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Classic Ras Proteins Promote Proliferation and Survival Via Distinct Phosphoproteome Alterations in Neurofibromin-Null Malignant Peripheral Nerve Sheath Tumor Cells

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Abstract

Neurofibromin, the tumor suppressor encoded by the neurofibromatosis type 1 (*NFI*) gene, potentially suppresses the activation of H-Ras, N-Ras and K-Ras. However, it is not known whether these classic Ras proteins are hyperactivated in *NFI*-null nerve sheath tumors, how they contribute to tumorigenesis and what signaling pathways mediate their effects. Here we show that H-Ras, N-Ras and K-Ras are coexpressed with their activators, (guanine nucleotide exchange factors), in neurofibromin-null malignant peripheral nerve sheath tumor (MPNST) cells and that all 3 Ras proteins are activated. Dominant negative (DN) H-Ras, a pan-inhibitor of the classic Ras family, inhibited MPNST proliferation and survival, but not migration. However, *NFI*-null MPNST cells were variably dependent on individual Ras proteins. In some lines, ablation of H-Ras, N-Ras and/or K-Ras inhibited mitogenesis. In others, ablation of a single Ras protein had no effect on proliferation; in these lines, ablation of a single Ras protein resulted in compensatory increases in the activation and/or expression of other Ras proteins. Using mass spectrometry-based phosphoproteomics, we identified 7 signaling networks affecting morphology, proliferation and survival that are regulated by DN H-Ras. Thus, neurofibromin loss activates multiple classic Ras proteins that promote proliferation and survival by regulating several distinct signaling cascades.

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Keywords

Malignant peripheral nerve sheath tumor; Neurofibromatosis; Neurofibromin; Phosphoproteomics; Ras

INTRODUCTION

Mutations affecting one of the genes encoding the classic Ras proteins, i.e. H-Ras, K-Ras and N-Ras, are extraordinarily common in a variety of human cancers and, when present, they may promote tumorigenesis (1). In non-neoplastic cells, Ras proteins regulate diverse cellular functions such as proliferation, survival, migration and differentiation in a context-specific manner. Ras proteins are normally activated when guanine nucleotide exchange factors (GEFs), which are themselves activated by stimuli such as growth factor signaling (2-4), cause Ras to release GDP and bind GTP. The Ras activation that results from this exchange is in turn limited by GTPase activating proteins (GAPs), which stimulate an intrinsic GTPase activity in Ras, triggering GTP hydrolysis and Ras inactivation (5, 6). In neoplastic cells, Ras mutations disrupt these regulatory mechanisms, resulting in Ras hyperactivation. This most commonly occurs because Ras mutations alter amino acids that are critically important for GAP binding, rendering the mutated Ras proteins resistant to GAP-mediated inactivation.

Mutation of the neurofibromatosis type 1 (*NF1*) gene, which encodes the ubiquitously expressed Ras GAP neurofibromin, can also produce Ras hyperactivation and tumorigenesis. As evidence of this, patients with neurofibromatosis type 1 (NF1), who have germline *NF1* mutations, are susceptible to the development of several tumor types including neurofibromas, malignant peripheral nerve sheath tumors (MPNSTs) (7), and pilocytic astrocytomas (8); these tumors demonstrate Ras hyperactivation, and inhibition of downstream Ras signaling targets in cells derived from these tumors has been shown to affect phenotypes such as proliferation, survival and migration. In addition, it is increasingly evident that *NF1* mutations contribute to the pathogenesis of a variety of sporadic neoplasms such as glioblastomas (9-12), serous ovarian adenocarcinomas (13), adult acute myelogenous leukemia (14, 15), pulmonary adenocarcinomas (16), breast carcinomas (17), neuroblastomas (18) and intracranial germ cell tumors (19). Notably, however, Ras activation in neurofibromin-null neoplasms potentially differs from that seen in tumors with activating Ras mutations in one critical manner. Neurofibromin negatively regulates the activity of all 3 classic Ras proteins (20-22). Consequently, neurofibromin loss can potentially activate H-Ras, N-Ras and K-Ras simultaneously. In this circumstance, it is unclear whether all 3 classic Ras proteins contribute to neoplasia or whether a particular Ras protein predominantly drives tumorigenesis. Because these 3 Ras proteins differ in their susceptibility to the drugs currently being tested as Ras inhibitors, it is essential to determine which of the 3 classic Ras proteins promote the pathogenesis of neurofibromin-null neoplasms and what effects this polypeptide exerts on tumor cell physiology to promote tumorigenesis. The fact that neurofibromin loss can potentially simultaneously activate multiple classic Ras proteins also raises the question of whether therapies targeting a

specific Ras protein will be rendered ineffective in neurofibromin-null tumors as a result of these proteins compensating for one another.

Malignant peripheral nerve sheath tumors (MPNSTs), the most common malignancy and the leading cause of death in NF1 patients, are an ideal model system for deciphering the role that different neurofibromin-regulated Ras proteins play in tumor pathogenesis. Here, we use MPNST cells to test the hypothesis that neurofibromin loss results in the simultaneous activation of multiple classic Ras proteins and examine the implications that this has for Ras-targeted therapeutics. To address these questions, we determined which of the classic Ras proteins and the GEFs required for their activation are expressed in MPNST cells and whether these Ras proteins are simultaneously activated in *NF1*-null MPNST cells. Given the possibility that the closely related members of the classic Ras subfamily might compensate for one another, we have both introduced a pan-inhibitor of the classic Ras subfamily (a dominant negative [DN] H-Ras mutant) into MPNST cells and ablated the expression of individual classic Ras proteins and then determined what effect each of these manipulations has on proliferation, migration, and survival. Finally, we used mass spectrometry-based phosphoproteome analyses to identify key signaling pathways potentially mediating the distinct effects of the classic Ras proteins.

MATERIALS AND METHODS

Antibodies and Reagents

Antibodies for H-Ras (sc-520, sc-833), N-Ras (sc-30, sc-31) and the HA epitope (sc-805) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A mouse monoclonal antibody recognizing K-Ras (MCA3223Z) was from AbD Serotec (Kidlington, UK). Mouse monoclonal antibodies against green fluorescent protein (GFP) (G1546) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (RDI-TRK5G4-6C5) were purchased from Sigma-Aldrich (St. Louis, MO) and Fitzgerald Industries International (Concord, MA), respectively. Antibodies against phosphorylated Erk1/2^{Thr202/Tyr204} (#9101), Akt^{Thr308} (#9275), JNK^{Thr183/Tyr185} (#9251) and the Myc epitope tag (#2276) were from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA).

MPNST Cell Lines

We have previously described the sources of the human MPNST cell lines used in this study (23, 24). We routinely assessed the morphology and doubling times of these cell lines and regularly tested them for *Mycoplasma*. Karyotypic analyses revealed only human chromosomes, eliminating the possibility of contamination with non-human cell lines.

Immunoblotting

Human MPNST cell lysates were immunoblotted and probed as previously described (25). To verify equal loading, all blots were reprobed with an antibody directed against GAPDH. Immunoreactive species were detected using a SuperSignal Pico Chemiluminescence kit (Thermo Scientific, Rockford, IL).

Conventional and Quantitative PCR

Conventional PCR was performed using our previously described methodology (26) with primers designed from reference sequences for each GEF (Supplemental Table 1). Real-time quantitative PCR with TaqMan primers (Applied Biosystems, Carlsbad, CA) was performed per our previous description (24), using an Applied Biosystems 7500 Real-Time PCR System. The resulting data were analyzed with Applied Biosystems Sequence Detection Software (version 1.4).

Ras DN Mutant and Epitope-tagged Ras Expression Plasmids

A plasmid expressing an HA-epitope tagged H-Ras (S17N, #RASH00TND0) DN mutant was obtained from the Missouri S&T cDNA Resource Center (Rolla, MO). The insert from this plasmid was cloned into the *KpnI* and *EcoRV* sites of the bidirectional doxycycline-inducible vector pBIG2i (27) to produce a doxycycline-inducible DN H-Ras plasmid (pSLC703). The insert from pEGFP-N1 (Clontech, Mountain View, CA) was cloned into *BamHI*-*NotI* digested pBIG2i to yield a doxycycline-inducible GFP control plasmid (pSLC460).

