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Phosphoproteomic Analyses of NRAS(G12) and NRAS(Q61) Mutant Melanocytes Reveal Increased CK2α Kinase Levels in NRAS(Q61) Mutant Cells

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Abstract

In melanoma, mutant and thereby constantly active neuroblastoma rat sarcoma (NRAS) affects 15—20% of tumors, contributing to tumor initiation, growth, invasion, and metastasis. Recent therapeutic approaches aim to mimic RAS extinction by interfering with critical signaling pathways downstream of the mutant protein. This study investigates the phosphoproteome of primary human melanocytes bearing mutations in the two hot spots of NRAS, NRAS(G12) and NRAS(Q61). Stable isotope labeling by amino acids in cell culture followed by mass spectrometry identified 14,155 spectra of 3,371 unique phosphopeptides mapping to 1,159 proteins (false discovery rate < 2%). Data revealed pronounced PI3K/AKT signaling in NRAS(G12V) mutant cells and pronounced mitogen-activated protein kinase (MAPK) signaling in NRAS(Q61L) variants. Computer-based prediction models for kinases involved, revealed that CK2α is

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CONFLICT OF INTEREST

The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL

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significantly overrepresented in primary human melanocytes bearing NRAS(Q61L) mutations. Similar differences were found in human NRAS(Q61) mutant melanoma cell lines that were also more sensitive to pharmacologic CK2α inhibition compared with NRAS(G12) mutant cells. Furthermore, CK2α levels were pronounced in patient samples of NRAS(Q61) mutant melanoma at the mRNA and protein level. The preclinical findings of this study reveal that codon 12 and 61 mutant NRAS cells have distinct signaling characteristics that could allow for the development of more effective, mutation-specific treatment modalities.

INTRODUCTION

Mutations in the rat sarcoma (RAS) oncogenes are found in one-third of human malignancies and are frequently associated with unfavorable clinical characteristics (Bucheit et al., 2013; Devitt et al., 2011; Ekedahl et al., 2013; Ellerhorst et al., 2011; Jakob et al., 2012). Up to 18% of melanomas harbor activating mutations in the neuroblastoma rat sarcoma viral homolog (NRAS) oncogene. RAS cycles between the guanosine triphosphate (GTP)-bound active state and the guanosine diphosphate-bound inactive state that is catalyzed by guanine nucleotide exchange factors, such as SOS1, and GTPase-activating proteins, such as NF1 (Bos et al., 2007; Plowman and Hancock 2005). Most alterations are located in codon 61 (approximately 84% of cases) and less frequently in codons 12 and 13 (approximately 7% and 5% of cases), all causing continuous activation and aberrant NRAS cycling (Bos 1989; Burd et al., 2014; Curtin et al., 2005; Smith et al., 2013). Single nucleotide changes in codon 61 involve the switch II of NRAS and impair the intrinsic catalytic activity. Such mutations lock NRAS in its GTP-bound active conformation. Mutants of codon 12 affect the Walker A motif of NRAS and impair the phosphate binding site. This renders the protein insensitive to physiological deactivation catalyzed by GTPase-activating proteins (Curtin et al., 2005; Fedorenko et al., 2013; Rajalingam et al., 2007; Scheffler et al., 1994). Even though both mutations are activating, NRAS(G12) and NRAS(Q61) display opposing defects in exchange rate, intrinsic hydrolysis rate, and sensitivity to guanine nucleotide exchange factors as well as GTPase-activating proteins (Smith et al., 2013).

Interestingly, it has also been shown that certain downstream effectors of RAS and GTPase-activating proteins compete for the same Ras binding domains (Scheffler et al., 1994). Thus, it is possible that mutation-specific changes in NRAS and consequent mutation-specific variations in the availability of binding sites influence downstream signaling events. The hypothesis that NRAS(G12) and NRAS(Q61) mutations have distinct signaling patterns is further supported by differences in the structural and chemical properties of RAS variants and findings in a highly homologous protein, KRAS (mutant in approximately 1% of melanomas), where even closer related genetic alterations in codons 12 and 13 differentially impact cell characteristics in model systems (Buhrman et al., 2011; Fetics et al., 2015; Guerrero et al., 2000; Morelli and Kopetz 2012). Using global phosphoproteomic analyses, we found that mutant NRAS(G12V) and NRAS(Q61L) differently affect downstream signaling in a model of transduced primary human melanocytes (PHMs). A phosphorylation motif search followed by a kinase prediction model revealed that NRAS(Q61) mutations induce CK2α kinase activity. Further, we found high CK2α kinase expression in a collection

of human NRAS mutant melanoma cell lines and patient samples of NRAS(Q61) mutant melanoma. This is particularly interesting because CK2α kinase is a potential therapeutic target for which specific inhibitors are readily available.

