q; CDPred score 1) and *Ets1* (c.305G > A:p.G102E; CDPred score −11). CDPred is an algorithm designed to predict the effect of amino acid substitutions (Johnston et al., 2010) with scores above −3, as observed for the *Mtmr2* variant, predicted to have a mild/neutral effect and scores below −7, as observed for *Ets1*, predicted to have a severe effect on protein function. The CDPred predictions are consistent with SIFT (Kumar et al., 2009), a second tool which predicts the *Mtmr2* variant to be tolerated (SIFT score = 0.07) compared to the deleterious *Ets1* variant (SIFT score = 0). To confirm *Ets1* as the gene responsible for the *variable spotting* phenotype we used an independent targeted *Ets1* allele to confirm that *Ets1* deficiency alone is sufficient to cause hypopigmentation, providing strong evidence that the *Ets1* variant is causative for the phenotype observed in *variable spotting* mice.

DNA samples from 2 heterozygous and 2 homozygous *variable spotting* mutant mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). A 496 bp DNA fragment surrounding the suspected mutation was amplified *via* PCR using the primers 5′-

AAGGTGTAGAGTAACTAGCATCGTCAG and 5′-AACTCCTAAGGCAGAGAAGAAAATAAG. PCR products were purified using ExoSAP-IT (Affimetryx), following the manufacturer's instructions, and directly sequenced. Sequences were analyzed using the Applied Biosystems Sequence Scanner v1.0 software (Applied Biosystems) and sequence alignments against the *Mus musculus* genomic database were carried out *via* the Nucleotide BLAST server (NCBI, NIH). Multiple alignments to the *Ets1* protein sequences for human, rat, zebrafish, dog, and mouse were carried out using ClustalW (via ExPASy.org).

Animals and genotyping

Ets1 mutant mice were a kind gift from Dr. Eric Svensson (University of Chicago, Chicago, IL). They were generated as previously described (Barton et al., 1998), and re-derived by outcrossing to C57BL/6J mice for eight generations (Gao et al., 2010). They have been maintained on C57BL/6J genetic background by intercrossing and by outcrossing to C57BL/6J mice (Jackson Laboratories). Mice carrying the *Dct-LacZ* transgene (*Tg(Dct-lacZ) #Ove*) were obtained from Dr. Paul Overbeek (Baylor College of Medicine, Houston, TX) and generated on an FVB/N genetic background, as previously described (Zhao and Overbeek, 1999). *Dct-LacZ* mice were maintained by intercrossing and bred with Ets1 heterozygotes to generate $Ets1^{+/-}$*::Dct-LacZ*$^{+}$ mice, which were intercrossed for the generation of embryos. $Sox10^{tm1Weg}/Sox10^{+}$ mice (hereafter referred to as $Sox10^{LacZ/+}$) were obtained from Dr. Michael Wegner (Institut für Biochemie, Universität Erlangen-Nürnberg, Germany) and generated on a mixed genetic background (129S1/Sv; C3HeB/FeJ; C57BL/6J), as previously described *via* insertion of the *LacZ* gene into the *Sox10* coding sequence (Britsch et al., 2001). $Sox10^{LacZ/+}$ mice were maintained by intercrossing and bred with *Ets1* heterozygotes to generate double heterozygotes.

Genomic DNA was isolated from tail biopsies or yolk sacs, and used for genotyping by PCR. Genotyping for *Ets1* was performed as previously described (Gao et al., 2010). For $Sox10^{LacZ}$, genotyping of was performed using the primers 5′-CAGGTGGGCGTTGGGCTCTT, 5′-CAGAGCTTGCCTAGTGTCTT, and 5′-TAAAAATGCGCTCAGGTCAA (Britsch et al., 2001). *Dct-LacZ* mice were genotyped *via* β-galactosidase staining of ear tips, using standard protocols (Nagy et al., 2003).

β-Galactosidase and Lysotracker Red staining of whole mouse embryos

Embryos from intercrosses between $Ets1^{+/-}$*::Dct-LacZ*$^{+}$ mice were harvested between embryonic days (E) 10.75–E15.5. Embryos from intercrosses between $Ets1^{+/-}$*::*$Sox10^{LacZ/+}$ mice were harvested at E9.5, E11.5, and E12.5. Whole embryos were subjected to β-galactosidase (LacZ) staining following standard protocols (Nagy et al., 2003). For each stage, at least 3 embryos of each genotype were stained and photographed. The staining was visualized under a Leica MZ6 dissecting scope and photographed using a Leica DC500 camera. In the case of E10.75 embryos, Lysotracker Red staining was carried out prior to β-galactosidase staining by incubating embryos in 5 μM Lysotracker Red DND-99 (Life Technologies) in PBS, protected from light, at 37 °C for 30 min. Subsequently, embryos were washed with PBS (pH7.2) and LacZ staining was performed.

Immunofluorescence

Freshly dissected E10.25 and E12.5 embryos were fixed in 4% paraformaldehyde for 3 and 16 h, respectively, at 4 °C, while E10.75, LacZ and Lysotracker Red-stained, embryos were post-fixed in 4% paraformaldehyde for 20 min at room temperature. Embryos were then washed with PBS and incubated at 4 °C in 10% sucrose for 4 h and in 20% sucrose, overnight. Embryos were embedded in Tissue-Tek Tissue Freezing Medium (Sakura), frozen at −80 °C, and sectioned at 10 μm. Sections were blocked with 1% Bovine Serum Albumin (BSA), 0.1% Triton X-100 in PBS for 45 min at room temperature and incubated in primary antibodies (diluted in blocking solution) for 1 h at room temperature. Primary antibodies include rabbit anti-phospho-Histone H3 (Ser10) (1:100; Millipore, 06-570), goat polyclonal anti-Sox10 (1:100; Santa Cruz Biotechnology, sc-17342), and rabbit anti-cleaved Caspase-3 (1:200; Cell Signaling, 9664S). After washing, sections were incubated with a suitable fluorescently-tagged secondary antibody (goat anti-rabbit Alexa Fluor 488 (1:200; Life Technologies, A11008), or donkey anti-goat Alexa Fluor 594 (1:200; Life Technologies, A11058)) for 1 h at room temperature. Sequential incubation in secondary antibodies (donkey anti-goat Alexa Fluor 594 followed by goat anti-rabbit Alexa Fluor 488) was carried out for Sox10 and cleaved Caspase-3 double-labeling in sections from E10.25 embryos. Cell nuclei were counterstained with 1 μg/ml Hoechst Dye (BioRad). The staining was visualized using a Leica DMRB compound fluorescent microscope. Sections were photographed using a Leica DC500 camera.

For E10.75 $Ets1^{-/-}$*::Dct-LacZ*$^{+}$ ($n=3$) and $Ets1^{+/-}$*::Dct-LacZ*$^{+}$ ($n=3$) embryos, melanoblasts (LacZ positive cells), dying melanoblasts (LacZ and Lysotracker Red positive cells), and proliferating melanoblasts (LacZ and phospho-Histone H3 positive cells) were counted in 80 sections spanning the trunk region of each embryo. For E10.25 $Ets1^{-/-}$($n=3$) and $Ets1^{+/-}$ ($n=3$) embryos, NC cells (Sox10 positive cells) and dying NC cells (Sox10 and cleaved Caspase-3 positive cells) were counted in 35 sections spanning the anterior half of the trunk region of each embryo. For E12.5 $Ets1^{-/-}$ ($n=3$) and $Ets1^{+/+}$ ($n=3$) embryos, Sox10 positive cells in the dorsal root ganglia were counted in 15 sections spanning the trunk region of each embryo. Data are shown as mean ± standard deviation. The Student's *t*-test was used to determine statistical significance. Data were considered statistically significant at $p < 0.05$

Phenotypic and statistical analyses

$Ets1^{+/-}$ mice were crossed to $Sox10^{LacZ/+}$ to generate progeny that were either $Ets1^{+/-}$, $Sox10^{LacZ/+}$, or $Ets1^{+/-}$*::*$Sox10^{LacZ/+}$. The single heterozygous progeny of the first generation (F_1) were intercrossed to generate the second generation (F_2) animals. Third generation animals (F_3) were generated by intercrossing single heterozygous as well as double heterozygous F_2 mice. For each generation, the ventral aspects of each mouse were photographed at 6 weeks of age. The areas of hypopigmentation were measured using the ImageJ 1.44p program (NIH).

Statistical differences for the frequency of belly spot appearance were determined using the Chi-square test. In order to compare the mean areas of hypopigmentation among the different genotypes, a Box–Cox transformation was applied to the belly spot area data (measured in mm^2). For each generation, the transformed areas of hypopigmentation of $Ets1^{+/-}$*::*$Sox10^{LacZ/+}$ mice were compared to the sum of the transformed areas of hypopigmentation of $Ets1^{+/-}$ and $Sox10^{LacZ/+}$ mice using a One-Way ANOVA with a contrast. Scatter plots of the hypopigmentation areas showing the mean and standard deviations for each group were generated using GraphPad Prism 6 (GraphPad Software, Inc.). Data were considered statistically significant at $p < 0.05$.

Colon dissection and Acetylcholinesterase staining

$Ets1^{+/-}$, $Sox10^{LacZ/+}$, and $Ets1^{+/-}$*::*$Sox10^{LacZ/+}$ mice were crossed to generate progeny of the following genotypes: $Ets1^{+/-}$*::*$Sox10^{+/+}$, $Ets1^{+/-}$*::*$Sox10^{LacZ/+}$, $Ets1^{-/-}$*::*$Sox10^{+/+}$, and $Ets1^{-/-}$*::*$Sox10^{LacZ/+}$.