Plasmids expressing Myc-tagged wild-type H-Ras (#RASH00MN00) and K-Ras (#RASK20MN00) and untagged wild-type N-Ras (#RASN000000) were also from the Missouri S&T cDNA Resource Center. Untagged wild-type N-Ras sequences were PCR amplified using primers containing *NotI* or *XhoI* sites, digested with *NotI* and *XhoI* and cloned into *NotI/XhoI* digested Myc-tagged pcDNA3.1+ plasmid to create a Myc-tagged N-Ras expression vector (pSLC816).

Transfection of MPNST Cell Lines

Transient transfections were performed in serum-free DMEM using FuGENE 6 Transfection Reagent (Roche, Indianapolis, IN) as recommended by the manufacturer. To stably transfect the doxycycline-inducible dominant negative H-Ras plasmid, transfected cells were split into tetracycline-free DMEM-10 supplemented with hygromycin (50-100 µg/ml, empirically determined for each line). Clones were picked 2 weeks later and screened by immunoblotting for the HA-epitope tag; appropriate clones expressed this epitope when cultured in media supplemented with 2 µg/ml doxycycline, but not when maintained in tetracycline-free media. Cell lines stably transfected with doxycycline-inducible vectors were maintained in DMEM, 10% tetracycline-free fetal bovine serum (FBS), 10 U/ml penicillin, 10 µg/ml streptomycin (TF DMEM10), supplemented with the optimal concentration of hygromycin.

Ras Activation Assays

Human MPNST lines transiently transfected with Myc-tagged wild-type Ras plasmids or Myc-tagged DN H-Ras control were lysed in magnesium-containing lysis buffer [25 mM HEPES (pH 7.5), 150 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 1% Igepal CA-630, 10% glycerol] supplemented with protease (Sigma #P8340, 1:100 dilution) and HALT phosphatase (Thermo Scientific #78420, 1:100 dilution) inhibitor cocktails. Lysates were clarified by centrifugation at 20,000 × g for 10 minutes, and protein concentrations then

measured using a DC Assay kit (Bio-Rad, Hercules, CA) per the manufacturer's recommendations. Activated Ras was captured from 250 µg of these lysates with 20 µl Ras Assay Reagent (Raf-1 Ras-binding domain agarose beads, Millipore, Billerica, MA), per the manufacturer's instructions. After washing, beads were boiled for 15 minutes in 40 µl 2x Stop Buffer [250 mM Tris-HCl (pH 6.8), 5 mM EDTA, 5 mM EGTA, 2% SDS, 10% glycerol, 25 mM dithiothreitol, 300 µM bromophenol blue] prior to loading on 12% SDS-PAGE gels; clarified lysate samples were run in parallel. Immunoblotting was performed as described above.

For Raf-1 Ras-binding domain agarose bead capture of endogenous activated Ras the following modifications were made. Lysates from log phase T265-2c human MPNSTs cell lines transduced with either a non-targeting shRNA, or a N-Ras targeting shRNA were used. Activated Ras was captured from 56 mg of lysate and 280 µl Raf-1 beads and K-Ras specific activation levels were assessed by immunoblot analysis using a K-Ras specific antibody.

Knockdown of Ras Expression

In some experiments, the expression of individual Ras proteins was knocked down using doxycycline-inducible pSLIK lentiviral vectors (28), which express shRNAs coordinately with green fluorescent protein (GFP). Lentiviruses containing cassettes expressing shRNAs targeting N-Ras (pSLC 748, 749, 750), K-Ras (pSLC 751, 752, 753) or a nonsense sequence (pSLC 727) were constructed and packaged as previously described (29). To create stable cell lines containing doxycycline-inducible shRNA vectors, cells were transduced by culturing in lentivirus-containing media supplemented with 6 µg/ml Polybrene (Sigma-Aldrich; St. Louis, MO). After 3 days, cells were split into fresh DMEM supplemented with 10% tetracycline-free fetal calf serum, 10 U/ml penicillin, 10 µg/ml streptomycin, and the optimal concentration of hygromycin selection antibiotic (50-100 µg/ml; determined empirically for each line). Colonies were picked after 2 weeks and screened by immunoblotting. Colonies with appropriate shRNA expression demonstrated knockdown of the targeted protein when cultured in media supplemented with 2 µg/ml doxycycline, but not in tetracycline-free media. Cell lines stably transfected with doxycycline-inducible vectors were maintained in TF DMEM-10 supplemented with the optimal concentration of hygromycin.

Alternatively, the expression of individual Ras proteins was also knocked down using Mission shRNAs (Sigma-Aldrich) targeting H-Ras (#s TRCN0000040088, TRCN0000040091, TRCN0000040092), N-Ras (#s TRCN0000033256, TRCN0000033257, TRCN00000300367) or K-Ras (#s TRCN0000033262, TRCN0000033263, TRCN0000033260). These lentiviral vectors are constructed in the pLKO.1-puro vector, which allows for stable transduction via puromycin selection. A puromycin concentration of 1.5 µg/ml was used to select for these vectors in T265-2c, S462 and 90-8 cells.

³H-Thymidine Proliferation Assays

Cells stably transfected with doxycycline-inducible vectors were plated in tetracycline-free DMEM-10 with or without 2 µg/ml doxycycline (5,000 cells/well, 48-well plates). Forty-eight hours post-plating, ³H-thymidine incorporation assays were performed per our

previously described protocol (24). Equal volumes of solution from each well were counted, with 16 replicates counted per condition. Data were analyzed using Student t-test, with p values < 0.05 considered significant.

Transwell Migration Assays

Cell lines stably transfected with doxycycline-inducible vectors were cultured for 48 hours in tetracycline-free DMEM-10 with or without 2 µg/ml doxycycline to induce expression. Cells were then serum-starved in Schwann cell-defined medium (30, 31) with or without 2 µg/ml doxycycline for an additional 24 hours. At the end of this period, migration assays were performed on poly-L-lysine/laminin coated Transwell filter inserts (Becton Dickinson Labware, Franklin Lakes, NJ), per our previously described protocol (32). Three replicate filters were performed for each condition, with 9 fields counted per filter. Data were analyzed using Student t-test, with p values < 0.05 considered significant.

Cell Viability (Calcein) Assays

MPNST lines stably transfected with doxycycline-inducible plasmids were plated at 20,000 cells per well in DMEM-10 overnight and then cultured in either DMEM-10 or DMEM alone for an additional 24 hours in the presence or absence of 2 µg/ml doxycycline. After 2 rinses in Hanks' Balanced Salt Solution (HBSS), cells were incubated for 30 minutes at room temperature in 200 µl of 4 ng/ml calcein acetoxymethyl diluted in HBSS. Signals were measured using a fluorescent plate reader.

Mass Spectrometric Phosphoproteome Analyses

The Invitrogen SILAC (stable-isotopic labeling using amino acids in culture) Protein and Quantitation Media Kit with DMEM-flex (MS10030) was used to label MPNST cells stably transfected with the doxycycline-inducible dominant negative H-Ras mutant with light ($^{12}\text{C}_6$) or heavy ($^{13}\text{C}_6$) L-lysine. Cells were maintained in log-phase growth in SILAC Light or Heavy medium for 5 days under doxycycline-free conditions. Two µg/ml doxycycline was then added to heavy-labeled cells only, and both heavy- and light-labeled cells were cultured for an additional 48 hours. Phosphoproteins were isolated using the Pierce Phosphoprotein Enrichment Kit (Thermo Scientific #90003). In brief, cells were lysed in the provided lysis buffer supplemented with 1% CHAPS, 1:100 Sigma protease inhibitor cocktail (#P8340) and 1:100 HALT phosphatase inhibitor cocktail (Thermo Scientific #78420). Equal quantities of protein isolated from heavy- and light-labeled cells were mixed together and incubated in the provided phosphoprotein enrichment column. The column was washed 3 times with lysis buffer. The provided elution buffer was then used to detach bound phosphoproteins from the Ga^{2+} immobilized metal ion affinity chromatography (IMAC) column. Eluted fractions were concentrated using an ICON concentrator and then resolved by SDS-PAGE. Gels were stained with GelCode Blue Stain Reagent (Thermo Scientific) to visualize protein bands. Gel lanes were divided into slices of equal size, which were destained overnight with a 1:1 mix of 100 mM ammonium bicarbonate and acetonitrile. Following destaining, gel slices were incubated with 25 mM dithiothreitol at 50°C for 30 minutes to reduce disulfide bonds and then incubated with 55 mM iodoacetamide for 30 minutes in the dark to alkylate free thiol groups. The gel slices were next washed twice with 100 mM ammonium bicarbonate (30 minutes/wash) and then dried in a SpeedVac (Savant).