RESULTS

Mutant NRAS(Q61) and NRAS(G12) differentially affect the phosphoproteome of PHMs

We analyzed nonimmortalized PHMs transduced with mutant NRAS(G12V), NRAS(Q61L), and empty vector controls. Mutant variants displayed equal NRAS mRNA levels, unchanged proliferation rates, and cell morphology compared with controls. Decreased pigmentation of cells was observed in NRAS(G12V) and NRAS(Q61L) mutants (Supplementary Figure S1 online). The global phosphoproteome of NRAS(G12V), NRAS(Q61L), and empty vector controls was investigated using stable isotope labeling by amino acids in cell culture (SILAC), phosphopeptide enrichment, and high accuracy mass spectrometry (Figure 1a). Computational analyses using Protein Prospector (v 5.12.4) identified a total of 14,155 spectra, establishing the sequences and phosphorylation sites for each phosphopeptide. In total, 3,371 unique phosphopeptides mapping to 1,159 different proteins (FDR < 2%) were identified. The majority of the detected phospho-sites mapped to serines (Supplementary Table S1 online). Changes of relative phosphopeptide levels of more than 2-fold between cells bearing NRAS(G12V), NRAS(Q61L), and empty vector controls were considered to represent phosphorylation sites differentially regulated (Figure 1b). A total of 132 proteins at 163 different phospho-sites were differentially phosphorylated between NRAS(G12V) mutant cells and empty vector controls (83 upregulated and 80 downregulated). NRAS(Q61L) mutant PHMs differentially regulated phosphopeptides in 150 proteins with a total of 202 different phospho-sites (73 upregulated, 129 downregulated) compared with empty vector controls (Supplementary Table S2 online). A total of 126 proteins and 163 phospho-sites were 2-fold differentially regulated between NRAS(G12V) and NRAS(Q61) (118 upregulated, 45 downregulated). SILAC results were validated by immunoblotting of select phosphoproteins (Figure 2). Annotated spectra of the SILAC data set can be accessed via: http://prospector2.ucsf.edu/prospector/cgi-bin/mssearch.cgi?report_title=MS-Viewer&search_key=l5qviepxcl&search_name=msviewer, using the search key lvp5pkzqt1.

NRAS(G12V) and NRAS(Q61L) mutant PHMs harness distinct downstream pathways

Gene ontology (GO) analysis of differentially regulated genes revealed that proteins in all cell compartments were affected by mutant NRAS (Figure 2a). Assessment of the canonical pathways regulated by the respective NRAS variants showed overrepresentation of “14-3-3-mediated signaling” in NRAS(G12V) cells (Supplementary Table S3 online) (“14-3-3-mediated signaling,” $P = 0.0001$; Fisher’s exact test). 14-3-3 proteins modulate PI3K signaling, are known to regulate several biological processes and the cancerous transformation of cells (Neal et al., 2012). In contrast, canonical pathway analysis for NRAS(Q61L) cells was enriched for mitogen-activated protein kinase (MAPK) signaling (“UVB-induced MAPK signaling,” $P = 4.67 \times 10^{-5}$; “UVC-induced MPAK signaling,” $P = 0.0003$; Fisher’s exact test). To validate SILAC results and in silico biological annotations, select proteins such as p-TPX2(S738), p-AKT1S1(T246), and members of the PI3K/mTOR/AKT and the RAF/MAPK/extracellular signal-regulated kinase (MEK)/extracellular

signal-regulated kinase (ERK) pathway signaling cascades were assessed by immunoblotting (Figure 2b and c) (Madhunapantula et al., 2007; Neumayer et al., 2014; Wiza et al., 2012). Cells used for validation of SILAC results represent biological replicates. Immunoblots confirmed an induction of PI3K/AKT/rS6 signaling in NRAS(G12V) mutant PHMs with increased levels of p-AKT and p-S6 (Figure 2c). On the other hand, NRAS(Q61L) mutant PHMs showed induction of the MEK/ERK pathway. These results match findings of the GO analyses. Annotation of the biological function of differentially regulated proteins showed that the GO terms “enzyme,” “transcription regulator,” and “kinase” were overrepresented comparing NRAS(G12V) and NRAS(Q61L) mutant PHMs (Supplementary Table S3).

NRAS mutations induce pro-oncogenic changes in the kinase activation loop

In many cases, kinase activity is regulated by phosphorylation events in the activation loop (a-loop) (Kunz et al., 2000). Phosphorylation of the a-loop changes tertiary structures of the protein and allows substrate access to the active site cleft. This enhances the catalytic activity of the kinases for phosphoryl transfer (Nolen et al., 2004). To determine phosphorylation events within the a-loop of kinases detected by SILAC, we first identified kinases within our data set and grouped them by kinase family. Next, we used the highly conserved DFG-start and APE-stop amino acid sequences of a-loops to screen for phosphorylation events within these domains (Nolen et al., 2004). Using this algorithm, we identified 36 kinases with a-loops, mapping to 7 different kinase families (Supplementary Table S4 online). Consistent with previous reports, a-loops were between 22 and 42 amino acids in length (Kunz et al., 2000). Four kinases (PAK4[S474], MAPK14[Y182], MAPK1[Y187], CDK11A[Y575/S577]) were differentially phosphorylated within the a-loop comparing NRAS(G12V) and NRAS(Q61L) mutant cells with their respective controls. Three of four kinases belonged to the human CMGC (cyclin-dependent kinase, MAPK, glycogen synthase kinase 3, CDC-like kinase) group of protein kinases that has been shown to control cell cycle regulation, activity of human tumor suppressors, and cell fate decisions (Varjosalo et al., 2013). PAK4 is a member of the STE Ser/Thr protein kinase family consisting of the homologs of the yeast STE7, STE11, and STE20 genes forming the MAPK cascade. Phosphopeptide levels of MAPK14 and PAK4 in the SILAC data set were at comparable levels in NRAS(G12V) and NRAS(Q61L) cells, but only MAPK14(Y182) levels were significantly lower than in the controls. The cell cycle regulator CDK11A(Y575/S577) showed a trend toward higher levels of phosphorylation in NRAS(Q61L) compared with NRAS(G12V) mutant cells, whereas phosphorylation of the a-loop in MAPK1 at Y187 showed a trend toward higher levels in NRAS(G12V) mutant cells.

