Utilizing a Novel Giant Congenital Melanocytic Nevus Murine Model to Investigate Therapeutic Strategies and Model Tumorigenesis

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Abstract

Giant congenital melanocytic nevi (gCMN) are oncogene-driven proliferations of melanocytes present since birth that are greater than 20 cm in projected adult size, and have a melanoma conversion frequency of ranging from 5-15%. Patients with gCMN typically develop melanoma in early childhood that is extremely aggressive and almost universally fatal. Therefore, targeted therapies to induce nevus regression would be enormously beneficial. NRAS activating mutations are postulated to be the driver mutation gCMN. Described here are two novel gCMN preclinical murine models that harbor an NrasQ61R mutation. Evaluation of both the constitutive nevus model (Dct promoter-driven constitutive Cre with NrasQ61R mutation) and the inducible nevus model (Tyr promoter-driven tamoxifen-inducible CreERT2 with NrasQ61R mutation) demonstrate that both models recapitulate human gCMN histological architecture and model spontaneous tumorigenesis. Of the various drug candidates tested, topical administration of a combination of the MEK inhibitor binimetinib and the c-KIT inhibitor imatinib was superior in causing almost complete nevus regression, as measured by a reduction of melanin deposition and melanocytes in the dermal layer. This may represent a potential topical treatment strategy for the regression of gCMN that avoids both the more deleterious side effects of either systemic drug administration or large-scale surgical procedures. Ultimately, this preclinical murine model may help generate new information about the rare, but deadly gCMN that may aid in improving daily symptoms from these lesions as well as hopefully reduce overall melanoma risk.
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**Glossary of Abbreviations**

- **BRAF**: v-Raf murine sarcoma viral oncogene homolog B
- **CMN**: congenital melanocytic nevus/nevi
- **gCMN**: giant congenital melanocytic nevus/nevi
- **MAPK**: Mitogen-activated protein kinase
- **NCM**: neurocutaneous melanosis
- **NRAS**: neuroblastoma RAS viral oncogene homolog
- **NYU-LCMN Registry**: Registry of Large Congenital Melanocytic Nevi of the New York University School of Medicine
- **PI3K**: phosphoinositide 3-kinase
- **UV**: ultraviolet
Introduction

Congenital melanocytic nevi (CMN) are oncogene-driven, benign proliferations of melanocytes that often involve the epidermis, dermis, and hypodermis. (1) These lesions develop in utero and in the absence of UV exposure, unlike the more common acquired melanocytic nevi. Larger CMN tend to lie along an axial distribution, and most commonly involve the trunk, head, and neck. (2) In newborns, CMN present as a light brown macule or papule with few or no hair follicles. With age, CMN often darken in color and develop hypertrichosis as well as a rough, cerebriform surface. (3) CMN are classified into four groups according to the projected adult size: small (<1.5 cm), medium (1.5-10 cm), large (11-20 cm), and giant (>20 cm). (4) The incidence of CMN of any size in newborns ranges from 0.2 to 2.1%. The incidence of giant CMN (gCMN) is estimated to be around 1 in 20,000 births, and these lesions are considered precursors of melanoma. (5) In addition to the extremely worrisome melanoma risk, gCMN are also associated with severe pruritus, superficial infections, and significant socio-aesthetic problems for the affected children.

Given the relative rarity of these lesions, most of the information on patient clinical data and outcomes available today are derived from case reports or gCMN registries. Gathering information about outcomes and melanoma incidence from case reports is not ideal, as these reports suffer from issues like publication bias. However, registries for people with gCMN have been particularly useful in monitoring melanoma development throughout life, and more specifically, in characterizing risk factors for melanoma. One of the best-known registries in the United States is the Registry of Large Congenital Melanocytic Nevi of the New York University School of Medicine (NYU-LCMN Registry). *(6) This registry was created in 1979 to answer the

* The NYU-LCMN follows patients with a CMN >20 cm, which is the definition used for gCMN in this manuscript.
previously controversial question of whether a gCMN carries an inherent risk of malignant transformation. Answering this question is of utmost importance to children with gCMN and their families, as the answer helps inform the parents about whether their child needs intervention or can instead go under close observation.(2) Although this registry has been tremendously helpful in gathering data on these measures, it suffers from a shorter follow-up time than is ideal (5.5 years) for estimating a lifetime melanoma risk.

The size of a CMN and corresponding severity of overall clinical phenotype have been found to be associated with melanoma risk, and those with a gCMN are statistically at much higher odds of developing melanoma.(7) The frequency of malignant conversion from benign nevus to melanoma is estimated to be ~1 in 100,000, whereas gCMN harbor a higher lifetime risk (~4.5-12%). (2,7–9) More specifically, CMN that are larger than 40 cm are thought to have a lifetime melanoma transformation risk of 10-15%.(7,10) Of the patients followed by the NYU-LCMN Registry that developed cutaneous or central nervous system (CNS) melanomas, 93% of them had gCMN that were 50 cm or larger in diameter.(2) Presence of neurologic abnormalities on MRI within the first year of life, such as neurocutaneous melanosis (defined as additional melanocytic tumors in the leptomeninges of the CNS), is the strongest predictor of melanoma risk.(5) It is unclear whether this is because neurocutaneous melanosis indicates a higher burden of mutated cells and thus overall risk or whether this indicates an earlier mutation. Approximately 70% of melanomas in patients with gCMN are diagnosed before puberty, with the median and mean age of cutaneous melanoma development estimated to be 3.0 years and 5.8 years, respectively.(7,11) Median survival following diagnosis is estimated to be 10.5 months.(11) Melanomas associated with gCMN are considered to be universally fatal.

In addition to the elevated risk of melanoma transformation, gCMN are associated with
several other conditions. Persons with gCMN often have dozens of satellite nevi scattered throughout their body. In addition, MRI studies of gCMN patients frequently detect neurocutaneous melanosis. The proportion of gCMN patients that have neurocutaneous melanosis as detected by MRI is estimated to be around 17-34%, and some of these patients exhibit neurologic symptoms like hydrocephalus and seizures. (5) Interestingly, an association between neurologic symptoms and male sex has also been reported. (10) The term “CMN syndrome” has been proposed within the past few years as a way to describe gCMN patients with these additional findings. These patients have been additionally observed to have characteristic facial features and minor endocrine dysfunction. (5) Studies on these patients have shown that same mutation within the NRAS gene is present consistently within the primary CMN, the satellite nevi, as well as in neurocutaneous melanosis-associated tissue. However, this mutation is absent in other tissue types. (12) These findings are suggestive that post-zygotic mutations may occur in multipotent progenitor cells during embryogenesis, which can result in multiple affected tissue types to create a mosaic phenotype.