Littermates of the genotypes listed above, ranging from 4 to 7 months of age, were euthanized and their colons dissected. Approximately 30 mm of the distal aspect of the colons of each animal were removed, cleaned, and fixed in 4% paraformaldehyde, containing 10 mM $CaCl_2$, overnight, at 4 °C. Subsequently, the colons were briefly washed with PBS (pH7.4) and incubated in 5% sucrose for 24 h. The colons were then washed with PBS and subjected to Acetylcholinesterase staining as previously described (Gariepy et al., 1998). The staining was visualized under a Leica MZ6 dissecting scope and photographed using a Leica DC500 camera. Colons from at least 5 animals of each genotype were stained and photographed. The extent of enteric innervation was quantified using the images captured, by measuring the white (nerve-free) area in 8 squares of 0.25 mm^2 each (for a total of 2 mm^2 per colon), within a region located 15 mm anterior to the anus. The ImageJ 1.44p program (NIH) was used to measure the white areas in each segment of colon. The Student's
t-test was used to compare the nerve-free areas of colons from mice of the various genotypes listed above. Scatter plots of the nerve-free areas in 2 mm^2 of colon for each genotype, showing the mean and standard deviations were generated using GraphPad Prism 6 (GraphPad Software, Inc.).

Vectors for luciferase assays

The *Ets1* overexpression vector (pCMV-HA-Ets1) and the pCMV-HA empty vector (Clontech) were kindly provided by Dr. Satrajit Sinha (State University of New York (SUNY) at Buffalo, Buffalo, NY). The pCMV-HA-Ets1 vector contains a Hyaluronic acid (HA)-tagged version of the full length mouse *Ets1* coding sequence (Nagarajan et al., 2009). The *Ets1* overexpression vector carrying the *variable spotting* mutation (pCMV-HA-Ets1Mut) was prepared from the PCMV-HA-Ets1 vector, using the Phusion Site-Directed Mutagenesis Kit (Thermo Scientific), following the manufacturer's instructions. The purified plasmid was sequenced to confirm the presence of the mutation. The *Sox10*-MCS4 luciferase vector (*Sox10*-MCS4 fragment in pLGF-E1b vector) was generated as previously described (Antonellis et al., 2008). The pRL-TK Renilla luciferase control vector (Promega) was used as an internal control for transfection efficiency.

Cell culture, transfections, and luciferase activity assays

The B16-F10 mouse melanoma cell line (ATCC, CRL-6322) was cultured under standard conditions. Cells were seeded in 12 well plates at a density of 1.5×10^5 cells per well, roughly 24 h prior to transfection. Once the cells reached 70–90% confluency they were co-transfected with 0.5 μg/well *Sox10*-MCS4 luciferase reporter vector, 0.5 μg/well pCMV-HA-Ets1, pCMV-HA-Ets1Mut or pCMV-HA empty vector, and 0.05 μg/well pRL-TK Renilla luciferase control vector, using the Lipofectamine 2000 lipid transfection reagent (Life Technologies), following the manufacturer's instructions. The cells were then incubated at 37 °C, 5% CO_2 for 24 h. After 24 h, the cells were gently washed with PBS (Thermo Scientific) and lysed with 1 × Passive Lysis Buffer (Promega), at room temperature. For each well, 20 μl of cell lysate were used to measure firefly and Renilla luciferase activities with the Dual Luciferase Reporter Assay System (Promega) following the manufacturer's instructions. Firefly and Renilla luciferase activities were measured using a TD-20/20 Luminometer (Turner Designs). For each experiment, the ratio of firefly to Renilla luciferase activity and the fold increase in this ratio over the ratio for the cells transfected with the PCMV-HA empty vector were calculated. Data represent mean fold activation over the PCMV-HA empty vector (bar height) and standard deviation (error bars) of three independent experiments carried out in quadruplicates. The Student's *t*-test was used to compare the mean fold activation in cells transfected with the *Ets1* overexpression vector to that of cells transfected with the *Ets1* overexpression vector carrying the *variable spotting* mutation.

Results

Variable spotting is caused by a novel missense mutation in the Ets1 gene

The spontaneous *variable spotting* mouse mutant exhibits a similar pigmentation phenotype to that of mice carrying a targeted deletion of the *Ets1* gene (Barton et al., 1998), which were used in this study. *Variable spotting* mutants present with white feet and tail, and occasionally head blaze and ventral spotting, which may be large (Jackson Laboratories). The *Ets1* null mutants always have large belly spots, hypopigmented tail and feet, and occasionally head blaze, while only roughly 40% of *Ets1* heterozygotes have belly spots, which tend to be small in size. As part of an exome sequencing project at the National Institutes of Health (NIH), a possible mutation within the open reading frame (ORF) of *Ets1* was identified in *variable spotting*. Through direct sequencing of heterozygous and homozygous mutants, we confirmed a nucleotide G-to-A transition in exon 3 of the *Ets1* gene (Fig. 1A), which results in a Glycine to Glutamate substitution at residue 102 (G102E) of the Ets1 protein. Multiple sequence alignment showed that this Glycine residue is highly conserved among different species (Fig. 1B), which suggests that it may be important for Ets1 protein function.

Ets1 is required for melanoblast and neural crest cell survival

The hypopigmentation phenotype observed in *Ets1* heterozygous and null mutant mice hinted to a potential role for *Ets1* in melanocyte development. In order to establish the earliest time point at which *Ets1* deficiency results in impaired melanocyte development, *Dct-LacZ* transgenic mice, in which *LacZ* expression is driven to melanoblasts under the control of the Dopachrome tautomerase (Dct) promoter (Zhao and Overbeek, 1999), were used to label melanoblasts *via* β-galactosidase activity in *Ets1* null, heterozygous, and wild type embryos. An apparent decrease in the number of trunk melanoblasts was observed in E11.5–E15.5 $Ets1^{-/-}$ embryos compared to $Ets1^{+/-}$ (Figs. 2, S1) and wild type littermates (data not shown).

The marked decrease in melanoblast numbers observed in $Ets1^{-/-}$ embryos implied that melanoblast survival and/or proliferation were likely impaired as a result of *Ets1* deletion. To establish the mechanism of action of *Ets1* in murine melanocyte development, the total numbers of trunk melanoblasts, as well as the numbers of dying and proliferating melanoblasts, were quantified at E10.75 (Fig. 3A – G). This embryonic stage was chosen because it was the earliest time point during which an apparent difference in melanoblast numbers could still be detected *via* LacZ staining. A significant decrease in the number of trunk melanoblasts was observed in $Ets1^{-/-}$ embryos compared to $Ets1^{+/-}$ littermates ($p=0.018$, Fig. 3E). At this stage, the percent of proliferating melanoblasts, which were identified by LacZ and phospho-Histone H3 double-labeling, did not differ significantly between $Ets1^{+/-}$ and $Ets1^{-/-}$ littermates ($p=0.852$, Fig. 3F). These results indicate that *Ets1* deficiency does not result in impaired melanoblast proliferation at E10.75. A significant increase in the percent of dying melanoblasts, which were labeled by LacZ and Lysotracker Red staining, was observed in $Ets1^{-/-}$ embryos compared to $Ets1^{+/-}$ littermates ($p=0.023$, Fig. 3G), suggesting that *Ets1* is required for melanoblast survival at this stage.

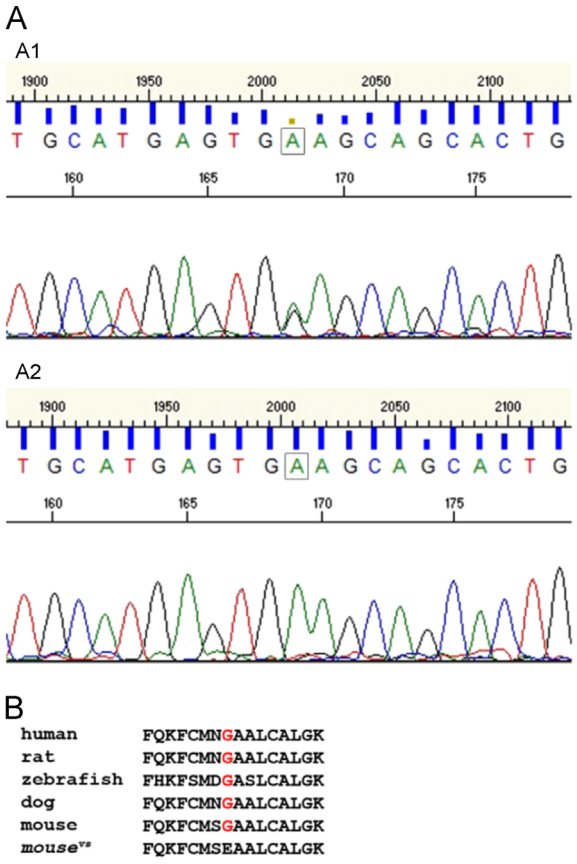

Fig. 1. *Variable spotting* is caused by a novel missense mutation in the *Ets1* gene. (A) Mutation analysis of *variable spotting* mice. Sequencing of heterozygous (A1) and homozygous (A2) *variable spotting* mutant mice revealed a G-to-A transition (boxes) in exon 3 of the *Ets1* gene at nucleotide position 647 (RefSeq NM_011808.2). This mutation results in a Glycine to Glutamate change at amino acid 102 (G102E) of the Ets1 protein. (B) Multiple sequence alignment of the Ets1 protein shows that the substituted Glycine residue (red font) is highly conserved among different species.

