A sensitized mutagenesis screen identifies \textit{Gli3} as a modifier of \textit{Sox10} neurocristopathy

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Haploinsufficiency for the transcription factor \textit{SOX10} is associated with the pigmentary deficiencies of Waardenburg syndrome (WS) and is modeled in \textit{Sox10} haploinsufficient mice (\textit{Sox10}$^{lacZ+/−}$). As genetic background affects WS severity in both humans and mice, we established an \textit{N-ethyl-N-nitrosourea} (ENU) mutagenesis screen to identify modifiers that increase the phenotypic severity of \textit{Sox10}$^{lacZ+/−}$ mice. Analysis of 230 pedigrees identified three modifiers, named modifier of \textit{Sox10} neurocristopathies (\textit{Mos1}, \textit{Mos2} and \textit{Mos3}). Linkage analysis confirmed their locations on mouse chromosomes 13, 4 and 3, respectively, within regions distinct from previously identified WS loci. Positional candidate analysis of \textit{Mos1} identified a truncation mutation in a hedgehog(HH)-signaling mediator, GLI-Kruppel family member 3 (\textit{Gli3}). Complementation tests using a second allele of \textit{Gli3} (\textit{Gli3}$^{Xt-J}$) confirmed that a null mutation of \textit{Gli3} causes the increased hypopigmentation in \textit{Sox10}$^{lacZ+/−}$;\textit{Gli3}$^{Mos1+/−}$ double heterozygotes. Early melanoblast markers (\textit{Mitf}, \textit{Sox10}, \textit{Dct}, and \textit{Si}) are reduced in \textit{Gli3}$^{Mos1+/−}$ embryos, indicating that loss of GLI3 signaling disrupts melanoblast specification. In contrast, mice expressing only the GLI3 repressor have normal melanoblast specification, indicating that the full-length GLI3 activator is not required for specification of neural crest to the melanocyte lineage. This study demonstrates the feasibility of sensitized screens to identify disease modifier loci and implicates \textit{GLI3} and other HH signaling components as modifiers of human neurocristopathies.

INTRODUCTION

Waardenburg syndrome (WS) describes a specific group of neurocristopathies whose phenotypes are primarily caused by melanocyte deficiencies. The features of WS include skin hypopigmentation (leukoderma), pigment defects of the choroid and iris, and deafness from loss of inner ear melanocytes. The presence of additional phenotypic anomalies results in four clinically distinct WS types that have been associated with mutations in at least six genes essential for the development of the neural crest (NC), a population of pluripotent cells that differentiates into many functionally diverse cell types. The identified WS genes include paired box gene 3 (\textit{PAX3}), microphthalmia-associated transcription factor (\textit{MITF}), snail homolog 2 (\textit{SNAI2}), endothelin-3 (\textit{EDN3}), endothelin B receptor (\textit{EDNRB}), and Sry-like HMG box 10 (\textit{SOX10}) (1–9). In particular, mutations in \textit{SOX10} have been identified in type II WS (WS2) and type IV WS (WS4), which combine features of WS with absence of enteric neurons (aganglionosis) that is characteristic of Hirschsprung disease (HSCR; OMIM 142623).

\textit{Sox10} is an HMG-box containing transcription factor that is expressed during embryonic development in pre-migratory NC and in a subset of migrating NC cells including those that give rise to the glial and melanocyte lineages (reviewed in 10). The melanocyte precursor cells, melanoblasts, express \textit{Mitf} as they migrate along a dorso-lateral route away from the neural tube. Further differentiation is marked by the subsequent expression
of the melanosomal protein silver (Si, also known as Pmel17) and the melanogenic enzyme dopachrome tautomerase (Dct) as melanoblasts proceed along their route to the ectoderm where a subset of these colonize the basal epidermis and hair follicles and are readily visible as terminally differentiated melanocytes due to their pigment production (reviewed in 11). Because of its role in multiple NC lineages, Sox10 is essential for development, and mice carrying heterozygous Sox10 mutations have NC defects resembling those of W54, including hypopigmentation and aganglionic megacolon (9,12,13). Similar to humans with WS, the mouse models of WS (14) show phenotypic variability among individuals with the same mutation. Together with WS patients where no mutation has been identified, this phenotypic complexity suggests that additional WS loci remain to be discovered. Mice with Sox10 mutations offer a unique tool to identify these additional WS loci and explain their mode of action.

In this study we designed a sensitized ENU modifier screen to identify loci that increase the NC defects, specifically white spotting (hypopigmentation), associated with mutations that modify the severity of hypopigmentation in affected founder animal, providing evidence for linkage to Chr 4 in a genome scan utilizing 91 SSLP markers in this region (0/9 recombinant; 0.01). For mapping Mos1, nine mice were used for an initial genome scan (six affected Sox10LacZ+/Gli3+/- mice plus three obligate Mos1+/+ heterozygous carriers). Genotyping of 220 SNPs with distinct alleles in C57BL/6J and BALB/cJ identified a 20 Mb region of proximal mouse Chr 13 where all nine animals had inherited the BALB/cJ allele from the affected founder animal, providing evidence for linkage to markers in this region (0/9 recombinant; P < 0.01).