Trypsin digestion was performed overnight at 37°C using Promega (Madison, WI) Gold Mass Spectrometry Grade Trypsin at a final concentration of 12.5 ng/μl. Digested gel slices were incubated twice (15 minutes/incubation) in a 1:1 mixture of 5% formic acid and 50% aqueous acetonitrile to extract peptides. Peptides were pooled, dried, and then resuspended in 20 μl of 0.1% formic acid prior to mass spectrometry analysis.

A 2-μl aliquot of each digest was loaded at 2 μl/min onto a 2 cm × 75 μm in diameter PepMap100 C₁₈ reverse-phase trap cartridge (Dionex, Sunnyvale, CA), using an autosampler (Eksigent, Dublin, CA). The cartridge was washed for 4 minutes with 0.1% formic acid in ddH₂O, after which the bound peptides were flushed onto a 15 cm × 75 μm PepMap100 C₁₈ reverse-phase analytical column (Dionex) with a 40-minute linear (5-50%) acetonitrile gradient in 0.1% formic acid at 300 nl/min using an Eksigent Nano1D+ LC. The column was washed with 90% acetonitrile-0.1% formic acid for 15 minutes and then re-equilibrated with 5% acetonitrile-0.1% formic acid for 30 minutes. The protein digest was analyzed using an Applied Biosystems 5600 Triple-ToF mass spectrometer (AB-Sciex, Toronto, Canada). The IonSpray voltage was 2300 V and the declustering potential was 60 V. IonSpray and curtain gases were set at 10 psi and 20 psi, respectively. The interface heater temperature was 120°C. Eluted peptides were subjected to a time-of-flight survey scan from 400-1250 *m/z* to determine the top 20 most intense ions for MS/MS analysis. Product ion time-of-flight scans at 50 msec were carried out to obtain the tandem mass spectra of the selected parent ions over the range from *m/z* 400-2000. Spectra were centroided and de-isotoped using Analyst software (AB Sciex; version TF). A β-galactosidase trypsin digest was used to establish and confirm the mass accuracy of the mass spectrometer. ProteinPilot Software version 4.0 (AB Sciex) was used to determine the relative abundance of heavy vs. light-labeled proteins from tryptic peptide spectra using the Paragon algorithm. Results were manually bias corrected in ProteinPilot (AB Sciex) using the median heavy-to-light (H:L) ratio of all proteins in the data set for a given experiment (after removing all contaminating proteins, reversed proteins, and proteins with only one quantifiable peptide) as a correction factor. Reported proteins were identified with 99% confidence by ProteinPilot (unused score ≥ 2) with a false discovery rate <1%. Network analyses were performed with Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA) using only those proteins in which the |log₂ (heavy-to-light ratio)| >0.33. The Fisher exact test was used to determine the probability that the changes in the networked proteins could occur by random chance. The -log of this *p* value is reported as the Ingenuity Pathway Analysis score.

RESULTS

All 3 Classic Ras Subfamily Members are Expressed and Activated in Human MPNST Cells

To establish which proteins in the classic Ras subfamily potentially contribute to MPNST pathogenesis, we examined the expression of all 3 of these neurofibromin-regulated proteins in a panel of eight human MPNST cell lines. Six of these lines were derived from neoplasms arising in NF1 patients (ST88-14, 90-8, NMS2, NMS2-PC, S462, T265-2c), while the other 2 originated from sporadic MPNSTs (STS-26T, YST-1). To verify the specificity of the antibodies we were using, ST88-14 MPNST cells were transiently transfected with plasmids

directing the expression of Myc-tagged wild-type Ras proteins and lysates of these cells were probed with our antibodies (Fig. 1A). We found that our antibodies against H-Ras, K-Ras and N-Ras did not cross-react with other Ras proteins and thus were specific for their intended antigens. Consequently, we used this panel of antibodies to probe immunoblots of lysates from all 8 MPNST cell lines. We found that H-Ras, K-Ras and N-Ras were uniformly present in these cell lines (Fig. 1B).

To determine whether the proteins necessary to activate the Ras proteins expressed in MPNST cells were present, we examined the expression of the 10 known Ras GEFs in these same 8 MPNST cell lines. Transcripts encoding nine of these ten GEFs were detected in at least some MPNST cell lines (Fig. 2A). Five of the detected GEFs (Sos1, Sos2, RasGRP3, BCAR3 and C3G) were universally expressed, whereas the other 4 (Ras GRF1, Ras GRF2, Ras GRP1 [CalDAG-GEF II] and Ras GRP2 [CalDAG-GEF I]) displayed more variable patterns of expression. Sos1 and RasGRP3 can activate both classic Ras and R-Ras subfamily proteins, whereas Sos2 has only shown affinity to H-Ras; BCAR3 and C3G have the ability to activate R-Ras but not classic Ras proteins (33-35). Considered together with the Ras expression data presented above, these data indicate that human MPNSTs are potentially capable of activating members of both the classic Ras and the R-Ras subfamilies. It is also notable that the profile of Ras GEFs expressed in MPNST cells differs in some regards from what has been previously found in *Nf1* null murine Schwann cells (36). Although C3G and the BCAR3 murine homolog AND34 were present in these non-neoplastic glia, *Nf1*^{-/-} Schwann cells, unlike MPNST cells, did not express Ras GRF1, Ras GRP1 or Ras GRP3. The other Ras GEFs we detected in MPNST cells were not examined in this earlier study.

To directly demonstrate that Ras was hyperactivated in NF1-null MPNST cells, we examined Ras activation in 2 representative NF1-associated MPNST lines (ST88-14 and T265-2c cells) and a sporadic MPNST line (STS-26T). In these experiments, the Raf-1 Ras binding domain (RBD) affinity reagent, which only binds activated Ras, was used to pull down activated Ras from lysates of these MPNST cell lines. Protein captured with the Raf-1 RBD reagent was then immunoblotted and probed with a pan-Ras antibody that recognized all 3 members of the classic Ras family (Fig. 2B). Activated Ras protein was detected in all 3 of the MPNST cell lines we tested. Notably, however, the amount of activated Ras present in ST88-14 and T265-2c cells was much higher than that evident in the sporadic STS-26T line.

To establish which classic Ras subfamily members are activated in neurofibromin-null MPNST cells, we next examined Ras activation in lysates of ST88-14 cells transiently transfected with Myc epitope-tagged wild-type Ras plasmids or a Myc-tagged dominant negative H-Ras control. In these experiments, the Raf-1 RBD affinity reagent readily pulled down Myc-tagged wild-type H-Ras, N-Ras, and K-Ras (Fig. 2C). In contrast, the Myc-tagged dominant negative H-Ras mutant, which is predominantly found in the inactive GDP-bound conformation due to its altered nucleotide-dissociation kinetics (37), did not bind this reagent. Thus, ST88-14 cells activate all of the classic Ras proteins that they express.

An H-Ras Dominant Negative Mutant Differentially Affects the Proliferation, Migration, and Survival of Human MPNST Cells

We next wished to determine the effect that classic Ras proteins exert on functions essential for MPNST pathogenesis. In designing these experiments, we considered the possibility that the Ras proteins expressed in MPNST cells would compensate for single-isoform loss by increasing the expression or activation of another Ras family member; in this circumstance, it would be necessary to target 2 or more Ras proteins simultaneously. As multiprotein ablation with siRNA is technically challenging, we instead chose to use a DN H-Ras mutant, which has been previously shown to inhibit the activation of H-Ras, N-Ras and K-Ras (38), as a pan-inhibitor of the classic Ras subfamily. ST88-14, T265-2c and STS-26T MPNST cell lines were stably transfected with plasmids expressing HA epitope-tagged DN H-Ras or a GFP negative control under the control of a doxycycline-inducible promoter. In preliminary experiments, we found that maximal induction occurred in these lines 36-48 hours after addition of 2 µg/ml doxycycline and that all lines demonstrated appropriate doxycycline-dependent induction of the target proteins (Fig. 3A). Neither DNA synthesis (Supplemental Fig. 1A) nor migration (Supplemental Fig. 1B) of the MPNST parent lines was affected when their media was supplemented with 2 µg/ml doxycycline, indicating that doxycycline had no effect on these experimental measures.