Mutant NRAS(G12V) activates PIM2 kinases, and NRAS(Q61L) activates CK2α kinases

For the kinase prediction model, all phosphorylation sites for NRAS(G12V) and NRAS(Q61L) mutant cells detected by SILAC were considered for analysis. Sequences of 13 amino acids with the centered phosphorylation site were used for the detection of overrepresented motifs using Maximal Motif Finder for Phosphoproteins (Wang et al., 2012). Because of the limited number of detected p-Y sites, analyses were only successful for p-S and p-T sites. A total of 22 p-S and 3 p-T motifs were detected. These motifs were used for the kinase prediction model using the networKIN analysis tool (Linding et al.,

2008). NRAS(G12V) and NRAS(Q61L) motifs formed distinct clusters supporting signaling differences of the two variants (Figure 3a). Analyses revealed that the top, statistically significant, and differentially involved kinases between the two conditions were PIM2 and CK2α (Supplementary Table S5 online). In silico predictions were validated by immunoblotting (Figure 3b and c). In addition to PIM2 being overrepresented, kinases related to the PI3K/AKT/rS6 pathway were predicted to be significantly more active in mutant NRAS(G12V) signaling, matching SILAC, immunoblot, and GO analyses in these cells. CK2α kinases were significantly overrepresented in NRAS(Q61L) PHMs (Supplementary Table S5). These findings are particularly interesting as all kinases that were predicted to be activated are related to cancer development, progression and metastasis (Ha et al., 2013; Meja et al., 2014; Nakahata et al., 2014). Furthermore, most of these kinases can be targeted by selective kinase inhibitors (Intemann et al., 2014; Meja et al., 2014; Wang et al., 2014).

NRAS(Q61L) mutations and pronounced MAPK signaling sensitize cells toward pharmacologic CK2α inhibition

Analysis of nine human NRAS mutant melanoma cell lines revealed CK2α protein expression in all cells tested (Figure 4a and Supplementary Figures S2, S3, and S4 online). CK2α protein expression was pronounced in NRAS(Q61) mutant cells (Figure 4a and Supplementary Figures S2 and S3). To investigate the biological role of CK2α in NRAS mutant melanoma cells, we used CX4945 for pharmacological inhibition. The inhibitor has previously demonstrated activity in different malignancies and was more potent than several other small molecule compounds currently available for selective inhibition of CK2α (Intemann et al., 2014; Wang et al., 2014). Cell viability assays revealed that NRAS mutant melanoma cell lines are sensitive to CK2α inhibition. NRAS(Q61) mutant cells D04, Sk-Mel-2, MM415, MM485, and Sk-Mel-30 required lower amounts of CX4742 than the NRAS(G12) mutant cell lines MaMel27II and 501 Mel (Figure 4b and Supplementary Figure S5 online). Sensitivity to CX4945 correlated with CK2α protein expression and also MAPK pathway activation. CK2α inhibition also effectively reduced cell viability in NRAS(G12) mutant cells WM3629 and WM3670 harboring additional low activating BRAF(D549G) and BRAF(G469E) mutations that are contributing to increase MAPK signaling (Supplementary Figure S4). BRAF(V600E) mutants Sk-Mel-28, MM466, and MM537, which are known to hyperactivate the MAPK pathway, also showed growth inhibition after CX4945 incubation (Supplementary Figure S5). Similarly, a reduction of cell viability was found in the NF1 (E1832X, I500S, and P2509S) mutant cell line M257 and the c-KIT(L576P) mutant line M230, both displaying high MAPK activity. In contrast, c-KIT(S476) mutant MaMel144al cells, showing low p-ERK levels, only had a minor reduction of cell viability at equimolar concentrations of CX4945 (Supplementary Figure S5). Genetic characteristics of cell lines used in this study are listed in Supplementary Table S6 online.

CK2α is expressed in clinical tumors of NRAS mutant melanoma and pronounced in NRAS(Q61) mutants

We analyzed 22 clinical samples of cutaneous NRAS mutant melanoma by immunohistochemistry for CK2α protein expression. A total of 18 tumors were NRAS(Q61)

mutant; 2 tumors harbored mutant NRAS(G12). One tumor carried a NRAS(C80R) mutation and one tumor had a mutation in NRAS(G12D) and NRAS(G60R) (Supplementary Table S7 online). Of the 18 NRAS(Q61) mutant samples, 16 showed CK2α protein expression, whereas only one of the 2 NRAS(G12) mutant melanoma samples had CK2α positive tumor cells. Even though results did not reach statistical significance because of the small number of NRAS(G12) mutant tumor samples, NRAS(Q61) mutant tumors showed higher levels of CK2α expression compared with NRAS(G12) mutant melanomas (average staining intensity [AU]: NRAS(Q61L) = 1.65; NRAS(G12D) = 0.5) (Figure 4c and d). Interestingly, the sample carrying the NRAS(C80R) mutation showing low p-MEK protein levels did not show any CK2α staining. In contrast, the sample harboring both mutant NRAS(G12D) and NRAS(G60R) with high levels of p-MEK showed strong CK2α staining (Supplementary Table S7).

