**Ras-Driven Transcriptome Analysis Identifies Aurora Kinase A as a Potential Malignant Peripheral Nerve Sheath Tumor Therapeutic Target**

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**Abstract**

**Purpose:** Patients with neurofibromatosis type 1 (NF1) develop malignant peripheral nerve sheath tumors (MPNST), which are often inoperable and do not respond well to current chemotherapies or radiation. The goal of this study was to use comprehensive gene expression analysis to identify novel therapeutic targets.

**Experimental Design:** Nerve Schwann cells and/or their precursors are the tumorigenic cell types in MPNST because of the loss of the NF1 gene, which encodes the RasGAP protein neurofibromin. Therefore, we created a transgenic mouse model, CNP-HRas12V, expressing constitutively active HRas in Schwann cells and defined a Ras-induced gene expression signature to drive a Bayesian factor regression model analysis of differentially expressed genes in mouse and human neurofibromas and MPNSTs. We tested functional significance of Aurora kinase overexpression in MPNST in vitro and in vivo using Aurora kinase short hairpin RNAs (shRNA) and compounds that inhibit Aurora kinase.

**Results:** We identified 2,000 genes with probability of linkage to nerve Ras signaling of which 339 were significantly differentially expressed in mouse and human NF1-related tumor samples relative to normal nerves, including Aurora kinase A (**AURKA**). AURKA was dramatically overexpressed and genomically amplified in MPNSTs but not neurofibromas. Aurora kinase shRNAs and Aurora kinase inhibitors blocked MPNST cell growth in vitro. Furthermore, an AURKA selective inhibitor, MLN8237, stabilized tumor volume and significantly increased survival of mice with MPNST xenografts.

**Conclusion:** Integrative cross-species transcriptome analyses combined with preclinical testing has provided an effective method for identifying candidates for molecular-targeted therapeutics. Blocking Aurora kinases may be a viable treatment platform for MPNST.

**Introduction**

Malignant peripheral nerve sheath tumors (MPNST) are aggressive soft tissue sarcomas that frequently metastasize and rarely respond to chemotherapy. Patients with Neurofibromatosis type 1 (NF1) inherit mutations in the NF1 gene and are predisposed to developing MPNST, identified in approximately 10% of NF1 patients. MPNSTs, having a 20% to 50% 5-year survival rate, are the major cause of mortality in adult NF1 patients (1). Approximately 50% of MPNST cases are sporadic, and some sporadic MPNSTs have mutations in the NF1 gene (2). The most common manifestation of NF1 is the development of benign peripheral nerve sheath tumors. Approximately 95% of NF1 patients harbor smaller benign dermal neurofibromas, and at least 30% of NF1 patients develop larger benign plexiform neurofibromas, typically associated with deeper nerve trunks. It is believed that plexiform neurofibromas can transform to MPNSTs (1).

The protein encoded by the NF1 gene, neurofibromin, is a Ras GTPase activating protein (RasGAP) for all Ras isoforms, negatively regulating the Ras signal transduction pathway. Ras GAPs accelerate the hydrolysis of active Ras-GTP to inactive Ras-GDP (1). Therefore, having loss of function mutations in NF1 in NF1-derived MPNSTs (3, 4)
Translational Relevance

Malignant peripheral nerve sheath tumors (MPNST) are aggressive sarcomas that arise sporadically or in the context of neurofibromatosis type 1 (NF1), especially in NF1 patients with benign plexiform neurofibromas. MPNSTs respond poorly to chemotherapy or radiation and are a major source of mortality in NF1 patients. No effective treatments for MPNST are proven, and molecular events contributing to malignant transformation of NF1 tumors are incompletely understood. However, identification of tumorigenic signaling pathways may facilitate the development of effective treatments. Using a combined gene expression database of mouse and human neurofibromas and MPNSTs, we identified overexpression of Aurora kinases in MPNSTs. Furthermore, we provide in vitro and in vivo evidence supporting the use of Aurora kinase inhibitors in MPNST treatment strategies. The combination of cross-species transcriptionome analyses with in vitro and in vivo MPNST models for preclinical testing provides a powerful translational pipeline for candidate molecular-targeted therapeutics into human clinical trials.

have elevated levels of Ras-GTP. Although in benign tumors, prolonged Ras activation can be associated with oncogene-induced senescence, in malignancy aberrant activation of Ras signaling generally leads to promotion of tumor cell proliferation and/or survival (5). It is unclear which Ras isoforms are essential in NF1 tumorigenesis, and the effects of specific isoforms seem to be cell-type dependent (6).