We first assessed the impact of DN H-Ras mutant expression on DNA synthesis in these MPNST cell lines using ³H-thymidine incorporation assays. We found that expression of the DN H-Ras mutant significantly reduced ³H-thymidine incorporation in the NF1-associated ST88-14 and T265-2c lines as well as in STS-26T cells, which are derived from a sporadic MPNST (Fig. 3B). Interestingly, however, inhibition of DNA synthesis by the DN H-Ras mutant was greatest in the 2 NF1-associated MPNST lines that we examined. In comparison, the sporadic STS-26T MPNST cell line, which expresses wild-type neurofibromin and does not show Ras hyperactivation (39) (Fig. 2B), showed a more modest inhibition of ³H-thymidine incorporation in the presence of DN H-Ras (Fig. 3B). Doxycycline-induced expression of GFP had no significant effect on MPNST proliferation (Fig. 3B). Thus, one or more of the classic Ras proteins promote the proliferation of MPNST cells.

We next examined the effect that the H-Ras DN mutant exerted on MPNST migration. This assessment was performed using Transwell migration assays with experimental parameters that we have previously optimized for MPNST cells (32). In contrast to the results we observed in our proliferation assays, we found that inducing expression of the DN H-Ras mutant had no effect on the migration of ST88-14, T265-2c or STS-26T cells (Fig. 3C). As with proliferation, doxycycline-induced expression of GFP also had no significant effect on the migration of these MPNST cell lines (Fig. 3C).

We also examined the effects that the H-Ras DN mutant exerted on the survival of MPNST cells. In serum-containing medium, ST88-14 cell viability, as assessed by calcein cleavage, was not affected by induction of GFP expression (Fig. 4A). However, induction of the DN H-Ras mutant resulted in a modest, but statistically significant, reduction in the number of viable ST88-14 cells (Fig. 4A; Supplemental Fig. 2A). The ability of DN H-Ras to reduce MPNST cell viability in serum-containing media was confirmed in T265-2c cells (Supplemental Fig. 2B). To determine whether inhibition of classic Ras signaling further

enhanced cell death under conditions of cellular stress, we repeated this same experiment in serum-deprived ST88-14 and T265-2c cells. When cultured in serum-free DMEM, expression of the DN H-Ras mutant resulted in an exaggerated loss of cell viability in both MPNST cell lines (Fig. 4B; Supplemental Fig. 2A, B), whereas induction of GFP expression had no effect. To determine whether the death of ST88-14 cells expressing DN H-Ras was apoptotic in nature, we examined the cleavage of caspase-3, the major effector caspase expressed in the nervous system, in ST88-14 cells maintained in DMEM alone. We found that the accumulation of cleaved caspase-3 increased in parallel with the expression of the H-Ras DN mutant 12 and 24 hours post-induction (Fig. 4C). In the presence of a 50 μ M concentration of the broad caspase inhibitor BAF, the DN H-Ras mutant had no effect on ST88-14 viability (Fig. 4D). We conclude that inhibition of classic Ras protein signaling induces caspase-dependent apoptosis in this MPNST line.

The Proliferation of MPNST Cell Lines is Variably Dependent on Individual Ras Proteins

Although the experiments described above indicated that one or more classic Ras proteins promoted proliferation, it was not clear whether a specific classic Ras protein was primarily responsible for this effect and, if so, which one. Findings from a clinical trial in NF1 patients with plexiform neurofibromas (the benign precursors of MPNSTs) indicated that H-Ras is not required for the proliferation of neoplastic Schwann cells. In this trial, it was found that the farnesyltransferase inhibitor tipifarnib, which inhibits H-Ras but not N-Ras or K-Ras signaling, did not inhibit the time to progression of plexiform neurofibromas (40). Consequently, we initially focused on examining the contribution that K-Ras and N-Ras make to MPNST proliferation by using shRNAs to individually ablate the expression of each of these Ras proteins. As we were interested in Ras activation in the setting of *NF1* loss, we also confined our studies to the NF1-associated MPNST cell lines we had tested with the H-Ras DN mutant.

In initial experiments, we were unable to obtain robust knockdown of N- or K-Ras with transient transfection of shRNA-expressing plasmids. Consequently, we stably transduced ST88-14 and T265-2c cells with lentiviruses containing doxycycline-inducible shRNA cassettes. Optimal target knockdown was observed in stable lines following incubation for 4 to 5 days in media supplemented with 2 μ g/ml doxycycline. Induction of nonsense control shRNA expression did not affect H-Ras, N-Ras, or K-Ras expression (Figs. 5A, 6A). Further, we found that the shRNA targeting K-Ras appropriately distinguished between different Ras proteins as the K-Ras shRNA reduced K-Ras expression without affecting N-Ras or H-Ras protein levels. Likewise, N-Ras shRNA expression did not reduce K-Ras or H-Ras expression (Fig. 5A). However, we did note that K-Ras expression was actually increased in the presence of N-Ras shRNA expression (Fig. 5A, 6A), suggesting that ST88-14 and T265-2c cells compensated for decreased N-Ras expression by increasing K-Ras expression.

After validating the effectiveness and specificity of the shRNAs, we examined the effects that ablating N-Ras or K-Ras had on MPNST mitogenesis. We found that knockdown of N-Ras expression did not produce statistically significant changes in proliferation in either ST88-14 (Fig. 5B) or T265-2c (Fig. 6B) cells. Curiously, K-Ras knockdown was similarly

ineffective in reducing the proliferation of ST88-14 cells (Fig. 5B). In contrast, doxycycline-induced expression of 2 different shRNAs targeting K-Ras reduced the proliferation of T265-2c cells (Fig. 6B). Further, the extent to which the K-Ras shRNAs inhibited T265-2c mitogenesis correlated with the extent of K-Ras knockdown; shK-Ras2, which more potently reduced K-Ras levels (Fig. 6A), inhibited T265-2c proliferation more profoundly than shK-Ras1 (Fig. 6B).

We also examined the effect that knockdown of N-Ras and K-Ras expression exerted on the migration of ST88-14 and T265-2c cells using Transwell migration assays performed as described above. We found that the migration of both ST88-14 and T265-2c cells was unaffected by either N-Ras or K-Ras ablation (Figs. 5C, 6C). Of note, this result is consistent with our demonstration that the DN H-Ras mutant, which inhibits the action of H-Ras, N-Ras and K-Ras, similarly fails to impede MPNST migration.

To expand our examination of which classic Ras proteins mediate proliferation, we next examined the effects of ablating N-Ras or K-Ras in S462 and 90-8 MPNST cells by stably transducing these cells with shRNA-expressing lentiviruses. As in our earlier experiments, we found that these MPNST lines differed in their dependence on specific classic Ras proteins. In S462 cells, all 3 K-Ras shRNAs and one N-Ras shRNA potently knocked down their targets, with the other 2 N-Ras shRNAs knocking down their target to a lesser degree. As we observed in ST88-14 and T265-2c cells, potent knockdown of N-Ras expression in S462 cells resulted in increased K-Ras expression (Fig. 7A). Knockdown with all 3 K-Ras shRNAs and 2 of the 3 N-Ras shRNAs also inhibited the proliferation of S462 cells, indicating that both of these classic Ras proteins contribute to mitogenesis in this line (Fig. 7A). In contrast, none of the N-Ras shRNAs and 2 of the 3 K-Ras shRNAs had a significant effect on the proliferation of 90-8 cells (Fig. 7B). We did observe that one of the K-Ras shRNAs (K63) significantly inhibited 90-8 mitogenesis. However, for reasons that are not clear, this shRNA also reduced N-Ras expression in 90-8 cells and so this result cannot be clearly ascribed to K-Ras loss.