Unfortunately, monitoring of CMN for melanoma transformation is difficult, and more importantly, it has not shown to have an effect on outcomes. (5) CMN-associated melanomas typically arise deep in the dermis of skin or even within the subcutaneous tissue, making early detection difficult. (13,14) At presentation, CMN-associated melanomas typically have a high Breslow thickness. (11) Melanomas may also arise in other areas of the body, such as the leptomeninges or, more rarely, other extracutaneous sites (it is unclear whether these melanomas develop from direct extension of the gCMN to surrounding tissue, or whether they represent metastasis). (2,15) CMN also often have benign proliferative nodules that can be worrisome to both the patient and providers. For providers, proliferative nodules pose a diagnostic challenge,
as they can be difficult to differentiate them from malignant melanoma both visually and histologically. However, within the past year improved markers to differentiate between proliferative nodules and malignant melanoma have been discovered.

There are few treatment options available, most of which are surgical. The choice of treatment method depends on nevus size, location, and structure, as well as surgeon availability. The primary method is tissue expansion of adjacent normal skin followed by staged excision with flap reconstruction. Other surgical options include skin grafts and excision without tissue expansion. Surgery and tissue expansion carry several risks, including large amounts of scarring, pain, infection, and seroma formation. The remaining options only improve appearance and fail to completely remove the melanocytic cells. These include dermabrasion and laser therapy. Repigmentation and hypertrophic scarring are common. Therapeutic decisions rely heavily upon discussion between the child, the parents, and a multidisciplinary care team. Of the subjects followed by the NYU-LCMN Registry, approximately 12% underwent complete excision, 46% had partial removals, and 41% elected to undergo observation.

Understanding the genetic basic of CMN has been historically challenging. Familial cases of CMN are rare, and they do not follow a Mendelian pattern. Rather, a somatic mutation appears to be causative. Human CMN have been individually reported to harbor mutations in NRAS, BRAF, TP53, MC1R, and GNAQ but it was originally unclear if these mutations had any role in CMN pathogenesis. Researchers have determined that most human CMN contain mutations in either the NRAS or BRAF genes, which are typically mutually exclusive. Smaller size lesions almost exclusively carry BRAFV600 mutations. Additionally, most nevi acquired during lifetime also harbor BRAF mutations. However, BRAF mutation in gCMN is rare. Rather, several studies support the theory that gCMN are driven by a single postzygotic
mutation in the *NRAS* gene. Activating *NRAS* mutations have been detected in 80-95% of gCMN. In another study, among patients that had multiple CMN, all of them had a mutation in codon 61 of the NRAS protein, most commonly either Q61K or Q61R amino acid substitutions. There is no phenotypic difference in persons with p.Q61K versus p.Q61R. NRAS is one of the three isoforms of the RAS family of GTPases proteins. Both the Q61K and Q61R codon 61 mutations result in oncogenic RAS proteins with reduced intrinsic GTPase activity, leaving them bound to GTP and constitutively active. NRAS is thought to have dual activation of both the mitogen-activated protein kinase (MAPK) and phosphatidylinositide 3 kinase (PI3K) signaling pathways, which are largely responsible for promoting cell growth and division, and suppressing apoptosis.

Although gCMN are believed to result from post-zygotic mutations, a family history of CMN has been reported by some patients. Researchers have wondered whether mutations in certain genes passed on through generations may have a modifying effect on CMN. Interestingly, a significant percentage of patients with CMN have been found to have carry compound heterozygous or homozygous *MC1R* variants that confer a red-head phenotype. Presence of these *MC1R* variants was also found to be associated with severity of clinical phenotype. However, the mechanism relating *MC1R* variants to congenital melanocyte nevogenesis is unknown. Whether there is an increased risk of melanoma for gCMN patients with MC1R variants is also not yet known.

Most nevi are inactive for decades. It is theorized this state occurs after mutation-induced activation of an oncogene (*NRAS* or *BRAF*). Following activation, DNA hyper-replication occurs, triggering a DNA damage response, and ultimately causing the cell to enter a state of quiescence that is termed oncogene-induced senescence. Markers of oncogene-induced
senescence (p16INK4a and β-galactosidase) have been found in melanocytic nevi, further supporting the theory of nevi as senescent neoplasms.(29) Nevi are also known to regress with aging, a phenomenon thought to be mediated by immune-based destruction. This is an interesting (and apparently common) phenomenon, particularly in light of the recent major successes observed in immune-based therapies of melanoma, and suggests that immune modulation might represent a therapeutic strategy for giant nevi.(30)

Another question is whether congenital melanocytic nevi cells have a higher intrinsic propensity to convert to melanoma, or if merely the higher number of melanocytes causes a greater statistical chance for transformation. When comparing acquired nevi that typically carry BRAF mutations to melanocytes in normal skin, there is no difference in conversion rates to melanoma. Thus, carrying a BRAF mutation does not intrinsically cause melanocytes to become more cancerous. However, most gCMN carry NRAS mutations instead. While the incidence rate of melanoma conversion in NRAS-mutated melanocytes has not been studied, melanomas with NRAS mutations are more likely to be thicker and to have a higher mitotic rate than BRAF-mutated or NRAS/BRAF wild-type melanomas.(31)

It is known that malignant progression of gCMN to melanoma requires additional mutations. Loss of heterozygosity has been observed in one patient who developed melanoma.(12) Genetic analysis on another infant with a gCMN that transformed to aggressive melanoma demonstrated loss of the normal NRAS allele and amplification of a mutant NRASQ61R allele. However, this loss was not the result of a deletion but instead likely due to post-mitotic recombination.(32) Culture of melanoma cells from this patient showed a five-fold increase in NRAS expression as compared to normal melanocytes. Additional genetic changes observed in gCMN-associated melanoma include changes in copy number, with large gains and/or losses of
either parts of or whole chromosomes.\textsuperscript{(33,34)}