Given the increase in melanoblast cell death observed in $Ets1^{-/-}$ mutant embryos at E10.75, we investigated the possibility that *Ets1* may be required for NC cell survival prior to the specification of melanoblasts. To this end, we labeled NC cells and dying cells *via* Sox10 and cleaved Caspase-3 immunostaining at E10.25 in $Ets1^{-/-}$ and $Ets1^{+/-}$ littermates (Fig. 3H and I). The transcription factor *Sox10* was chosen as a NC cell marker because it is initially expressed in NC cells as they begin to delaminate from the neural tube and persists in cells that will give rise to peripheral nervous system components (Kuhlbrodt et al., 1998) and melanocytes (Southard-Smith et al., 1998). The total number of NC cells (Sox10 positive cells) present in the anterior half of the trunk region, did not differ significantly between $Ets1^{-/-}$ and $Ets1^{+/-}$ embryos ($p=0.848$, Fig. 3J). Nonetheless, the percent of dying NC cells in this region (Sox10 and cleaved Caspase-3 positive cells) was significantly higher in $Ets1^{-/-}$ embryos compared to $Ets1^{+/-}$ littermates ($p=0.033$, Fig. 3K), which suggests that Ets1 is required for NC cell survival at this stage.

Sox10 expression was also examined at E9.5 *via in situ* hybridization (Fig. S2) and LacZ staining (Fig. 6A and B) of whole embryos from intercrosses between $Ets1^{+/-}$ mice or $Ets1^{+/-}$::$Sox10^{LacZ/+}$ mice, respectively. At this stage, the pattern and levels of *Sox10* expression do not appear to differ between $Ets1^{-/-}$ embryos and $Ets1^{+/+}$ or $Ets1^{+/-}$ littermates. Nevertheless, *Sox10 in situ* hybridization revealed ectopic *Sox10* expression in the frontal aspect of the head in $Ets1^{-/-}$ embryos (Fig. S3B, red arrowhead). The lack of a significant difference in the numbers of NC cells between $Ets1^{-/-}$ and $Ets1^{+/-}$ embryos at E10.25, in spite of the increased number of dying NC cells in $Ets1^{-/-}$ embryos, implies that *Ets1* appears to act precisely around this time point so that the effects of *Ets1* deficiency become apparent soon after, when a sharp decrease in melanoblast numbers is observed. The fact that *Sox10* expression did not appear to be affected in *Ets1* null mutant mice at E9.5, suggests that the critical time point at which *Ets1* regulates NC cell and melanoblast survival is somewhere between E10 and E11.

Ets1 genetically interacts with Sox10 in melanocyte and enteric ganglia development

The increase in melanoblast and NC cell apoptosis observed in *Ets1* null mutants, indicates that Ets1 is required early for NC cell and melanoblast survival. Other genes required for melanoblast survival, include those that code for the transcription factors *Mitf* (*microphthalmia-associated transcription factor*), *Sox10*, and *Pax3* (*Paired-box 3*), and for the signaling molecules *Endothelin-3* (*Edn3*) and *Kit-ligand* (*Kitl*), and their receptors *Endothelin receptor b* (*Ednrb*) and *Kit* (reviewed in Silver et al. (2006)). Evidence from studies in the chick embryo pointed to a potential genetic interaction between *Ets1* and *Sox10* in melanocyte development. In the chick embryo, Ets1, along with Sox9 and c-Myb, directly binds to and activates a *Sox10* enhancer element that drives *Sox10* expression to cranial NC cells (Betancur et al., 2010). In order to determine whether a genetic interaction exists between *Ets1* and *Sox10* in murine melanocyte development, $Ets1^{+/-}$ mice were crossed to $Sox10^{LacZ/+}$ mice and the hypopigmentation phenotypes of the progeny were examined for three generations. For all generations, the frequency of belly spot appearance in double heterozygotes ($Ets1^{+/-}$::$Sox10^{LacZ/+}$) was significantly higher than in single heterozygotes ($Ets1^{+/-}$ and $Sox10^{LacZ/+}$) ($p<0.001$ for all generations, Fig. 4A). In the three generations examined, almost all $Ets1^{+/-}$::$Sox10^{LacZ/+}$ mice had belly spots (F_1: 97%, F_2: 96%, F_3: 100%), compared to approximately 40% for $Ets1^{+/-}$ mice and 48% of $Sox10^{LacZ/+}$ mice. In addition to showing increased hypopigmentation frequency, double heterozygous mice had belly spots that were significantly larger than would be expected from the addition of the areas of the belly spots of the single heterozygotes ($p<0.001$ for all generations, Fig. 4B–G). These results suggest that a synergistic genetic interaction exists between *Ets1* and *Sox10* in melanocyte development.

Mutations in the transcription factor *Sox10* are responsible for Waardenburg syndrome type IV (WS4), which is characterized by pigmentation defects and aganglionosis of the distal colon (Hirschsprung's disease) (Pingault et al., 1998). Mutations in *Sox10* have also been associated with non-syndromic Hirschsprung's disease (Sánchez-Mejías et al., 2010). Given the importance of *Sox10* for proper enteric innervation, and the synergistic genetic interaction between *Ets1* and *Sox10* in melanocyte development, we hypothesized that *Ets1* deficiency might also negatively affect enteric innervation. Megacolon was observed in 2 *Ets1* null mice, both of which were heterozygous for *Sox10* ($Ets1^{-/-}$::$Sox10^{LacZ/+}$) (Fig. S3). In both instances, the megacolon manifested early (3.5 and 12 weeks of age) and was found to be the result of severe hypoganglionosis of the distal colon (Fig. S3B and D). Nevertheless, megacolon was only observed in 2 of the 7 animals that were obtained for this genotype.

To further characterize the effect of *Ets1* deficiency on enteric ganglia patterning, the distal colons of animals from crosses between $Ets1^{+/-}$, $Sox10^{LacZ/+}$, and $Ets1^{+/-}$::$Sox10^{LacZ/+}$ mice were dissected and subjected to Acetylcholinesterase staining to label enteric ganglia (Fig. 5A–D). For each colon, the nerve-free (white) area in 2 mm^2 within a region located 15 mm anterior to the anus, was measured (Fig. 5E). The mean nerve-free area of colons from $Ets1^{-/-}$::$Sox10^{+/+}$ mice ($n=8$, mean $=1.402\pm0.048$ mm^2) was significantly larger than