Although the clinical trial findings noted above indicated that H-Ras was not essential for the proliferation of neoplastic Schwann cells in plexiform neurofibromas, we wanted to confirm that this was also true for MPNST cells. To determine whether H-Ras contributed to MPNST mitogenesis, we therefore attempted to ablate H-Ras expression in T265-2c, S462 and 90-8 cells and determine the effect this had on the proliferation of these lines. However, H-Ras proved to be surprisingly resistant to knockdown. After finding that H-Ras could not be effectively knocked down with four different stably transduced doxycycline-inducible lentiviral vectors, we tried stably transducing MPNST cells with 3 pLKO.1-puro lentiviral vectors expressing H-Ras shRNAs. We achieved a partial knockdown (Fig. 7C) with 2 of these vectors (shH91, shH92). Interestingly, we found that the H-Ras knockdown we achieved with these vectors was sufficient to reduce the proliferation of T265-2c (Fig. 7C) and S462 cells (Fig. 7D). In contrast, this degree of H-Ras knockdown had no effect on the proliferation of 90-8 cells (Fig. 7E). Thus, the proliferation of MPNST cells is also variably dependent on the action of HRas.

In MPNST Lines That Do Not Require N-Ras, Ablation of N-Ras Results in Compensatory Activation of K-Ras

K-Ras expression is much higher in T265-2c cells than in ST88-14 cells (Fig. 1B). This suggested that ablation of K-Ras expression might have no effect on ST88-14 mitogenesis because this cell line is not dependent on K-Ras for proliferation. Alternatively, ST88-14 cells might evade the consequences of K-Ras loss by a compensatory increase in the activation of another classic Ras protein; a third possibility, namely that there is a compensatory increase in the expression of K-Ras protein levels in response to N-Ras loss, may also facilitate evasion of the consequences of N-Ras knockdown as we noted that such an increase occurs following N-Ras knockdown in at least some MPNST lines (Figs. 5A, 6A, 7A, B). To determine whether knockdown of one Ras protein in ST88-14 cells results in enhanced activation of another Ras protein, ST88-14 cells stably transduced with a doxycycline-inducible lentivirus targeting either a nonsense control or N-Ras sequences were transiently transfected with a plasmid directing the expression of Myc-tagged K-Ras. We then assessed Ras activation in these cells in the presence and absence of shRNA expression using Raf-1 RBD pulldown assays. Although the nonsense control shRNA had no effect on Ras activation, we found that knocking down N-Ras expression resulted in an increase in the level of activation of the Myc-tagged K-Ras (Fig. 8A). These observations suggest that ST88-14 cells evade the deleterious effects of knocking down an individual Ras protein by enhancing the activation of another Ras protein. We further confirmed this observation by showing that knocking down N-Ras expression in T265-2c cells similarly resulted in an increase in the activation of endogenous K-Ras (Fig. 8B). However, this compensatory mechanism is not universally operant and/or effective in MPNST cells because knocking down K-Ras expression does impair the proliferation of T265-2c and S462 MPNST cells.

Inhibition of Classic Ras Protein Signaling Induces Distinct Changes in the Phosphoproteome

The findings described above indicate that inhibition of classic Ras signaling with the DN H-Ras mutant inhibits MPNST mitogenesis and survival, but has no effect on the migration of these cells. This suggests that hyperactivated classic Ras proteins regulate key signaling pathways in MPNST cells that control mitogenesis and survival. To identify these pathways, we performed mass spectrometry-based phosphoproteome analyses in ST88-14 cells stably transfected with plasmids expressing DN H-Ras under the control of a doxycycline-inducible promoter. We chose to perform these experiments using a novel approach based on the isolation of phosphoproteins rather than phosphopeptides as this allowed us to monitor phosphoprotein recovery throughout the procedure using antibodies that recognize representative phosphoproteins.

Uninduced cells were labeled with media containing a light ($^{12}\text{C}_6$) lysine isotope, while doxycycline-induced cells were labeled with media containing a heavy ($^{13}\text{C}_6$) lysine isotope. Equal quantities of light- and heavy-labeled cell lysates were mixed together and fractionated on gallium (Ga^{2+}) IMAC columns, which bind proteins containing negatively charged phosphate groups (Fig. 9A). In these experiments, we found that approximately 10% of the protein loaded onto the column bound to the matrix, which is consistent with

estimates of the number of phosphorylated proteins present in the cytoplasm. Immunoblotting for representative phosphoproteins (phosphorylated Erk1/2^{Thr202/Tyr204}, Akt^{Thr308}, and JNK1-3^{Thr183/Tyr185}) confirmed that these proteins were enriched in the protein fraction binding to the IMAC column (Fig. 9B).

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was then used to determine the identity and relative abundance of each light-labeled versus heavy-labeled protein captured by IMAC based on the relative intensities of the detected heavy and light tryptic peptides. The phosphorylation status of most of the detected proteins did not change following the induction of DN H-Ras (Fig. 10A), as indicated by the tight clustering of heavy-to-light (H:L) ratios around 1 ($\log_2(\text{H:L}) = 0$) in these populations of proteins. However, there was a subpopulation of proteins present that both demonstrated substantial differences in phosphorylation after induction of DN H-Ras ($|\log_2(\text{H:L})| > 0.33$) and met criteria for statistically significant detection (i.e. multiple peptides from each protein were detected, providing statistical confidence that they were present. Table 1 contains a list of the most significant hits.

To identify the cytoplasmic signaling networks potentially regulated by the classic Ras subfamily, we applied Ingenuity Pathway Analysis to the population of proteins the levels of which changed ($|\log_2(\text{H:L})| > 0.33$) in response to the induction of DN H-Ras. Seven interconnected protein networks were identified that changed following induction of DN H-Ras expression (Figs. 10B, 11). The highest ranked of these networks included proteins involved in RNA and protein synthesis, stability and modification (Fig. 11). Proteins involved in the regulation of actin cytoskeletal dynamics also appeared in the top 3 networks affected by DN H-Ras induction (Fig. 11), clustering in ways that suggested effects on cellular morphology and organization rather than on migration. Many of the detected actin dynamic proteins fell downstream of the RhoA/ROCK1 signaling axis, suggesting that DN H-Ras may enhance actin stress fiber formation through RhoA/ROCK1 rather than impacting dynamic actin structures in the lamellipodia. Other protein networks altered by DN H-Ras regulate proliferation, cell death and metabolism (Fig. 11). Although not implicated by Ingenuity Pathway Analysis as part of an affected cell death network, we noted that the apoptosis inhibitor 5 (API-5) protein showed significantly decreased phosphorylation following DN H-Ras induction (Table 1). API-5 phosphorylation is inversely correlated with levels of apoptosis (41), suggesting that DN H-Ras induced reduction in API-5 phosphorylation promotes the apoptosis observed following the expression of this DN mutant. Alternatively, the pro-apoptotic effects of DN H-Ras may reflect lower level changes in multiple factors rather than extreme changes in a single factor, as proteins in multiple networks that we found to be altered by DN H-Ras expression have secondary roles in apoptosis induction.

DISCUSSION

We hypothesized that multiple members of the classic Ras subfamily are hyperactivated in MPNST cells as a result of neurofibromin loss and that these proteins promote tumorigenesis by activating distinct cytoplasmic signaling pathways that elicit different physiologic effects. In keeping with this hypothesis, the MPNST cell lines we examined

express all 3 neurofibromin-regulated Ras proteins: H-Ras, N-Ras and K-Ras. These lines also express a complement of RasGEFs sufficient to activate each of these molecules, and we demonstrated that all of the classic Ras proteins expressed in MPNST cells can be activated. The effects of our H-Ras DN mutant indicate that classic Ras subfamily proteins contribute to neoplasia but that they do so by affecting distinct cellular functions – classic Ras proteins promote tumor cell mitogenesis and survival, but not migration. Curiously, we found that knocking down H-Ras, K-Ras and/or N-Ras in some MPNST lines inhibited proliferation while K-Ras and N-Ras can apparently compensate for one another in other cell lines. Using phosphoproteomics, we identified several signaling networks that are differentially regulated downstream of the classic Ras subfamily. Considered together, these findings provide some intriguing insights into the mechanisms involved in the pathogenesis of neurofibromin-null neoplasms.