To further assess a potential association between mutant NRAS and CK2α, we ran analyses in “The Cancer Genome Atlas” (TCGA) provisional skin cutaneous melanoma data set (n = 470) (Cerami et al., 2012; Gao et al., 2013). For each sample, NRAS mutation status and relative CK2α mRNA expression levels were reported. Across all samples, NRAS was mutated in 18% of cases (n = 86) with an alteration at Q61 being the most frequent (n = 75). Of the 11 alternate mutation sites, mutations at G13 (n = 3) and G12 (n = 2) were the first and second most common. The remaining samples had no reported mutations in the NRAS gene (NRM; n = 384). Comparing the mean mRNA levels between these three cohorts revealed significantly higher CK2α expression in NRAS(Q61) mutant melanoma samples compared with the NRM cohort (NRAS(Q61) = 0.94; NRM = 0.13; $P = 7.23^{-5}$) (Figure 4e). NRAS(G12) mutant patient samples also showed a trend toward increased CK2α expression compared with melanomas with NRM (NRAS(G12) = 0.88; NRM = 0.13; $P = 0.17$), however, at lower levels than NRAS(Q61) mutant tumors (NRAS(Q61) = 0.94). Statistical analyses of clinical NRAS(G12) mutant melanomas did not reach significance because of the small number of samples in the TCGA data set (n = 2).

DISCUSSION

Mutations in RAS are among the most frequent oncogenic alterations in all cancers (Bos 1989; Schubbert et al., 2007). Single nucleotide polymorphism are almost exclusively found in two biologically distinct hot spots, codons 12 (or 13) and 61 (Ellerhorst et al., 2011; Fedorenko et al., 2013). Both mutations are NRAS activating, but little is known whether NRAS(G12) and NRAS(Q61) variants differentially affect downstream signaling. Recent research supports that such small differences in RAS oncogenes can have substantial impact on protein function (Guerrero et al., 2000; Haigis et al., 2008; Smith et al., 2013; Whitwam et al., 2007).

In this study, we focused on changes in the phosphoproteome after transduction of mutated NRAS variants in PHMs. Phosphorylation of proteins is one of the most important posttranslational modifications and is involved in almost every cellular process in eukaryotic cells. Thus, studying the phosphoproteome on an omic scale allows investigating critical biological changes attributed to mutant NRAS. Indeed, this approach highlighted differences in downstream signaling in a model of NRAS(G12V) and NRAS(Q61L) mutant PHMs.

However, translating such findings to melanoma is challenging as the complex genetic background of malignant cells is likely to influence signaling patterns. In addition, NRAS(G12) mutant melanoma is rare and even big databases such as TCGA contained only two NRAS(G12) mutant melanoma patient samples. This underscores the need of model systems to point toward potential signaling variations caused by different NRAS mutations. Data revealed that PHMs bearing NRAS(G12V) mutations preferentially activate the PI3K/AKT pathway. This is consistent with previous findings where codon 12 mutant RAS variants were found to induce PI3K signaling by directly activating PI3K itself (Staruschenko et al., 2004) or the activation of glycogen synthase kinase 3α (Fedorenko et al., 2015). NRAS(Q61) mutant PHMs showed MAPK pathway hyperactivation. This signaling cascade is one of the most important pathways for cancer initiation, proliferation, and metastasis (Chin et al., 1997; Posch and Ortiz-Urda 2013; Posch et al., 2013; Vujic et al., 2014; Whitwam et al., 2007). It is possible that the strong MAPK pathway activation in NRAS(Q61) mutant cells, including other factors, contributes to the clinical observation that NRAS(Q61) mutations are more frequent than NRAS(G12) in melanoma (Supplementary Table S7) (Burd et al., 2014; Devitt et al., 2011; Ellerhorst et al., 2011; Jakob et al., 2012).

Functional classification of proteins by GO annotation revealed that kinases were among the top regulated proteins in NRAS transduced PHMs. Kinases are typically expressed in an inactive state and converted into a fully active form by the addition of a phosphate to the activation loop (Kunz et al., 2000; Nolen et al., 2004). Analyses of our data set revealed that most kinases with phosphorylation of the activation loop are members of the CMGC group of proteins. This group of kinases control critical cellular processes and have been implemented in the regulation of human tumor suppressors and control of cell fate decisions. Enriched CMGC kinase networks were also reported in human cancers, which suggests a role of the CMGC interaction proteome in controlling tumor cell growth (Varjosalo et al., 2013).

Reduced phosphorylation in the a-loop of MAPK14 (p38α) at Y182 has been described with increased proliferation and impaired differentiation of cells and supports the oncogenic potential of both NRAS(G12) and NRAS(Q61) mutations (Hui et al., 2007). Additionally, data revealed increased phosphorylation of PAK4 at S474. Even though levels did not reach statistical significance, data showed a trend toward higher PAK4 a-loop phosphorylation compared with controls. PAK4 stimulates cell survival through various effectors such as the phosphorylation of BAD, which was also found to be increased in the SILAC data set of this study. PAK4 is overexpressed in several cancers and required for RAS-dependent, anchorage-independent growth of tumor cell lines (Whale et al., 2011). It is activated by growth factor receptors, active CDC42 and interestingly RAC1, a known regulator of NRAS signaling (Li et al., 2012).

Studying the phosphoproteome of cells also enables us to use knowledge-based computer models to predict kinases that are most likely involved in phosphorylation events detected by mass spectrometry. The search for over-represented phosphorylation motifs followed by kinase prediction revealed that PIM2 kinase activity is pronounced in NRAS(G12V) mutant PHMs, whereas CK2α is increased in NRAS(Q61L) mutants. Immunoblotting revealed that human NRAS(Q61) mutant melanoma cell lines express CK2α and that its expression tends

