RASopathy Gene Mutations in Melanoma

Ruth Halaban1,4 and Michael Krauthammer2,3,4

Next-generation sequencing of melanomas has unraveled critical driver genes and genomic abnormalities, mostly defined as occurring at high frequency. In addition, less abundant mutations are present that link melanoma to a set of disorders, commonly called RASopathies. These disorders, which include neurofibromatosis and Noonan and Legius syndromes, harbor germline mutations in various RAS/mitogen-activated protein kinase signaling pathway genes. We highlight shared amino acid substitutions between this set of RASopathy mutations and those observed in large-scale melanoma sequencing data, uncovering a significant overlap. We review the evidence that these mutations activate the RAS/mitogen-activated protein kinase pathway in melanoma and are involved in melanomagenesis. Furthermore, we discuss the observations that two or more RASopathy mutations often co-occur in melanoma and may act synergistically on activating the pathway.

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Exome and genome sequencing have unraveled a large number of genetic and genomic changes in melanoma (Hodis et al., 2012; Krauthammer et al., 2012; Krauthammer et al., 2015; The Cancer Genome Atlas Network, 2015). The results confirmed the presence of frequent activating mutations in BRAF and NRAS and inactivating mutations in CDKN2A and TP53, and they unraveled additional lower frequency “drivers,” including the recurrent RAC1P295S and IDH1R132C and the frequently modified PPP6C, ARID1, and ARID2. The most recent findings highlight the numerous NF1 (neurofibromin 1) mutations affecting up to approximately 12% of all melanomas, with higher frequency (45%) in melanomas that are wild type (WT) for BRAF and RAS, with abundant inactivating mutations, such as early termination, insertions/deletions, and splice variants (Krauthammer et al., 2015; The Cancer Genome Atlas Network, 2015). Consequently, the consensus is that melanomas can be subdivided into four categories: BRAFmut, RASmut, NF1mut, and triple WT (Krauthammer et al., 2015; The Cancer Genome Atlas Network, 2015). Other cancers with large number of NF1 mutations include glioblastoma (14%) (The Cancer Genome Atlas Network, 2008) and squamous cell carcinoma (11%) (The Cancer Genome Atlas Network, 2012).

The “NF1 discovery” draws attention to the autosomal-dominant genetic disorder neurofibromatosis type 1 (NF1), caused by haploinsufficiency of neurofibromin, a RAS guanosine triphosphate (GTP)ase-activating protein that affects 1 in 2,500 to 1 in 3,500 individuals (Aoki et al., 2016; Ratner and Miller, 2015; Smokou et al., 2015). The classic manifestations of NF1 include café-au-lait macules (observed in 95% of patients), skinfold freckling, neurofibromas, brain tumors, iris hamartomas, and characteristic bony lesions. NF1 early-termination mutations in patients’ germlines are frequent (~80%), leading to release of constraints on RAS, followed by mitogen-activated protein kinase (MAPK) activation (Ratner and Miller, 2015), recapitulating the observations in melanoma (Krauthammer et al., 2015).

Neurofibromatosis is one of many autosomal-dominant genetic disorders with overlapping sets of symptoms, currently termed RASopathies (including Noonan and Legius syndromes), that have germline nonsynonymous mutations in genes encoding proteins in the RAS/MAPK signaling cascade. In addition to NF1, the list includes BRAF, RAF1, NRAS, KRAS, HRAS, RASA2, PTPN11, SPRED1, SOS1, CBL, SHOC2, MAP2K1, MAP2K2, and RITI (Ratner and Miller, 2015; Aoki et al., 2016) (Figure 1 and Table 1). Somatic mutations in these genes are also observed in cancer, where they may be functionally relevant, as assessed by their ability to activate the RAS/MAPK pathway and/or enhance cell proliferation. Often, these somatic mutations alter the very same amino acid present in the germline of RASopathy patients (Table 1). In melanoma, this relationship and functional consequences are most clearly established for changes in BRAF, NRAS, MAP2K1, and RASA2.