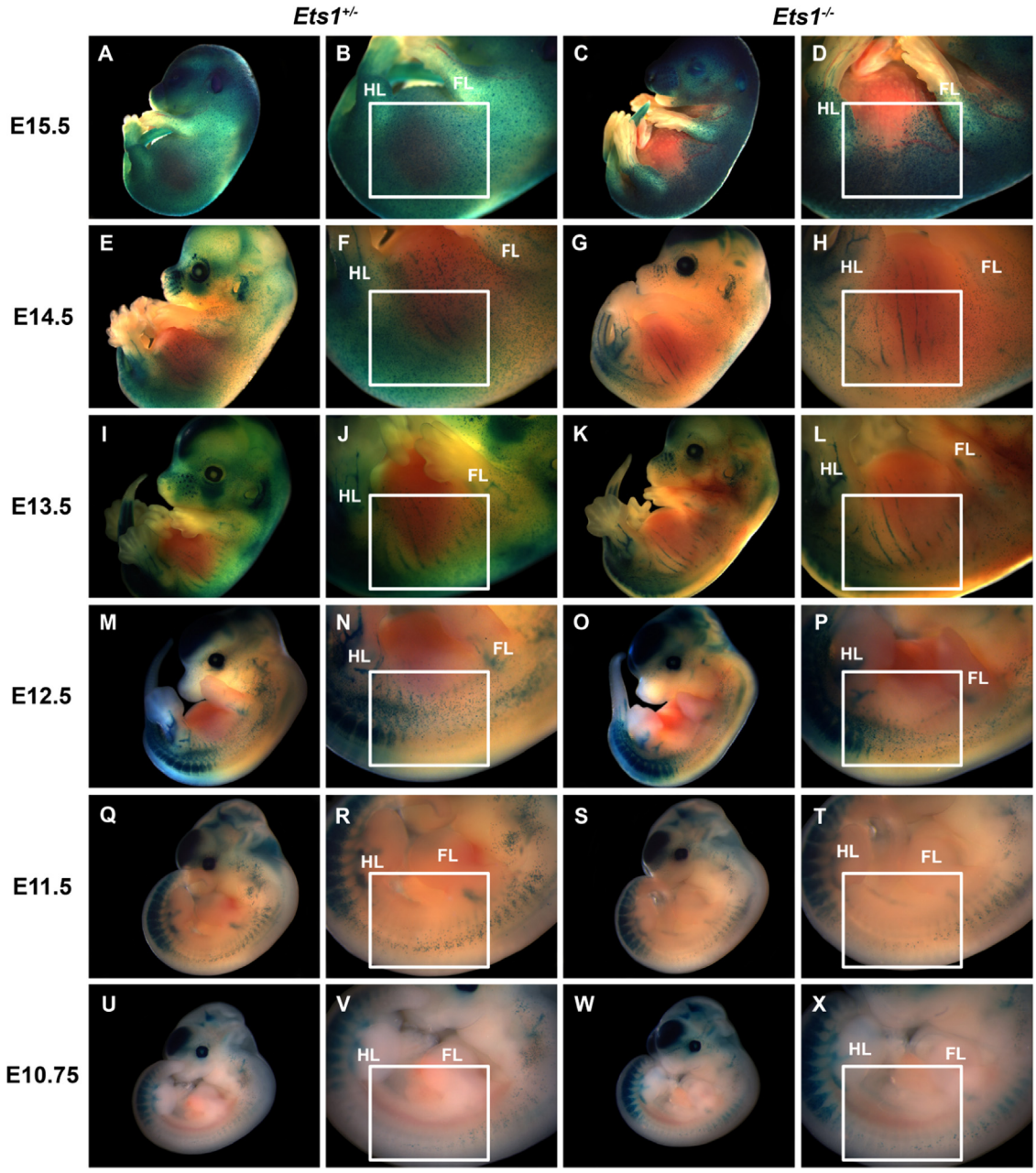

Fig. 2. *Ets1* null mutant embryos have decreased numbers of melanoblasts**.** *Dct-LacZ* transgenic mice, in which *LacZ* expression is driven by the *Dopachrome tautomerase (Dct)* promoter that drives expression to melanoblasts, were crossed to $Ets1^{+/-}$ mice to produce $Ets1^{+/-}$::*Dct-LacZ*$^{+}$ mice. Embryos from intercrosses between $Ets1^{+/-}$::*Dct-LacZ*$^{+}$ mice were harvested between embryonic days (E)10.75 – 15.5, subjected to LacZ staining and analyzed for the numbers and position of melanoblasts (blue dots) in the trunk region. At all these ages, $Ets1^{-/-}$ embryos have reduced numbers (predominantly in the areas contained within the white frames) of melanoblasts (C, D, G, H, K, L, O, P, S, T, W, X) compared to $Ets1^{+/-}$ (A, B, E, F, I, J, M, N, Q, R, U, V) and wild type (not shown) littermates (HL: Hindlimb, FL: Forelimb).

the mean nerve-free areas of colons from $Ets1^{+/-}$::$Sox10^{+/+}$ ($n=6$, mean $= 1.260 \pm 0.048$ mm^2, $p=0.001$) and $Ets1^{+/-}$:: $Sox10^{LacZ/+}$ ($n=5$, mean $= 1.291 \pm 0.075$ mm^2, $p=0.010$) mice (Fig. 5E), suggesting that *Ets1* deficiency results in impaired enteric ganglia patterning in the distal colon.

In order to explore the effect of *Sox10* heterozygosity, we compared the nerve-free areas of colons from $Ets1^{+/-}$ and $Ets1^{-/-}$ mice that were either wild type or heterozygous for *Sox10*. The mean nerve-free area in colons from $Ets1^{+/-}$::$Sox10^{+/+}$ mice did not differ significantly ($p=1.000$) from that of colons from $Ets1^{+/-}$::$Sox10^{LacZ/+}$ mice (Fig. 5E). Although *Sox10* heterozygosity did not significantly impact enteric ganglia patterning in *Ets1* heterozygotes, the colons of *Ets1* null mutants that were also heterozygous for *Sox10* had significantly larger nerve-free areas ($n=5$, mean $= 1.501 \pm 0.081$ mm^2) compared to those of *Ets1* null mutants that were wild type for *Sox10* ($p=0.017$, Fig. 5E). Thus, *Ets1* null mutants had nerve free areas that were significantly larger than *Ets1* heterozygotes when they were wild type or heterozygous for *Sox10*.

These results provide evidence to support the presence of a genetic interaction between *Ets1* and *Sox10* in the development of enteric ganglia. Although the colons from the two $Ets1^{-/-}$::$Sox10^{LacZ/+}$ animals in which megacolon was detected (Fig. S2) were not included in this analysis, as these were considered extreme cases, the occurrence of megacolon in these mice further supports the possibility that *Ets1* and *Sox10* interact in enteric ganglia development.

The synergistic genetic interaction observed between *Ets1* and *Sox10* pointed to a potential functional interaction between these

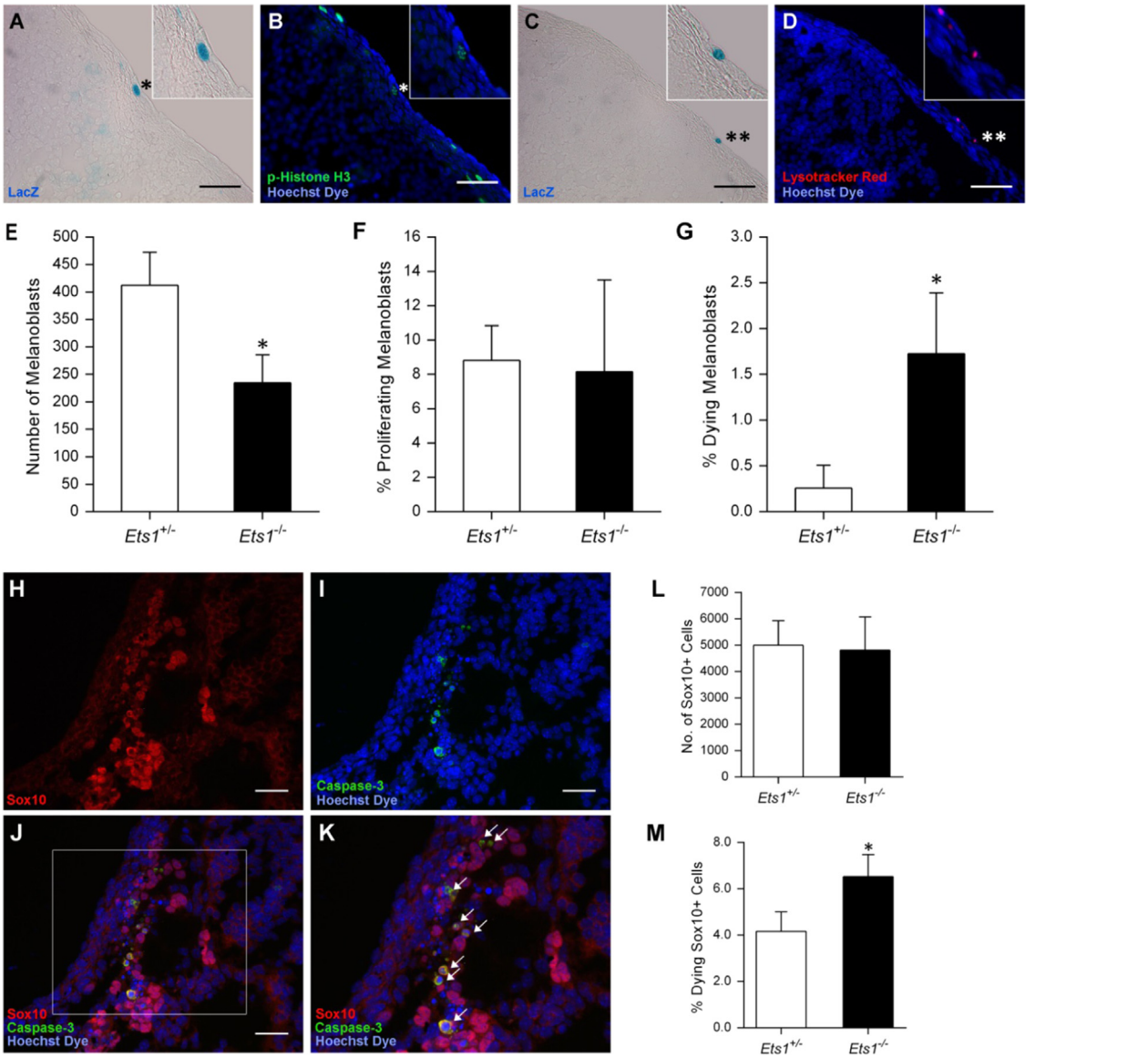

Fig. 3. *Ets1* is required for melanoblast and neural crest cell survival. (A, C) Melanoblasts traveling along the surface of the embryo are marked *via* LacZ staining (black asterisks). (B) Proliferating cells are positive for the phospho-Histone H3 (p-Histone H3) antibody (green fluorescence). (D) Lysotracker Red marks lysosomes in dying cells (red fluorescence). (A, B) Proliferating melanoblasts (*) are LacZ and p-Histone H3 positive. (C, D) Dying melanoblasts (**) are LacZ and Lysotracker Red positive. (E) Melanoblasts, (F) proliferating melanoblasts and (G) dying melanoblasts were counted in 80 sections for E10.75 $Ets1^{+/-}$ ($n=3$) and $Ets1^{-/-}$ ($n=3$) embryos. (E) $Ets1^{-/-}$ embryos have significantly reduced numbers of melanoblasts compared to $Ets1^{+/-}$ littermates ($^*p<0.05$). (F) The percent of proliferating melanoblasts did not differ significantly between $Ets1^{-/-}$ and $Ets1^{+/-}$ littermates ($p=0.852$), (G) while the percent of dying melanoblasts was significantly higher ($^*p<0.05$) in $Ets1^{-/-}$ embryos compared to $Ets1^{+/-}$ littermates. (H–K) Sox10 and cleaved Caspase-3 double-label immunofluorescence of E10.25 mouse embryos. Neural crest (NC) cells were labeled *via* Sox10 antibody staining (red fluorescence). Dying cells were labeled using a cleaved Caspase-3 antibody (green fluorescence). (J, K) Dying NC cells are positive for Sox10 and cleaved Caspase-3 (white arrows). (L) NC cells and (M) dying NC cells were counted in 35 sections spanning the anterior half of the trunk region for E10.25 $Ets1^{+/-}$ ($n=3$) and $Ets1^{-/-}$ ($n=3$) embryos. (J) The number of NC cells in $Ets1^{-/-}$ embryos is not significantly different from that of $Ets1^{+/-}$ littermates ($p=0.848$). (K) The percent of dying NC cells is significantly higher ($^*p<0.05$) in $Ets1^{-/-}$ embryos compared to $Ets1^{+/-}$ littermates (scale bars=50 μm).