A similar mapping strategy was used to localize Mos2 and Mos3 to Chr5 and 3, respectively. For Mos2, we detected linkage to Chr4 in a genome scan utilizing 91 SSLP markers to genotype eight affected animals. Subsequent genotyping of affected animals (N = 31) in this region localized Mos2 to 35 Mb region of Chr4 flanked by markers D4Mit9 (1/31 recombinant; P < 0.0001) and D4Mit203 (8/31 recombinant; P < 0.01). For Mos3, genotyping 408 informative SNPs in five affected animals revealed weak linkage to two regions of the genome. Subsequent genotyping of SSLPs spanning those regions in additional affected animals (N = 32) confirmed linkage to a 20 Mb region of Chr3 flanked by markers D3Mit178 (2/32 recombinant; P < 0.001) and D3Mit65 (3/32 recombinant; P < 0.0001). The genomic locations of Mos1, Mos2 and Mos3 did not overlap with orthologs of WS genes (Fig. 1B), suggesting they are potentially novel WS modifier loci.

**RESULTS**

A sensitized mutagenesis screen to identify modifiers of SOX10

A breeding strategy was designed to identify ENU-induced mutations that modify the severity of hypopigmentation in Sox10LacZ+ mice (Supplementary Material, Fig. S1). BALB/cJ male mice were given 3-weekly ENU injections, allowed to recover fertility, and then mated with C57BL/6J females to generate first generation (G1) offspring. G1 males were subsequently mated with Sox10LacZ+/5,1/lacZ+/- heterozygous females (13), herein referred to as Sox10LacZ+/-, because they contain a targeted disruption of the endogenous Sox10 locus that drives expression of a β-galactosidase reporter gene from the Sox10 promoter and predisposes the mice to NC defects. Second generation (G2) offspring were then examined for increased severity of neurocristopathies in Sox10LacZ+ mice, as measured by increased hypopigmentation.

Incorporation of two different inbred strains in this screen made efficient mapping of the BALB/cJ mutagenized allele possible in subsequent crosses to C57BL/6J. Since genetic background could affect the hypopigmentation of the Sox10LacZ+ animals, we performed a control-cross in which non-ENU-treated BALB/cJ males were substituted for the ENU-treated BALB/cJ males. For these and all subsequent crosses, the extent of ventral hypopigmentation was scored according to a standardized scale (0–4), with 0 representing no hypopigmentation and 4 representing the most severe ventral hypopigmentation (Supplementary Material, Fig. S2). The control-cross demonstrated minimal variation in hypopigmentation among Sox10LacZ+ G2 offspring on a BALB/cJ; C57BL/6J mixed genetic background. The control G2 offspring (N = 111) received ventral hypopigmentation scores of 0 (79%) or 1 (21%) and dorsal hypopigmentation was never observed. These results showed that genetic background effects from allelic variance between BALB/cJ and C57BL6/J would not interfere with identification of novel, ENU-induced gene mutations affecting pigmentation.

In total, 230 G1 male offspring were generated and at least three litters of G2 offspring from each G1 male were screened for increased hypopigmentation. Seven G1 pedigrees produced one or more Sox10LacZ+ G2 offspring with increased ventral hypopigmentation (score ≥2) accompanied by dorsal hypopigmentation, neither of which was observed in the control cross. In three of these seven G1 pedigrees the observed phenotype was reproducible in subsequent generations, indicating a heritable, mendelian phenotype. These three loci were named modifier of Sox10 1, 2 and 3 (Mos1, Mos2, and Mos3) (Fig. 1A).
Mos1 increases the severity of hypopigmentation in Sox10LacZ+/mice

Sox10LacZ+/;Mos1/+ affected mice exhibited severe ventral hypopigmentation that often extended onto the dorsal surface forming a belt in the lumbar region (Fig. 2A). To confirm that Mos1 exacerbated the NC defects in Sox10LacZ+/mice, the penetrance and expressivity of Mos1 alone and in conjunction with Sox10LacZ+/+ was analyzed after breeding the Mos1/+ founder onto a C57BL/6J background (Fig. 2B and C). Analysis of G3 and G4 offspring showed that with respect to hypopigmentation, Mos1 alone acts as a semidominant mutation that is not fully penetrant. A portion (37.7%) of heterozygous Mos1/+ mice exhibited ventral hypopigmentation ranging in size from 1 to 3 (Fig. 2). Double heterozygous Sox10LacZ+/;Mos1/+ mice exhibited a significant, synergistic increase in hypopigmentation compared with either single heterozygous Mos1/+ or single heterozygous Sox10LacZ+/+ mice (P < 0.05) (Fig. 2). Unlike the majority (75.8%) of Sox10LacZ+/+ heterozygotes, minimal hypopigmentation (0–1) was not seen in any double heterozygous mice. Ventral hypopigmentation that extended over the dorsal surface to form a belt was seen in 17% of Sox10LacZ+/;Mos1/+ mice, but was never observed in Mos1/+ mice or in Sox10LacZ+/+ mice. These results indicate that the ENU-induced mutation Mos1 enhances the melanocyte defects of Sox10LacZ+/mice, acting in synergy with Sox10 haploinsufficiency to produce more severe NC abnormalities.