The complement of Ras proteins that we detected in our panel of human MPNST cell lines is similar to that previously reported in wild-type and *Nf1*^{-/-} mouse Schwann cells (36). Notably, however, several of the GEFs expressed in human MPNST cells are not normally present in non-neoplastic murine Schwann cells. GEFs serve as key intermediates between cell surface signaling molecules such as growth factor receptors and Ras proteins. These upstream regulators of Ras signaling remain relevant even in the context of neurofibromin loss, as demonstrated by the ability of growth factor stimulation to increase Ras activation in neurofibromin-null cells (39). Consequently, the presence of certain GEFs in neoplastic Schwann cells but not their non-neoplastic counterparts raises the question of whether the upstream signals driving Ras activation in MPNST cells are distinct from those operant prior to transformation. Given this, it will be of considerable interest to determine which of the GEFs identified in our studies are particularly important for Ras activation in MPNST cells and to establish whether specific growth factor receptors are essential for the activation of key GEFs.

We also demonstrated that all of the classic Ras proteins expressed in MPNST cells are activated, contradicting previous findings that N-Ras and K-Ras, but not H-Ras, were activated in MPNST cells (42). As the authors of this earlier study did not detect H-Ras expression in ST88-14, 90-8 or STS-26T cells – all of which demonstrated H-Ras expression in our hands – this discrepancy may be explained by the relative specificity and sensitivity of the different H-Ras antibodies used in these studies. It should also be noted that the spectrum of classic Ras proteins activated by neurofibromin loss in MPNST cells may differ from that observed in other neurofibromin-null cell types. For example, studies in *Nf1*^{-/-} astrocytes have shown that K-Ras is the sole activated classic Ras protein in these glia (43), whereas activation of both N-Ras and K-Ras is evident in neurofibromin-null acute myelogenous leukemia cells (44). The reason for such differences is at present unclear but may include factors such as the profile of GEFs expressed in different cell types.

Our demonstration that a DN H-Ras mutant inhibits MPNST proliferation and survival implicates the classic Ras subfamily in the regulation of these responses in MPNST cells. However, precisely which classic Ras proteins promote proliferation and survival appears to differ among individual MPNST cell lines. For instance, in T265-2c and S462 cells, we found that ablation of K-Ras inhibited mitogenesis. K-Ras also plays a key role in survival

in the former line, as we have previously shown that reducing K-Ras expression leads to death in T265-2c cells (45). In contrast, neither K-Ras nor N-Ras knockdown impeded the proliferation of ST88-14 cells and we found that ablating N-Ras expression resulted in a compensatory increase in K-Ras activation. At present, it is unclear why K-Ras is particularly important for the proliferation and survival of T265-2c cells, especially since there appears to be some degree of functional redundancy among the classic Ras proteins in ST88-14 cells. This disparity may suggest that some MPNSTs, such as the tumor from which T265-2c cells were derived, have defects in signaling pathways that coordinate the activation of different Ras proteins. These observations also have important clinical implications because they suggest that MPNSTs differ in their dependence on specific Ras proteins. Thus, defining which Ras proteins drive proliferation and survival in an individual tumor will be critically important if we are to develop and apply new therapies targeting Ras in MPNST cells.

The differential effects of DN H-Ras on MPNST proliferation, survival and migration suggest that classic Ras proteins control specific cellular signaling cascades regulating proliferation and survival, but not those essential for migration. To address this postulate, we utilized a global phosphoproteomics approach that allowed us to perform a global examination of the phosphorylation events that were impacted by DN H-Ras. Phosphoproteomics has previously been used to investigate alterations induced by individual classic Ras proteins with oncogenic mutations (46). Despite different enrichment procedures (phosphopeptide versus phosphoprotein enrichment), quantitation methodologies (internal standard versus SILAC labeling) and cell types, many of the proteins whose phosphorylation status was altered by DN H-Ras in our study were also altered by either activated N-Ras or K-Ras in this previous study (46). Lamin-A/C phosphorylation, for example, was observed to decrease following DN H-Ras induction, indicating that classic Ras signaling promotes the phosphorylation of this molecule. Increased lamin-A/C phosphorylation was observed in cells with either N-Ras or K-Ras activating mutations (46), confirming the validity of our results. Interestingly, some of the other proteins identified in both our study and the earlier examination of N- and K-Ras effects were altered in different ways by N-Ras versus K-Ras activation. Cortactin phosphorylation was increased following DN H-Ras induction, suggesting that classic Ras proteins inhibit its phosphorylation. This was similarly observed in H1299 cells (large cell carcinoma cells with an activating N-Ras mutation), whereas, in contrast, cortactin phosphorylation was increased in A549 lung adenocarcinoma cells, which have an activating K-Ras mutation (46). API5 shows a similar but reversed trend—its phosphorylation was observed to decrease following DN H-Ras induction and increased in cells with activated N-Ras, but not K-Ras. This may indicate that N-Ras is a stronger influence on cortactin and API5 phosphorylation than K-Ras in the context of simultaneous classic Ras isoform activation in MPNST cells. The latter is particularly interesting because N-Ras activation has a stronger link to steady-state anti-apoptotic signaling than K-Ras activation in many systems (47-49).

Although we found that DN H-Ras induced profound changes in the phosphorylation of some proteins regulating cytoskeletal dynamics, we also found that this mutant did not affect migration. As an explanation for this apparent discrepancy, we would note that the proteins whose phosphorylation was affected by DN H-Ras expression were grouped by Ingenuity

Pathway Analysis into networks affecting cellular morphology rather than cellular migration. The 2 cytoskeletal dynamic proteins the phosphorylation status of which was most strongly and significantly affected by DN H-Ras induction were vimentin and cofilin. While vimentin has been implicated in a variety of cellular functions, its most well-studied roles are in maintaining cellular integrity (50, 51) and the intracellular distribution of organelles (52, 53). Only a few of the vimentin phosphorylation sites identified from in vitro experiments have been validated in vivo; and phosphorylation at these residues has been linked to filament disassembly during mitosis (54-56), which is consistent with our finding that DN H-Ras induction affects proliferation but not migration. In contrast, regulation of cofilin phosphorylation has a well-established role in migration, i.e. activation of cofilin by Ser³ dephosphorylation at the leading edge of a cell is a critical early step in this process. However, this dephosphorylation must also be paired with phosphorylation (inactivation) of cofilin in the cell body (57, 58) to restrict cofilin activation spatially for polarized cell motility. Global cofilin status is thus unlikely to correlate directly to migratory capacity (59), and so the increased cofilin phosphorylation observed following DN H-Ras induction may reflect alterations in cofilin phosphorylation in the cell body that would not affect the function of this molecule in lamellipodia. Indeed, cofilin inhibition is necessary for RhoA-induced actin stress fiber formation (60, 61), and increased stress fiber formation has been previously reported in MPNST cells following treatment with farnesylthiosalicylic acid (39), another broad spectrum inhibitor of the classic Ras subfamily.

Actin dynamic proteins, which were also impacted by the DN H-Ras mutant, are likewise intimately involved in cytokinesis, suggesting that these changes may affect proliferation. Further, DN H-Ras affected the phosphorylation of molecules involved in protein synthesis, a process that is intimately intertwined with cellular proliferation (62, 63). Although we did not observe changes in the phosphorylation of nuclear proteins directly controlling mitogenesis in our phosphoproteomics experiments, this was not unexpected. As noted above, the phosphoproteomics methodology we employed is geared towards the analysis of cytoplasmic proteins—we have confirmed that our procedure does not lyse the nuclear envelope and consequently nuclear proteins were not represented in our lysates.

We recognize that there are some potential caveats to the interpretation of our data. Because dominant negative mutants function by sequestering GEF molecules, the inhibitory specificity of the H-Ras DN mutant will be determined by the specificity of the GEF molecules themselves. GEFs can have secondary affinities to other proteins within the Ras superfamily so it is possible that inhibition of non-Ras proteins such as Rap may contribute to the phenotypes we observed following DN H-Ras expression in MPNST cells. In addition, because some GEF molecules can potentially activate both classic Ras and members of the closely related R-Ras subfamily, it is possible that some cross-subfamily inhibition by DN H-Ras may occur. However, as R-Ras subfamily members have been implicated in Schwann cell migration (36), the lack of a DN H-Ras effect on migration argues that such cross-reactivity is minimal. Ablation of the expression of individual R-Ras proteins or analogous studies with a DN R-Ras mutant will likely be necessary to confirm this and expand upon our results in future work.

