

ADDENDUM

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## Pigmentation-based insertional mutagenesis is a simple and potent screening approach for identifying neurocristopathy-associated genes in mice

Nicolas Pilon<sup>a,b</sup>

<sup>a</sup>Molecular Genetics of Development Laboratory, Department of Biological Sciences and BioMed Research Center, Faculty of Sciences, University of Quebec at Montreal (UQAM), Montreal, Quebec, Canada; <sup>b</sup>UQAM Research Chair on Rare Genetic Diseases, Montreal, Canada

### ABSTRACT

Neurocristopathies form a specific group of rare genetic diseases in which a defect in neural crest cell development is causal. Because of the large number of neural crest cell derivatives, distinct structures/cell types (isolated or in combination) are affected in each neurocristopathy. The most important issues in this research field is that the underlying genetic cause and associated pathogenic mechanism of most cases of neurocristopathy are poorly understood. This article describes how a relatively simple insertional mutagenesis approach in the mouse has proved useful for identifying new candidate genes and pathogenic mechanisms for diverse neurocristopathies.

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### A forward genetic screen for neurocristopathy-associated genes

Neural crest cells (NCC) are a multipotent embryonic cell population that migrates extensively from the dorsal region of the neural tube (the central nervous system primordium). Such characteristics allow NCC to colonize several regions of the developing embryo and generate a wide array of specialized cell types such as melanocytes (pigment cells), peripheral neurons and glia, adrenal chromaffin cells, cranio-facial chondrocytes and osteoblasts as well as specific cell subsets of the heart, inner ear and thymus.<sup>1</sup> Accordingly, abnormal NCC development results in congenital malformations (e.g. aganglionic megacolon, cleft palate and conotruncal heart malformations) and cancers (e.g., neuroblastoma, melanoma and multiple endocrine neoplasia) that may concomitantly affect several tissues as seen in Waardenburg and CHARGE syndromes. These diverse pathological entities (>40) are collectively regrouped under the name

neurocristopathy.<sup>2,3</sup> Neurocristopathies have also in common that a large number of cases are currently unexplained at the genetic level.

In two of our latest publications,<sup>4,5</sup> we show that the wide developmental potential of NCC can be used in screening efforts aimed at identifying new neurocristopathy-related genes. These articles clearly demonstrate the simplicity and usefulness of using pigmentation anomalies as a proxy for other NCC defects. The general idea of our approach is based on the facts that pigmentation is controlled via the secretion of melanin pigments by the NCC-derived melanocytes and that albino mice are devoid of pigmentation because of an inactivating mutation in the key melanin-producing enzyme Tyrosinase (Tyr).<sup>6</sup> In this regard, the development of a co-injection strategy involving the rescue of the albino mutation with a *Tyr* minigene has proved to be a clever way for facilitating identification of transgenic animals by simple visual inspection of coat color.<sup>7,8</sup> The description of