Mos1 is a novel, nonsense mutation of Gli3

Because 95% (84/88) of Mos1/+ animals had limb defects resembling the semidominant polydactyly previously identified in Extratoes (Gli3<sup>x</sup>) (15,16), a classic mouse mutant located on Chr 13, we assessed if the Gli3 transcription factor was mutated in Mos1 animals. Genomic DNA from an affected Mos1/+ mouse was analyzed by direct sequencing of all coding exons and intron/exon junctions of Gli3 and compared with sequences of BALB/cJ and C57BL6/J control samples. A single sequence variation was identified in which Mos1 DNA differed from the parental DNA at a single nucleotide in exon 8 (Fig. 3A).
The *Mos1* allele carried a nucleotide substitution (1148C>A) predicted to replace Tyr350 with a stop codon (Tyr350Stop; Y350X). Using a real-time PCR genotyping assay, we confirmed that this mutation was not detected in BALB/cJ or C57BL/6J control DNAs and consistently segregated with the *Mos1* phenotype in 100% of animals tested (N = 130). The location of the *Gli3*^Mos1^ nonsense mutation is upstream of the zinc-finger binding domain, suggesting it would disrupt both the activator and repressor forms of GLI3 (Fig. 3B). This is supported by comparison with two other *Gli3* mouse alleles, *Gli3*^Xt-J^ and *Gli3*^D699^, and to published human GLI3 mutations in Pallister-Hall syndrome (PHS, OMIM 146510) (17) and Greig cephalopolysyndactyly syndrome (GCPS, OMIM 175700) (18). Comparison shows that *Gli3*^Mos1^ is most similar to *Gli3*^Xt-J^ and to GCPS patients with deletions, translocations and point mutations within or upstream of the zinc-finger binding domain (19). Therefore, we propose that *Gli3*^Mos1^ acts as a loss of function allele.

**Analysis of *Gli3*^Xt-J^ confirms that *Mos1* phenotypes result from mutation of *Gli3***

The *Gli3*^Xt-J^ allele of *Extratoes* is a deletion that results in phenotypes similar to those in human GLI3-associated disorders,
including craniofacial defects, brain abnormalities and polydactyly (20). Gli3<sup>Xt-J</sup> homozygotes die in utero or at birth with gross polydactyly, multiple craniofacial defects, and exencephaly while Gli3<sup>330</sup>-<sup>690</sup> heterozygotes are viable but exhibit an enlarged interfrontal bone and preaxial polydactyly. To determine whether the ENU-induced mutation in Gli3 causes the Mos1 phenotype, several analyses were performed to compare the Mos1 phenotype with the Gli3<sup>330</sup>-<sup>690</sup> phenotype. First, to examine if Gli3<sup>Mos1/Mos1</sup> homozygotes show embryonic lethality similar to that reported for Gli3<sup>Xt-J/Xt-J</sup>, Gli3<sup>Mos1</sup>/<sup>Mos1</sup> mutant mice were intercrossed and genotype classes were examined at weaning. Of 24 progeny recovered at weaning, no homozygotes were identified, indicating that the Gli3<sup>Mos1</sup> mutation is lethal in homozygotes (0/24 offspring; P < 0.005). Next, allelism was assessed by genotyping the viable offspring from a complementation cross between mice heterozygous for each allele (Gli3<sup>Mos1/+</sup> X Gli3<sup>Xt-J/+</sup>). Gli3<sup>Mos1</sup>-<sup>330</sup>-<sup>690</sup> compound heterozygotes were never observed at weaning (0/32 offspring; P < 0.01), confirming that Gli3<sup>Mos1</sup> and Gli3<sup>Xt-J</sup> are allelic. Finally, heterozygous Gli3<sup>Xt-J</sup>/+ mice were mated with Sox10<sup>-</sup><sup>lacZ/</sup> mice, and offspring of each genotype class was analyzed for hypopigmentation. Similar to Sox10<sup>LacZ/</sup>;Gli3<sup>Mos1/</sup> mice, Sox10<sup>LacZ/</sup>;Gli3<sup>Xt-J/</sup> double heterozygous mice showed a significant increase in the penetrance and severity of hypopigmentation compared with Gli3<sup>Xt-J/</sup> or Sox10<sup>LacZ/</sup> heterozygous animals (Fig. 4). Taken together these results show that Gli3 is functionally disrupted in Mos1 mice causing the increased hypopigmentation observed in Sox10<sup>LacZ/</sup>;Gli3<sup>Mos1/</sup> mice.