to be higher than in the NRAS(G12) cell lines MaMel27II and 501Mel. NRAS(Q61) mutant melanoma cells were also more sensitive to pharmacologic CK2α inhibition supporting an important role of the CK2α kinase for survival in NRAS(Q61) mutant cells (St-Denis and Litchfield 2009). A limitation of this finding is that only two solely NRAS(G12) mutant cell lines could be tested. The high abundance of additional MAPK activating mutations such as low activating BRAF mutations in NRAS(G12) mutant melanoma cell lines obscures the effects of mutant NRAS(G12) on this signaling cascade. Results suggest that pronounced MAPK signaling also sensitizes NRAS mutant cells toward CK2α inhibition (Heidorn et al., 2010; Pedersen et al., 2013). Interestingly, cells bearing genetic alterations that are known to hyperactivate the MAPK pathway such as mutant BRAF(V600) and dysregulated NF1 were also sensitive toward pharmacological CK2α inhibition (Supplementary Figures S2, S3, and S5). This observation highlights the critical role of the MAPK pathway in melanoma cell homeostasis and further suggests that CK2α inhibition might also be effective in tumors with increased MAPK activity mediated by molecular changes other than NRAS mutations. In addition, it shows that BRAF mutations in loci other than BRAF(V600E) are potentially important for melanoma cell homeostasis.

Our findings are in line with recent research by Burd et al. (2014), whose study centered around nucleotide binding, GTPase activity as well as protein stability, and showed increased tumorigenicity of mutant NRAS(Q61) in melanoma. They also report on significantly increased engagement of the PI3K signaling cascade in NRAS(G12/G13) mutant melanoma cells and a trend toward increased MAPK signaling in NRAS(Q61) mutants. It is possible that the lack of significance of the latter correlation can be attributed to the low activating BRAF mutations in NRAS(G12/G13) mutant melanoma cell lines included in this comparison.

Because of the limited number of available NRAS(G12) mutant melanoma samples, it is currently challenging to address mutation-specific differences in CK2α mRNA or protein expression in clinical tumors. Yet, immunohistochemical analyses of cutaneous NRAS mutant melanoma in this study point into the direction that CK2α is expressed at higher levels in clinical NRAS(Q61) mutant melanoma compared with NRAS(G12) mutants. Importantly, analyses of mRNA expression levels in the TCGA data set show that CK2α expression is also significantly higher in NRAS(Q61) mutant melanomas compared with non-NRAS mutant disease (Figure 4).

Data of this study offer a comparative insight into signaling differences of two NRAS variants representing hot-spots for genetic alterations in NRAS mutant melanoma. The preclinical findings presented herein show that codon 12 and 61 mutant NRAS cells have distinct signaling characteristics that might allow for mutation-specific pharmacologic interference.

MATERIAL AND METHODS

All cells were propagated in their recommended medium and analyzed when reaching 80—90% confluency. A pool of early passage (<5), infant-foreskin-derived, PHMs were used in indicated experiments. A detailed protocol for PHM extraction is provided in Supplementary

Material and Methods online. NRAS plasmids were generated by site directed mutagenesis in a gateway/lenti virus transduction system. Coexpressed green fluorescent protein was used as a transduction efficacy reporter. Comparable NRAS expression levels between the mutated variants were confirmed by quantitative PCR (Supplementary Figure S1). SILAC was performed as previously described (Ong et al., 2002). Nonimmortalized, transduced PHMs were grown for at least eight doublings in the respective SILAC medium, lysed and subjected to further processing following established protocols. Protein Prospector (v 5.12.2) was used for identification of mass spectrometry spectra, peptides, and corresponding proteins (Clauser et al., 1999). Further statistical analyses were performed using Maximal Motif Finder of Phosphoproteins and networKIN (v2) (Linding et al., 2008; Wang et al., 2012). Predicted kinases were ranked by hierarchical clustering and statistical significance was assessed by chi-square tests (2df; probability less than critical value 0.99). Activation loops were identified using previously described consensus motifs. Inhibitors used in the study were purchased from Selleck Chemicals. Relative viability was assessed using CellTiter-Glo (Promega, USA; G7570) and CalcuSyn software (Biosoft, USA, version 2.1) following the manufacturers' recommendations (Chou and Talalay 1984). Immunoblotting and immunohistochemistry were performed as previously described (Posch et al., 2013). All research involving patient tissue was approved by the Institutional Review Board of the University of California San Francisco (IRB#12-09483), and written, informed patient consent was obtained. Detailed information on material and methods used in this study can be found in Supplementary Material and Methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

a-loop	activation loop
ERK	extracellular signal-regulated kinase
GO	gene ontology
GTP	guanosine triphosphate
MAPK	mitogen-activated protein kinase

MEK	MAPK/ERK kinase
NRAS	neuroblastoma rat sarcoma viral homolog
PHM	primary human melanocyte
NRM	no reported mutations
RAS	rat sarcoma
SILAC	stable isotope labeling by amino acids in cell culture
TCGA	the Cancer Genome Atlas