COMPARISONS OF SPECIFIC GENES

BRAF

The canonical V600E/K substitutions lead to BRAF-kinase activation, the first to be targeted by specific inhibitors (Bollag et al., 2012). Other changes in BRAF (L245F, F468S, G469R, L485F, N583S/T, K601E) are shared between melanoma and the RASopathies cardio-facio-cutaneous and Noonan syndromes (Rodriguez-Viciana and Rauen, 2008) (Table 1). Many of these noncanonical alterations are located within the kinase domain (amino acids 457–713) and are activating mutations that lead to increased kinase activity over BRAFWT and extracellular signal-regulated kinase (ERK) activation in transfected COS cells (Rodriguez-Viciana and...
Rauen, 2008; Wan et al., 2004). Furthermore, \textit{BRAF} G469E, D594G, and K601E mutant melanomas display increased ERK phosphorylation over nonmutant control cell lines (Smalley et al., 2009).

\textbf{NRAS} Melanomas typically harbor changes in the Q61 position of \textit{NRAS} and, to a much lesser degree, in G12 and G13. Mice knock-in studies showed that expression of \textit{Nras}Q61R but not \textit{Nras}G12D promoted melanoma formation in vivo in \textit{p16INK4A}-deficient mice (Burd et al., 2014). Functional studies showed that the basis for these differences is \textit{Nras}Q61R enhanced GTP binding, decreased intrinsic GTPase activity, and increased stability when compared with \textit{Nras}G12D (Burd et al., 2014). Germline mutations in Q61 were not reported, but Noonan syndrome patients and those with melanomas share the very same G12 and G13 \textit{NRAS} amino acid substitutions (Table 1).

\textbf{MAP2K1} Recurrent \textit{MAP2K1}P124L mutations are present in melanoma tumors (Krauthammer et al., 2015; Nikolaev et al., 2012), and \textit{MAP2K1}P124L is also observed in the RASopathy cardio-facio-cutaneous syndrome. The mutation confers increased kinase activity (Carlino et al., 2015; Emery et al., 2009). The effect of the mutation on drug response is likely to be cell specific. The \textit{MAP2K1}P124L appeared in the tumor of a patient who relapsed after treatment with the MEK inhibitor selumetinib (Emery et al., 2009). In addition, pre-existing \textit{MAP2K1}P124L diminished, but did not preclude, the clinical response to \textit{BRAF} inhibitors of \textit{BRAF}mut melanomas (Carlino et al., 2015; Johnson et al., 2015). In culture, two double mutant melanoma cells lines showed intermediate sensitivity to dabrafenib but were exquisitely sensitive to the downstream MAPK/ERK kinase and ERK inhibitors trametinib and VX-11e (Carlino et al., 2015). Likewise, in our studies, treatments with the MAPK/ERK kinase inhibitor selumetinib showed that one patient-derived melanoma cell line carrying both \textit{BRAF}V600K and \textit{MAP2K1}P124L mutations was relatively resistant (YUKSI melanoma line, half maximal inhibitory concentration = 374 nmol/L), whereas another one with \textit{BRAF}V600R and \textit{MAP2K1}P124L was highly sensitive (YUZEAAL melanoma line, half maximal inhibitory concentration = 15 nmol/L) (Krauthammer et al., 2015).