**Figure 3.** A nucleotide substitution in Mos1 lies in the N-terminal region of the Gli3 coding sequence. (A) Sequencing of BALB/c control and Mos1 heterozygote genomic DNA reveals a C to A substitution at nucleotide position 1148 (arrows) resulting in a nonsense mutation at codon 350 (Tyr350Stop) in the Mos1 allele. The heterozygous Mos1 trace shown contains both C (wild-type sequence of BALB/c) and A (mutant sequence of Mos1) at position 1148. (B) Graphic representation of full-length Gli3 protein and 3 truncated proteins resulting from mutant mouse alleles of Gli3. The zinc finger domain (ZFD) (34) and proteolytic cleavage site (C) (26) are indicated along with a region of the protein important for transactivation (TA) that spans several fragments independently shown to function in transactivation (64–66). While the Gli3<sup>Mos1</sup>-<sup>330</sup>-<sup>690</sup> allele results in a truncated protein that lacks the full ZFD and is a loss of function allele, Gli3<sup>Mos1</sup>-<sup>330</sup>-<sup>690</sup> retains the ZFD, and thus functions as a transcriptional repressor. Mutations in the middle third of the human GLI3 gene (bracketed) are predicted to produce truncated functional repressor proteins causing Pallister-Hall syndrome (PHS). The location of the Mos1 mutation would result in a truncated protein lacking the ZFD, and is predicted to be a loss of function allele similar to Gli3<sup>Mos1</sup>-<sup>330</sup>-<sup>690</sup> and also to human Greig cephalopelysyndactyly, which is caused by mutations in the human gene that fall outside the bracketed PHS region (19).

**Figure 4.** Gli3 deficiency disrupts development of Sox10<sup>-</sup><sup>lacZ/</sup>-expressing cells

To further assess the effects of the Gli3<sup>Mos1</sup> mutation as a modifier of NC defects, Sox10<sup>LacZ/</sup>;Gli3<sup>Mos1/</sup> mice were crossed to either Sox10<sup>LacZ/</sup>;Gli3<sup>Mos1/</sup> or Gli3<sup>Mos1/</sup> mice, and embryos were collected for morphological and histological analyses. At E11.5, we observed craniofacial anomalies including microphthalmia and malformation of the diencephalon and mesencephalon in Sox10<sup>LacZ/</sup>;Gli3<sup>Mos1/Mos1</sup> embryos (Fig. 5). These phenotypes are consistent with those in Sox10<sup>LacZ/</sup>;Gli3<sup>Mos1/Mos1</sup> embryos and what has been previously described for Gli3<sup>Mos1/Mos1</sup> embryos (15). The patterning of the Sox10<sup>LacZ/</sup>-expressing dorsal root ganglia (DRG), sympathetic ganglia and cranial ganglia, visualized by LacZ staining, appeared unaffected in Sox10<sup>LacZ/</sup>;Gli3<sup>Mos1/Mos1</sup> embryos as compared with Sox10<sup>LacZ/</sup> control embryos (Fig. 5). In contrast, there was a striking reduction in the number of Sox10<sup>LacZ/</sup>-expressing melanoblasts within the medial lateral trunk of Sox10<sup>LacZ/</sup>;Gli3<sup>Mos1/Mos1</sup> embryos (Fig. 5D–I). Notably, in regions lacking Sox10<sup>LacZ/</sup>-expressing melanoblasts, there were ectopically located Sox10<sup>LacZ/</sup> expressing cells positioned dorsal to the DRG that were larger than Sox10<sup>LacZ/</sup>-expressing melanoblasts (Fig. 5E and H, arrowheads). Sox10<sup>LacZ/</sup>;Gli3<sup>330</sup>-<sup>690</sup> mutant embryos also exhibited reduced Sox10<sup>LacZ/</sup>-expressing melanoblasts and large ectopic Sox10<sup>LacZ/</sup>-expressing cells in their trunks, confirming that these phenotypes resulted from GLI3 deficiency (Fig. 5F and I).

**Mos1 mutant embryos show dramatic reduction in early stage melanoblasts**

To further investigate the reduction in melanoblasts observed in the mid-trunk of Sox10<sup>lacZ/</sup>;Gli3<sup>Mos1/Mos1</sup> mutants, we next evaluated expression of the early melanoblast markers Mitf and Si in homozygous Gli3<sup>Mos1/Mos1</sup> embryos (Fig. 6). In E11.5 wild-type embryos, numerous melanoblasts were present in the head, cervical region, tail and trunk. As was observed with Sox10<sup>LacZ</sup> expression, Mitf- and Si-positive melanoblasts were reduced in number in the mid-trunk of mutant Gli3<sup>Mos1/Mos1</sup> embryos compared with wild-type embryos (Fig. 6). These data demonstrate that GLI3 deficiency alone disrupts normal trunk melanoblast specification. No ectopic expression was observed for Mitf or Si, suggesting that the ectopic Sox10-positive cells in Sox10<sup>lacZ/</sup>-expressing Gli3<sup>Mos1/Mos1</sup> embryos (Fig. 5) were not specified melanoblasts, but instead represent other SOX10-expressing NC derivatives, potentially melanoblast precursors.

**Table 1.** Allelic complementation analysis

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<td>Sox10&lt;sup&gt;LacZ/&lt;/sup&gt;;Gli3&lt;sup&gt;Mos1/Mos1&lt;/sup&gt;</td>
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To determine if the melanoblast deficiency in GLI3-deficient embryos persists beyond E11.5, the effect of the GLI3Mos1 mutation on the melanoblast lineage was examined at multiple developmental stages. For this the Tg(Dct-LacZ) line of transgenic mice was used in which the Det promoter drives expression of LacZ in melanoblasts, caudal embryonic DRG, and the telencephalon (21–23). As with other melanoblast markers, the GLI3Mos1/Mos1, Tg(Dct-LacZ) embryos demonstrated a severe reduction in melanoblast numbers in the mid-trunk region, throughout the ventral and dorsal surfaces at E11.5 (Fig. 7). This deficiency persisted through E14.0 and E16.0, at which time GLI3Mos1/Mos1, Tg(Dct-LacZ) embryos presented with a large ventral region devoid of melanoblasts that extended around the lumbar trunk area (Fig. 7G–J). Heterozygous GLI3Mos1+/+, Tg(Dct-LacZ) embryos were less severely affected than their homozygous littermates and at E16.0 exhibited a small ventral region devoid of melanoblasts (data not shown), consistent with the appearance of a small white spot of ventral hypopigmentation in a subset of heterozygous animals (Fig. 2A).