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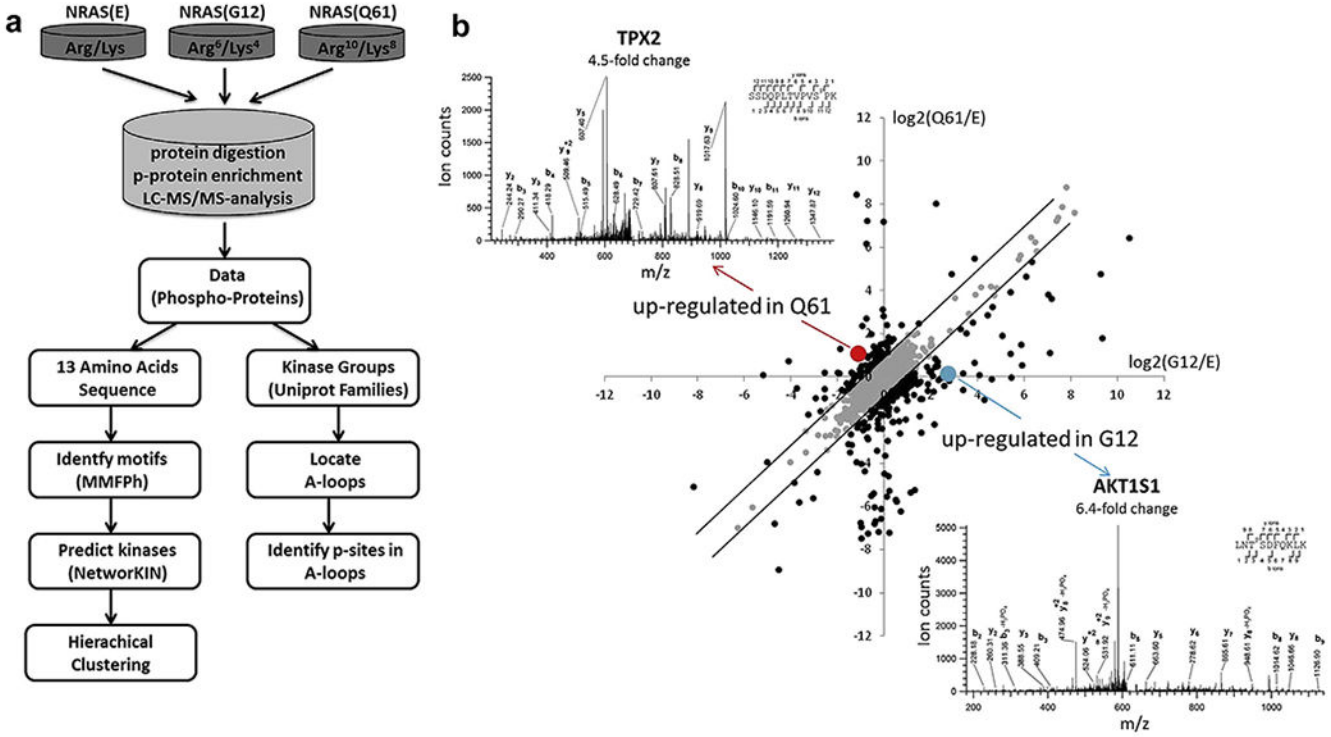

Figure 1. Differential regulation of phosphoproteins by NRAS(G12) and NRAS(Q61) mutations.

(**a**) Schematic diagram of the workflow for phosphoproteomic analyses. (**b**) Dot-blot diagram of differentially regulated phosphopeptides between NRAS(Q61) and NRAS(G12) (log2 scale). Black dots: >2-fold Upregulated phosphopeptides in Q61 and G12. Gray dots: unregulated phosphopeptides. Red dot: phosphopeptide mapping to p-TPX2(S738) and CID tandem mass spectra obtained from precursor ions with m/z values 717.8506^{+2} (*X*) and 637.3127^{+2} (*Y*). Blue dot: phosphopeptide mapping to p-AKT1S1(T246) with an expectation value for the sequence of 5.4×10^{-4} and 1.1×10^{-2}. SLIP localization scores are 19 and 15, respectively. Sequence ions are labeled in the figure, and indicated with marks over (C-terminal ions) and below (N-terminal ions) the sequence of the peptide. CID, collision-induced dissociation; NRAS, neuroblastoma rat sarcoma; SLIP, site localization in peptide (SLIP) score.