Activating \textit{NRAS} mutations have been explored in mouse models by other groups. Transgenic mice expressing human \textit{NRAS}\textsuperscript{Q61R} from a mouse tyrosinase (\textit{Tyr}) promoter have been used by several groups, but mainly to study melanoma formation in the context of \textit{p16}\textsuperscript{INK4A} deficiency or neurocutaneous melanosis promoted by an additional WNT pathway activating mutation.\textsuperscript{(35–38)} In another model, mice expressing melanocyte-specific \textit{NRAS}\textsuperscript{G12D} via an upstream LSL cassette and constitutive \textit{Tyr}-Cre yielded mice with CNS melanoma,\textsuperscript{(39)} limiting survival or an ability to study cutaneous nevi. To create this mouse model, LoxP-Stop-LoxP ("LSL")-\textit{Nras}\textsuperscript{Q61R} knock-in mice were crossed with mice transgenic for either of two melanocyte-targeted Cre alleles: 1) dopachrome tautomerase (\textit{Dct}) promoter-driven constitutive Cre or 2) \textit{Tyr} promoter-driven tamoxifen-inducible CreER\textsuperscript{T2}. Both models display numerous key attributes of human giant congenital nevi, from histologic features to sporadic melanoma progression. These models will be hopefully be valuable for developing improved therapies to spare children the highly morbid extensive skin resections.

The focus of the work within this project was to primarily validate that the preclinical murine models accurately simulate the features and behaviors of corresponding human cGMN, and to secondly screen for locally-delivered (e.g., topically or intralesionally) therapeutic agents that can cause nevus regression. Specifically, we hypothesized that constitutive NRAS activation might be important for maintenance of congenital melanocytic nevi, and that inactivation of pathways constitutively stimulated by this mutation may provide a therapeutic opportunity for the children with gCMN. Coupled with the morbidity and poor effectiveness of current therapies, utilization of these novel preclinical models of cGMN can pave a pathway to badly needed innovative remedies for children with giant congenital nevi.
Methods

Mouse Models: Melanocyte-specific Nras$^{Q61R}$ expression was induced by crossing LSL-Nras$^{Q61R}$ mice (kindly provided by Dr. Norman Sharpless, University of North Carolina) with two melanocyte-targeted Cre alleles: Dct promoter Cre (Dct-Cre mice, kindly provided by Dr. F. Beermann, ISREC, Lausanne, Switzerland), or Tyr promoter CreER$^{T2}$ (Tyr-CreER$^{T2}$, kindly provided by Dr. M. Bosenberg, Yale). The following strains were used for nevus characterization and study of tumorigenic potential: Tyr-CreER$^{T2}$ LSL-Nras$^{Q61R/+}$ and Tyr-CreER$^{T2}$ LSL-Nras$^{Q61R/Q61R}$ for inducible nevi; and Dct-Cre Nras$^{Q61R/+}$ and Dct-Cre Nras$^{Q61R/Q61R}$ for constitutive nevi; both sets on either Mc1r$^{E/E}$ and Mc1r$^{e/e}$ C57BL/6 backgrounds (8 total groups). Mice with the Tyr-CreER$^{T2}$ promoter were treated on the ears bilaterally with 4-hydroxytamoxifen (4HT, Sigma #H6278, 10 µL of a 25 mg/ml solution in DMSO) for 5-7 days, starting at postnatal day 1 to induce nevogenesis. Male and female mice were used in all experiments. Controls included LSL-Nras$^{Q61R/+}$ mice, Dct-Cre mice, and Tyr-CreER$^{T2}$ mice. The Cre-only mice were included to control for possible nonspecific Cre effects such as Cre excision of pseudo loxP sites, which have been shown to occur in some cells.(41,42)

Histological study of gCMN tissue: Tissue from mice with phenotypically apparent gCMN was collected, fixed in neutral buffered formalin, and embedded in paraffin wax. Hematoxylin & eosin (H&E) staining was performed.

Fontana-Masson staining: Sections were deparaffinized and hydrated in distilled water. Slides were placed step-wise in 2.5% Ammoniacal Silver Working Solution, 0.1% gold chloride solution, 2% sodium thiosulfate, and finally nuclear-fast red solution, with washing with distilled water between steps.
**Immunofluorescence:** Primary antibodies included the following: anti-MITF at 1:20 (Vector Laboratories # VP-M650), anti-TRP2 at 1:200 (Santa Cruz, sc-10451) anti-SOX10 at 1:200 (Biocare Medical # ACI3099A), and anti-S100 at 1:200 (Abcam ab76729). Secondary antibodies included the following: donkey anti-mouse IgG H&L (Alexa Fluor 488) (ThermoFisher A-21203), donkey anti-goat IgG H&L (Alexa Fluor 594) (ThermoFisher A-11058), donkey anti-goat IgG H&L (Alexa Fluor 488) (ThermoFisher A-11055), and Donkey anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 488 (ThermoFisher A-21206). All secondary antibodies were diluted to 1:250.

**Quantification of tumorigenesis:** All mouse strains were monitored for tumorigenesis weekly. Dates of tumor discovery for each mouse were recorded, along with location of tumor. Of the Tyr-CreERT2 LSL-NrasQ61R/Q61R mice that developed tumors, eight representative tumors were histologically examined with the assistance of expert dermatopathologist Dr. M. Mihm. Tumor tissue sections were individually stained with H&E as well as Fontana-Masson. They were additionally stained with the immunofluorescence melanocyte-specific markers MITF, DCT, SOX10, and S100. Tumor incidences and latencies were quantified for each strain. Kaplan-Meier curves were generated using the software GraphPad Prism 6. Differences in melanoma incidence were assessed by the log-rank test, with a significance level of 0.05 (two-tailed).