In addition to melanoblasts, LacZ is expressed in the retinal pigment epithelium (RPE), DRG, and telencephalon of Mos1 mutant embryos. In Mos1 mutant embryos, we did not observe any changes in the Tg(Dct-LacZ) expression in the RPE or DRG, but Tg(Dct-LacZ) expression was absent in the telencephalon of GLI3Mos1/Mos1, Tg(Dct-LacZ) embryos in comparison with GLI3+/+, Tg(Dct-LacZ) embryos at E11.5, E14.0 and E16.0 (Fig. 7, E16.0 data not shown). The loss of Tg(Dct-LacZ) expression in the telencephalon is not due to altered Sox10 expression in GLI3Mos1/Mos1, Tg(Dct-LacZ) embryos, as Sox10LacZ/LacZ, Tg(Dct-LacZ) embryos, which lack any functional SOX10, retain expression of Tg(Dct-LacZ) in the telencephalon (data not shown). The loss of the normal dorsal Tg(Dct-LacZ) expression in the GLI3Mos1/Mos1, Tg(Dct-LacZ) telencephalon is consistent with the ventralization of the telencephalon previously reported in GLI3 mutants (24,25).

**GLI3 deficiency does not prevent melanocyte differentiation**

As GLI3 deficiency results in lethality, it was not clear whether GLI3 is required for the end stages of melanocyte differentiation such as melanin pigment production. To address this, NC cultures grown from E9.5 wild-type, GLI3Mos1+/− (N = 3), and GLI3Mos1/Mos1 (N = 3) embryos were grown in media supportive of melanocyte differentiation for 14 days. These cultures can be highly variable, however, it was clear that regardless of the genotype, all cultures generated large numbers of differentiated, highly pigmented melanocytes indicating that NC derived from GLI3Mos1+/− and GLI3Mos1/Mos1 embryos can produce fully differentiated melanocytes (Supplementary Material, Fig. S3). Additionally, skin was isolated from GLI3Mos1/Mos1 embryos and grafted onto nude mice. Skin grafts from both wild-type (N = 2) and GlI3Mos1/Mos1 embryos (N = 3) produced pigmented hairs indicating that GLI3-deficient melanoblasts are able to terminally differentiate, enter hair follicles and produce pigmented hairs (Supplementary Material, Fig. S3).

**GLI3 repressor is sufficient for melanoblast specification**

Because GLI3 can act as either a transcriptional activator or repressor in response to HH signaling (26), we sought to determine which GLI3 function was active during melanoblast development and therefore responsible for the modulation of the Sox10 haploinsufficiency defects. Our data strongly suggests that GLI3Mos1 is a null allele predicted to lack both the GLI3 activator and the GLI3 repressor. Therefore, we used GLI3Mos1+/−, GLI3Mos1++/+ mice that express a C-terminally truncated form of GLI3 that acts only as a transcriptional repressor (27), and analyzed GLI3+/+, GLI3Mos1+/− and GLI3Mos1+/+/+ embryos for S1 expression at E12.5. Unlike the severe reduction in melanoblasts in the trunk of GLI3Mos1/Mos1 embryos (Fig. 6 D and H), melanoblast numbers in
Gli3D699/D699 homozygote embryos (N = 4) appeared grossly normal compared with Gli3D699/+ heterozygote (N = 2) and Gli3+/+ wild-type embryos (N = 2) (Fig. 8). The normal melanoblast specification in Gli3D699/D699 embryos suggests that the GLI3 repressor promotes melanoblast specification in the absence of full-length GLI3 activator.

**DISCUSSION**

In Sox10 haploinsufficient mice and other disease models, quantitative trait loci (QTL) analysis has been a successful approach to map a number of loci that modify the severity of disease traits, however, identification and confirmation of the causative sequence variation is labor intensive and has only been completed for a small number of mapped QTLs (28). Additionally, recent data suggest that variation among the classical inbred strains is limited and not evenly distributed throughout the genome (29), thus leaving large regions of the genome without the sequence diversity needed to be screened using current QTL methodologies. One way to increase the repertoire of genetic variation that can be tested for modifier effects is to introduce genome wide mutations into genetic...
crosses where the phenotype of interest has been well characterized, thus allowing potential modifier effects for every locus in the genome to be screened. Historically, this type of mutagenesis screen to identify enhancers and suppressors of phenotypes has been extremely successful in lower model organisms (30) and more recently applied in mice to identify factors involved in selective biological processes (31,32). Indeed, these mutagenesis screens have some distinct advantages over QTL analysis for the identification of specific sequence variations that modify a phenotype of interest (33) including the relative ease with which the causative sequence variation can be identified.