transcription factors in melanocyte and enteric ganglia development. Given that *Ets1* is required for *Sox10* expression in the chick cranial NC (Betancur et al., 2010), it is possible that it could also be an important regulator of *Sox10* expression in the mouse embryo. In order to visualize gross changes in *Sox10* expression in *Ets1* null mutants, embryos from intercrosses between $Ets1^{+/-}$::$Sox10^{LacZ/+}$ mice were harvested at E9.5, E11.5, and E12.5 and *Sox10* expression was analyzed *via* LacZ staining. At E9.5, no apparent changes in LacZ staining were detected in $Ets1^{-/-}$ embryos compared to $Ets1^{+/+}$ (Fig. 6A and B) and $Ets1^{+/-}$ littermates (not shown). At E11.5, *LacZ* staining was reduced in 75% of the $Ets1^{-/-}$ ($n=4$) embryos (Fig. 6D) examined compared to $Ets1^{+/+}$ ($n=3$) (Fig. 6C) and $Ets1^{+/-}$ ($n=3$) (not shown) littermates. At E12.5, a decrease in LacZ staining was observed in 50% of $Ets1^{-/-}$ ($n=6$) embryos (Fig. 6F) compared to $Ets1^{+/+}$ ($n=3$) (Fig. 6E) and $Ets1^{+/-}$ ($n=9$) (not shown) littermates. The changes in LacZ staining observed in *Ets1* null mutant embryos suggest that *Ets1* may regulate *Sox10* expression during embryonic development. However, these changes could also be the result of a decrease in the number of NC cell derivatives, thus the numbers of Sox10 positive cells in the dorsal root ganglia, where LacZ staining was reduced, were quantified for E12.5 $Ets1^{-/-}$ ($n=3$) and $Ets1^{+/+}$ ($n=3$) embryos. The average number of Sox10 positive cells in the dorsal root ganglia was not significantly different ($p=0.753$) between $Ets1^{-/-}$ and wild type embryos, indicating that the decreased intensity in LacZ staining observed in $Ets1^{-/-}$ embryos is

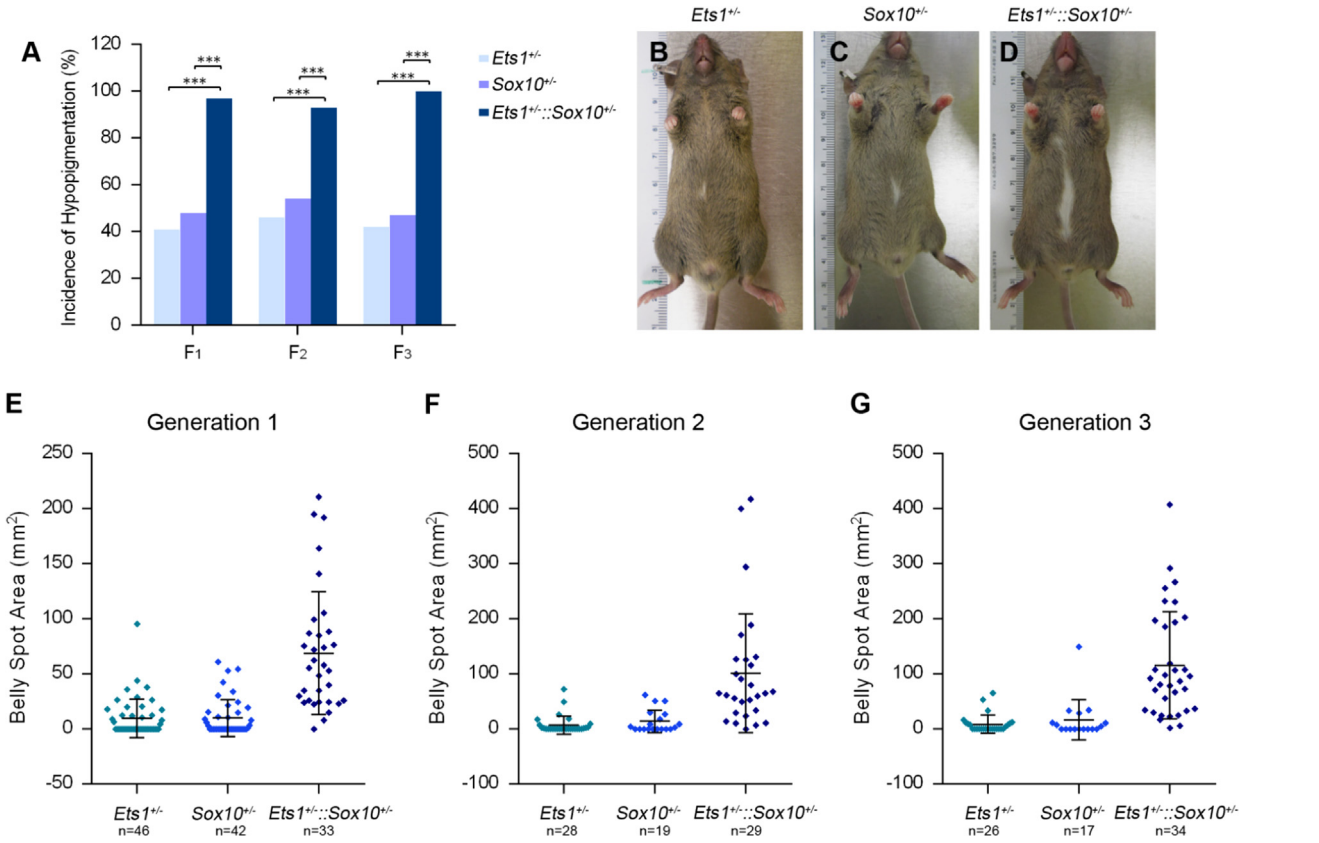

Fig. 4. *Ets1* and *Sox10* interact synergistically in melanocyte development. (A) The incidence of hypopigmentation in $Ets1^{+/-}$::$Sox10^{LacZ/+}$ mice was significantly greater than the incidence of hypopigmentation in $Ets1^{+/-}$ and $Sox10^{LacZ/+}$ mice ($p < 0.001$) for all three generations analyzed ($n \geq 17$ for each genotype, for all generations). (B, C, D) Representative pictures of ventral hypopigmentation for each genotype show that double heterozygous mice had larger areas of hypopigmentation compared to *Ets1* and *Sox10* single heterozygotes. (E, F, G) The areas of hypopigmentation in $Ets1^{+/-}$, $Sox10^{+/-}$, and $Ets1^{+/-}$::$Sox10^{LacZ/+}$ were measured for progeny from 3 generations. The transformed areas of the belly spots of $Ets1^{+/-}$::$Sox10^{LacZ/+}$ mice were significantly greater than the sum of the transformed areas of the belly spots of $Ets1^{+/-}$ and $Sox10^{LacZ/+}$ mice ($p < 0.001$) in all three generations examined.

not caused by a decrease in the number of Sox10-positive NC derivatives.

Ets1 transactivates a neural crest-lineage specific Sox10 regulatory region

Although many transcriptional targets of *Sox10* have been identified, the various aspects of *Sox10* regulation are only now beginning to be understood. The *Sox10* promoter, which has not been well characterized, does not direct the expression of *Sox10* to most of its typical expression sites (Deal et al., 2006). However, 14 multiple conserved sequences (MCS) that can drive *Sox10* expression to various cell lineages have been described (Antonellis et al., 2006, 2008; Deal et al., 2006). A subset of these sequences was first identified by mutation analysis of the transgene-insertion $Sox10^{Hry}$ mutant, a mouse model of WS4. In this mutant, a 16 kb area located 47 kb upstream of *Sox10* is deleted (Antonellis et al., 2008). This deletion severely impairs but does not completely abrogate *Sox10* expression, suggesting that this 16 kb region contains cis-regulatory elements necessary for *Sox10* expression. Of these elements, *Sox10*-MCS1c, *Sox10*-MCS4, *Sox10*-MCS5, *Sox10*-MCS7, and *Sox10*-MCS9 can drive reporter gene expression in cultured melanocytes. Furthermore, both *Sox10*-MCS4 and *Sox10*-MCS7 are able to recapitulate *Sox10* expression *in vivo*, in almost all NC-derived structures, including melanocytes and enteric ganglia, where *Sox10* is normally expressed (Antonellis et al., 2008). Analysis of the 820 bp sequence of the *Sox10*-MCS4 enhancer using the MatInspector software (Genomatix, Inc.) revealed the presence of 4 potential Ets1 binding sites (Table S1). The ability of Ets1 to activate the *Sox10*-MCS4 enhancer was tested *via* luciferase reporter assay in B16-F10 mouse melanoma cells. Compared to cells transfected with the empty PCMV-HA vector, cells transfected with an *Ets1* overexpression vector (PCMV-HA-Ets1) showed approximately a 6.5-fold increase in luciferase activity (Fig. 7).

In light of the fact that Ets1 is able to transactivate the *Sox10*-MCS4 enhancer in mouse melanoma cells, *Sox10* expression, as well as the expression of other melanoblast-specific genes, was evaluated in mouse melanocytes (melan-a) and melanoma cells (B16-F10) in which *Ets1* was either overexpressed or knocked-down. In mouse melanocytes, we failed to detect substantial changes in the expression levels of *Kit*, *Ednrb*, *Pax3*, *Mitf*, and *Sox10* in cells transfected with an *Ets1* overexpression vector, compared to cells transfected with an empty vector (Fig. S4B). Similarly, no considerable changes in the expression levels of *Kit*, *Pax3*, *Mitf*, and *Sox10* were detected in mouse melanoma cells overexpressing *Ets1* (Fig. S4D).