**Therapeutic agent screening for nevus regression:** Therapeutic agents (NRAS-signaling targeted agents and melanocyte-selective toxic agents) were tested on induced nevi of both the ears and paws of Tyr-CreER T2 NrasQ61R/Q61R mice. Agents that were tested include the MEK inhibitors binimetinib, trametinib, and selumetinib; the PI3K inhibitor omipalisib; and the c-KIT inhibitor imatinib (see Table 1 for concentrations and manufacturing information). These agents were tested both alone and in specific combinations. To induce paw nevi, mice were treated on
the paws bilaterally with 25 mg/ml of 4HT beginning at postnatal day 1, for six times total over the course of two weeks (Figure 7A). To induce ear nevi, mice were treated on the ears bilaterally with 25 mg/ml of 4HT beginning at postnatal day 1, for seven times total over the course of two weeks (Figure 7B). For topical ear nevi treatments, 5 µl of tested agents [binimetinib (50 µg/5 µl), omipalisib (25 µg/5 µl), and imatinib (500 µg/5 µl)] were applied topically to the right ear, and 5 µl of DMSO control vehicle was applied to the left ear five times per week for nine weeks total. Tissue sections were harvested 15 weeks after the final drug treatment and stained with H&E. For intralesional paw nevi treatments, 10 µl of tested agents [trametinib (1 µg/10 µl) and selumetinib (10 µg/10 µl)] were injected subcutaneously to the right paw 5-6 times per week for two weeks total (from age 6-8 weeks-old). Tissue was harvested at 27 weeks of age and stained with H&E. Immunofluorescence staining for SOX10 and brightfield microscopy of unstained slides for gross melanin detection were performed. Combination therapies [trametinib (15 µg/5 µl) and imatinib (250 µg/5 µl); binimetinib (25 µg/5 µl) and imatinib (250 µg/5 µl)] were also tested on ear nevi, with 5 µl total of combination agents painted on the right ear and 5 µl of DMSO as a control painted on the left ear. Immunofluorescence staining for SOX10 and brightfield microscopy of unstained slides for gross melanin detection were performed on these slides as well. Topical treatment doses were determined from published oral doses.(43–45)

Results

Gross phenotype of the inducible and constitutive murine gCMN models

Newborn mice harboring Dct-Cre-mediated NrasQ61R expression displayed significant widespread cutaneous hyperpigmentation (Figure 1A). Mice heterozygous for the NrasQ61R
mutation displayed a stronger phenotype than did mice heterozygous for the mutation. The Tyr-CreER<sup>T2</sup> LSL-Nras<sup>Q61R</sup> mice responded to topical 4HT with significant hyperpigmentation on the plantar surface of the 4HT-treated paw, whereas the control did not (Figure 1B).

**Histological analysis of murine nevus tissue**

Dermatopathology expert Dr. Martin Mihm extensively reviewed histology from these models and confirmed that they accurately recapitulate histologic features of human giant congenital nevi. Fontana-Masson staining of tissue from a visible nevus in the footpad of a 16-day-old Tyr-CreER<sup>T2</sup> LSL-Nras<sup>Q61R/Q61R</sup> mouse showed abundant ectopic dermal melanin following 4HT induction, whereas an age-matched LSL-Nras<sup>Q61R/+</sup> mouse lacking Cre had no visible dermal melanin (Figure 2A). Immunofluorescence of four melanocytic markers (DCT, Sox10, MITF, S100) demonstrated ectopic dermal melanocytes within the skin of the mutant mouse, whereas no ectopic dermal melanocytes were visible within control skin (Figure 2B-D).

**Tumorigenic potential of inducible versus constitutive gCMN mouse models**

Tyr-CreER<sup>T2</sup> LSL-Nras<sup>Q61R</sup> were observed to develop spontaneous amelanotic tumors (Figure 3A). Tumor samples were collected for histological evaluation. Bright field microscopy evaluation of an H&E-stained tumor tissue sample from a 17-month-old Tyr-CreER<sup>T2</sup> LSL-Nras<sup>Q61R/Q61R</sup> mouse showed melanin deposition clustered within certain regions of the tumor, despite tumors being grossly unpigmented (Figure 3B). The melanocytic marker DCT, SOX10, and S100 were detected in numerous cells within the tumor tissue (Figure 3C-D). MITF, however, was not apparent.

During observation of Tyr-CreER<sup>T2</sup> LSL-Nras<sup>Q61R/Q61R</sup> mice (n=22), 72.7% eventually developed melanoma, with a median latency of 31.0 weeks following 4HT induction. Of the Tyr-CreER<sup>T2</sup> LSL-Nras<sup>Q61R/+</sup> mouse group (n=23), 34.8% developed melanoma with a median
latency of 89.3 weeks following 4HT induction. No tumors developed in the control group (n=19). Both strains demonstrated statistically significant differences in tumor-free survival Kaplan-Meier curves when compared to the control group by log-rank test (Figure 4).

Dct-Cre Nras\textsuperscript{Q61R} mice were also observed to develop melanoma. Within the Dct-Cre Nras\textsuperscript{Q61R/Q61R} group (n=22), 27.3% of mice developed melanoma with a median latency of 12.3 weeks after birth. Within the Dct-Cre Nras\textsuperscript{Q61R/} group (n=52), 11.5% of mice developed melanoma with a median latency of 37.7 weeks after birth. No tumors developed in the control group (n=12). Only the Dct-Cre Nras\textsuperscript{Q61R/Q61R} group had a statistically significant difference in tumor-free survival when compared to the control group by the log-rank test (Figure 5).

Effect of black versus red background on tumorigenesis in mice with gCMN

Red-hair phenotype Dct-Cre Nras\textsuperscript{Q61R} Mc1r\textsuperscript{e/e} mice were also monitored for melanomagenesis and compared to the black-haired Dct-Cre Nras\textsuperscript{Q61R} Mc1r\textsuperscript{E/E} (these are the same group of mice mentioned in the previous section). Within the Dct-Cre Nras\textsuperscript{Q61R/Q61R} Mc1r\textsuperscript{e/e} (n=5), 60% developed melanoma with a median latency of 7.6 weeks (Figure 6). When comparing the Dct-Cre Nras\textsuperscript{Q61R/Q61R} Mc1r\textsuperscript{e/e} versus the Dct-Cre Nras\textsuperscript{Q61R/Q61R} Mc1r\textsuperscript{e/e}, differences in tumor-free survival by the log-rank test were not significant (p=0.1089).

Screen of therapeutic agents:

NRAS-signaling targeted agents and melanocyte-selective toxic agents (MEK inhibitor binimetinib, PI3K inhibitor omipalisib, and c-KIT inhibitor imatinib) were tested topically on 4HT-induced ear nevi of Tyr-CreER\textsuperscript{T2} LSL-Nras\textsuperscript{Q61R/Q61R} mice. Brightfield microscopy comparisons of H&E stained tissue of DMSO versus therapeutic agent showed reduction of melanin deposition of varying degrees in the ears treated topically with therapeutic agents. Binimetinib generated the largest reduction in melanin as compared to the control vehicle (with
only scant melanin visible), whereas omipalisib and imatinib created more modest reductions in melanin (Figure 7B). Additionally, there is a significant visible reduction in pigmentation following treatments intraleisional administration of MEK inhibitors (trametinib and selumetinib) on 4HT-induced paw nevi of Tyr-CreERT2 LSL-NrasQ61R/Q61R mice, with slightly more gross pigment reduction by trametinib than selumetinib (Figure 7D-E). Brightfield microscopy shows a significant amount of melanin deposition within the selumetinib-treated paw nevus, whereas the trametinib-treated paw nevus has only scant melanin remaining. Immunofluorescence evaluation of melanocytic markers demonstrates few SOX10-positive melanocytes within the trametinib-treated paw nevus, with more SOX10-positive melanocytes within the selumetinib-treated paw nevus.