In this paper, we present a sensitized mutagenesis screen to identify candidate genes for human neurocristopathies. Using this dominant screen we identified three loci that increased the severity of neurocristopathy in Sox10 haploinsufficient mice and have mapped these loci to regions not previously attributed to known WS loci. By including Sox10 haploinsufficiency in the screen, we can identify mutations with no heterozygote phenotype on their own, which otherwise could only be detected with an additional generation of breeding (~10 weeks in mice) that is required for a traditional three-generation recessive screen. In addition, our screen retains mutations that could be missed in a three-generation recessive screen due to embryonic lethality, which was in fact observed for all 3 of the loci reported here. The use of two different inbred strains in the screen eliminated the outcrossing required to map loci, thus allowing us to quickly determine if our phenotypes are caused by mutations in novel loci or represent mutations in previously identified NC development and disease genes.

One of the pedigrees identified in our ENU screen, Mos1, showed semidominant hypopigmentation and polydactyly
carrying heterozygous mutations in either single gene suggesting a synergistic interaction between the two loci. Subsequent linkage and candidate gene analysis identified a causative point mutation in Gli3. We showed that a previously published null allele of Gli3, Gli3Xt-J, had similar effects on pigmentation and survival. Additionally, a complementation test done by intercrossing Gli3Xt-J/+ and Gli3Mos1/+ mice failed to produce any viable compound heterozygous mice carrying both mutations, thus providing strong evidence that the stop mutation identified in Gli3Mos1 results in a functionally null allele that is responsible for the hypopigmentation in the Mos1 mice. Double heterozygous mice carrying mutations in both Sox10 and Gli3 show a significant increase in hypopigmentation compared with the Sox10LacZ/+ heterozygous mice. In particular, double heterozygous Sox10LacZ+/Gli3Mos1/+ mice exhibit extensive ventral hypopigmentation that can extend over the dorsal surface to form a belt. Using Mitf, Si, DctLacZ, and Sox10LacZ expression analysis, we showed that Gli3 deficiency results in a vast reduction of melanoblasts in the trunk.

Gli3 is a member of the GLI family of C2H2-type zinc finger transcription factors whose members are vertebrate homologs of the Drosophila Cubitus interruptus (Ci) gene (34). GLI family members mediate the final stages of HH signaling and their regulation of HH target genes plays an important role during embryogenesis (reviewed in 35). The timing and location of Gli3 expression in the dorsal neural tube overlaps with where NC cells form (20,36), consistent with a role for Gli3 in specification of NC derivatives. However, the reduction in melanoblast specification in Gli3Mos1/Mos1 embryos is unlikely to result from an overall reduction of NC, since DRG and sympathetic ganglia appear relatively normal in these regions. Our observation that Gli3 deficiency does not impair later stages of melanocyte differentiation is consistent with an early role for Gli3 during specification of the melanocyte lineage. Interestingly, melanoblast specification outside the trunk region of Gli3Mos1/Mos1 embryos was not noticeably reduced. This embryonic phenotype is consistent with the phenotype in Sox10LacZ+; Gli3Mos1/+ adult animals where hypopigmentation is limited to the trunk. This region-specific effect on melanoblast specification could be reflective of normal, wild-type melanoblast distribution, where lower melanoblast numbers are seen in the mid-trunk region (21,22,37). Alternatively, there could be independent pathways that compensate for the loss of Gli3 in regions outside the trunk. Collectively, our data provides strong evidence supporting a role for Gli3 in early specification of the melanocyte lineage.

During development, Gli3 can act as either a transcriptional activator or repressor, and HH signaling regulates this dual activator/repressor function. In the presence of HH, full-length GLI3 activates target genes, while in the absence of HH, posttranslational cleavage of the C-terminus of GLI3 produces an N-terminal form of GLI3 that acts as a repressor (26) (reviewed in 38 and 39). Given that the NC is induced at the neural cord level (21,22,37). Alternatively, there could be independent pathways that compensate for the loss of Gli3 in regions outside the trunk. Collectively, our data provides strong evidence supporting a role for Gli3 in early specification of the melanocyte lineage.

and resulted in homozygous embryonic lethality. The extent of hypopigmentation was significantly increased in Mos1/+; Sox10LacZ/+ double heterozygotes compared with mice...
had normal numbers of trunk melanoblasts. These results show that the repressor form of GLI3 promotes melanoblast specification, and suggest that a low level of HH signaling is required for normal melanoblast specification.

The precise mechanism through which the GLI3 repressor acts to facilitate melanoblast specification remains to be determined. Because Sox10 interacts with a number of genes during melanocyte development, including Mitf (40–43) and components of the wingless-related MMTV integration site (Wnt) signaling pathway (44,45), the genetic interaction we observe between Gli3 and Sox10 mutants could be mediated through these other pathways rather than a direct interaction with Sox10. Recent work reveals that canonical Wnt signaling may directly activate Gli3, thereby helping to establish dorsal ventral patterning within the spinal cord (46). Interestingly, the GLI3 repressor has been shown to inhibit canonical Wnt signaling by physically interacting with and antagonizing active forms of β-catenin (47). Thus, Gli3 appears to be both a target of and a regulator of canonical Wnt signaling. The careful balance of HH and Wnt signaling was shown to affect neuronal subtypes within the developing spinal cord (46) suggesting that perhaps interactions between the HH and Wnt signaling cascades could also play a role in melanocyte progenitor cell fate. Wnt signaling is known to influence the proliferation and specification of melanocyte precursors (48–52), and it is possible that loss of GLI3 repressor in Gli3<sup>Mos1/Mos1</sup> mutants perturbs the balance of HH and Wnt signaling, resulting in a ventralization of cell fate that affects the melanocyte lineage.