Given the lack of substantial changes in the expression of melanoblast-specific genes as a result of *Ets1* overexpression, we assessed the effect of *Ets1* knockdown on the expression of these genes. For both cell lines, no substantial changes on the expression levels of *Pax3*, *Mitf*, *Sox10*, and *Kit* were detected (Fig. S5). We observed a slight upregulation in *Ednrb* expression (fold change $= 2.006 \pm 0.087$), in mouse melanocytes transfected with

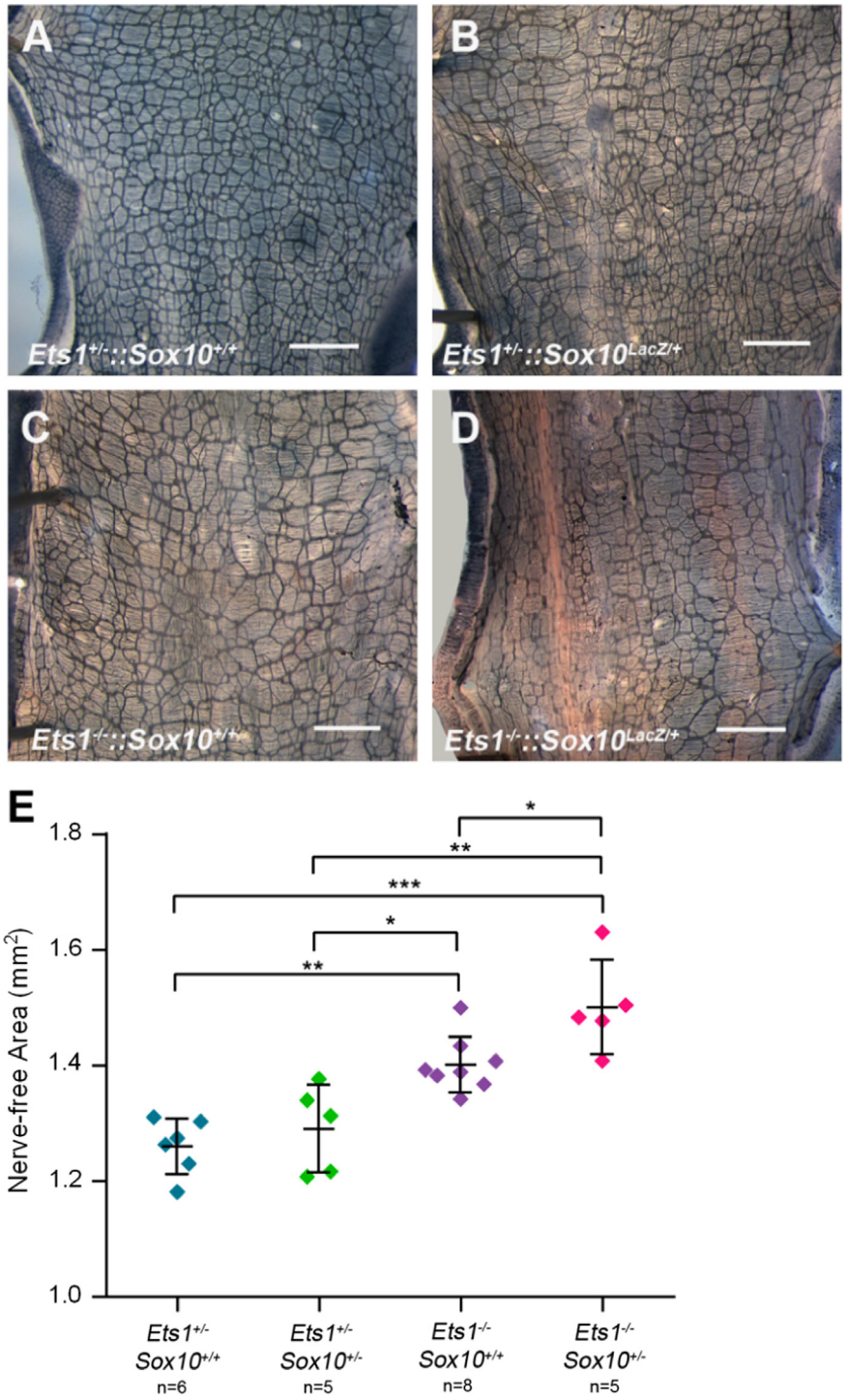

Fig. 5. *Ets1* null mutants show deffects in enteric ganglia patterning. (A–D) Representative images of enteric ganglia labeled *via* Acetylcholinesterase staining in the distal colons from (A) $Ets1^{+/-}$::$Sox10^{+/+}$, (B) $Ets1^{+/-}$::$Sox10^{LacZ/+}$, (C) $Ets1^{-/-}$::$Sox10^{+/+}$, and (D) $Ets1^{-/-}$::$Sox10^{LacZ/+}$ mice (scale bars = 1 mm). Scatterplot showing the nerve-free areas (including means and standard deviations) in 2 mm^2 regions of the distal colons from mice of each genotype. The mean nerve-free area in colons from $Ets1^{+/-}$::$Sox10^{+/+}$ mice did not differ significantly from that of $Ets1^{+/-}$::$Sox10^{LacZ/+}$ ($p = 1.000$) mice. The mean nerve-free area of colons from $Ets1^{-/-}$::$Sox10^{+/+}$ mice was significantly larger than the mean nerve-free areas of colons from $Ets1^{+/-}$::$Sox10^{+/+}$ (**$p < 0.005$) and $Ets1^{+/-}$::$Sox10^{LacZ/+}$ (*$p < 0.05$) mice. The mean nerve-free area of the colons from $Ets1^{-/-}$::$Sox10^{LacZ/+}$ mice was significantly greater compared to that of colons from $Ets1^{+/-}$::$Sox10^{+/+}$ (***$p < 0.001$), $Ets1^{+/-}$::$Sox10^{LacZ/+}$ (**$p < 0.005$), and $Ets1^{-/-}$::$Sox10^{+/+}$ (*$p < 0.05$) mice.

2 individual Ets1 siRNAs (Fig. S5C); nonetheless, this upregulation was not observed in cells transfected with an Ets1 siRNA pool (Fig. S5B). These results suggest that at least within the context of melanoma cells and differentiated melanocytes, Ets1 does not appear to regulate the expression levels of *Sox10*, *Pax3*, *Mitf*, *Kit*, and *Ednrb*.

The variable spotting mutation inhibits Sox10-MCS4 enhancer activation

The *variable spotting* mutation results in the substitution of a highly conserved Glycine (G102) residue. The G102 residue is present within the Pointed (PNT) domain of the Ets1 protein. This domain