Interestingly, NF1-associated MPNSTs and sporadic MPNSTs show similar pathology and share similar gene expression signatures, possibly indicating a convergence to similar pathways downstream of Ras signaling (7, 8). Multiple effector pathways lie downstream of Ras, many of which have been implicated in NF1 tumorigenesis (1). Neurofibromas and MPNSTs from NF1 patients and mouse models with Nf1 mutations have elevated levels of phosphorylated ERK, the downstream effector of the canonical Ras/Raf/MEK/ERK pathway, and a MEK inhibitor provided a modest reduction in human MPNST cell survival in xenografts [(9); unpublished data]. The mTOR signaling pathway is also activated in MPNST, but blocking mTOR signaling with rapamycin or its analogs only transiently delayed tumor growth (9, 10).

MPNSTs have a complex karyotype with amplifications and deletions of many alleles, some of which may represent therapeutic targets. Multiple studies have identified individual molecular alterations in addition to Nf1 mutation in MPNST (1). Early alterations in premalignant tumors have included loss of the CDKN2A/B locus, which normally encodes proteins that negatively regulate the cell cycle (11), and loss of function mutations in the common tumor suppressor gene TP53 have been frequently observed (1).

Somatic inactivation of additional tumor suppressor genes has been used to model MPNSTs in mice. Null mutations in Nf1 and p53 in cis produced tumors in mice characteristic of human MPNST (GEM-PNST; refs. 12 and 13). Amplification and/or overexpression of potential oncogenes, especially those encoding receptor tyrosine kinases, has also been implicated in NF1 tumorigenesis, including PDGFR, CKIT, EGF (1), and IGF1R (14).

Despite these significant contributions to understanding the molecular etiology of NF1, thus far, no chemotherapeutic approach blocking any molecular target, including growth factor receptors upstream of Ras, Ras itself, Ras downstream effectors, or combinations of targets, has prevented or arrested neurofibroma formation or more than transiently delayed MPNST growth (15). However, a recent combinatorial study including rapamycin, an inhibitor of mTOR downstream of Ras, and an HSP90 inhibitor, enhancing proteotoxic stress, showed synergistic efficacy in the Nf1:p53 cis MPNST mouse model (16). The results of this study suggest that combining a Ras pathway inhibitor with a cytotoxic agent may be an effective treatment strategy for MPNST, an idea not yet tested in human clinical trials.

As additional candidates for MPNST chemotherapies are needed, we focused on molecular alterations downstream of H-Ras activation in Schwann cells, utilizing a Schwann cell–specific H-Ras gene expression signature derived from a novel transgenic mouse model to identify mechanisms contributing to tumorigenesis and potential therapeutic targets in NF1 tumors. We identify overexpression and amplification of a Ras target gene, AURKA, in MPNSTs. Furthermore, we show that blocking AURKA diminishes MPNST cell growth in vitro and in vivo.

Materials and Methods

CNP-HRas12V expression analysis

The CNP-HRas12V data set consisted of 8 control nerves encompassing 2 genotypes 4 Nf1 flox/flox; 4 P0Cre (controls) and 6 CNP-HRas12V nerves. All statistical comparisons and data visualization were performed using GeneSpring GX v7.3.1 (Agilent Technologies). All statistical tests were corrected for multiple testing effects by applying the Benjamini and Hochberg false discovery rate correction. We used a t test and FDR ≤ 0.01 to compare groups.

Bayesian sparse latent class factor modeling

Gene orthologs of transcripts differentially expressed in CNP-HRas12V peripheral nerves were used as seeds in a Bayesian factor regression modeling analysis. The model includes 6 categorical responses: 4 species-specific tumor states (mouse neurofibroma, mouse MPNST, human neurofibroma, human MPNST) and 2 species-independent tumor states (neurofibroma, MPNST). By design, the 6 categorical factors are associated with the phenotypic outcome of NF1 tumor progression. Using a threshold of 0.85 for factor inclusion probability and 0.95 for variable inclusion probability, and constraining such that a minimum of
10 genes are required to exceed the factor threshold to include that factor in an expanded model, the analysis terminated with a model on 2,000 gene orthologs distributed across \( k = 100 \) latent factors and 6 categorical response factors.

**Gene interaction network**
A total of 339 gene orthologs from the 2,000 genes linked to Ras signaling as determined by Bayesian factor regression modeling analysis were also identified as similarly regulated transcripts using frequentist statistics (Supplementary Fig. S1). We used GeneGo’s MetaCore to construct a gene interaction network using the direct interactions network building algorithm, which resulted in a single multipnode connected network of 85 gene orthologs (Supplementary Fig. S2).