As a well-established downstream target and mediator of Wnt signaling (53–55), Mitf provides an intriguing direct target for GLI3 repression within the melanocyte lineage. However, a preliminary search for GLI3 binding sites did not identify any highly conserved GLI3 binding sites within the Mitf promoter suggesting that the GLI3 repressor acts indirectly to regulate Mitf expression. While it is likely that GLI3 interacts with a number of target genes during NC lineage specification, Foxd3 is a promising candidate for a GLI3 target and we hypothesize that this binding could indirectly regulate Mitf expression. Fox genes have been implicated in mediating HH signaling in craniofacial NC derivatives (56) and the zebrafish foxd3 directly binds the mitfa promoter, thereby repressing transcription and inhibiting mitfa-positive melanoblast specification (57). Therefore it is possible that in Gli3<sup>Mos1/Mos1</sup> mutants, Foxd3 expression is deregulated, thus disrupting melanoblast specification. While significant future work remains to determine the mechanism through which the GLI3 repressor affects melanoblast specification, our findings clearly implicate GLI3 as a participant in these networks and uncover a new role for GLI3 in the melanocyte lineage.

In conclusion, we have identified three loci that act as modifiers for Sox10-dependent melanocyte defects, increasing both penetrance and severity of the defects in Sox10<sup>LacZ/+</sup> heterozygous mice. We have shown that one of these loci is a mutation in Gli3 and have demonstrated the phenotype caused by a reduction in normal Gli3 gene dosage is significantly exacerbated by a reduction in Sox10 gene dosage.
(Sox10<sup>LacZ</sup>± mice), suggesting that the two genes cooperate, directly or indirectly, during specification of the melanocyte lineage. These data highlight the role of Gli3 signaling in melanocyte development, and predict the importance of future studies investigating the role of GLI3 and/or SOX10 in other human disorders that combine digit and melanocyte defects.

**MATERIALS AND METHODS**

**Mouse husbandry**

BALB/cJ and C57BL/6J inbred mouse strains were purchased from The Jackson Laboratory. Engineered mice with a LacZ cassette replacing the endogenous Sox10 locus (Sox10<sup>LacZ</sup> or Sox10<sup>tmWeg</sup>) (13) were obtained on a mixed genetic background and maintained at NHGRI by crossing to C57BL/6J. The Gli3<sup>Xt-J</sup> mice that carry a spontaneous mutation in Gli3 were purchased from The Jackson Laboratory (stock B6.C3-Gli3<sup>tm1Urt</sup>/J) and maintained at NIH by crossing to C57BL/6J. Gli3<sup>tm1Urt</sup>, herein referred to as Gli3<sup>D699</sup> mice, were provided by Ulrich Ruther (27). All other mice described in the ENU screen and embryology studies were bred and housed in an NHGRI animal facility according to NIH guidelines. For genotyping, genomic DNA was prepared from tail biopsies or yolk sacs using a PUREGENE DNA purification kit (Gentra Systems, Inc., Minneapolis, MN, USA) according to the manufacturer’s instructions. Noon on the day of vaginal plug observation was designated E0.5 for timed pregnancies.

**ENU injections**

ENU was prepared and injected carried out as previously described (58). Briefly, 1-N-ethyl-N-nitrosourea (ENU) (Sigma; St. Louis, MO, USA) was dissolved at 100 mg/ml in 95% ethanol and then diluted to 5 mg/ml in a sterile phosphate/citrate buffer (0.1 M sodium phosphate, 0.05 M sodium citrate, pH 5.0). A spectrophotometer reading at a wavelength of 398 nm was used to confirm the ENU concentration, and BALB/cJ male mice were given weekly intraperitoneal injections of 0.1 mg/g of body weight for three consecutive weeks. Mice were allowed to recover for 8 weeks post-injection and loss of fertility was confirmed by mating with C57BL/6J females. Five males that lost and subsequently regained fertility (G0) were bred to C57BL/6J females beginning at 12 weeks post-injection. In total, 230 resulting first generation progeny (G1) were crossed to Sox10<sup>LacZ</sup>/± mice and all subsequent offspring (G2) were observed at weaning for hypopigmentation that extended beyond the ventral surface.

**Quantitation of hypopigmentation**

To quantify the extent of ventral hypopigmentation in Sox10<sup>LacZ</sup>± mice in G2 mice bred from non-mutagenized BALB/cJ males, the ventral surface of mice was photographed and the area of hypopigmentation was quantitated as a proportion of the total ventral surface area. Analysis was performed using the public domain NIH Image software developed at the U.S. National Institutes of Health and freely available (http://rsb.info.nih.gov/nih-image). Once the extent of hypopigmentation was shown to be consistent in the G2s from the BALB/cJ and C57BL/6J mixed genetic background, all mice bred for this study were assigned a numerical score of 0–4, with 1 representing the smallest spots of visible hypopigmentation, to quantitatively represent the extent of ventral hypopigmentation observed.