Combination therapies of MEK inhibitors (trametinib and binimetinib) and the c-KIT inhibitor imatinib were also tested. Brightfield microscopy of 4HT-induced ear nevi of Tyr-CreERT2 NrasQ61R/Q61R mice demonstrates significantly less melanin (although not complete disappearance) in the imatinib and trametinib-treated ear as compared to the control DMSO-treated ear (Figure 8A). Melanin reduction with imatinib and binimetinib is more dramatic as assessed by brightfield microscopy, with complete absence of melanin in the combination-treated ear compared to the DMSO-treated control ear. Interestingly, the combination-treated ear also had significant epidermal thickening compared to the control ear. A more thorough investigation of the ear sample shows that melanin reduction is more effective in areas nearby large epidermal thickening. Immunofluorescence staining for the melanocyte-specific marker SOX10 demonstrates several melanocytes within the dermis of imatinib and trametinib-treated mice. Significant melanin deposits are visible by brightfield microscopy (Figure 8B). Fewer melanocytes are detected by SOX10 positivity within the dermis of imatinib and binimetinib-
treated mice.

**Discussion, Conclusion, and Suggestions for Future Work**

Described here are two novel giant nevus preclinical murine models with Nras\(^{Q61R}\) mutation, paralleling the primary mutation present in most human gCMN (as well as a subset of human melanomas). Evaluation of both the constitutive nevus model (*Dct* promoter-driven constitutive Cre with Nras\(^{61R}\) mutation) and the inducible nevus model (*Tyr* promoter-driven tamoxifen-inducible CreER\(^T2\) with Nras\(^{61R}\) mutation) demonstrate that both models recapitulate human gCMN histological architecture and model spontaneous tumorigenesis. Of the various drug candidates tested, topical administration of a combination of the MEK inhibitor binimetinib and the c-KIT inhibitor imatinib was superior in causing almost complete nevus regression. This may represent a potential topical treatment strategy for the regression of gCMN that avoids both the more harmful side effects of systemic drug administration as well as highly morbid surgical procedures.

In humans, giant congenital nevi exhibit distinct histologic features that differentiate them from common acquired nevi. Large and giant CMN are characterized by melanocytic proliferations within the deeper dermal and subcutaneous layers, whereas the melanocytic proliferations of small CMN are largely restricted to the epidermal layer. Within cGMN, melanocytes are found either in isolated groups or are found oriented linearly along collagen bundles of the reticular dermis. They can also be found around and within hair follicles, sebaceous glands, eccrine apparatus, vessel walls, and nerves. Histological evaluation of nevi tissue from these preclinical murine models by a pigmented-lesion dermatopathology expert indicates that these models accurately recapitulate histologic features of human gCMN. Gross
phenotypic exam also shows stable hyperpigmentation of cutaneous tissue.

These mouse models also demonstrated sporadic melanoma transformation, a key feature of human gCMN. Melanomas were grossly amelanotic, but histological analysis demonstrated occasional melanin deposition as well as cells positive for the melanocytic markers DCT, S100, and SOX10. Tumor incidence rates and tumor latency varied widely between mouse strains. This may be a result of various factors, including melanocytic promoter strength, total area of nevus tissue, zygosity of the $N_{ras}^{Q61r}$ allele, age of nevogenesis, and variant status of the $McIr$ gene. Tumor incidence was highest in the $Tyr$-CreER$^{T2} N_{ras}^{Q61R/Q61R}$ group (72.7%), which was incidentally the model used for drug screening experiments. This is somewhat surprising given that the activated $N_{ras}$ gene product should be localized to only 4HT-treated skin, as opposed to the mice with constitutive nevi that have $N_{ras}$ expression in all Dct-expressing melanocytes. However, initial studies for the creation of the murine mouse model suggest that $Tyr$ is a particularly strong promoter, as mouse strains with constitutive $Tyr$-Cre $N_{ras}^{Q61R/Q61R}$ expression were almost universally fatal in utero or died shortly after birth with severe hydrocephalus. Within the $N_{ras}$-activated $Tyr$-CreER$^{T2}$ group, tumor incidence is also statistically significantly associated with zygosity, suggesting that overall expression of $N_{ras}$ may influence overall tumor incidence. This is interesting given a recent genetic study on an infant with gCMN-associated melanoma, in which amplification of a mutant $NRAS^{Q61R}$ allele and increased expression of the mutant $NRAS$ was detected. (32) A final explanation for the high tumor incidence within this strain is the longer period of monitoring as compared to the constitutive Dct-promoter driven mice.

Mice within the $N_{ras}$-activated Dct-Cre group had lower rates of tumor incidence, albeit with a much smaller tumor latency. Homozygosity of the $NRAS^{Q61R}$ allele similarly led to the
highest rates of melanoma formation in this group. Interestingly, evidence of hydrocephalus or cranial abnormalities was frequently seen in Dct-Cre Nras<sup>Q61R/Q61R</sup> mice, perhaps suggestive of neurocutaneous melanosis, which might suggest an overall higher burden of mutated melanocytes globally and may explain the shorter tumor latency. While mice heterozygous for the NRAS<sup>Q61R</sup> allele developed melanoma at a rate of 11.5%, which is most similar to the rate of melanomagenesis associated with human gCMN, survival curves were not significantly different from the control. This may be due to low power of this analysis given the smaller rate of melanoma formation in this group, and the low number of controls. Phenotypically and genetically, Dct-Cre Nras<sup>Q61R/+</sup> mice may also be a more accurate model for gCMN, given the congenital development of nevi as well as the heterozygosity of the Nras mutation. Most human gCMN are found to contain heterozygous mutations of Q61K or Q61R for the NRAS protein (although one person has been reported to be homozygous for the NRAS Q61K mutation).(12)