**AURKA locus copy number analysis**
For SNP-array analysis, raw data from Illumina’s cnv370-duo and h660w-quad SNP array chips were processed using GenomeStudio (Illumina). B-allele frequency and log ratio were calculated using the reference cluster file provided by Illumina. Quantitative PCR analysis of DNA, AURKA gene amplification was determined by quantitative PCR carried out on a LightCycler 480 Instrument (Roche Applied Science), using Universal Probe Library Technology. A total number of 37 samples were analyzed: 13 MPNSTs, 5 MPNST-derived cell lines, 8 neurofibromas, and 11 normal samples (which presented a diploid status of AURKA and were used as controls). For additional details, see Supplementary Materials and Methods.

**Lentiviral short hairpin RNA infection**
MPNST cell lines were cultured as described (7, 17). Cells were evaluated by STR DNA profile analysis of 15 loci plus Amelogenin to exclude cross contamination. For lentiviral short hairpin RNA (shRNA) infection, MPNST cells at 50% to 60% confluence were infected with lentiviral particles containing shRNAs targeting AURKA, AURKB, or shNon-targeting (Sigma-Aldrich; TRC library). The CCHMC Viral Vector Core produced virus using a 4-plasmid packaging system (http://www.cincinnatichildren.org/research/div/exp-hematology/translational/vpf/vvc/default.htm). Lentiviral particles were incubated with MPNST cells (MOI ~10) in the presence of polybrene (8 μg/mL; Sigma) for 24 hours followed by selection in 2 μg/mL puromycin.

**Quantitative real-time PCR-PCR**
Total RNA was isolated from cells using the RNasy kit (Qiagen) and used as a template for cDNA synthesis (high-capacity cDNA archive kit; Applied Biosystems) and quantitative real-time PCR (qRT-PCR; ABI 7500 Sequence Detection System) as described (7). Primers for AurkA were forward-TAGCCCTCGCTATCGCCACTGCT and reverse-ACAAGACCTGGTTGTGTCAGT and for AurkB forward-TGAGGAGGAGCAGAATATGTTGCGCA and reverse-AGGTCTCGTTGTGATGCACTCT.

**Western analysis**
Cell lysates were created and Western blotting conducted as described (7), membranes were probed with several different antibodies including anti-AurkA antibodies (Cell Signalling Technology Inc., #3092), anti-AurkB antibody (Cell Signalling Technology, Inc., #3094), anti-histone 3 (Cell Signalling Technology, Inc., #9715), anti-p-histone 3 (Cell Signalling Technology, Inc., #9701), anti-PARP (Cell Signalling Technology Inc., #9542) and then, stripped and reprobed with anti-β-actin antibodies (Cell Signalling Technology, Inc., #4967) as a loading control. Signals were detected using horseradish peroxidase-conjugated secondary antibodies (BioRad) and the ECL Plus developing system (Amersham Biosciences).

**In vitro cytotoxicity assay**
MPNST cell lines as described in ref. 8 were plated in quadruplicate for each dose of Aurora kinase inhibitor on 96-well plates at 500 cells per well in serum-containing growth medium. Plates were incubated at 37°C and 5% CO₂. Twenty-four hours after plating, cells were treated with carrier alone [10% FBS (Hyclone) in DMEM] or inhibitors (LC laboratories). MLN8237 compound was provided by Millennium Pharmaceuticals, Inc. The amount of proliferation was quantified at 2 and 4 days after addition of drug by a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay using Cell Titer 96 Proliferation Kit (Promega). Absorbance was read at 490 nm in a Spectramax M2 plate reader (Molecular Devices).

**β-Galactosidase staining**
Cells were washed in PBS, fixed for 3 minutes (room temperature) in 2% formaldehyde/0.2% glutaraldehyde (or 3% formaldehyde), washed, and incubated at 37°C (no CO₂) with fresh senescence associated 3-Gal (SA-3-Gal) stain solution: 1 mg of 5-bromo-4-chloro-3-indolyl P3-D-Galactoside (X-Gal) per mL (stock = 20 mg of dimethylfornamide per mL)/40 mmol/L citric acid/sodium phosphate, pH 6.0/5 mmol/L potassium ferrocyanide/5 mmol/L potassium ferricyanide/150 mmol/L NaCl/2 mmol/L MgCl₂. Staining was evident in 12 to 16 hours.

