See related article on pg 2041

NRAS, NRAS, Which Mutation Is Fairest of Them All?

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In 28% of melanomas, *NRAS* is mutated in one of two hotspots: G12 or Q61. Phosphoproteomic analysis of primary human melanocytes transduced with G12 and Q61 showed different phosphorylation events in the phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways. Surprisingly, $NRAS^{G12}$ modulates the PI3K pathway and overexpresses the kinase PIM2, whereas $NRAS^{Q61}$ is associated with the MAPK pathway and overexpression of CK2α.

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Reversible protein posttranslational modifications regulate the activity of most proteins, and phosphorylation is the modification that has been most studied (de Graauw et al., 2006). Protein kinases and phosphatases are crucial to regulating many protein qualities, including stability, activity, subcellular localization, and protein-protein interaction. These modifications are essential for the control of cellular functions, including proliferation, differentiation, and migration (de Graauw et al., 2006). The absence of appropriate regulation of phosphorylation may cause human disease, including cancer. It appears to be important to identify all the proteins that can be phosphorylated in normal and pathologic contexts for specific cell lineages. Novel technologies allow these types of analyses to unravel signaling pathways in great depth to understand and target proteins for therapeutic purposes (de Graauw et al., 2006).

Melanoma remains a fatal disease once it becomes metastatic, even though a profound revolution has occurred recently with immunotherapies and targeted therapies. To date, the most successful targeted treatment is associated with targeting of $BRAF^{V600E}$, which is present in 50% of melanomas and which activates the mitogen-activated protein kinase (MAPK) pathway constitutively. A second gene, *NRAS,* is also commonly mutated in melanoma, occurring in 27.7% of all patients (Berger et al., 2012; Hodis et al., 2012; Krauthammer et al., 2012; TCGA Research Network, 2016). Unfortunately, we do not know how to target this protein therapeutically. Thus, better knowledge of this protein would bring ideas and opportunities to the design of useful therapeutic approaches.

NRAS belongs to the RAS superfamily and is a guanosine diphosphate (GDP)-binding protein, which switches between an inactive guanosine diphosphate-bound state to an active GDP-bound state, depending on growth factor stimulation (Zhang and Cheong, 2016). Activated NRAS is capable of activating the MAPK and PI3K pathways (Zhang and Cheong, 2016). Mutations at G12 and Q61 lock NRAS in an active form, and they are present in various cancers, including melanoma (Figure 1). The G12 and Q61 mutations have been found in 4.9% and 88.1% of NRAS melanoma patients, respectively. The most frequent mutation in codon G12 is the missense mutation G12D, present in 2.1% of patients, and in codon Q61 the most frequent mutation is Q61R, present in 41.3% of patients. Either of these two *NRAS* mutations, *G12* and *Q61*, induces abnormal phosphorylation of downstream molecules.

Posch et al. (2016) took on the challenge of investigating two additional mutations in *NRAS,* G12V and Q61L. They analyzed transduced non-immortalized primary human melanocytes expressing $NRAS^{G12V}$ or $NRAS^{Q61L}$, and used an empty vector as control. The mRNA levels for $NRAS^{G12V}$ and $NRAS^{Q61L}$ were similar in both transduced primary human melanocytes. No obvious cellular modification was observed; the rate of proliferation was similar, and the morphology of the cells was not affected. However, cell pellets of the two mutants were hypopigmented compared with empty vector controls, suggesting that melanogenesis was affected. To investigate the global phosphoproteome of $NRAS^{G12V}$, $NRAS^{Q61L}$, and empty vector transduced cells, they first labeled the three cell lines using stable isotope labeling by amino acids in cell culture (SILAC), generated and enriched phosphopeptides after protein digestion, and finally analyzed the three types of samples by high-accuracy mass spectrometry.

The study by Posch et al. (2016) identified 126 proteins and 163 phosphosites, which were 2-fold differentially regulated between $NRAS^{G12V}$ and $NRAS^{Q61L}$. Subsequent gene ontology analysis revealed the most interesting fact of their study: namely, that the $NRAS^{G12V}$ mutation regulates PI3K signaling that and $NRAS^{Q61L}$ modulates the MAPK pathway (Posch et al., 2016). These findings were validated by immunoblotting, showing that $NRAS^{G12V}$ primary human melanocytes produced more pAKT than $NRAS^{Q61L}$, and conversely, that $NRAS^{Q61L}$ hyperactivated phosphorylated MAPK/extracellular signal–regulated kinase (pMEK) and phosphorylated extracellular signal–regulated kinase (pERK) compared with $NRAS^{G12V}$.