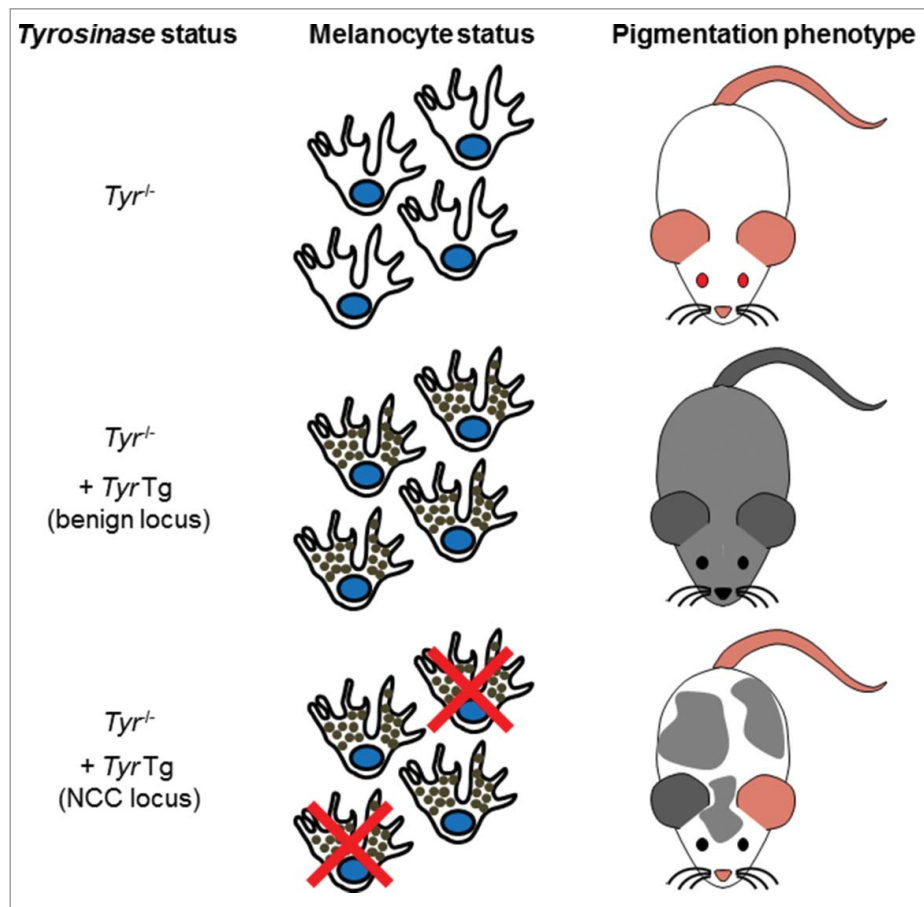
**CONTACT** Nicolas Pilon  [pilon.nicolas@uqam.ca](mailto:pilon.nicolas@uqam.ca)  Biological Sciences, University of Quebec at Montreal, 141 President-Kennedy Ave Montreal, Quebec H2X 3Y7, Canada.

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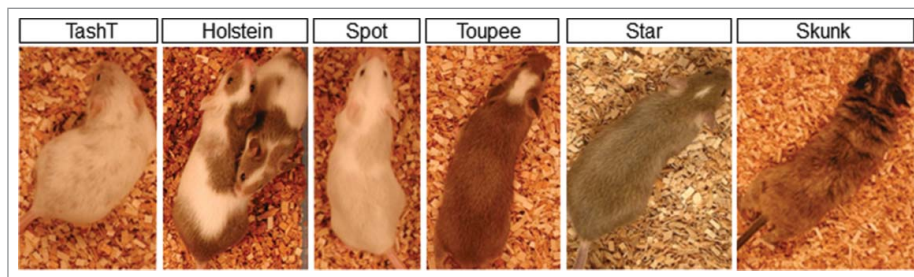
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**Figure 1.** Description of the pigmentation-based forward genetic screen in albino mice. Albino mice are unpigmented due to a G to C nucleotide change at position 308 of the *Tyr* gene, causing a cysteine to serine mutation at amino acid 103 of the Tyrosinase protein. Reintroduction of a functional *Tyr* gene via transgenesis can rescue the albino phenotype. *Tyr* transgene-induced pigmentation is uniform if inserted in a locus not important for NCC and not uniform if inserted in a locus important for NCC.

the TashT<sup>4</sup> and Holstein<sup>5</sup> mouse lines are now the proof of concept that the *Tyr* minigene can also be used as a potent screening tool for insertional mutagenesis-mediated identification of loci with key roles in NCC. Indeed, when the *Tyr* minigene is inserted in a locus important for NCC development, rescued pigmentation is non-uniform due to an insufficient number of melanocytes in the epidermis (Fig. 1).

As of today, we have obtained five hypopigmented mutant lines out of a total of ~200 lines generated. As shown in Figure 2, these lines have been named after their distinctive pigmentation pattern observed in heterozygous animals. Importantly, when these heterozygotes are crossed to generate homozygotes, other NCC-related phenotypes show up. Among these, aganglionic megacolon is the most frequently



**Figure 2.** Overview of the mutant lines obtained via pigmentation-based forward genetic screening. For each line, a characteristic pigmentation pattern can be observed in heterozygous animals.

observed, being present in three lines (TashT, Holstein and Spot). While this phenotype appears isolated – apart from the pigmentation defects – in the TashT and Holstein lines, it is observed in combination to inner ear defects in the Spot line. Consequently, this makes TashT<sup>4</sup> and Holstein<sup>5</sup> new models for Hirschsprung's disease and Spot (to be described elsewhere) a new model for Waardenburg syndrome type 4. In the Toupee line (to be described elsewhere), homozygous animals display a complex phenotype that is reminiscent of CHARGE syndrome and which notably includes multiple congenital malformations affecting the face, palate, eyes, inner ears, heart, thymus and cranial nerves. Finally, in the Star line (to be described elsewhere), homozygosity appears to be embryonic lethal. Of note, a sixth line, named Skunk (to be described elsewhere) was also identified based on the presence of hyperpigmented patches.