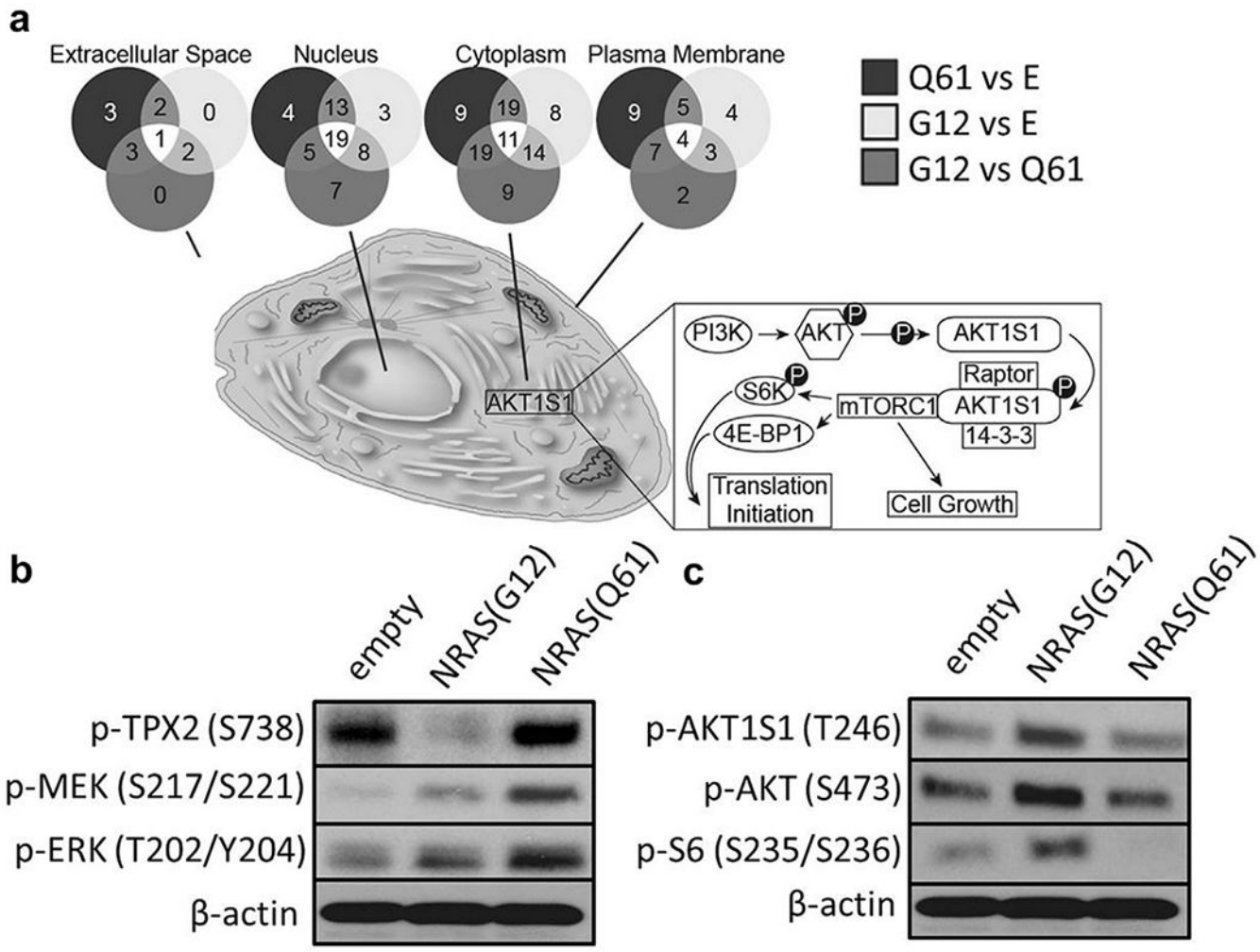

Figure 2. NRAS(G12) and NRAS(Q61) mutations affect all cell compartments.

(**a**) Schematic of a cell and Venn diagrams for differentially regulated proteins mapping to their respective cell compartments. Graphic representation of the PI3K/AKT/mTOR pathway and the involvement of p-AKT1S1. (**b**, **c**) Immunoblot analyses of PHMs expressing mutant NRAS(G12V), NRAS(Q61L) and empty vector controls representing biological replicates of the cells used for the SILAC analysis. The MEK/ERK pathway is induced in NRAS(Q61L) mutant cells and the PI3K/AKT/mTOR signaling cascade is activated in cells bearing the NRAS(G12V) mutation. E, empty; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; NRAS, neuroblastoma rat sarcoma; PHM, primary human melanocyte; SILAC, stable isotope labeling by amino acids in cell culture.

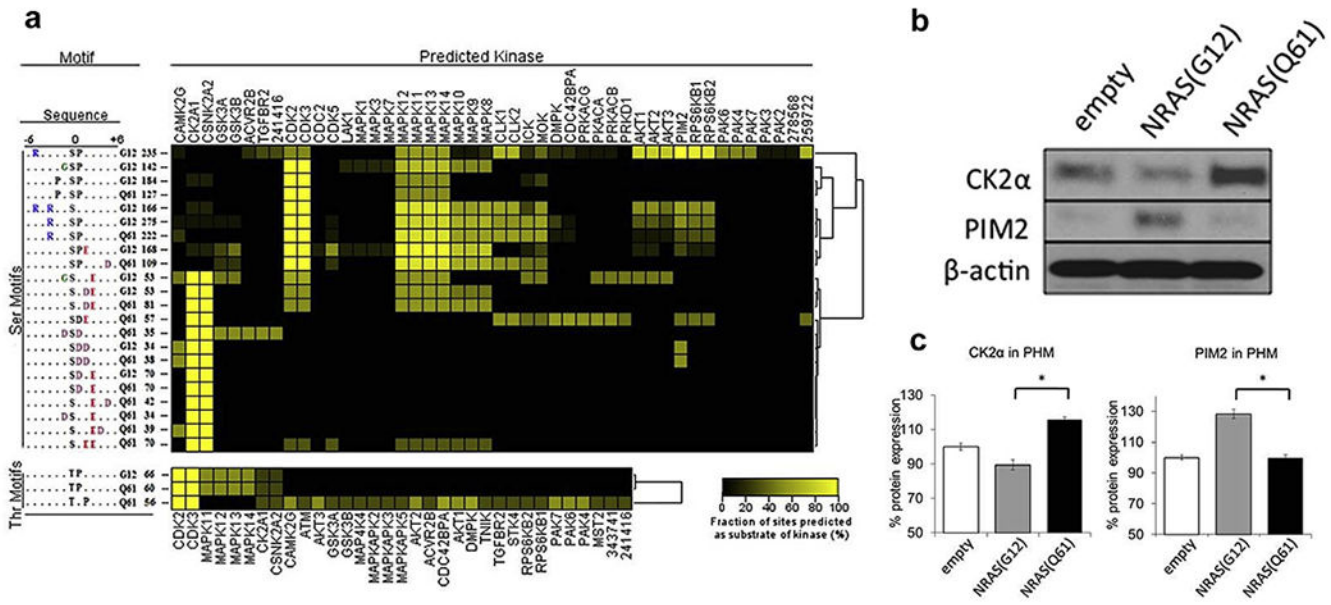

Figure 3. Kinases predicted to be overrepresented in NRAS(G12V) and NRAS(Q61L) signaling.

