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NF1 is a tumor suppressor in neuroblastoma that determines retinoic acid response and disease outcome

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Summary

Retinoic acid (RA) induces differentiation of neuroblastoma cells in vitro and is used with variable success to treat aggressive forms of this disease. This variability in clinical response to RA is enigmatic, as no mutations in components of the RA signaling cascade have been found. Using a large-scale RNAi genetic screen, we identify crosstalk between the tumor suppressor *NF1* and retinoic acid induced differentiation in neuroblastoma. Loss of *NF1* activates RAS-MEK signaling, which in turn represses ZNF423, a critical transcriptional co-activator of the retinoic acid receptors. Neuroblastomas with low levels of both *NF1* and *ZNF423* have extremely poor outcome. We find *NF1* mutations in neuroblastoma cell lines and in primary tumors. Inhibition of MEK signaling downstream of NF1 restores responsiveness to RA, suggesting a therapeutic strategy to overcome RA resistance in *NF1* deficient neuroblastomas.

Keywords

Neurofibromatosis; differentiation; retinoic acid; neuroblastoma; RAS

Introduction

Neuroblastoma is a malignancy of early childhood that arises from the developing autonomic nervous system (Maris et al., 2007). It is the most frequently diagnosed cancer in the first year of life and shows a remarkably heterogeneous clinical behavior. Some tumors regress spontaneously, whereas others progress to highly aggressive metastatic disease with a poor overall survival rate. Amplification of the *MYCN* oncogene, and/or loss of the chromosomal regions 1p36 and 11q23 correlate with poor disease outcome (Attiyeh et al., 2005; Caron et al., 1996; Seeger et al., 1985). Recurrent gene mutations are relatively rare in neuroblastoma. Somatic and germ line activating mutations in the *ALK* kinase have been

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identified in 7% of neuroblastomas, providing the basis for a promising molecular targeted therapy in this subgroup (reviewed in Mosse et al., 2009). *PHOX2B* and *PTPN11* mutations were found in 2% and 3% of neuroblastomas, respectively (Bentires-Alj et al., 2004; van Limpt et al., 2004).

Neuroblastomas frequently transdifferentiate into more benign ganglioneuroblastomas and ganglioneuromas. Progression in the opposite direction is also observed in patients. The vitamin A metabolite retinoic acid (RA) is an important morphogen for the developing nervous system in vivo and capable of differentiating neuronal cells in vitro (Duester, 2008). Various neuroblastoma cell lines cease proliferation, differentiate into neuronal-like cells or undergo apoptosis upon exposure to RA (Sidell et al., 1983). These observations established the basis for the clinical application of RA in the treatment of neuroblastoma. RA is one of the few targeted therapeutics currently used in the clinic for aggressive neuroblastoma, but the benefit is limited. A phase III randomized trial showed that treatment with 13-cis-RA given after completion of intensive chemo-radiotherapy yields a slight but significant improvement in event-free survival in high-risk neuroblastoma (Matthay et al., 2009; Matthay et al., 1999).

The physiological functions of retinoids are primarily exerted through the regulation of specific target genes mediated by retinoid acid receptors (RARs). RARs are nuclear hormone receptors that function as ligand dependent transcription factors (reviewed in Rochette-Egly and Germain, 2009). Their activity requires hetero-dimerization with the retinoid X receptors (RXR) that can also associate with several other nuclear hormone receptors. RAR/RXR heterodimers constitutively bind to retinoic acid response elements (RAREs) in the promoter regions of target genes and actively repress transcription in the absence of ligand. This process involves recruitment of the corepressors NCoR and SMRT and histone deacetylases (HDAC). RA binds to RAR and triggers conformational changes that release the corepressors and in turn promote the assembly of coactivator complexes. Subsequently, transcription of target genes is initiated. Many of the coactivators, including CBP/p300, PCAF and SRC1-3 (NCOA1-3), possess histone acetylase (HAT) activity that promotes transactivation of RAR/RXR. In contrast, ligand-dependent corepressors such as LCoR and PRAME recruit HDACs or PcG proteins to ligand-bound RAR/RXR complexes to repress their activities (Epping et al., 2005; Fernandes et al., 2003). Therefore, co-activators/repressors play crucial roles for the context dependent action of RA and may be important determinants and biomarkers for RA based therapies in the clinic.

Recently, we have identified ZNF423 as a critical co-factor required for RAR/RXR function using a large-scale RNA interference (RNAi) based screen in F9 mouse teratocarcinoma cells, a widely used model system to study RA signaling (Huang et al., 2009). We found that ZNF423 is also crucial for RA mediated growth arrest and differentiation in human neuroblastoma cell lines and its reduced expression was a powerful marker of poor prognosis in neuroblastoma patients, independent of *MYCN* amplification. However, we have not observed loss of heterozygosity at the *ZNF423* locus in primary neuroblastomas, nor could we restore *ZNF423* levels in neuroblastoma cell lines with low *ZNF423* by DNA demethylating agents. These results indicate that *ZNF423* expression is determined by transcriptional regulation, rather than by epigenetic silencing or genetic loss. The signaling pathways that regulate *ZNF423* expression could therefore be the critical components governing the RA response.