**Table 1. Melanoma and RASopathy Shared Gene Mutations**

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Shared Amino Acid Change</th>
<th>RASopathy Syndrome Type</th>
</tr>
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<tbody>
<tr>
<td>\textit{NF1}</td>
<td>R1241*, R1362*, R1870Q, and other nonsense mutations causing premature truncation</td>
<td>Neurofibromatosis 1</td>
</tr>
<tr>
<td>\textit{NRAS}</td>
<td>G12D/R/V, G13D, T30R</td>
<td>Noonan syndrome</td>
</tr>
<tr>
<td>\textit{KRAS}</td>
<td>G12A/D/R (S), G13D (E/R/L), Q61R</td>
<td>Cardio-facio-cutaneous syndrome, Noonan syndrome</td>
</tr>
<tr>
<td>\textit{HRAS}</td>
<td>G13R/D (C), Q61R</td>
<td>Costello syndrome</td>
</tr>
<tr>
<td>\textit{RAF1}</td>
<td>S257L, P261L (H/T/A/S), T491R</td>
<td>Noonan syndrome, LEOPARD syndrome</td>
</tr>
<tr>
<td>\textit{MAP2K1}</td>
<td>P124L (D)</td>
<td>Cardio-facio-cutaneous syndrome</td>
</tr>
<tr>
<td>\textit{MAP2K2}</td>
<td>F57L/V (C)</td>
<td>Cardio-facio-cutaneous syndrome, Noonan syndrome</td>
</tr>
<tr>
<td>\textit{RASA2}</td>
<td>R311C</td>
<td>Noonan syndrome</td>
</tr>
<tr>
<td>\textit{SPRED1}</td>
<td>R117Q (*) and other nonsense mutations causing premature truncation</td>
<td>Neurofibromatosis 1-like syndrome, Legius syndrome</td>
</tr>
<tr>
<td>\textit{PTPN11}</td>
<td>P71L, V279C, A461T, T468M, P499L, Q506F, Q510H</td>
<td>Noonan syndrome, LEOPARD syndrome</td>
</tr>
<tr>
<td>\textit{SOS1}</td>
<td>P102S (R), M269K (T/R), G434R, R552K (T/S/M/G), D1200E</td>
<td>Noonan syndrome</td>
</tr>
<tr>
<td>\textit{CBL}</td>
<td>L493F</td>
<td>Noonan-like syndrome</td>
</tr>
</tbody>
</table>

\(^1\)The melanoma mutations are from Yale, Broad Institute, and The Cancer Genome Atlas data; the RASopathy syndrome mutations are from the Human Gene Mutation Database (Stenson et al., 2012) and ClinVar (Landrum et al., 2014). The additional amino acid substitutions in RASopathy genes not shared with melanomas are indicated in parenthesis. An asterisk indicates early termination.
RASA2
Melanomas carry 25 different RASA2 substitutions, five of which are of the early-termination type present also in other cancers (cervical, head and neck cancers), suggesting a tumor suppressor function (Figure 2a). The recurrent RASA2R511C somatic mutation is one of three RASA2 variants described in patients with Noonan syndrome (Chen et al., 2014)( Figure 2a). The R511C amino acid change located in the RAS-GTPase activating protein (GAP) domain abolishes RASA2 activity and increases the activity levels of RAS-GTP and ERK (Chen et al., 2014). The protein, like NF1, functions as a suppressor by activating RAS GTPase and converting RAS-GTP to RAS (Figure 1). Similarly, suppression of RASA2 in melanoma cells by small interfering RNAs increased the levels of activated RAS (Arafeh et al., 2015). The impact of RASA2 mutants is likely to be synergistic with NF1, because they occur mostly in NF1-mutant tumors that are BRAF/RAS WT (Krauthammer et al., 2015).

SPRED1
SPRED1 acts as a tumor suppressor because it enhances NF1 inhibitory activity by recruiting the protein to the plasma membrane and to RAS (Hirata et al., 2016; Stowe et al., 2012) (Figure 1). Mutations in SPRED1 act in an autosomal-dominant manner in Legius and NF1-like syndromes, mild forms of NF1 carrying skin features such as multiple café-au-lait macules, but no neurofibromas (Brems et al., 2012). Like NF1, this is another RASopathy gene that carries high frequency of early-termination mutations in both melanoma (71%) and the germline of patients (65%). In addition, NF1-like syndrome and melanomas share the SPRED1 R117Q substitution (Table 1). Interestingly, mutational analysis of melanocytes isolated from café-au-lait lesions of a patient with germline SPRED1-R24* carried another SPRED1 mutation, T102fsX6. The two SPRED1 mutations were located on different alleles, suggesting that SPRED1 function was completely absent, allowing increased MAPK activity and enhancing the rate of melanocyte proliferation, providing in vivo confirmation of its importance in melanocyte biology (Brems et al., 2007).

