RASopathy Gene Mutations in Melanoma

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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 $RAC1^{P29S}$ and IDH^{R132C} 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: $BRAF^{mut}$, RAS^{mut}, $NF1^{mut}$, 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; Smpokou 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 *RIT1* (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, N581S/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 $BRAF^{WT}$ and extracellular signal-regulated kinase (ERK) activation in transfected COS cells (Rodriguez-Viciana and

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Abbreviations: ERK, extracellular signal-regulated kinase; GTP, guanosine triphosphate; MAPK, mitogen-activated protein kinase; NF1, neurofibromatosis type 1; WT, wild type

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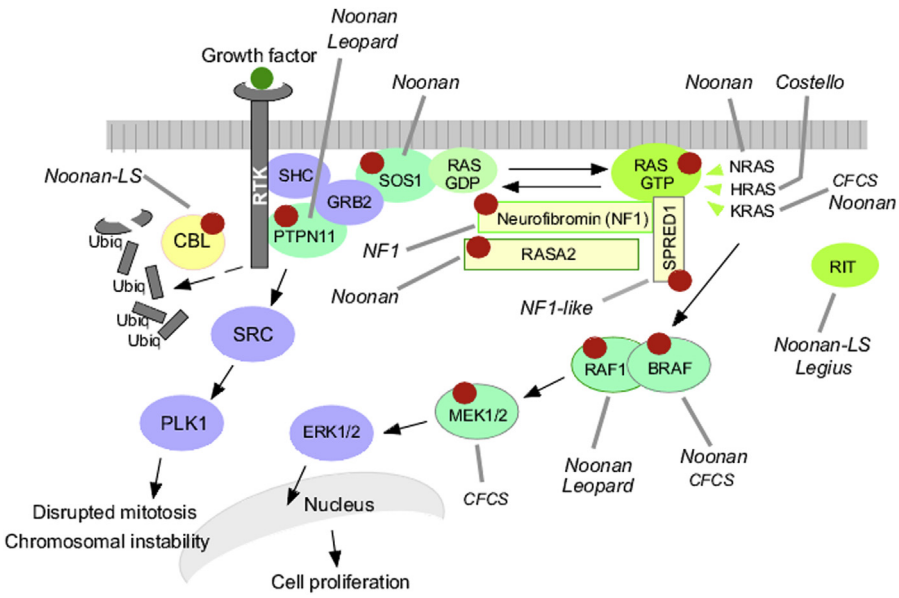

Figure 1. Mitogen-activated protein kinase pathway indicating RASopathy mutant genes and those shared with melanoma. The green and yellow bars are RASopathy genes with (presumably) activating and inactivating mutations, respectively. Those with amino acid changes shared with melanoma are marked with a red dot. CFCS, cardio-facio-cutaneous syndrome; GDP, guanosine diphosphate; GTP, guanosine triphosphate Noonan-LS, Noonan-like syndrome; RTK, receptor tyrosine kinase.

Rauen, 2008; Wan et al., 2004). Furthermore, *BRAF* G469E, D594G, and K601E mutant melanomas display increased ERK phosphorylation over nonmutant control cell lines (Smalley et al., 2009).

Table 1. Melanoma and RASopathy Shared Gene Mutations[1]

Gene Symbol	Shared Amino Acid Change	RASopathy Syndrome Type
NF1	*R1241*, R1362*, R1870Q,* and other nonsense mutations causing premature truncation	Neurofibromatosis 1
BRAF	L245F, F468S, G469R, L485F, N581H (K/D), V600G	Cardio-facio-cutaneous syndrome, Noonan syndrome
NRAS	G12D/R/V, G13D, T50I	Noonan syndrome
KRAS	G12A/I/D/R (S), Q22K (E/R/L), Q61R	Cardio-facio-cutaneous syndrome, Noonan syndrome
HRAS	G13R/D (C), Q61K (R)	Costello syndrome
RAF1	S257L, P261L (H/T/A/S), T491I (R)	Noonan syndrome, LEOPARD syndrome
MAP2K1	P124L (D)	Cardio-facio-cutaneous syndrome
MAP2K2	F57L/V (C)	Cardio-facio-cutaneous syndrome, Noonan syndrome
RASA2	R511C	Noonan syndrome
SPRED1	R117Q (*) and other nonsense mutations causing premature truncation	Neurofibromatosis 1-like syndrome, Legius syndrome
PTPN11	F71L, Y279C, A461T, T468M, P491L, Q506P, Q510H	Noonan syndrome, LEOPARD syndrome
SOS1	P102S (R), M269K (T/R), G434R, R552K (T/S/M/G), D1200E	Noonan syndrome
CBL	L493F	Noonan-like syndrome

[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 alternative amino acid substitutions in RASopathy genes not shared with melanomas are indicated in parenthesis. An asterisk indicates early termination.