Next, from the 126 identified proteins, Posch et al. (2016) focused on DFG-start (ASP-PHE-GLY) and APE-stop (ALA-PRO-GLU) amino acid sequences that mark the activation loop of kinases. They identified 36 kinases with activation loops, of which PAK4, MAPK14, MAPK1, and CDK11A were

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Clinical Implications

- *NRAS* mutation is a common driver of melanoma (28%).
- $NRAS^{G12}$ and $NRAS^{Q61}$ lead to different phosphorylation events, inducing the PI3K and MAPK pathways, respectively.
- $NRAS^{Q61}$ induces CK2α expression, suggesting the latter kinase as a newtherapeutic target.

differentially phosphorylated in the activation loop comparing $NRAS^{G12V}$ and $NRAS^{Q61L}$ cells with empty vector controls. These four kinases have been shown to be involved in cell cycle control, activity of human tumor suppressors, and cell fate decisions.

Investigating serine/threonine motifs, Posch et al. (2016) computationally predicted kinases that are involved predominantly in the phosphorylation events detected by SILAC. It appeared that PIM2 was highly phosphorylated by $NRAS^{G12V}$, and CK2α by $NRAS^{Q61L}$. This is certainly interesting, because these two kinases are involved in tumor initiation, progression, and metastasis in leukemia and prostate cancer. Thus far, PIM2 has not been shown to be involved directly in melanoma biology; CK2α was found to be involved in melanoma resistance (Zhou et al., 2016). Based on these results, Posch et al. used a CK2α inhibitor, CX4945, on $NRAS^{G12}$- and $NRAS^{Q61}$-mutant melanoma cell lines in vitro and showed that $NRAS^{Q61}$ cells were more sensitive than $NRAS^{G12}$ cells. The inhibition of PIM2 by inhibitors was not performed.

Finally, Posch et al. (2016) completed this study by evaluating the clinical relevance of their findings using their own tumors and The Cancer Genome Atlas (TCGA) melanoma data set (Cerami et al., 2012; Gao et al., 2013). They analyzed 22 cutaneous *NRAS*-mutant melanoma biopsy samples from their own library by immunohistochemistry. It appeared that the level of CK2α was higher in $NRAS^{Q61}$ tumors than in $NRAS^{G12D}$ tumors. These results are in agreement with their in vitro findings, and they connect N-Ras^{Q61} with CK2α in vivo. Using the TCGA data set and comparing mRNA levels of CK2α in $NRAS^{Q61}$ tumors with no reported mutations (no reported mutation; indicating a lack of $NRAS^{Q61}$ mutations) showed significantly higher levels of CK2α mRNA in $NRAS^{Q61}$ tumors than in tumors with no reported mutations. Unfortunately, because of the low number of $NRAS^{G12}$ tumors, the comparison of expression levels for CK2α between $NRAS^{Q61}$ and $NRAS^{G12}$ was not statistically different. However, there was a trend toward a difference for CK2α expression.

Posch et al. (2016) performed their analysis using G12V and Q61L mutations. These two mutations are found in melanoma, but they are not the most frequent. It would have been of interest to perform such studies with G12D and Q61K/R mutations as well. It has been shown that combined targeting of Raf and PI3K, Ras-effector arms, effectively reduced tumor growth in *NRAS*-mutant tumors (Q61L and Q61K); thus, it is possible that these two mutants might behave in a similar way (Jaiswal et al., 2009). With respect to the mutation G12D, it has been shown that it alone does not induce melanomagenesis in adult mouse melanocytes (Pedersen et al., 2014). However, more than one third of mice carrying one copy of $NRAS^{G12D}$ and one copy of $BRAF^{D594A}$, (Tyr::CreERT2/°, $NRas^{G12D/+}$, $BRaf^{D594A/+}$) developed melanoma within 24 months. Furthermore, almost all mice (95%) carrying two copies of $NRas^{G12D}$ and one copy of $BRAF^{D594A}$ (Tyr::CreERT2/°, $NRas^{G12D/G12D}$, $BRaf^{D594A/+}$) developed melanoma within 13 months. This suggests that $NRas^{G12D}$ and $BRaf^{D594A}$ cooperate to initiate melanomagenesis (Pedersen et al., 2014). According to the Posch et al. and Pedersen et al. articles, it might not be farfetched to speculate that $NRAS^{G12V}$ could also cooperate with $BRAF^{D594A}$. Taken together, these results suggest that it may not be a specific amino acid substitution by itself that contributes to certain changes involved in melanomagenesis, but rather the location within the protein itself. The $NRAS^{G12V}$ mutation affects the phosphate-binding loop (or Walker A motif) within the NRAS protein (Figure 1), whereas the $NRAS^{Q61L}$ mutation affects the Switch II motif involved in intrinsic catalytic activity. These mutations occur in the two mutational hotspots of *NRAS*. Therefore, Posch et al. thought it might be worthwhile to characterize the effects of alterations in these two *NRAS* domains represented by mutant $NRAS^{G12V}$ and $NRAS^{Q61L}$.