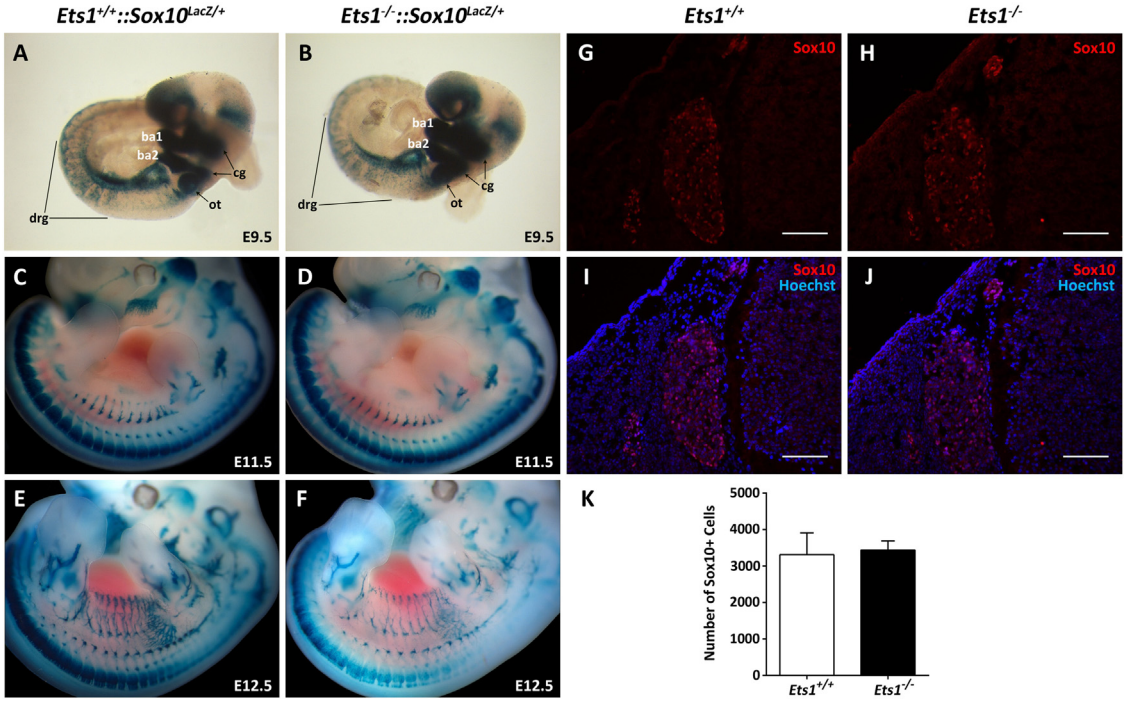

Fig. 6. *Sox10* expression is not altered at E9.5 but is reduced at E11.5 and E12.5 in some *Ets1* null mutant embryos. (A–F) *Sox10* expression was visualized *via* LacZ staining in embryos from intercrosses between $Ets1^{+/-}$::$Sox10^{LacZ/+}$ mice. (A, B) At E9.5, no gross differences were observed in LacZ expression between $Ets1^{-/-}$ and $Ets1^{+/+}$ or $Ets1^{+/-}$ (not shown) littermates. (C, D) At E11.5, LacZ expression was reduced in 75% of $Ets1^{-/-}$ embryos ($n=4$) (D), compared to $Ets1^{+/-}$ ($n=3$) (not shown) and $Ets1^{+/+}$ ($n=3$) (C) littermates. (E, F) At E12.5, a decrease in LacZ expression was observed in 50% of $Ets1^{-/-}$ ($n=6$) (F) embryos compared to $Ets1^{+/-}$ ($n=9$) (not shown), and $Ets1^{+/+}$ (E) ($n=3$) littermates. (G – K) Sox10 positive cells in the dorsal root ganglia were quantified in 15 sections spanning the trunk region for $Ets1^{+/+}$ ($n=3$) (G, I) and $Ets1^{-/-}$ ($n=3$) (H, J) embryos at E12.5. (K) At this stage, no significant differences in the numbers of Sox10 positive cells present within the dorsal root ganglia were observed between $Ets1^{+/+}$ and $Ets1^{-/-}$ littermates ($p=0.753$). (Magnifications: *A*, *B*=40 ×, *C*, *D*=25 ×, *E*, *F*=21 ×) (cg: cranial ganglia, ot: otocyst, drg: dorsal root ganglia, ba1: first branchial arch, ba2: second branchial arch) (scale bars=100 μm).

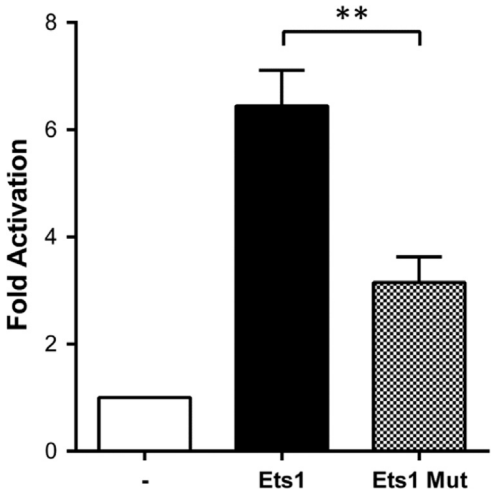

Fig. 7. Activation of the *Sox10*-MCS4 enhancer by wild type and mutant Ets1. B16 mouse melanoma cells were co-transfected with the *Sox10*-MCS4 luciferase reporter vector, the pRL-TK Renilla luciferase control vector, and either the PCMV-HA empty vector (–), or an expression vector encoding an HA-tagged version of the full length wild type (Ets1) or mutant (Ets1Mut) mouse *Ets1* coding sequence. Ets1 was found to activate the *Sox10*-MCS4 enhancer. The presence of the *variable spotting* mutation resulted in a significant decrease in *Sox10*-MCS4 enhancer activation (**$p < 0.005$). The firefly luciferase activity was normalized by Renilla luciferase activity. Data represent mean fold activation over the PCMV-HA empty vector (bar height) and standard deviation (error bars) of 3 independent experiments carried out in quadruplicates.

enhances the transactivation activity of Ets1 and contains a docking site for the mitogen-activated protein kinase (MAPK) ERK2 (Seidel and Graves, 2002). Docking of ERK2 at this site is critical for the phosphorylation of a threonine residue (T38) that is required for enhanced transcriptional activation by Ets1 *via* recruitment of the transcription factor CREB-binding protein (CBP) (reviewed in Garrett-Sinha (2013)). In order to test whether the *variable spotting* mutation affects the ability of Ets1 to activate the *Sox10*-MCS4 enhancer, the mutation was inserted into the *Ets1* ORF *via* site-directed mutagenesis. A significant decrease in relative luciferase activity was observed in cells transfected with the *Ets1* over-expression vector carrying the *variable spotting* mutation compared to those transfected with the wild type *Ets1* overexpression vector ($p=0.0022$) (Fig. 7). This decrease corresponds to approximately a 49% reduction in *Sox10*-MCS4 enhancer activation.

Discussion

In the present study, we provide evidence to support a role for the transcription factor *Ets1* in NC cell and melanoblast survival, and show that it interacts with *Sox10* to promote proper melanocyte and enteric ganglia development. In vivo analysis revealed a significant increase in melanoblast cell death and a concomitant decrease in melanoblast numbers as early as E10.75 in *Ets1* null mutants compared to heterozygous littermates. The early survival defect observed in the melanoblast population in *Ets1* null mutant mice suggests that *Ets1* may be involved in the regulation of one or more early melanoblast-specific genes. Given the increase in melanoblast cell death observed in *Ets1* null mutants at E10.75, it was plausible that *Ets1* may regulate the survival of NC cells prior to their specification into the melanocyte lineage. Although no significant differences in the numbers of trunk NC cells were observed between E10.25 *Ets1* null and heterozygous littermates, a significant increase in the number of dying NC cells was observed in *Ets1* null mutants. These results indicate that *Ets1* acts as a

survival factor for both NC cells, prior to their differentiation into the melanocytic lineage, and melanocyte precursors. Although the presence of viable melanocytes in adult *Ets1* null mutants indicates that *Ets1* is not essential for proper melanocyte differentiation, it is possible that the large decrease in melanoblast cell numbers observed in these mice could in part arise as a consequence of altered NC cell fate decisions. The presence of ectopic NC cell-derived cartilage nodules in the hearts of *Ets1* null mutant mice suggests that *Ets1* may act as a negative regulator of cartilage fate in NC cells (Gao et al., 2010). Consequently, in spite of the melanoblast survival defect observed in *Ets1* null mutants, we cannot discard the possibility that in addition to being required for melanoblast survival, *Ets1* may also be involved in NC migration and or in cell lineage specification.

Other pigmentation mutants in which melanoblast survival defects are observed as early as E10.5–E11.5 include mutants of the transcription factors *Sox10*, *Pax3*, and *Mitf* as well as mutants of the G-coupled Endothelin receptor b (*Ednrb*) and the tyrosine kinase receptor *Kit*. Mutations in these genes not only affect melanoblast survival, but also proliferation, migration and final differentiation (reviewed in Baxter et al., (2004) and Silver et al. (2006)). The transcription factors *Pax3* and *Sox10* are initially expressed in NC cells at E8.5 prior to their commitment into the melanocytic fate. *Mitf*, *Kit*, and *Ednrb*, on the other hand, are first expressed between E10 and E10.5, around the time of melanoblast specification from the NC (reviewed in Silver et al. (2006)). All of these genes are required for melanoblast survival and it is possible that the Ets1-induced effect on melanoblast survival is mediated *via* one or more of these critical melanocytic genes. Of these genes, we considered *Sox10* as the most likely downstream effector of *Ets1* based on evidence from studies in the avian system. In the chick embryo, Ets1 drives *Sox10* expression in cranial NC cells by directly binding to and activating the Sox10E2 enhancer, which is critical for the initiation of *Sox10* expression in cranial NC cells (Betancur et al., 2010). The hypopigmentation phenotype observed in *Ets1* null mutant mice results from defects in the development of melanocytes derived from trunk NC cells. As such, it is conceivable that in the mouse *Ets1* regulates *Sox10* expression in the trunk NC. In order to determine whether a genetic interaction exists between *Ets1* and *Sox10* in melanocyte development, we compared the pigmentation phenotypes of single and double heterozygous progeny from crosses between $Ets1^{+/-}$ and $Sox10^{LacZ/+}$ mice. $Ets1^{+/-}$ and $Sox10^{LacZ/+}$ mice were generated in different genetic backgrounds; for this reason, three generations of mice were examined in order to take into account the potential effect of background modifiers. A significant increase in the frequency of belly spot appearance was observed in double heterozygous mice compared to single heterozygotes. Analysis of the belly spot areas of each mouse revealed that the mean areas of hypopigmentation in double heterozygous mice were significantly larger than the sum of the areas of hypopigmentation in single heterozygotes. These results were consistent and significant across all generations and indicate that a synergistic genetic interaction exists between *Ets1* and *Sox10* in melanocyte development. The fact that the results were consistent across all generations suggests that the presence of strain-specific alleles did not seem to significantly affect the penetrance of the hypopigmentation phenotype in these mice.

Whether synergistic genetic interactions indicate the presence of an underlying functional relationship remains controversial (Perez-Perez et al., 2009). Synergistic genetic interactions identified through the study of double heterozygous pigmentation mutants have often been found to be the result of molecular interactions between the genes in question. The presence of a synergistic genetic interaction between the transcription factor *Mitf* and the tyrosine kinase receptor *Kit* was the first indication that these genes work together in the modulation of pigment production. Mice heterozygous for a semi-dominant *Mitf* mutation ($Mitf^{mi\text{-}wh/+}$) have a uniform ventral spot and a light coat color. Crossing $Mitf^{mi\text{-}wh/+}$ mice to mice heterozygous for a semi-dominant mutation in *Kit* ($Kit^{W\text{-}36H}$), which only have ventral spots and occasionally white feet and tail, resulted in an exacerbated pigmentation phenotype, which was more severe than expected if the hypopigmented areas of the single heterozygotes were added (Beechey and Harrison, 1994). Similar results were obtained when $Mitf^{mi\text{-}wh/+}$ mice were crossed to heterozygous mice in which the *LacZ* gene was inserted into the *Kit* locus ($Kit^{W\text{-}LacZ/+}$). Additional experiments demonstrated that signaling *via* the *Kit* receptor modulates *Mitf* and is required for Mitf-dependent induction of Tyrosinase (*Tyr*), a rate limiting enzyme in melanin production (Hou et al., 2000). The study of double heterozygous pigmentation mutants has also revealed the presence of synergistic interactions resulting from underlying molecular interactions between *Mitf* and the anti-apoptotic factor *Bcl2* (McGill et al., 2002) and between the transcription factors *Pax3* and *Kit* (Guo et al., 2010).