**MPNST xenograft**
The subcutaneous NF1−/−, S462TY human MPNST xenograft model has been previously described (17). S462TY is a derivative of S462 MPNST cells, and once daily Saturday and Sunday beginning when tumors reached 250 mm³. We measured tumors and weighed mice twice weekly. Tumor volume was calculated...
by: \( L \times W_2(\pi/6) \), where \( L \) is the longest diameter and \( W \) is the width. Mice were treated until tumors reached 2,500 mm\(^3\) or a maximum of 66 days. Control tumors (9/11) reached 2,500 mm\(^3\) by day 66 of treatment. The remaining 2 mice had tumors that were >1,800 mm\(^3\) and actively growing. Survival was analyzed by log-rank test using GraphPad Prism.

**Pharmacokinetics**

Blood was collected by cardiac puncture in nontumor bearing mice. Blood was drawn at 2, 4, and 16 hours after dosing from 3 to 5 mice per time point. Plasma was separated by centrifugation at 2,200 \( \times g \) for 10 minutes and MLN8237 concentration quantified by LC/MS/MS (18, 19).

**Immunohistochemistry**

Teased sciatic nerves from adult wild-type or CNP-HRAS12v mice were prepared for immune labeling as described (20). Schwann cells were labeled with anti-S100\(\beta\) antibodies (Dako) followed by FITC-conjugated secondary antibodies (green); expression of the HA-tagged CNP-HRAS12V transgene was detected with anti-HA antibodies (Santa Cruz Biotechnology, Inc.) followed by TRITC-conjugated secondary antibodies (red; Fig. 1A).

Paraffin sections were deparaffinized, hydrated, and transferred to 0.1 M citrate buffer (pH 6.0) for antigen retrieval. Slides were boiled for 10 \( \times ^\circ C \) in citrate buffer, cooled at room temperature for 30 minutes, rinsed in water twice and in PBS 3 times. Sections were quenched with 3% hydrogen peroxide for 10 minutes, rinsed in PBS, and blocked in 10% normal goat serum with 0.3% Triton-X-100. Sections were incubated overnight in primary antibody; rabbit cleaved caspase-3 (1:8,000, Cell Signaling, #9661), rabbit p-histone 3 (1:800, Cell Signaling, #9701), rabbit Ki-67 (1:5,000 NCL-Ki67-P; Novocastra), rabbit cyclinB1 (1:500, Cell Signaling, #4138). Sections were then incubated in biotinylated goat anti-rabbit secondary antibodies (Vector, at a dilution of 1:200). Sections were counterstained with Harris hematoxylin and cover glassed in histo-mount. For nuclear staining, sections were incubated with propidium iodide/RNase staining solution (Cell Signaling, #4087) for 15 minutes at room temperature in the dark, rinsed in PBS 3 times and then cover glassed with Prolong Gold Antifade Reagent (Life Technologies, #P36930). Sections were photographed on the Zeiss LSM 510 scanning confocal microscope equipped with an argon multiline, HeNe 543, and HeNe 633 lasers. For nuclear staining with DAPI (Sigma), sections were incubated in DAPI (1:10,000) for 5 minutes, rinsed in PBS 3 times, and cover glassed in Fluoromount G (EM Sciences, #17984-25) mounting medium.

**Results**

**Bayesian factor model analysis and latent structure linked to Ras signaling**

It seemed plausible that specific Ras targets would be reexpressed in the setting of MPNST to overcome...
suppressive signaling characteristic of senescence in benign tumors (3). Ras signaling can be cell-type dependent (6), and global transcriptional changes directly downstream of Ras activation specifically in Schwann cells or their precursors, the pathogenic cell type(s) in NF1 (21, 22), had not been reported previously. Therefore, to focus our analysis, we first identified genes in peripheral nerves associated with Ras-GTP expression in Schwann cells.

The starting point for our analysis was to generate a mouse expressing a constitutively active, oncogenic allele of Ras (HRAS12V) in Schwann cells, driven by the CNP promoter (23). The CNP promoter drives expression early in Schwann cell progenitors, and expression is maintained in mature Schwann cells (23). We chose to express H-Ras (in contrast to K- or N-Ras) as all 3 Ras proteins are expressed by mouse Schwann cells (24) yet the proliferation of Nf1 mutant Schwann cells in vitro is blocked by farnesyl protein transferase inhibitors, which affect H- but not N- or K-Ras (25). The expression of the HA-tagged HRAS12V transgene was validated in myelinating and nonmyelinating Schwann cells using an anti-HA antibody by immunolabeling teased sciatic nerve preparations (Fig. 1A). These mice were viable and fertile, lived a normal lifespan, and did not form neurofibromas or MPNSTs. It remains possible that expression of Ras from an endogenous locus would cause nerve tumors. No peripheral nerve defects were noted on gross or pathologic examination. Analysis of CNP-HRAS12V expressing peripheral nerve by electron microscopy shows unmyelinated fiber bundles that are slightly disorganized (Fig. 1B) with a minor decrease in the number of axons per Remak bundle (Fig. 1C). The phenotype resembles a milder version of the nerve disruption observed in CNP-EGFR mice (26), and the phenotype was not enhanced in the Nf1+/− background (data not shown).