NRAS

Melanomas typically harbor changes in the Q61 position of *NRAS* and, to a much lesser degree, in G12 and G13. Mice knock-in studies showed that expression of $Nras^{Q61R}$ but not $Nras^{G12D}$ promoted melanoma formation in vivo in *p16INK4A*-deficient mice (Burd et al., 2014). Functional studies showed that the basis for these differences is $Nras^{Q61R}$ enhanced GTP binding, decreased intrinsic GTPase activity, and increased stability when compared with $Nras^{G12D}$ (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 *NRAS* amino acid substitutions (Table 1).

MAP2K1

Recurrent $MAP2K1^{P124L/S}$ mutations are present in melanoma tumors (Krauthammer et al., 2015; Nikolaev et al., 2012), and $MAP2K1^{P124L}$ 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 $MAP2K1^{P124L}$ appeared in the tumor of patient who relapsed after treatment with the MEK inhibitor selumetinib (Emery et al., 2009). In addition, pre-existing $MAP2K1^{P124L}$ diminished, but did not preclude, the clinical response to *BRAF* inhibitors of $BRAF^{mut}$ 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 kinsase and ERK inhibitors trametinib and VX-11e (Carlino et al., 2015). Likewise, in our studies, treatments with the MAPK/ERK kinsase inhibitor selumetinib showed that one patient-derived melanoma cell line carrying both $BRAF^{V600K}$ and $MAP2K1^{P124L}$ mutations was relatively resistant (YUKSI melanoma line, half maximal inhibitory concentration = 374 nmol/L), whereas another one with $BRAF^{V600R}$ and $MAP2K1^{P124L}$ was highly sensitive (YUZEAL melanoma line, half maximal inhibitory concentration = 15 nmol/L) (Krauthammer et al., 2015).

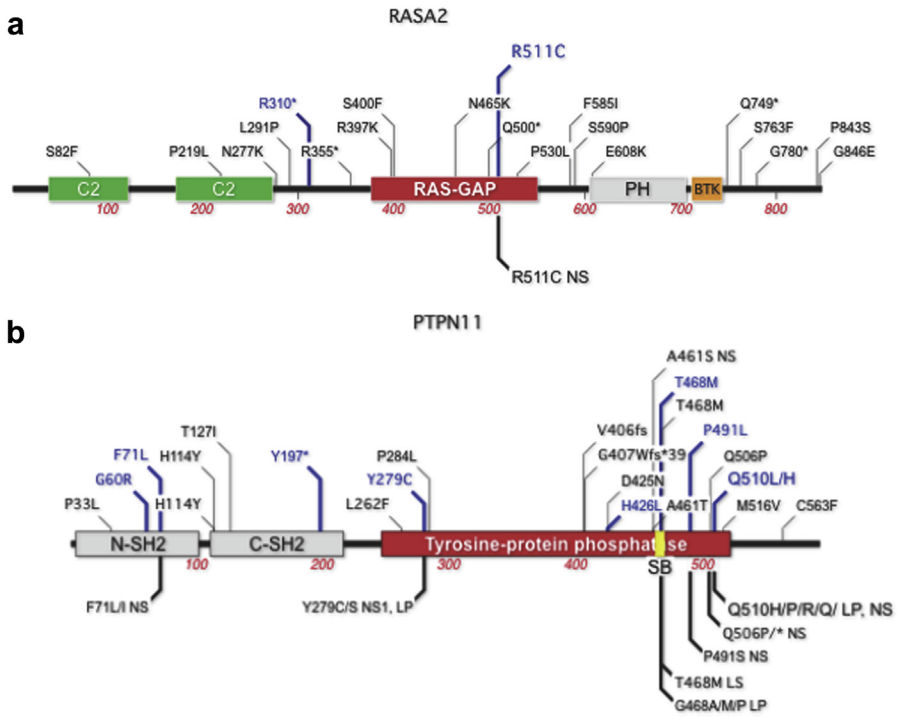

Figure 2. Schematic representation of *RASA2* and *PTPN11* mutations in melanomas and RASopathies. (**a**) *RASA2*. (**b**) *PTPN11*. The mutations indicated above and below the bar are those identified in melanoma and those shared with RASopathy syndromes, respectively. In blue are mutations shared 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.

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 *RASA2*^{R511C} 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, T468M, P491L, 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

et al., 2014). 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, that 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 *PTPN11*, 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 *PTPN11*, in one patient leading to death during early infancy (Nystrom et al., 2009; Prada et al., 2011). In another case, a child with double genetic defects in *NF1* and *PTPN11* 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^{F28L} (Stites et al., 2015). Likewise, we identified two cases of *NF1*-mutant melanomas, one with $KRAS^{A146T}$ and the other with $KRAS^{Q22K}$, 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 *PTPN11* and *NF1* mutations in melanoma (Krauthammer et al., 2015) is strengthened by a recent report of the presence of *PTPN11* 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 $Nras^{G12DS}$ transformed normal melanocytes to melanoma much less efficiently when compared with $Nras^{Q61R}$ in $p16^{INK4a}$ 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; Smpokou 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 $NF1^{L847P}$ 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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