Because of the molecular complexity of melanoma, treatment of advanced disease remains a challenge. Several inhibitors exist targeting specific proteins of signaling pathways such as BRAF, MEK, ERK, and mTOR. Unfortunately, patients' responses remain transient because of the development of resistance (Wellbrock and Arozarena, 2016). Posch et al. (2016) suggest that the kinase CK2α, overexpressed in patients with $NRAS^{Q61}$-mutant melanoma, can be targeted. Comparing different cancers using the data sets of the TCGA, cross-cancer alteration for *NRAS* showed that several cancers show *NRAS* mutations (TCGA Research Network, 2016). For instance, thyroid, colorectal, and bladder cancers; rhabdomyosarcoma; multiple myeloma; and leukemia have mutations in the two hotspots of $NRAS^{G12}$ (phosphate-binding loop domain) and $NRAS^{Q61}$ (Switch II domain). Posch et al. open new avenues for the treatment of melanoma patients carrying *NRAS* mutations, using CK2α as a potential target.

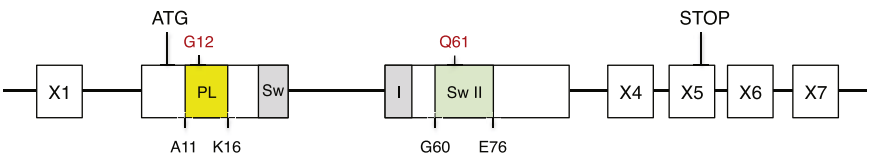

Figure 1. Schematic representation of the primary structure of *NRAS*. *NRAS* contains seven exons. ATG is located in exon 2 and stop codon in exon 5 (X5). *NRAS* is commonly mutated at the level of amino acid G12 (X2) and Q61 (X3) located in the phosphate-binding loop domain (PL, yellow) and Switch domain II (Sw II, light green), respectively. Switch domain I (Sw I) is encoded by X2 and X3.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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See related article on pg 2080

Mineralocorticoid Receptor Antagonists—A New Sprinkle of Salt and Youth

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Skin atrophy and impaired cutaneous wound healing are the recognized side effects of topical glucocorticoid (GC) therapy. Although GCs have high affinity for the glucocorticoid receptor, they also bind and activate the mineralocorticoid receptor. In light of this, one can speculate that some of the GC-mediated side effects can be remedied by blocking activation of the mineralocorticoid receptor. Indeed, according to Nguyen et al., local inhibition of the mineralocorticoid receptor via antagonists (spironolactone, canrenoate, and eplerenone) rescues GC-induced delayed epithelialization and accelerates wound closure in diabetic animals by targeting epithelial sodium channels and stimulating keratinocyte proliferation. These findings suggest that the use of mineralocorticoid receptor antagonists coupled with GC therapy may be beneficial in overcoming at least some of the GC-mediated side effects.

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Interplay of the glucocorticoid receptor and mineralocorticoid receptor in skin and wound healing

In dermatology, we have long capitalized on the presence of receptors that mediate anti-inflammatory effects. For example, the effects of the glucocorticoid receptor (GR) may be seen in the use of topical and systemic steroids to treat a myriad of inflammatory skin disorders. However, the efficacy of topical glucocorticoid (GC) use does not come without unintended side effects, including skin atrophy and delayed wound healing. For these reasons, it has been the topic of intense scientific inquiry in an attempt to delineate the mechanisms underlying these sequelae of corticosteroid use. Promiscuous activation of cutaneous mineralocorticoid receptors (MR), due to high-affinity binding of excess cortisol, may be one potential driver. However, it has been shown recently that topical inhibition of the MR attenuates glucocorticoid-induced epidermal atrophy (Maubec et al., 2015). Nguyen et al. (2016) propose that cutaneous MR antagonism improves healing in pathological wounds treated with topical corticosteroids by promoting re-epithelialization. Although much is known about GR function in the skin, the importance of competition by activation of the MR and the implications thereof are just beginning to be recognized.

Both GR and MR belong to the steroid hormone nuclear-receptor superfamily of ligand-dependent transcription factors. GR is found in virtually every cutaneous compartment: epidermal and follicular keratinocytes, epithelial cells of eccrine and apocrine glands, sebocytes, melanocytes, immune cells within the epidermis and dermis, dermal fibroblasts, and smooth muscle cells. Cortisol produced systemically and locally, within the epidermal compartment, serves as the primary ligand for GR, thus potentiating its well-known downstream anti-inflammatory properties. Cortisol-bound GR homodimers mediate GC anti-inflammatory effects through a diverse array of mechanisms, including, but not limited to, transcriptional regulation that results in downstream blockade of prostaglandin production and physical interaction and inhibition