To determine whether the synergistic genetic interaction between *Ets1* and *Sox10* in melanocyte development could be the result of an underlying functional interaction, we examined *Sox10* expression in *Ets1* mutant mice using the $Sox10^{LacZ}$ transgenic system. A decrease in LacZ expression was observed in at least half of all E11.5 and E12.5 *Ets1* null embryos compared to *Ets1* heterozygous and wild type littermates suggesting that *Ets1* may play a role in the regulation of *Sox10* expression. In order to gain a better understanding of the underlying functional interaction that may exist between *Ets1* and *Sox10*, the ability of wild type and mutated Ets1 protein to activate the *Sox10*-MCS4 enhancer, which is critical for *Sox10* expression in mouse melanocytes (Antonellis et al., 2008) was investigated. Analysis of the *Sox10*-MCS4 sequence revealed the presence of four potential binding sites for Ets1 suggesting the possibility that Ets1 could regulate *Sox10* expression *via* this enhancer. Through luciferase reporter assays we showed that Ets1 is able to drive reporter gene expression through *Sox10*-MCS4. We also showed that the *variable spotting* mutation, which we determined to be a mutation in the *Ets1* ORF, results in a significant reduction in *Sox10*-MCS4 enhancer activation. These data suggest that the pigmentation defect observed in *Ets1* mutant mice may, at least in part, be the result of decreased *Sox10* expression. In spite of this, we did not observe substantial changes in *Sox10* expression in mouse melanocytes or melanoma cells in which Ets1 was knocked-down or overexpressed. It is possible that in the mouse melanocyte and melanoma cell lines used for these experiments other factors might be able to compensate for Ets1 deficiency. In the case of *Ets1* overexpression, the absence of a significant change in the expression of *Sox10* or other potential downstream targets could result if endogenous Ets1 protein is sufficient to drive the expression of said targets, and thus the regulatory regions of these genes are already saturated by the endogenous protein. Additional experiments in NC cells and melanoblasts, aimed at understanding the effects of Ets1 overexpression and knockdown on *Sox10* expression, are needed to further characterize the functional relationship between *Ets1* and *Sox10* in melanocyte development.

The presence of a spontaneous mutation in *Ets1* which results in a pigmentation phenotype provided the opportunity to validate the finding that Ets1 can drive expression through *Sox10*-MCS4. Although the G102E Ets1 mutant protein is able to activate the *Sox10*-MCS4 enhancer, reporter gene expression is reduced by approximately 49% compared to the wild type Ets1 protein. These results suggest that the *variable spotting* mutation is hypomorphic, because it reduces but does not completely abrogate Ets1 protein function. The Glycine residue mutated in the spontaneous *variable spotting* mutant is present within the PNT domain, an important functional domain of the Ets1 protein that enhances the transactivation activity of Ets1. The PNT domain contains a docking site for ERK2 (Seidel and Graves, 2002). Docking of ERK2 at this site is critical for the phosphorylation of a threonine residue that is required for enhanced transcriptional activation by Ets1 *via* recruitment of the

transcription factor CBP (reviewed in Garrett-Sinha, 2013). Mutating this ERK2 docking site was previously found to abrogate Ras/MAPK-mediated enhancement of Ets1 transactivation activity to the same degree as mutating the phosphoacceptor T38 residue (Seidel and Graves, 2002). Three residues within the PNT domain of Ets1, Leucine 114 (L114), L116 and Phenylalanine 120 (F120) have been shown to play a role in ERK2 binding (Seidel and Graves, 2002). Given the reduced Ets1 transactivation activity of the *variable spotting* mutant protein, it is possible that the G102 residue may be important for docking of ERK2 and T38 phosphorylation; nonetheless, the hypomorphic nature of the G102E mutation suggests that this residue although important, is likely not essential. It is also possible that the effect of the *variable spotting* mutation on *Sox10*-MCS4 activation is not related to ERK2 docking and subsequent T38 phosphorylation. Phosphorylation of T38 is important for Ets1 activation of Ras-responsive genes (Seidel and Graves, 2002). To date, no evidence exists to support a direct role for the MAPK pathway in *Sox10* activation in the melanocyte lineage. Additionally the G102E mutation is located outside of the (114) LXLXXXF(120) domain, which is crucial for ERK docking (Dittmer, 2003). Additional studies are needed to dissect the specific mechanism *via* which the *variable spotting* mutation results in decreased *Sox10*-MCS4 activation.

In addition to being required for proper melanocyte development, *Sox10* is also required for the development of enteric ganglia (Southard-Smith et al., 1998). In the spontaneous embryonic lethal $Sox10^{Dom/Dom}$ mutant mouse, NC cells fail to colonize the intestine as a result of increased apoptosis (Kapur, 1999). This may be the result, at least in part, of decreased activation of the tyrosine kinase receptor c-Ret, which is also required for the development of enteric ganglia, and has been shown to be activated by Pax3 and Sox10 (Lang et al., 2000). The requirement of *Sox10* for the survival of enteric ganglia precursors prompted us to examine the colons of *Ets1* null mutant mice to determine whether *Ets1* deficiency would negatively affect enteric innervation. *Ets1* null mice had significantly decreased enteric innervation in the distal colon, compared to heterozygotes, indicating that *Ets1* may also be important for the development of enteric ganglia. In view of the role of *Ets1* in NC cell survival, it is possible that the defect in enteric ganglia patterning observed in *Ets1* null mutant mice may be the result of a reduction in the number of NC cells available to colonize the distal colon.

Throughout the course of the present study, two instances of severe hypoganglionic megacolon were observed in *Ets1* null mutant mice, which were also heterozygous for *Sox10* ($Ets1^{-/-}::Sox10^{LacZ/+}$) (Fig. S2). Although no significant difference in the extent of enteric innervation was observed between $Ets1^{+/-}::Sox10^{+/+}$ and $Ets1^{+/-}::Sox10^{LacZ/+}$ mice, $Ets1^{-/-}::Sox10^{LacZ/+}$ mice were found to have colons with greater nerve-free areas compared to $Ets1^{-/-}$ mice that were wild type for *Sox10*. The significant decrease in enteric innervation observed in $Ets1^{-/-}::Sox10^{LacZ/+}$ mice compared to $Ets1^{-/-}::Sox10^{+/+}$ may be the result of a synergistic interaction between *Ets1* and *Sox10*. In light of the absence of a significant difference in enteric innervation between $Ets1^{+/-}::Sox10^{+/+}$ and $Ets1^{+/-}::Sox10^{LacZ/+}$ mice and the embryonic lethality of $Sox10^{LacZ/LacZ}$ mutants, it was not possible to directly test the presence of a synergistic genetic interaction using double null mutant mice. Nevertheless, the decreased enteric innervation observed in $Ets1^{-/-}::Sox10^{LacZ/+}$ mice compared to $Ets1^{-/-}::Sox10^{+/+}$ mice and the presence of hypoganglionic megacolon in 2 of the 7 $Ets1^{-/-}::Sox10^{LacZ/+}$ mice examined suggests that *Ets1* and *Sox10* may interact in the development of enteric ganglia. Our finding that *Ets1* promotes the survival NC cells is also consistent with this hypothesis. A decrease in enteric progenitor cell survival has been described in *Sox10* heterozygous mice that are also homozygous for the mutant $Ednrb^{sl/sl}$ (*piebald*) or $Edn3^{ls/ls}$ (*lethal spotting*) alleles. Compared to single mutants, these animals show a dramatic decrease in the number of enteric neuronal progenitor cells in the digestive tract, which results from increased apoptosis in vagal NC cells prior to the time when they begin to invade the digestive tract at E9.5–10 (Stanchina et al., 2006). In *Sox10* homozygous mutants, increased apoptosis of enteric ganglia precursor cells also results in the failure of these cells to colonize the intestines (Kapur, 1999).

Taken together, the results of this study suggest that *Ets1* is required for proper NC cell and melanoblast survival, possibly acting upstream of *Sox10*. Our data indicate that *Ets1* and *Sox10* act synergistically during melanocyte development and may also interact in enteric ganglia development. Furthermore, the ability of Ets1 to activate a *Sox10* regulatory region that is required for *Sox10* expression in melanocytes and enteric ganglia, suggests that the synergistic genetic interaction that exists between *Ets1* and *Sox10* may be the result of an interaction at the molecular level. Additional studies are needed to determine if this interplay also acts in other neural crest derivatives. In the present study, we also identified a spontaneous *Ets1* mouse pigmentation mutant, and characterized the negative effect of this mutation on the ability of Ets1 to activate a *Sox10* regulatory region. Additional studies are warranted to characterize the molecular aspects of the interaction between *Ets1* and *Sox10* and the mechanism *via* which the *variable spotting* mutation may impair this interaction. The results of this study in combination with the future work proposed will provide a better understanding of the gene regulatory network directing melanocyte development. A detailed characterization of this network is critical because alterations in genes required for the development of melanocytes and other NC cell derivatives have been recognized in several human pathological conditions, including cancer.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2015.04.012.

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