There were also observed differences in spontaneous melanoma formation in NRAS<sup>Q61R</sup>-driven giant nevi on both black- (Mc1r<sup>E/E</sup>) and red-haired (Mc1r<sup>e/e</sup>) backgrounds. The Mc1r<sup>e/e</sup> variant is associated with a nonfunctional melanocortin-1 receptor that is unable to activate the pigment production pathway. Persons and mice with this mutation are only able to produce the reddish-yellow pheomelanin, and cannot produce any of the more UV-protective black eumelanin. Interestingly, human MC1R polymorphisms have been shown to be associated with increased size of CMN. There was an observed increased spontaneous melanoma formation in a BRAF<sup>V600E</sup> mouse model with the pheomelanin-associated Mc1r<sup>e/e</sup> variant as compared to albino BRAF<sup>V600E</sup> mice, introducing the concept that pheomelanin is inherently carcinogenic.(46)

Preliminary data shown in this paper suggest higher penetrance and shorter latency on the red-hair background, perhaps reflecting more aggressive behavior, but a larger study is required to
confirm these results. It is unknown whether humans with MC1R polymorphisms and gCMN have elevated rates of melanomagenesis, and this mouse strain represents a potentially useful model to examine this question.

Following characterization of these preclinical mouse models, a screen of several potential topical and intralesional drug candidates for nevus regression was performed. An important distinction between the therapeutic strategy here and NRAS targeting in melanomas, is that this screen focused upon localized therapy, rather than systemic. Localized therapy employed may offer an opportunity for profoundly greater dose-intensity that may convert into significantly greater efficacy than seen for systemic treatments. Topical or intralesional therapy is also ideal in the pediatric population, as both laser and surgical procedures generally require anesthesia given the large area of a gCMN. Finally, while topical delivery is challenging due to limited human skin penetration, successful topical drug candidates represent a proof-of-principal approach that could support later enhanced penetration pharmaceutical strategies or injectable therapies for optimal local drug delivery.

Two categories of agents aimed at eradicating giant congenital nevi were tested: a) antagonists of NRAS downstream signaling pathways, b) agents targeting melanocyte lineage vulnerabilities. Specifically, several small molecules that target various molecules along the Ras-activated signaling pathways were selected, including the RAF/MEK/ERK and PI3K/AKT/mTOR cascades. These pathways are well-known to play key roles in melanocyte growth and survival. Many of these agents have shown efficacy in melanoma or in murine melanoma experiments, and these agents may serve as similarly good agents for targeting of gCMN cells. The MEK inhibitors trametinib (FDA approved) and binimetinib have both shown good activity in NRAS-mutated melanoma.(47) Combined targeting of mTOR within the PI3K
pathway with MEK has shown efficacy in treated NRAS-mutant melanoma in vivo.(48) Vitiligo and other pigmentation abnormalities is a well-reported side effect of oral imatinib therapy.(49) The protein c-KIT, a target of imatinib, has been found to be essential for melanocyte proliferation and survival, and may explain these clinical side effect observed in cancer patients.(50) For this reason, imatinib was included in the drug screen as a melanocyte-selective toxic agent. Combination therapies were also included in the screen, as this treatment strategy has been found to be superior in melanoma studies. Specifically, simultaneous targeting of both MEK and PI3K (with PI3K inhibitor GDC-0941) was found to be superior to MEK inhibition alone in nude mouse xenograft melanoma studies.(36)

Based upon the drug screen, a combination of binimetinib and imatinib was the most successful strategy in inducing nevus regression. Another group has had success with systemic MEK inhibition in an Nras-driven nevus model that also contains activated Wnt signaling, suggesting a dependency on MEK/ERK activity during the growth phase of the nevus.(36) Based on these findings, a group has used the MEK inhibitor trametinib as therapy in four patients with NRAS-mutated CNS melanoma, with good response to therapy between 6 weeks to 9 months before disease progression.(5) However, a key caveat of the results of the mouse model is that the study was focused only on suppression of nevus formation, rather than regression of stable giant nevi. Thus, this study does not inform whether systemic MEK inhibition would also work on senescent nevi. This study expands upon this study both specifically examining drug candidates in stable, senescent lesion, as well as studying the effect of localized therapy for cutaneous lesions. Persistent phosphor-ERK positivity has been demonstrated in senescent human gCMN with activated NRASQ61K.(51) Co-staining of p16INK4A and beta-galactidose was also present in these lesions. Data collected by Yeon Sook Choi demonstrates phosphor-ERK
staining in senescent $Nras^{Q61R}$ nevi in this preclinical model. Thus, preliminary data suggests that post-senescence treatment with topical signaling inhibitors produces selective ablation of nevus cells, suggesting dependency of even senescent melanocytes on MEK/ERK activity.

In the future, there are several steps that may help further characterize these preclinical models and expand upon therapeutic options. Particularly interesting is the role of pheomelanin in this mouse model, as in humans the red-head phenotype has been associated with gCMN. Preliminary results from this mouse model suggest increased melanoma burden and shorter tumor latency in red-haired background mice with nevi. This result is similar to previous experiments on pheomelanin-associated $BRAF^{V600E}$ nevi.(46)

Another well-known feature of gCMN that may be interesting to examine in the future is the association with hypertrichosis.(3) This suggests that nevus-directed events may modulate hair growth, perhaps resulting from signals downstream of the activated NRAS protein. Initial analysis of hair follicle cycle behavior in the nevi of $Dct-Cre Nras^{Q61R/Q61R}$ mice as compared to control $Dct-Cre Nras^{+/+}$ mice suggests that hair cycles are mismatched in the mutant mouse compared to the control. As the expression of mutation $Nras$ is restricted to melanocytes in our mouse model, this suggests that the altered hair follicle physiology may be driven in part by melanocytes, rather than keratinocytes.

Complete characterization of this model requires recognition that 30% of gCMN-associated melanomas arise in the CNS, rather than in the lesion itself. This is not wholly unsurprising, given the large number of persons with gCMN who have concomitant neurocutaneous melanosis. Closer examination of these mouse models is required to determine whether these models also develop CNS melanoma. Several mice suffer from neurologic abnormalities and symptoms, including seizures, hydrocephalus, and increased cranial perimeter.
Upon dissection, a *Dct-Cre Nras^{Q61R/Q61R}* mouse had gross pigmentation of the leptomeninges (whereas this was lacking in the age-matched control mouse) that appeared consistent with previously published images of neurocutaneous melanosis in mice. These findings were verified with Fontana-Masson stains.