We identified 308 genes (154 gene orthologs) differentially expressed between mouse nerves from the CNP-HRas12V and normal control mouse nerves (FDR ≤ 0.01; Fig. 1D). We did not observe significant functional enrichment of genes involved in Ras-MAPK (Raf/MEK/ERK) signaling or senescence after querying the 154 orthologs in the DAVID database or the GATACA knowledgebase.

Transcriptional changes in CNP-HRas12V nerves compared with wild-type nerves downstream should result, directly or indirectly, from altered Ras signaling in Schwann cells. These Schwann cell–specific Ras pathway gene orthologs were used as seeds in a Bayesian factor regression modeling analysis of an orthologous transcriptome data set including mouse and human benign neurofibromas and malignant peripheral nerve sheath tumors (MPNST; ref. 27). The model identified 2,000 genes with probability of linkage to Ras signaling (Fig. 2A).

To determine which, if any, of the 2,000 gene orthologs show significant deregulated expression in NF1 samples, we filtered the 2,000 gene orthologs linked to Ras signaling across genes upregulated or downregulated in mouse or human neurofibroma or MPNST relative to respective normal nerve controls. A total of 339 gene orthologs showed increased or decreased expression in mouse model and

Figure 2. AURKA is overexpressed and amplified in MPNST. A, Bayesian latent factor regression modeling of microarray gene expression data identified 2,000 gene orthologs linked to the Ras pathway in mouse and human NF1 tumors. B, AURKA and AURKB were expressed at high levels in MPNST cell lines and primary tumors relative to neurofibromas and normal nerves. Each sample is represented by a vertical line and is directly under the sample type categories represented in A. Sample types are separated by an open space. The vertical dashed line divides mouse samples (left) and human samples (right). Expression level of AURKA (top) or AURKB (bottom) was normalized to mouse or human normal nerve, respectively, and represented as 1.00 on a log scale. The red line represents the average expression level among samples within each sample type (C). Example of SNP-array analysis of an MPNST. This figure shows the log R ratio (LRR) of chromosome 20 for tumor MPNST K (see Supplementary Table S1). A blue line denotes the running average of LRR values. A value of 0 indicates the presence of 2 copies of each locus analyzed. A mean value above 0 indicates a copy number gain of the locus interrogated. A vertical yellow line localizes the AURKA locus.
human neurofibromas or mouse model and human MPNSTs (Supplementary Fig. S1).

We used the GeneGo MetaCore Systems Biology Analysis Platform MetaCore to explore the connections between the 339 genes and identified a major network (Supplementary Fig. S2). This network contained the over-expressed AURKA gene and its overexpressed downstream targets PLK1, cyclin B1, TPX2, HEC, and TACC3. Upstream activators of AURKA, E2F3, and FOXM1, as well as securin (PITG1), which regulates AURKA phosphorylation of Histone H3 within pericentromeric heterochromatin early in the G2 phase (28) and interacts with AURKA to regulate cellular responses to antineoplastic drugs (29), were also overexpressed genes within the network. Another prominent theme was decreased expression of CD36, cytochrome B, RBP7, MGST, PREG3, and SCD. A decrease in gene copy number at the PPARγ locus was not detected by SNP-array analysis (data not shown).

Notably, dramatic overexpression of AURKA (upregulated 7.9-fold) was observed in the filtered mouse and human MPNST sample data as compared with normal nerve (Fig. 2B) as well as within the Ras-driven latent factor search results (Fig. 2A) and K-means clustering of the cross-species NF1 data set (9). Expression of AURKB was moderately increased in human MPNST (1.9-fold; Fig. 2B), and AURKC was not differentially expressed in either mouse or human tumor samples.