Thanks to current high-throughput sequencing methods, both the site of transgene integration as well as the presence of associated small scale genomic rearrangements (insertions, duplications or deletions) have been precisely identified in four lines to date (TashT, Holstein, Spot and Toupee). Importantly, in all four cases, this resulted in the identification of a locus not previously associated with the modeled neurocristopathy. It is also noteworthy that, for three of these lines, the insertion has perturbed the function of nearby highly-conserved non-coding regulatory sequences. Considering that small-scale genomic rearrangements are more representative of the human disease state than single nucleotide polymorphisms,<sup>9,10</sup> this strongly suggests that a large number of neurocristopathy cases currently unexplained at the genetic level could be explained by similar outcomes. In this regard, although the ENCODE project has started to reveal the extent of the regulatory genome in both humans in mice,<sup>11,12</sup> the majority of it still awaits functional annotation. We are convinced that mouse models like ours will be instrumental for uncovering those regulatory elements that are relevant to human pathologies.

### Important breakthroughs for Hirschsprung's disease

Hirschsprung's disease – the best known neurocristopathy – is a life-threatening congenital malformation of the enteric nervous system that affects 1 in 5000

newborns.<sup>13</sup> This severe condition is caused by the failure of NCC-derived enteric neural progenitors to reach the terminal regions of the developing intestines.<sup>14</sup> As these progenitors notably generate the peristalsis-controlling myenteric neural ganglia, the affected segment (aganglionic) remains tonically contracted, resulting in functional intestinal obstruction and accumulation of fecal material (megacolon). Hirschsprung's disease is clinically subdivided in short-segment (i.e. restricted to the rectosigmoid colon) and long-segment forms.<sup>13</sup> The short-segment form represents the vast majority of cases and is more common in males than females, with an overall ratio of ~4:1. In addition to maleness, Down syndrome (trisomy 21) is another condition that predisposes to Hirschsprung's disease. Indeed, Down syndrome patients are estimated to be up to 100-fold more likely to develop Hirschsprung's disease in comparison to the general population.<sup>13</sup>

The vast majority of enteric neural progenitors are specifically derived from NCC of vagal origin – although a minor contingent is also provided by NCC of sacral origin.<sup>15</sup> The particular migration pattern of such “enteric” NCC (eNCC) is believed to be the main contributing factor to the pathogenesis of Hirschsprung's disease. In the mouse, eNCC of vagal origin initially reach the foregut mesenchyme around e9.5 and then migrate in the posterior direction to eventually reach the end of the hindgut by e14.5. Tight coordination of eNCC migration with cell proliferation, survival and differentiation mechanisms is critically required for completion of enteric nervous system formation.<sup>16</sup> Therefore, disruption of any of these processes can result in aganglionic segments of varying length.<sup>17</sup>

At the genetic level, Hirschsprung's disease is considered a complex disease and current estimations indicate that mutations in known genes collectively explains less than one-third of the cases.<sup>18</sup> The main Hirschsprung's disease-associated genes encode members of GDNF/RET and EDN3/EDNRB signaling pathways, both of which having been shown to regulate virtually all cellular processes involved in the formation of the enteric nervous system. Indeed, the study of mouse models bearing mutation of these genes revealed important roles for both of these signaling pathways in the control of migration, proliferation, survival and differentiation of eNCC.<sup>17,19</sup> On the other hand, both the male sex bias and the strong association with Down syndrome are intriguing hallmarks of Hirschsprung's disease that were – before our work with the TashT

and Holstein lines (see below) – very poorly understood at the molecular level.