(**a**) Heat map and hierarchical clustering of predicted kinases, mapped to their respective phosphorylation motif in NRAS(G12V) and NRAS(Q61L) mutant cells. CK2α and PIM2 kinases are predicted to be most differentially regulated ($P < 0.001$) (Supplementary Table S5 online). (**b**) Immunoblot analysis confirming CK2α overrepresentation in NRAS(Q61L) mutant cells and PIM2 overrepresentation in NRAS(G12V) mutant cells. (**c**) Quantitative analyses of protein expression (N = 3; error bars represent the SEM). NRAS, neuroblastoma rat sarcoma; SEM, standard error of the mean.

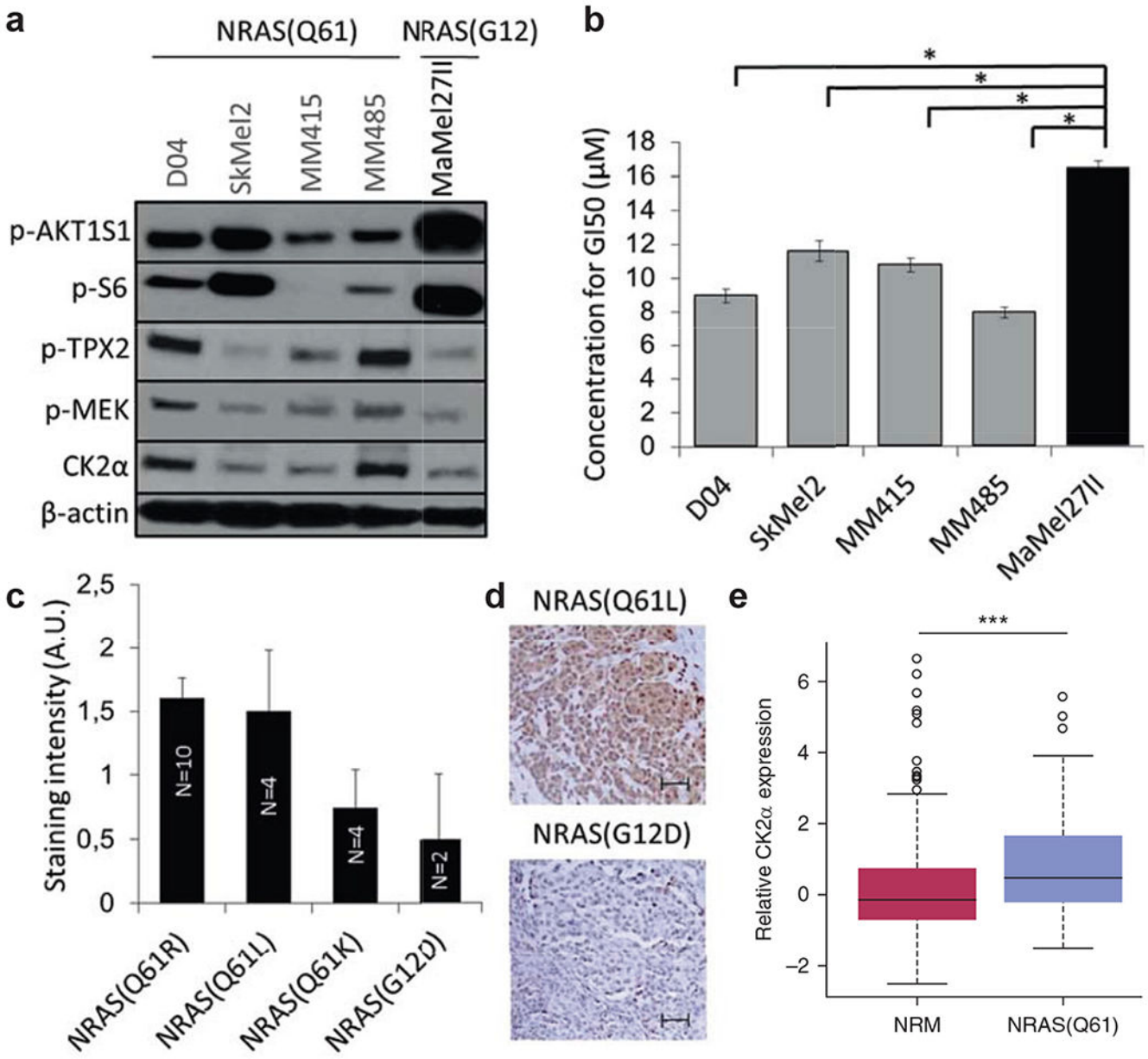

Figure 4. The role of CK2α in NRAS mutant melanoma cells.

(**a**) Ck2α protein expression in five human NRAS mutant melanoma cell lines with the NRAS(G12D) mutant cell line MaMel27II expressing low amounts of CK2α. (**b**) MaMel27II cells require higher concentrations of the CK2α inhibitor CX4945 to reach 50% growth inhibition (GI50) compared with NRAS(Q61) mutant cells. (**c**) Immunohistochemical staining intensity of 20 human NRAS mutant melanoma tissue samples, grouped by their respective NRAS mutation. (Bars represent the average staining intensity ranging from no staining = 0 to intensive staining = 3; error bars represent the SEM; $*P < 0.05$.) (**d**) Images of representative immunohistochemically stained clinical melanoma samples bearing NRAS(Q61L) or NRAS(G12D) mutations (scale bar = 50 μm). (**e**) Analyses of clinical melanoma samples in The Cancer Genome Atlas reveals increased

CK2α mRNA expression in NRAS(Q61) (N = 75) versus non-NRAS mutant (N = 384) melanoma (two-tailed Student's *t*-test, ***$P < 0.001$). MEK, MAPK/ERK kinase; NRAS, neuroblastoma rat sarcoma; SEM, standard error of the mean.