In conclusion, we have found that neurofibromin loss results in the activation of multiple members of the classic Ras subfamily in neoplastic Schwann cells. Our observations suggest that this subfamily serves distinct functions in MPNST cells, a hypothesis that is further supported by the differential effects of the H-Ras DN mutant on MPNST proliferation, migration, and survival. Our phosphoproteomics analyses indicate that classic Ras proteins also affect the phosphorylation of distinct collections of cytoplasmic signaling molecules, which begins to explain the distinct physiologic effects of this subfamily. Although the analyses presented in this manuscript provide useful and novel insights into the mechanisms operant in neurofibromin-null tumors, we caution that this study represents only an initial step towards deciphering the cytoplasmic signaling cascades downstream of the classic Ras subfamily. Studies with inhibitors such as the DN Ras mutants we employed examine the terminal consequences of inhibiting the signaling cascade—identifying the cytoplasmic signaling intermediates that are more proximal to each Ras protein will be essential as we move forward and will require a different experimental design. Answering questions such as these will provide the basic framework of information that is necessary for the future development of therapies that are effective against the growing number of tumor types that occur as a result of a loss of neurofibromin expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Human malignant peripheral nerve sheath tumors (MPNSTs) variably express classic Ras isoforms. **(A)** ST88-14 cells were transiently transfected with plasmids expressing Myc-tagged dominant negative (DN) H-Ras or wild-type H-Ras, N-Ras, or K-Ras. Lysates of these cells were immunoblotted and probed with antibodies directed against H-Ras (sc-833, 1:1000), N-Ras (sc-30, 1:1000), K-Ras (AbD Serotec MCA3223Z, 1:1000), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:100,000). Endogenous Ras (which migrates ~5kD below the Myc-tagged isoforms) is not shown. **(B)** Lysates from eight human MPNST cell lines probed with isoform-specific antibodies for classic Ras proteins using GAPDH as a loading control.

Figure 2.

Multiple classic Ras isoforms are activated in malignant peripheral nerve sheath tumor cells. (A) PCR analyses of Ras guanine nucleotide exchange factor expression in human MPNST cell lines. (B) Raf-1 Ras binding domain (RBD) assays performed on lysates of NF1-associated MPNST cell lines (ST88-14, T265-2c) and a sporadic MPNST line (STS-26T). Activated Ras captured with the Raf-1 RBD was immunoblotted and probed with a pan-Ras antibody recognizing all 3 members of the classic Ras family. (C) Raf-1 RBD assays performed on ST88-14 cells transiently transfected with Myc-tagged wild-type H-, N- or K-Ras plasmids or the Myc-tagged dominant negative H-Ras control plasmid.

Figure 3.

Dominant negative (DN) H-Ras mutant inhibits malignant peripheral nerve sheath tumor mitogenesis but not migration. (A) Expression of HA-tagged DN H-Ras and green fluorescent protein (GFP) control in ST88-14, STS-26T, and T265-2c doxycycline-inducible stable cell lines in the presence (+) or absence (–) of 2 µg/ml doxycycline. (B) Induction of DN H-Ras expression reduces ³H-thymidine incorporation in the ST88-14, STS-26T, and T265 doxycycline-inducible stable cell lines. (C) Induction of DN H-Ras does not affect Transwell migration in the ST88-14, STS-26T, and T265 doxycycline-inducible stable cell lines. *p < 0.05.

Figure 4.

Dominant negative (DN) H-Ras induces apoptotic cell death, which is enhanced under conditions of cellular stress. **(A)** Calcein cleavage is modestly, but significantly, reduced following induction of DN H-Ras but not green fluorescent protein (GFP) in ST88-14 cells cultured in DMEM-10. **(B)** Calcein cleavage is even further reduced following induction of DN H-Ras but not GFP in ST88-14 cells cultured in DMEM alone. **(C)** Cleaved caspase-3 (Cell Signaling #9661, 1:1000) is increased following induction of DN H-Ras in ST88-14 cells cultured in DMEM alone. HA epitope expression verifies DN H-Ras induction. **(D)** Induction of DN H-Ras does not decrease calcein cleavage in serum-deprived conditions in the presence of the broad caspase inhibitor BAF (50 μ M). *p < 0.05.

Figure 5.

Neither N-Ras nor K-Ras knockdown significantly affects the proliferation or migration of ST88-14 cells. (A) ST88-14 cells stably transduced with doxycycline-inducible shRNA lentiviruses were maintained for 5 days in DMEM, 10% tetracycline-free FBS, 10 U/ml penicillin, 10 µg/ml streptomycin. (TF DMEM-10) with or without 2 µg/ml doxycycline. Immunoblotting for classic Ras proteins shows specificity of K-Ras and N-Ras shRNAs. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. (B) Neither N-Ras nor K-Ras ablation significantly reduces ³H-thymidine incorporation in ST88-14 cells. (C) Neither N-Ras nor K-Ras ablation reduces migration in ST88-14 cells.

Figure 6.

K-Ras knockdown, but not N-Ras knockdown, inhibits the proliferation of T265-2c cells.

(A) T265-2c cells stably transduced with doxycycline-inducible shRNA lentiviruses were maintained for 5 days in DMEM, 10% tetracycline-free FBS, 10 U/ml penicillin, 10 µg/ml streptomycin. (TF DMEM-10) with or without 2 µg/ml doxycycline. Immunoblotting for classic Ras proteins shows specificity of K-Ras and N-Ras shRNAs. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. (B) Induction of K-Ras shRNA, but not N-Ras shRNA, reduces ³H-thymidine incorporation in ST88-14 cells. Two shRNAs targeting different sequences in the K-Ras isoform are shown. (C) Neither N-Ras nor K-Ras shRNA ablation reduces migration in T265-2c cells.

Figure 7.

H-, N- and K-Ras are variably required for malignant peripheral nerve sheath tumor (MPNST) proliferation. **(A)** S462 cells were stably transduced with lentiviruses targeting N-Ras or K-Ras. Immunoblotting for N-Ras and K-Ras proteins shows specificity of K-Ras and N-Ras shRNAs (right panel). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. N-Ras and K-Ras knockdown both inhibited S462 proliferation (left panel). **(B)** 90-8 cells were stably transduced with lentiviruses targeting N-Ras or K-Ras. Immunoblotting for N-Ras and K-Ras proteins again shows specificity of K-Ras and N-Ras shRNAs (right panel). GAPDH was used as a loading control. In contrast, neither N-Ras nor K-Ras knockdown inhibited 90-8 proliferation except for the K63 vector. This vector, however, also resulted in reduced N-Ras expression. **(C, D)** Three different H-Ras shRNAs inhibited the proliferation of T265-2c cells **(C)** and S462 cells **(D)**. **(E)** In contrast, H-Ras shRNAs had no effect on the proliferation of 90-8 cells. For all panels, * $p < 0.05$.

Figure 8.

K-Ras activation is increased following shRNA-mediated ablation of N-Ras in ST88-14 cells. **(A)** ST88-14 cells stably transduced with doxycycline-inducible shRNAs were transiently transfected with Myc-tagged wild-type K-Ras and cultured for 3 days in DMEM-10 with or without 2 μg/ml doxycycline. Activation of the ectopically expressed Myc-tagged K-Ras isoform was assessed with the Raf-1 Ras binding domain (RBD) assay. **(B)** The effect of N-Ras ablation on endogenous levels of activated K-Ras was assessed in T265-2c stable cell lines transduced with either control non-targeting or N-Ras specific shRNA using the Raf-1 RBD assay.

Figure 9. Phosphoprotein enrichment of stable-isotope labeled doxycycline-inducible cell lines. **(A)** Flow scheme of methodology used for phosphoprotein enrichment. **(B)** Immunoblotting for representative phosphoproteins verifies phosphoprotein enrichment in co-eluted fraction. Induction of dominant negative mutants in the Heavy preparations was verified by immunoblotting for the HA epitope. Antibody dilutions were as follows: pErk1/2 1:1200, pAkt 1:500, pJNK1-3 1:500, HA epitope 1:50,000; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 1:100,000. SILAC, stable-isotopic labeling using amino acids in culture).

Figure 10.

Different networks of phosphorylated proteins affected by dominant negative (DN) H-Ras. (A) Global distribution of heavy DN H-Ras to light (uninduced) ratios of detected phosphoproteins in ST88-14 DN H-Ras cells. Plotted values are $\log_2(H:L)$. (B) Ingenuity Pathway Analysis (IPA) analysis identified 7 networks affected by DN H-Ras induction with IPA scores ≥ 16 .