To assess the mechanism underlying high expression of AURKA, we determined copy number of the AURKA locus using SNP-array and qPCR analyses on DNA from dermal neurofibromas, MPNSTs, and MPNST cell lines (Fig. 2C; Supplementary Fig S3 and Tables S1 and S2). MPNSTs and MPNST cell lines but not neurofibromas showed copy number gains in the AURKA locus. All (5/5) MPNST cell lines tested, each previously analyzed by gene expression microarrays, and 3 of 14 (21%) primary MPNSTs (one analyzed by gene expression microarray, MPNST #19) exhibited ≥3n copies of the AURKA locus. The SNP-array technique can underestimate gene amplification in tumor cells (30) because of the presence of normal cells. Therefore, we used qPCR-DNA analysis to detect copy number gains with more sensitivity. Using this technique we confirmed copy number gains in 5/5 MPNST cell lines and detected copy number gains in 8 of 13 (62%) primary MPNSTs (data not shown). Increases in copy number were also identified in 1 of 8 neurofibroma samples, consistent with the low number of structural alterations in the AURKA locus in 19 dermal neurofibromas (31). These data suggest that amplification of AURKA could be an early event in progression to malignancy. Indeed, AURKA gene expression was elevated in some human neurofibroma samples (Fig. 2B).

Copy number alteration at the AURKA locus correlated with variations in mRNA expression levels across tumor samples (Fig. 2B and C), indicating that gene amplification may contribute to AURKA overexpression in MPNST. This supports the idea that increased AURKA expression relative to AURKB and the other mitotically-regulated genes listed earlier is not simply because of comparing proliferating cells to nonproliferating normal nerve cells leading to a proliferative signature, but that AURKA is distinctly upregulated.

Reducing Aurora kinase gene expression inhibits MPNST cell survival in vitro

To test whether Aurora kinases play a functional role in MPNST cells, AURKA or AURKB expression was blocked with lentiviral shRNAs. Three days after lentiviral infection of MPNST cells, shAURKA reduced AURKA mRNA expression 3.7-fold relative to a nontargeting shRNA (shNT); shAURKB reduced AURKB mRNA expression 5.8-fold (Fig. 3A). Downregulated expression of each gene was specific with respect to the other. Expression of Aurora kinases A and B were confirmed at the protein level by Western blotting in MPNST cells 3 days after lentiviral shRNA infection (Fig. 3B). Inhibiting either AURKA or AURKB expression with shRNA diminished expression of the specific aurora kinase (A or B) proteins, respectively. Knocking down expression of either AURKA or AURKB reduced MPNST cell accumulation relative to an shNT control as analyzed at 5 days after lentiviral infection in 4 MPNST cell lines using phase contrast microscopy (Fig. 3C) or an MTS assay (Fig. 3D and Supplementary Fig. S4A).

MLN8237 reduces AURKA activity and MPNST cell survival in vitro

These AURK gene expression knock down data suggested a role for Aurora kinases in MPNST cell survival. To verify this finding, human MPNST cells were separately treated with 3 different Aurora kinase inhibitors. VX-680 and ZM447439 have been shown to block Aurora kinases A, or B, and C at nanomolar concentrations (32). At concentrations defined for each inhibitor, both compounds effectively decreased cell survival (Supplementary Fig. S4B). In a 4-day assay, the IC50 values for VX-680 ranged from 40 nmol/L (T265) to 110 nmol/L (26T) (Supplementary Fig. S4B); the IC50 values for ZM447439 ranged from 450 nmol/L (T265) to 110 nmol/L (26T) (Supplementary Fig. S4B). A compound selectively targeting Aurora kinase A, MLN8237 (18), also significantly reduced MPNST cell survival with IC50 values ranging from 100 nmol/L (S462) to 210 nmol/L (ST8814) and averaging 164 nmol/L (Fig. 3E). MPNST cells stained positive for a marker of senescence (β-galactosidase staining) and displayed enlargement in cell size and flattening of the cell body characteristic of senescence at 48 hours posttreatment with 100 nmol/L MLN8237, as compared with vehicle-treated cells (Fig. 3F). Previous studies reported an average IC50 of 32 nmol/L for a panel of Ewing’s sarcoma and neuroblastoma cell lines (19). These values are well below the average trough concentration of 1 μmol/L reported in human phase I studies using the recommended phase 2 dose of 50 mg twice daily (33).

Inhibiting Aurora kinase A results in mitotic spindle defects, mitotic delay, and eventually apoptosis (34). Therefore, pharmacodynamic analysis of Aurora inhibitors often includes measuring markers of proliferation and apoptosis,
including p-histone 3 and PARP, respectively (35). Because phosphorylation of the serine 10 residue of the N-terminal tail of histone H3 is crucial for chromosome condensation and cell-cycle progression (36), protein levels for p-histone-3 were measured at 24 and 48 hours posttreatment with MLN8237, in vitro. Although there was no change in total Histone 3, there was significant reduction in p-histone 3 levels at 300 nmol/L and 1 μmol/L treated MPNSTs at both time points (Fig. 3G). Similar reduction in p-histone 3 was observed for cells treated with lentiviral shRNA against
AURKA (Fig. 3G), indicating a decrease in mitotic cells. In contrast, there was no measurable increase in cell death at either time point, as indicated by an absence of cleaved PARP, which was clearly visible after staurosporin exposure.