Although there is evidence that several small molecule inhibitors of the MAPK and PI3K pathways and melanocyte-selective toxic agents cause varying levels of nevus regression, screening of other categories of drug candidates may lead to additional options. Other candidate therapies may include additional small molecule agents targeting the MAPK/PI3K pathways (e.g., AKT and mTOR inhibitors), more melanocyte-selective toxic agents (e.g., tyrosinase inhibitors, HDACs, and additional c-KIT inhibitors), as well as immunotherapy agents (e.g., anti-PD1, anti-toll receptors, haptens, and STING agonists). Finally, although nevus regression was the primary outcome in this study, a perhaps more interesting outcome measure would be to see if there is reduced incidence of melanoma in treated mice.

While these models have proven to be valuable for studying gCMN, there remain myriad differences between genetically engineered mice and humans. Therefore, prior to introducing promising therapies in human subjects, such agents must be tested on human skin samples from gCMN. In the future, engraftment of living human, surgically resected gCMN tissue onto immunodeficient mice would ultimately permit rigorous preclinical assessment of treatments. Although there are many future directions in which these models can be furthered explored, the results presented here are promising and represent potential novel therapeutic options for treatment of gCMN in young individuals.
Summary:

In summary, described here are two novel gCMN preclinical murine models that harbor an Nras\textsuperscript{Q61R} mutation. Evaluation of both the constitutive nevus model (\textit{Dct} promoter-driven constitutive Cre with Nras\textsuperscript{61R} mutation) and the inducible nevus model (\textit{Tyr} promoter-driven tamoxifen-inducible CreER\textsuperscript{T2} with Nras\textsuperscript{61R} mutation) demonstrate that both models recapitulate human gCMN histological architecture and model spontaneous tumorigenesis. Of the various drug candidates tested, topical administration of a combination of the MEK inhibitor binimetinib and the c-KIT inhibitor imatinib was superior in causing almost complete nevus regression. This may represent a potential topical treatment strategy for the regression of gCMN that avoids both the more harmful side effects of systemic drug administration as well as highly morbid surgical procedures. Further characterization of these mouse models include exploring the effect of the red-hair background on gCMN phenotype and melanoma transformation, investigating the effect of \textit{Nras}-mutated melanocytes on hair cycle kinetics, and determining whether neurocutaneous melanoma is an associated feature. Additional future directions include expanding the drug screen to include additional agents that target the MAPK/PI3K pathways, melanocyte-selective toxic agents, and immunotherapy drugs. Ultimately, these preclinical murine models will hopefully help generate new information about the rare but deadly gCMN that may aid in improving daily symptoms from these lesions as well as hopefully reduce overall melanoma risk.

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histological characterization. ASD monitored mice for tumor formation and generated tumor incidence plots. ASD and YSC performed therapeutic agent screen on the preclinical mouse models. Both YSC and David E. Fisher (DEF) mentored ASD in the experimental design and execution. ASD wrote the thesis, with input by DEF.


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<th>Inhibitor source</th>
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<tr>
<td><em>Small molecules targeting NRAS-signaling pathways</em></td>
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<td>Trametinib (GSK1120212)</td>
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<td>PI3Ka,β,γ,δ; mTOR</td>
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<tr>
<td><em>Melanocyte-selective toxic agents</em></td>
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<tr>
<td>Imatinib (STI571)</td>
<td>c-KIT &amp; other RTKs</td>
<td>500 µg (54)</td>
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**Table 1.** Therapeutic agents tested either topically or intralesionally on the inducible nevi mouse model.

All agents were obtained from Selleckchem, in Houston, TX.
Figure 1: Gross phenotype of constitutive and inducible giant melanocytic nevus murine mouse models.

Panel A shows mice at postnatal day three, with varying levels of constitutive Nras expression on the Dct-promoter. Mouse #2 serves as a control mouse, with two wild-type Nras alleles. Mouse #1 is heterozygous for the p.Q61R mutation, and displays moderate hyperpigmentation along the dorsum. Mouse #3 is homozygous for the p.Q61R mutation, and displays intense hyperpigmentation along the dorsum. Panel B shows a comparison of two mice that had both paws topically painted with 25 mg/ml 4HT for six times total over the course of two weeks, pictured at postnatal day 16. Mouse #1 served as the control, with no visible hyperpigmentation following painting with 4HT due to absence of Tyr-CreER$^{T2}$ promoter. Mouse#2 developed significant hyperpigmentation on the plantar surface of the feet following topical treatment with 4HT. Figure adapted from Yeon Sook Choi.
Figure 2: Giant melanocytic nevus in the footpad of a 16-day-old Tyr-CreER$^{T2}$ LSL-
**Nras**$^{Q61R/Q61R}$ mouse compared with age-matched **LSL-Nras**$^{Q61R/+}$ mouse lacking Cre after 4HT induction.

(a) Fontana-Masson staining of melanin. Abundant melanin is located within the dermal layers of the footpad, whereas melanin is absent in the mouse footpad of the mouse lacking Cre. (b) and (c) Immunofluorescence of melanocytic markers MITF and DCT reveal ectopic dermal melanocytes (yellow arrows) in mutant skin. Stippled lines delineate epidermis (epi) and hair follicles. (d) Immunofluorescence of melanocytic markers SOX10 and S100 reveal ectopic dermal melanocytes (yellow arrows) in mutant skin. Epidermal melanocytes are also present in both the control and mutant skin. Stippled lines delineate epidermis (epi) and hair follicles. Figure adapted from Yeon Sook Choi.
Figure 2: Melanomas develop in Tyr-CreERT2 LSL-NrasQ61R/Q61R mice.

(a) Grossly unpigmented melanoma formation on the tail in 8-month-old Tyr-CreERT2 LSL-NrasQ61R/Q61R mouse with 4HT-induced tail pigmentation (below) compared to an age-matched control mouse (above). (b) Bright field microscopy evaluation of tumor tissue from a 17-month-old Tyr-CreERT2 LSL-NrasQ61R/Q61R mouse that developed a melanoma on the lower dorsum. Melanin deposition (blue arrow) is visible in certain regions of the tumor, despite tumors being grossly unpigmented. (c) The melanocytic marker DCT was detectable via immunofluorescence with the tumor tissue. MITF, however, was not. (d) The melanocytic markers SOX10 and S100 were abundantly present in the majority of cells within the tumor tissue.
Figure 4: Induced Tyr-CreER\textsuperscript{T2} LSL-Nras\textsuperscript{Q61R} mutant mice with inducible nevi develop melanoma.