**Genetic mapping and sequencing**

All three Mos loci were mapped using only affected animals for analysis. For the full genome scans, affected Mos<sup>1</sup> and Mos<sup>3</sup> mice were genotyped on the Illumina platform for SNPs polymorphic between the BALB/cJ and C57BL/6J parental strains used in this study (Brigham Women’s Hospital, Harvard University). Affected Mos<sup>2</sup> mice were genotyped at polymorphic SSLP markers (CIDR, The Johns Hopkins University). A standard χ<sup>2</sup> test was used to analyze backcross data for linkage (59) using the number of recombination events per number of meiotic opportunities for each marker. For Mos<sup>1</sup>, the linkage to Chr 13 was consistent with data from a previous genome scan where suggestive but not significant linkage to proximal Chr 13 was detected with fewer markers (91 SSLP markers).

Sequencing of Gli3 was carried out using standard techniques to PCR amplify each exon and surrounding splice sites from genomic DNA for sequence analysis (Harvard Partners Healthcare Center for Genetics and Genomics, Harvard Medical School). The sequence of an affected Mos<sup>1+/+</sup> heterozygote was compared with the sequence of the parental inbred strains to identify a single nucleotide change on the ENU-treated BALB/cJ chromosome.

**Genotyping**

Taqman® MGB probes (Applied Biosystems, Foster City, CA, USA) were designed across the Gli3<sup>Mos1</sup> mutation (1148C>A) specific for the wild-type BALB/cJ allele (VIC-CTTACACTACCCCTCC) and the ENU induced mutant allele (FAM-CTTCACCTACCCCTCC). PCR reactions were carried out in 1X Taqman® Universal PCR Master Mix (Applied Biosystems) containing 900 nM of each primer (TCCACAGCCCTGCATTGAG and AGGATCTGTTGATGCATGTGAAGG) and 200 nM of each allele-specific probe. Cycling conditions were 2 min at 50°C, 10 min at 95°C followed by 40 cycles of 92°C for 15 s, 60°C for 1 min. Relative quantitation of the two alleles was determined in an end-point assay.

Genotyping of the Gli3<sup>D699</sup> colony knock-in allele was confirmed by Thymidine kinase (TK) positive PCR amplification with TkFOR GATGCGGGCGGTGTTAATGAC and TkREV GATGCGGGCGGTGTTAATGAC and AGGATCTGTTGATGCATGTGAAGG, and 200 nM of each allele-specific probe. Cycling conditions were 2 min at 50°C, 10 min at 95°C followed by 40 cycles of 92°C for 15 s, 60°C for 1 min. Relative quantitation of the two alleles was determined in an end-point assay.

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C3R: GTGGGCTGCTGCTGAAACGTGAC, amplifying a 193 bp wild-type allele.

Sox10\textsuperscript{LacZ} and Tg(Dct-LacZ) genotyping was performed by PCR amplification of the LacZ cassette as previously reported (23).

**Beta-galactosidase staining**

Embryos from timed pregnancies were stained for beta-galactosidase activity using standard methods. Briefly, embryos were fixed (1 × PBS, 1% formaldehyde, 0.2% glutaraldehyde, 0.02% NP40) on ice for 2 h followed by three 15 min, room temperature washes (1 × PBS, 2 mM MgCl\textsubscript{2}, 0.02% NP40). Staining (1 × PBS, 12 mM K-Ferricyanide, 12 mM K-Ferrocyanide, 0.002% NP40, 4 mM MgCl\textsubscript{2} and 320μg/ml 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside in N,N-dimethyl formamide) was carried out overnight at 37°C followed by two 30 min, room temperature washes (1 × PBS, 0.2% NP40). Embryos were transferred to a final fixative solution (4% formaldehyde, 10% methanol, 100 mM sodium phosphate) for analysis and storage.

**Whole-mount in situ hybridization**

Mouse embryos were fixed overnight in 4% paraformaldehyde in PBS. Reverse-transcribed digoxigenin-conjugated probes were made from linearized plasmids. In situ hybridizations were performed by using published protocols (60) with the following modifications. After probe hybridization, Ribonuclease A digestion was omitted, and Tris-buffered saline was used in place of PBS. BM-purple substrate (Roche, Molecular Biochemicals) was used in place of 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium. 

**Skin grafting**

Skin grafting was performed according to standard procedures. Briefly, skin was grafted from late gestation embryos (approximately E16.5) onto 8–10 weeks old immune-compromised recipients (Albino Swiss Nude from Taconic; approximately E16.5) onto 8–10 weeks old immune-compromised recipients (Albino Swiss Nude from Taconic; Foxn1nu/nu). Full-thickness skin grafts (1c mm) were performed by using published protocols (60) with the following modifications. After probe hybridization, Ribonuclease A digestion was omitted, and Tris-buffered saline was used in place of PBS. BM-purple substrate (Roche, Molecular Biochemicals) was used in place of 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.

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