Cleaved caspase-3 was also not detected (data not shown). Posttreatment with MLN8237, A urkA protein levels increased as compared with vehicle-treated MPNST cell doses over 300 nmol/L at 24 hours posttreatment and for all the doses at the 48 hours posttreatment time point. Increase in A urkA protein is likely because of the accumulation of cells in the G2–M phase posttreatment with the drug (Fig. 3G).

MLN8237 inhibits MPNST cell growth in vivo

To test the effect of Aurora kinase inhibition on MPNST cells in vivo, S462TY MPNST cells derived from an NF1 patient were implanted into nu/nu mice and treated with MLN8237 (n = 11) or vehicle control (n = 11), at 20 mg/kg/dose, a dose 50% of the MTD in most mouse strains. Inhibiting Aurora kinase with MLN8237 diminished tumor volume (Fig. 4A–C) and increased mouse survival (P = 0.0005; Fig. 4D). Comparing vehicle treated (Fig. 4A) versus MLN8237 (Fig. 4B) shows that MLN8237 had a profound effect on 10/11 tumors. Notably only one of 11 mice treated with MLN8237 required sacrifice because of tumor burden within a prolonged (66 day) treatment period (Fig. 4B). On average, MLN8237 showed effects starting at 15 to 20 days after drug administration (Fig. 4C).

To confirm MLN8237 concentrations that inhibited MPNST xenograft growth were clinically relevant, pharmacokinetic analysis of MLN8237 was conducted (Fig. 4E). Mice were administered MLN8237 at 20 mg/kg twice daily for 5 days and blood plasma was collected at 2, 4, and 16 hours after dosing. $C_{\text{max}}$ was reached by 4 hours and measured 2.9 μmol/L; blood plasma levels reached 2.77 by 2 hours. The blood plasma levels measured at 2 and 4 hours are well above the 1 μmol/L dosage necessary to successfully inhibit Aurora kinase in vivo, as previously reported (18, 19).

MLN8237 induces cytomegaly in MPNST xenografts

To define cellular processes that may contribute to MLN8237-induced tumor stasis, we examined cell-cycle markers using immunohistochemistry on paraffin sections from MPNST xenografts. There was a marked decrease in the number of cells positively labeled for Ki67 in the MLN8237-treated tissue as compared with vehicle-treated tissue samples (Fig. 5A), indicating a reduction in actively proliferating cells. No difference was observed in the number of cells labeled for cleaved caspase-3 in vehicle versus MLN8237-treated xenografts (Fig. 5A), indicating no effect on apoptosis because of MLN8237. In spite of a decrease in Ki67, there was no change in percent of cells immunolabeled for phospho-histone-3 in the vehicle versus MLN8237-treated samples, indicating possible arrest in the G2–M phase of the cell cycle. CyclinB1 was used as a marker of G2–M. There was an increase in cyclinB1 in the nucleus and cytoplasm of cells from MLN8237-treated xenograft samples as compared with vehicle, supporting the idea that the A urkA inhibitor caused tumor cells to stall in G2–M. Consistent with G2–M arrest, xenografts treated with MLN8327 contained numerous enlarged cells with multiple nuclei, as observed with propidium iodide (Fig. 5B). Quantification of this effect...
indicated that 4 or 16 hours after the last dose of MLN8237, the population of xenograft tumor cells with multiple nuclei was significantly increased (Fig. 5B). As Aurora kinase A is a mitotic kinase that regulates mitotic spindle formation and segregation, the enlarged multinucleated cells indicate arrest in G2–M phase.

Discussion

The purpose of this study was to identify molecular alterations downstream of Ras signaling in Schwann cells relevant to NF1 tumorigenesis. We used a Schwann cell–specific Ras signature to drive Bayesian factor analysis, and gene expression data derived from human NF1 tumors and mouse NF1 GEM models to identify Ras target genes that may lead to tumor progression. Gene amplification of the downstream MAPK1/ERK2 target AURKA resulted in AURKA overexpression in MPNST. MPNST cells were dependent on AURKA overexpression for survival, as inhibition of AURKA reduced cell survival in vitro and caused tumor stasis in vivo. The cumulative results of these experiments suggest that AURKA amplification contributes to NF1 mutant Schwann cell progression to malignancy. Our preclinical data support advancement of the AURKA-specific inhibitor, MLN8237, as a molecular-targeted clinical candidate for the treatment of MPNST. Finally, our approach supports investigation of additional overexpressed Ras targets in NF1 tumorigenesis.