The majority of Tyr-CreER\textsuperscript{T2} LSL-Nras\textsuperscript{Q61R} mice eventually develop melanoma, with median tumor latency of 31.0 weeks. Tyr-CreER\textsuperscript{T2} LSL-Nras\textsuperscript{Q61R/+} mice also develop melanoma, but at about half the rate and with a much longer median tumor latency of 89.3 weeks. Kaplan-Meier survival curves are shown above (green line: Tyr-CreER\textsuperscript{T2} LSL-Nras\textsuperscript{Q61R/Q61R}, red line: Tyr-CreER\textsuperscript{T2} LSL-Nras\textsuperscript{Q61R/+}, and blue line: control). Comparison of tumor-free survival following 4HT induction of both of these mutant strains against the control group via log-rank test shows statistically significant differences for both groups.
Figure 5: Dct-Cre Nras<sup>Q61R</sup> mutant mice with constitutive nevi also develop melanoma.

A little more than a quarter of Dct-Cre Nras<sup>Q61R/Q61R</sup> mice eventually develop melanoma, with median tumor latency of 12.3 weeks. Dct-Cre Nras<sup>Q61R/+</sup> mice also develop melanoma, but at a much lower rate and with a longer median tumor latency of 37.3 weeks. Kaplan-Meier survival curves are shown above (blue line: Dct-Cre Nras<sup>Q61R/Q61R</sup>, red line: Dct-Cre Nras<sup>Q61R/+</sup>, and green line: control). Comparison of tumor-free survival for the Dct-Cre Nras<sup>Q61R/Q61R</sup> mutant group against the control group via log-rank test showed a statistically significant difference of p=0.0277.
Figure 6: The effect of red versus black background on melanomagenesis in mice with constitutive nevi.

Red-haired Dct-Cre \( Nras^{Q61R/Q61R} Mc1r^{e/e} \) mice develop tumors two times more than black-haired Dct-Cre \( Nras^{Q61R/Q61R} Mc1r^{E/E} \). Tumors in the red-haired mice almost five weeks earlier than black-mice develop tumors. A Kaplan-Meier survival curve demonstrates the difference in overall tumor-free survival of the red- (red line) versus black-haired (blue line) mice.
Figure 7: Screening NRAS-signaling targeted therapies and melanocyte-selective toxic agents.

(a) Schedule for ear nevus induction and drug treatment for \( \text{Tyr-CreER}^{T2 \text{ LSL-Nras}^{Q61R/Q61R}} \) mice. The ears of mice were treated with 25 mg/ml of 4HT bilaterally beginning at postnatal day 1, seven times total over the course of two weeks. Tested agents were applied topically to the right ear and DMSO control vehicle to the left ear 5 times/week for 9 weeks total. Tissue sections were harvested 15 weeks after the final drug treatment. (b) Brightfield microscopy comparisons of H&E stained tissue of DMSO versus therapeutic agent (first row: DMSO versus 50 ug/5 ul MEK inhibitor binimetinib, second row: DMSO versus 25 ug/5 ul PI3K inhibitor omipalisib, third row: DMSO versus 500 ug/5 ul c-KIT inhibitor imatinib). Binimetinib generated the largest reduction in melanin (brown deposits) as compared to the control vehicle. (c) Schedule for paw nevus induction and drug treatment for \( \text{Tyr-CreER}^{T2 \text{ LSL-Nras}^{Q61R/Q61R}} \) mice. The paws of mice were treated with 25 mg/ml of 4HT bilaterally beginning at postnatal
day 1, six times total over the course of two weeks. Tested agents were applied subcutaneously to the right paw 5-6 times per week for two weeks total. (d) Treatment results with intralesional trametinib (1 µg/10 µl). The first panel shows the mouse paw prior to treatment, and the second panel shows the paw 26 weeks following the final drug treatment. The third panel shows brightfield microscopy of H&E stained paw tissue, with minimal melanin deposition (blue arrow). The last panel demonstrates few cells positive for the melanocyte-specific marker SOX10 (yellow arrows) in the dermis by immunofluorescence staining. No melanin deposition was visible via brightfield microscopy. Panel E show treatment results with intralesional selumetinib (10 µg/10 µl). The first panel shows the mouse paw prior to treatment, and the second panel shows the paw 21 weeks following the final drug treatment. The third panel shows brightfield microscopy of H&E stained paw tissue, with abundant melanin deposition (blue arrow). The last panel demonstrates several cells positive for the melanocyte-specific marker SOX10 (yellow arrows for dermal location, white arrow for epidermal location) by immunofluorescence staining. Melanin deposition (white deposits) was visible via brightfield microscopy.
Figure 8: Testing combination therapies on ear nevi.

Combination therapies of MEK inhibitors and imatinib (a c-KIT inhibitor) were tested on induced ear nevi of Tyr-CreER\textsuperscript{T2} LSL-Nras\textsuperscript{Q61R/Q61R} mice. To induce ear nevi, the mice were treated on the ears bilaterally with 25 mg/ml of 4HT beginning at postnatal day 1, seven times total over the course of two weeks. (a) H&E-stained tissue sections under brightfield microscopy following treatment with control vehicle DMSO on the left ear as well as combination 250 ug/5 ul imatinib + 15 ug/5 ul trametinib on the right ear. There is significantly less melanin (brown deposits) in the imatinib+trametinib-treated ear as compared to the control-treated ear. The
second row shows H&E-stained tissue sections under brightfield microscopy following treatment with control vehicle DMSO on the left ear as well as combination 250 ug/5 ul imatinib + 25 ug/5 ul binimetinib on the right ear. There is less melanin (brown deposits) and greater epidermal widening (see arrows) in the imatinib+binimetinib-treated ear as compared to the control-treated ear. (b) Immunofluorescence staining is positive for the melanocyte-specific marker SOX10 (yellow arrows). Melanin deposits are also visible (red arrow, merged from brightfield microscopy picture of unstained slide). In the imatinib+binimetinib-treated ear (right panel) compared to the DMSO-treated ear (left panel), there are fewer cells positive for SOX10 as well as no visible melanin deposits.