PTPN11
The gene product, also known as Src homology phosphatase-2 (SHP2), is a nonreceptor protein tyrosine-phosphatase with multiple positive functions in signal transduction (Chan et al., 2008). It is a docking protein for the growth factor receptor-bound protein-2 (GRB2)/son of sevenless (SOS) complex, thereby promoting MAPK activation and cell division (Figure 1). The protein tyrosine phosphatase, non-receptor type 11 gene, PTPN11, is frequently altered in Noonan and Leopard syndromes and cancer cells (Zhang et al., 2015). Analyses of the D61G mutation, frequent in RASopathies, showed that it is gain-function-change, activating the proto-oncogene SRC tyrosine kinase, which in turn activates the serine/threonine kinase polo-like kinase-1 (PLK1), inducing chromosomal instability and disruption of mitosis (Liu et al., 2016). Among the 20 mutation sites in melanoma, seven are shared with Noonan and Leopard syndromes (F71L, Y279C, A461T, R491L, Q506P, Q510H). Except for one that is located in the N-terminal SH2 domain (F71L), the rest are in the tyrosine-protein phosphatase (PTP) domain (Figure 2b). Other cancers, such as acute myeloid leukemia, sarcoma, and glioblastoma, also share PTPN11 mutations with melanomas and RASopathies, the most common being Q510H (Figure 2b, marked with blue). The somatic, like the germline, mutations are likely of the gain-of-function type because of disruption of the autoinhibitory interaction between the N-SH2 and PTP domains of the protein (Chan et al., 2008; Yu

Figure 2. Schematic representation of RASA2 and PTPN11 mutations in melanomas and RASopathies. (a) RASA2, (b) PTPN11. The mutations indicated above and below the bar are those identified in melanoma and those shared with RASopathy syndromes, respectively. In blue are mutations shared with other cancers. The bars indicate conserved domains. Numbers below the bars indicate the amino acid positions. BTK, Bruton’s tyrosine kinase Cys-rich motif; C2, protein kinase C conserved region 2; LP, LEOPARD syndrome; LS, Legius syndrome; NS, Noonan syndrome; PH, pleckstrin homology-like domain; RAS-GAP, GTPase-activator protein for Ras-like GTPases; SB, substrate binding site; SH2, Src homology 2 domain.
Indeed, a change in the N-SH2 domain (D61G) activates SHP2 and enhances tumor adhesion, proliferation, migration, and invasion of breast cancer cells (Hu et al., 2015).

**SOS1**

SOS1 is a guanine nucleotide exchange factor for RAS protein (RasGEF), catalyzing the transition of RAS-guanosine diphosphate to RAS-GTP, which is usually activated downstream of growth factor receptors (Figure 1). In melanomas, this gene carries five shared mutations with Noonan syndrome, two of them present in NF1-mutant/BRAF/RAS WT lesions (G434R, R552K) (Table 1). As with PTEN11, it is expected that the mutations are of the gain-of-function type, promoting an activated RAS-GTP status.

In total, melanomas share disease-causing, nonsilent amino changes in 13 out of 16 known RASopathy genes (Figure 1 and Table 1).

**EVIDENCE FOR FUNCTIONAL COOPERATION OF RASOPATHY GENES**

Looking at all mutations in RASopathy genes (other than NF1), we find that they are significantly enriched in NF1-mutant melanomas, that is, 57.7% of NF1-mutant, 15.6% of NRAS-mutant, 6.6% of triple WT, and 4.3% of BRAF-mutant melanomas harbor concurrent RASopathy mutations (Krauthammer et al., 2015). The observation that NF1 and other RASopathy gene mutations co-occur likely suggests that they act in a synergistic manner. Next-generation sequencing of DNA from 27 patients with NF1 showed additional variants in more than one gene in the RAS-MAPK pathway, likely contributing to the observed neurofibromatosis features (Chen et al., 2014). Noonan syndrome patients with atypical severe symptoms harbored coexisting mutations in NF1 and PTEN11, in one patient leading to death during early infancy (Nyström et al., 2009; Prada et al., 2011). In another case, a child with double genetic defects in NF1 and PTEN11 developed bilateral optic nerve gliomas, with other family members who carried only the NF1 mutation displaying mild neurofibromatosis symptoms (café-au-lait spots) (Thiel et al., 2009).