We identified 339 potential Ras target genes differentially expressed in mouse and human neurofibromas and/or MPNSTs relative to normal nerve. We hypothesized that expression of some or all of these specific Ras target genes might enhance the effects of the already activated Ras pathway, promoting tumorigenesis. Validating this idea, AURKA, a known transcriptional target of Ras-MAPK signaling (37), was identified by Bayesian analysis as a Schwann cell H-Ras target overexpressed in mouse and human MPNSTs but to a much lesser extent in neurofibromas.

Notably, AURKA can potentiate HRAS-mediated transformation correlating with increased phosphorylation of MEK and ERK (38, 39). Although the exact mechanism by which AURKA and Ras pathways cooperate is unknown, it is intriguing that Aurora kinases can directly interact with RasGAP, which is, like neurofibromin, an off signal for Ras proteins (40, 41). AURK genes encode Aurora kinases that regulate mitosis and promote cell-cycle progression (42). AURKA, AURKB, and AURKC have each been implicated as oncogenes in multiple types of cancer (42), and AURKA was robustly overexpressed in our mouse and human MPNST data sets.

AURKA gene amplification provides a mechanism to increase AURKA expression, and AURKA amplification is often observed in cancer (42). The level of AURKA mRNA overexpression (10-fold) in MPNST is greater than the copy number gains (2- to 5-fold) detected at the AURKA locus, suggesting that additional mechanisms, perhaps including Ras/Raf/MEK/ERK signaling and/or the overexpression of AURKA upstream activators, contribute to elevated AURKA expression in MPNST. In some studies, AURKA gene amplification showed an inverse relationship with response to MLN8237 (43). In contrast, MLN8237 exhibited significant antitumor activity in our NF1–/– MPNST model with AURKA amplification. This model differed from those previously reported, which showed increased phosphor-histone3 in response to MLN8237 and lacked cytomegaly (43), and is similar to that of Huck and colleagues, which shows senescence in response to MLN8237 (44).
Aurora kinase inhibitors, like many antimitic compounds, kill a variety of tumor cells, including some types of sarcomas (45, 46). MLN8237 has successfully treated leukemias that become resistant to the tyrosine kinase inhibitor, nilotinib (47) and enhanced cisplatin-induced cell death in esophageal cancer xenografts (43) and medulloblastoma cell lines (48). However, cells can become resistant to Aurora kinase inhibitors (45). Our preclinical studies using Aurora kinase inhibitors to diminish MPNST cell growth in vitro and in vivo provide evidence supporting investigation into effectiveness of Aurora kinase inhibitors in NF1 treatment. However, Aurora kinase inhibitors had a static effect on MPNST in vivo, and the addition of cytotoxic agents may be required. Importantly, clinical trials are ongoing using Aurora kinase inhibitors in the treatment of solid tumors (42). As Ras-driven tumors are often nonresponsive to conventional chemotherapeutics, the development of combinatorial therapeutic regimens is essential (16). Combining Aurora kinase inhibitors with other therapeutics, including MEK inhibitors or chemo-sensitizing agents, may be useful. The predominant dose-limiting toxicities associated with MLN8237 in 2 separate phase 1 studies in patients with advanced solid cancers were myelosuppression and gastrointestinal-associated toxicities including stomatitis (46). Therefore, careful consideration of dose and schedule will be needed when combining MLN8237 with other therapeutic agents that cause similar adverse events to avoid overlapping toxicities. Given the cell-cycle arrest in G2–M phase, compounds that target the G2 checkpoint may be attractive candidates.

In summary, our results support a molecular model in which amplification of AURKA drives cell-cycle progression in NF1 mutant Schwann cells, promoting malignant trans-

formation. Several lines of evidence make AURKA and attractive candidate for molecular-targeted MPNST therapeutics. AURKA expression is downstream of Ras signaling and AURKA and several of its substrates are overexpressed in MPNST. Furthermore, several Aurora kinase inhibitors have shown efficacy in preclinical studies and clinical trials for a variety of tumors. The recent development and assessment of the AURKA-specific inhibitor, MLN8237, is highly effective against leukemia and solid tumors in a panel of preclinical models of pediatric cancers and has proceeded to clinical trials (19, 49). In our preclinical model, MLN8237 increases survival of mice with MPNST xenografts. The results of this study support further investigation of Aurora kinase inhibitors, alone or in combination, in the treatment of MPNST.

Disclosure of Potential Conflicts of Interest
J A. Ecsedy and M.G. Qian are employees of Millennium Pharmaceuticals, Inc. No potential conflicts of interest were disclosed by the other authors.

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