The TashT line is the first mouse model to display a partially penetrant aganglionic megacolon phenotype in a strong male-biased manner.<sup>4</sup> In contrast to the megacolon phenotype, colonic aganglionosis is almost fully penetrant in homozygous TashT animals (TashT<sup>Tg/Tg</sup>). The sex bias in megacolon expressivity can be explained by the fact that the <sub>Male</sub>TashT<sup>Tg/Tg</sup> enteric nervous system ends around a “tipping point” of minimal colonic ganglionosis (~80% of colon length) while the <sub>Female</sub>TashT<sup>Tg/Tg</sup> enteric nervous system ends just beyond it. Detailed analysis of embryonic intestines – notably via time-lapse imaging as well as via a novel quantitative method developed in our lab<sup>20</sup> – revealed that aganglionosis in TashT<sup>Tg/Tg</sup> animals is due to slower migration of eNCC. The TashT insertional mutation is located in a Chr.10 gene desert containing multiple highly conserved elements that exhibit repressive activity in reporter assays. RNAseq analyses and 3C (chromosome conformation capture) assays revealed that these silencer elements normally repress *Fam162b* (at 3.6Mb from the transgenic insertion) and that the TashT insertion results, at least in part, in the NCC-specific relief of repression of this uncharacterized gene. We are currently testing the hypothesis that these silencer elements might regulate supplemental genes using circular chromosome conformation capture assays coupled to high-throughput sequencing (4C-seq). The transcriptional signature of eNCC from TashT<sup>Tg/Tg</sup> embryos is also notably characterized by the deregulation of genes encoding members of the most important signaling pathways for enteric nervous system formation – *Gdnf/Ret* and *Edn3/Ednrb*. Very interestingly, cross-analysis of this WT-*vs*-TashT<sup>Tg/Tg</sup> dataset (1243 genes differently expressed >2-fold at  $p < 0.001$ ) with a more recent <sub>Male</sub>TashT<sup>Tg/Tg</sup>/<sub>Tg-*vs*-Female</sub>TashT<sup>Tg/Tg</sup> data set (18 genes differently expressed >2-fold at  $p < 0.001$ ; unpublished) revealed that only one gene is significantly deregulated. This Y-linked gene is specifically overexpressed in <sub>Male</sub>TashT<sup>Tg/Tg</sup> eNCC and its candidacy for explaining the male bias of the TashT megacolon phenotype is currently being tested with new mouse models.

In the Holstein line, all homozygotes (Hol<sup>Tg/Tg</sup>) die from aganglionic megacolon around weaning age. No sex bias is observed for this phenotype and, accordingly, the enteric nervous system of both male and female Hol<sup>Tg/Tg</sup> animals fails to reach the minimal level of

colonic ganglionosis discovered with the TashT line. The Holstein transgene insertion site is located upstream of the *Collagen-6 $\alpha$ -4* (*Col6a4*) gene on Chr.9. We found that this insertion induces an eNCC-specific upregulation of *Col6a4* gene expression in the developing intestines, most likely via disruption of CTCF-mediated insulation from a large genomic region specifically active in NCC. This ultimately leads to an increase in total collagen VI protein levels in the extracellular matrix surrounding both the developing and the postnatal enteric nervous system. Other data – notably from time-lapse imaging and heterotopic transplantation studies – indicated that such increased collagen VI levels during development mainly results in slower migration of Hol<sup>Tg/Tg</sup> eNCC. Further *ex vivo* analyses revealed that collagen VI is simply a poor substratum for supporting eNCC migration and can even interfere with the migration-promoting effects of fibronectin. Finally, in total agreement with our findings made with Hol<sup>Tg/Tg</sup> mice, we also observed that the enteric ganglia from the ganglionated region of a majority of human Hirschsprung’s disease patients are specifically surrounded by abundant collagen VI microfibrils. Importantly, we further found that this outcome was accentuated in patients also suffering from Down syndrome. We believe that this latter finding can be explained by the facts that human *COL6A1* and *COL6A2* genes are jointly located on human Chr.21q and that the collagen VI  $\alpha 1$  and  $\alpha 2$  chains encoded by these genes are critically required for the assembly of every collagen VI microfibril – together with any one of the structurally related  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 5$ , or  $\alpha 6$  chains.<sup>21,22</sup> Therefore, this work not only allowed us to identify a new clinically-relevant pathogenic mechanism for Hirschsprung’s disease but also to offer a plausible explanation to the predisposition of Down syndrome patients to Hirschsprung’s disease.

## Conclusion

The work mentioned here clearly demonstrates that our pigmentation-based forward genetic screen approach can allow identifying candidate loci as well as pathogenic mechanisms for neurocristopathies. Through this work, we expect to significantly improve the way children suffering from such life-threatening diseases – as well as their family – are currently being taken in charge.

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.



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