A similar situation exists in cancer cells. Bioinformatics analyses of over 900 cell lines from the Cancer Cell Line Encyclopedia showed that 31% of cells containing noncanonical KRAS mutations also had an NF1 mutation (P < 0.005) (Stites et al., 2015). A mathematical model based on RAS signaling reactions applied to a neurofibromin-deficient condition predicted, and then was experimentally supported, that loss of NF1 enhances the activity of the noncanonical RAS<sub>G12D</sub> (Stites et al., 2015). Likewise, we identified two cases of NF1-mutant melanomas, one with KRAS<sub>A146T</sub> and the other with KRAS<sub>G22K</sub>, suggesting a similar situation. Our observation that RASA2 and NF1 significantly co-occur in melanoma (Krauthammer et al., 2015) is mirrored by a report that double-loss of Nf1 and Rasa1 in mice is required to enhance the development of T-cell acute lymphoblastic leukemia/lymphoma, supporting a synergistic effect on dysregulation of RAS signaling (Lubeck et al., 2015). Similarly, our data showing co-occurring PTEN11 and NF1 mutations in melanoma (Krauthammer et al., 2015) is strengthened by a recent report of the presence of PTEN11 mutations in NF1-mutant desmoplastic melanomas (Shain et al., 2015).

Co-occurring pairs of RASopathy mutations are present in melanoma lacking NF1 variants. For example, noncanonical RASopathy BRAF changes G469E and D594G coincide with NRAS G12D (Lin et al., 2008). However, the significance of these double mutations requires further studies because of the observations mentioned that knock-in Nras<sup>G12D</sup> transformed normal melanocytes to melanoma much less efficiently when compared with Nras<sup>G261R</sup> in p16<sup>INK4a</sup> knockout mice (Burd et al., 2014).

Functional cooperation was also observed in a subtype of KRAS/NRAS WT acute myeloid leukemia that is characterized by down-regulation of sprouty RTK signaling antagonist 4 (SPRY4), a SPRED1-related gene product that negatively regulates RAS-GTP, and co-occurring heterozygous deletions in TP53 and/or in other negative regulators of RAS signaling, such as NF1, RASA1, DUSP1, and DUSP14 (Geiger et al., 2015; Zhao et al., 2015). Altogether, the presence of more than one RASopathy gene mutation is likely to enhance RAS function and to induce growth advantage by enhancing the MAPK pathway.

An important question relates to the incidence of cancer in neurofibromatosis and other RASopathy patients. Individuals with germline NF1 alterations are at increased risk of developing various tumors, including malignant peripheral nerve sheath tumor, pheochromocytoma, leukemia, glioma, rhabdomyosarcoma, breast and ovary tumors, and rarely melanomas (Ratner and Miller, 2015; Smokou et al., 2015). Studies show that additional mutations are present in these tumors such as second hits in NF1 and TP53, multiple copy number alterations, and deletion of CDKN2A (Ratner and Miller, 2015). Whole-exome sequencing of tumors from NF1 patients with NF1<sup>F434R</sup> showed that each of the lesions (dermal neurofibromas, breast cancer, malignant peripheral nerve sheath tumor) harbored another mutation in NF1; the breast cancer and malignant peripheral nerve sheath tumor presented with additional mutations unique for each tumor (McPherson et al., 2015).

These results and our observations that NF1 mutations are more frequent in melanomas that carry a high number of mutations, suggest that suppression of NF1 alone is not sufficient to confer malignancy and that combined loss of multiple negative regulators of the RAS pathway are required for melanomagenesis.

**CONFLICT OF INTEREST**

The authors state no conflict of interest.

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**REFERENCES**