Dominant negative H-Ras Ingenuity Pathway Analysis networks

Molecules in Network	Score	Focus Molecules	Top Functions
<p>↑ACTG1*, ↓ACTN1*, ↓AHNAK, Alpha catenin, ↓ANXA2*, ↑CFL1*, ↑CTNNA1, ↓DDX39B*, ↑EIF5*, ↑EIF3A, Eif4g, ↓HIST1H1C*, ↓HIST1H1D, Histone H1, ↓HNRNPD, ↓HNRNPK*, Hsp27, ↑HSPA8*, ↓LDHA*, ↑NCL, NFAT (complex), NFkB (complex), ↑PKM2*, ↓PRDX2, ↑RAN*, Ribosomal 40s subunit, Rnr, ↑RPS2, ↑RPS16*, ↑RPS18, ↑RPS15A, ↑SRSF1, ↑SRSF3, ↓TCERG1, ↓TLN1*</p> <p>↓ACTR1A, Alpha tubulin, ↓ANXA6*, ↑ARPC2*, ↑BAG2, Beta Tubulin, ↑CCDC6, ↓CCT3*, ↑CCT7*, ↓CLIP1, ↑CTTN, ↓DCTN3, Dynamin, ERK1/2, Erm, ↓EZR*, EZR-MSN-RDX, F Actin, ↓FLNA*, ↑GAPDH*, IgG1, ↑IQGAP1, ↓MAP4, ↑MX1*, ↑PFN1, ↑PPIB*, ↓RDX*, ↓SH3GL1, ↑TCP1*, ↓TMSB10/TMSB4X, ↑TRIP10*, ↑TUBA1B*, ↑TUBB*, ↑TUBB2C*, Tubulin</p>	60	27	RNA Post-Transcriptional Modification, Protein Synthesis, Cellular Assembly and Organization
<p>↑ACOT13, AIMP2, ↓API5, ↑BZW1, ↑CAB39*, ↑EEF1E1, EGF, EPHA2, ERCC1, ↓FAM50A, FGF2, FN1, FOS, FZD1, HNF4A, ↑IARS, ↓LACRT, ↓MRPS23, PGGT1B, PIP5K1A, ↑POLDIP2, ↑PSMD12, ↑RARS*, RASGRP3, RHOA, ↓RPS10, SDC1, SH2D3C, ↑SNRPD3*, ↓TMSB10/TMSB4X, ↓TPR, TRAF6, ↓TUFGM, VAV2, VHL</p>	30	16	Cell Morphology, Connective Tissue Development and Function, Gene Expression
<p>26s Proteasome, 60S ribosomal subunit, Akt, ATPase, Ck2, ↓DTYMK, Estrogen Receptor, Fgf, ↓FKBP4, ↓FLNC, FSH, Histone h3, Histone h4, Hsp70, Hsp90, HSP, ↓HSP90AB1*, ↑HSPA5*, IL12 (complex), Interferon alpha, ↑ISG20, Lh, MHC Class I (complex), ↑PDIA3*, RNA polymerase II, ↑RPL12*, ↓RPL18, ↑RPL22*, ↑SND1*, ↑STAT1*, ↑STIP1*, ↓SUPT16H*, ↑UBA52*, Vegf, ↑WDR1</p>	29	16	Post-Translational Modification, Drug Metabolism, Endocrine System Development and Function
<p>Actin, Alpha actin, Alpha Actinin, ↑ANXA7, Calmodulin, Calpain, CaMKII, Caspase, Collagen type I, ↑EHD4, Focal adhesion kinase, ↓GPI*, ↑HRAS, ↓HSD17B4*, ↑HSPD1*, Immunoglobulin, Integrin, ↓LMNA, ↑MSN*, Nfat (family), Pdgf (complex), PI3K (complex), Pkc(s), Rac, Rap1, Ras, Ras homolog, Rb, Rho gdi, Rock, Rsk, ↑RSU1, TCR, ↑TRAP1*, ↑VIM*</p>	16	11	Cellular Assembly and Organization, Cellular Compromise, Cell Morphology
<p>6-dimethylaminopurine, ADRA2A, BRAP, CLCF1, Cytochrome c, ↓DDB1, DUSP6, ↑EIF5B*, ↑ENO1*, ERK, Fcer1, FSTL1, ↑GLUD1*, ↓HIST1H1C*, HISTONE, ↓HUWE1, ↓ICAM1, Insulin, Jnk, LDL, LGALS7/LGALS7B, MAPK12, MTCH1, P38 MAPK, p85 (pik3r), ↑PARP1*, PDGF BB, PLC gamma, ↓PTBP1*, SH2B2, SH3RF1, ↑TRIM28, Trk Receptor, TRPC3, TXNDC17</p>	16	10	Cellular Growth and Proliferation, Inflammatory Response, Cell Death
<p>↓AK3*, ↓ATP5O*, CD40, CHKA, CPT1A, CTSL2, DOK2, DUSP6, FBXW7, FSTL1, HTT, Ifn, IFNK, Ifnz (includes others), IRG1, ↓KYNU*, LEP, LXN, MYC, PPARGC1A, RGS14, RPLP1, SDHA, ↑SHMT2*, SLC2A2, ↑SRP9, ↑SRSF4, SRSF6, Tgtp1, ↓TMSB10/TMSB4X, TNF, TNFSF11, ↓UGGT1*, ↓UGP2*, ↓YARS*</p>	16	10	Carbohydrate Metabolism, Small Molecule Biochemistry, Cellular Development

Figure 11.

Dominant negative (DN) H-Ras Ingenuity Pathway Analysis (IPA) networks. Red gene symbols represent proteins that showed increased binding to the immobilized metal ion affinity chromatography (IMAC) column following DN H-Ras induction. Green gene symbols indicate proteins with decreased binding. Bolded gene symbols represent proteins whose quantitation was deemed to be significantly different between uninduced and DN H-Ras induced ST88-14 cells by ProteinPilot.

Table 1

Proteins With Phosphorylation Significantly Altered By Dominant Negative H-Ras Expression

Accession #	Name	Manual Bias Corrected H:L	Protein Pilot <i>p</i> value
sp P62328 TYB4	Thymosin beta-4 GN=TMSB4X	0.0327	<0.05
sp Q9GZZ8 LACRT	Extracellular glycoprotein lacritin GN=LACRT	0.0701	<0.01
sp P02545 LMNA	Prelamin-A/C GN=LMNA	0.3920	<0.05
sp P48047 ATPO	ATP synthase subunit O, mitochondrial GN=ATP5O	0.5134	<0.05
sp P54577 SYYC	Tyrosyl-tRNA synthetase, cytoplasmic GN=YARS	0.6323	<0.05
sp P27816 MAP4	Microtubule-associated protein 4 GN=MAP4	0.6790	<0.01
sp O14776 TCRG1	Transcription elongation regulator 1 GN=TCERG1	0.6963	<0.01
sp P07437 TUBB5	Tubulin beta chain GN=TUBB	0.7222	<0.05
sp P23919 KTHY	Thymidylate kinase GN=DTYMK	0.7374	<0.05
sp Q9BZZ5 API5	Apoptosis inhibitor 5 GN=API5	0.7653	<0.05
sp P31948 STIP1	Stress-induced-phosphoprotein 1 GN=STIP1	1.2607	<0.05
sp P62826 RAN	GTP-binding nuclear protein Ran GN=RAN	1.2969	<0.05
sp P23284 PPIB	Peptidyl-prolyl cis-trans isomerase B GN=PPIB	1.4474	<0.05
sp O43324 MCA3	Eukaryotic translation elongation factor 1 epsilon-1 GN=EEF1E1	1.5074	<0.05
sp P08670 VIME	Vimentin GN=VIM	1.7599	<0.001
sp P23528 COF1	Cofilin-1 GN=CFL1	2.5057	<0.01
sp Q9Y376 CAB39	Calcium-binding protein 39 GN=CAB39	3.5764	<0.05
sp P42224 STAT1	Signal transducer and activator of transcription 1-alpha/beta GN=STAT1	3.6030	<0.05
sp Q96AZ6 ISG20	Interferon-stimulated gene 20 kDa protein GN=ISG20	3.8073	<0.05