As pointed out above, the overall response rate to RA in neuroblastoma patients is low, suggesting that only a subgroup of patients benefits from the treatment. Currently, no predictive markers of RA responsiveness are available for clinical use. No alterations in any of the core components of the RA signaling pathway have been described in neuroblastoma

tumors, posing a conundrum how to explain the diversity in RA sensitivity. To gain more mechanistic insights in the factors that control RA responsiveness in neuroblastoma, we performed an unbiased large-scale RNAi screen in neuroblastoma cells. We identify here the tumor suppressor *NF1* as a major determinant of RA sensitivity in neuroblastoma cells and find genetic alterations in *NF1* in primary tumors and neuroblastoma cell lines. We provide proof of concept experiments for a therapeutic strategy to overcome RA resistance in *NF1* deficient neuroblastomas by the co-application of RA with MEK inhibitors.

Results

NF1 suppression confers resistance to RA in neuroblastoma

In response to RA the human neuroblastoma cell line SH-SY5Y ceases proliferation and differentiates into neuronal-like cells. To identify novel determinants of RA resistance in neuroblastoma, we performed a large-scale RNAi-based loss-of-function genetic screen using a collection of 24,000 short hairpin (shRNA) vectors targeting 8,000 human genes (Berns et al., 2004). We used a barcoding technology to identify genes whose suppression causes resistance to RA (Brummelkamp et al., 2006). The entire shRNA library was introduced into SH-SY5Y cells by retroviral infection and cells were plated at low density with or without RA (Figure 1A). After four weeks of RA selection, genomic DNA was isolated from both cultures. The stably integrated shRNA cassettes (19-mer bar code sequences) were recovered by PCR. The relative abundance of individual shRNA vectors was quantified by hybridization of the PCR products to microarrays harboring all 24,000 barcode sequences. The barcode screen was carried out in triplicate and the results are shown in Figure 1B. Each dot in the M/A-plot represents one individual shRNA vector in the library. M- and A-values reflect relative enrichment and hybridization signal intensity. Reproducible outliers are generally located in the right upper corner. Low-intensity spots are prone to technical artifacts and thus unreliable. Therefore we restricted our candidate selection by applying M/A cut-off values as indicated in Figure 1B. To rule out ‘off-target’ effects, we only consider a gene identified from the screen as a genuine hit, if at least two independent shRNAs suppress the expression of the target and also confer RA resistance. Within the list of 44 candidate hits fulfilling the cut-off criteria, the only gene that was represented by two shRNAs was the tumor suppressor gene *NF1* (neurofibromin 1) and was studied further.

To validate the results of the screen, we individually introduced the two *NF1* shRNAs (#1 and #2) from the library and one newly generated (#3) into SH-SY5Y cells. Vectors targeting GFP (sh*GFP*) or harboring only the empty vector (pRS) served as controls throughout the study. All three *NF1* knockdown vectors conferred resistance to RA in long-term colony formation assays and efficiently suppressed *NF1* mRNA and protein expression (Figure 1C). We also noted a growth advantage of *NF1* knockdown cells in the absence of exogenously added RA. This may result from resistance to low levels of RA present in the culture medium or from a RA-independent proliferation advantage. Therefore, we performed a long-term proliferation assay of SH-SY5Y cells expressing shRNAs against *NF1*, *GFP* or control vector (pRS) using the 3T3 protocol (Figure 1D). A slightly increased growth rate of *NF1* knockdown cells was indeed detected in the absence of exogenous RA. However, when exposed to RA, sh*NF1* expressing cells continued to proliferate, while control cells completely ceased proliferation after a latency of about 10 days and the total cell number even declined, indicating that prolonged RA treatment caused cell death of the control cells. Thus, *NF1* knockdown cells are clearly rescued from RA mediated differentiation and growth arrest. Identical results were obtained in other neuroblastoma cell lines having different levels of *MYCN* amplification (SK-N-SH, SJ-NB6, Kelly, HT-230 and LAN-5, Figure S1 and data not shown), indicating *NF1* knockdown can cause resistance to RA independent of *MYCN* amplification status in a range of neuroblastomas.

RAS signaling linked to RA response

It has been found that the neuroblastoma cell line SJ-NB-10 (NB90-9) harbors a homozygous deletion of some exons of the *NF1* gene, suggesting that these cells could be insensitive to RA (The et al., 1993). Figure 2 shows that SJ-NB-10 cells indeed lack *NF1* mRNA and protein (Figure 2A) and fail to respond to RA (Figure 2B). NF1 is a large protein that has GTPase activating activity (GAP) and functions as a negative regulator of RAS proteins by promoting their conversion from the active GTP bound to the inactive GDP bound form (Cichowski and Jacks, 2001; Martin et al., 1990). Loss of *NF1* expression results in hyperactive RAS signaling and therefore we reasoned that hyperactive RAS signaling could cause RA resistance in this *NF1* deficient neuroblastoma cell line.

We stably introduced the GAP-related domain of NF1 (NF1-GRD) into the *NF1* deficient SJ-NB-10 cells. Expression of the NF1-GRD is widely used to restore NF1 dependent GAP activity and thus inhibition of RAS activity in cells lacking NF1 (Martin et al., 1990). Expression of the NF1-GRD was determined by qRT-PCR using NF1-GRD specific primers (Figure 2D). SJ-NB-10 cells expressing NF1-GRD were exquisitely sensitive to RA and cell growth was impaired by almost 7-fold relative to the control cells. In the absence of RA, however, the inhibition was only 2.5-fold, underscoring the RA specific sensitization effect (Figure 2C).

Our results suggest that hyperactive RAS signaling is responsible for the RA resistance associated with a naturally occurring homozygous *NF1* deletion in a human neuroblastoma cell line. To substantiate the role of RAS signaling further, we tested if an activated RAS ($KRAS^{V12}$, Figure 2F) oncogene can cause RA resistance in sensitive SH-SY5Y cells. Similar to *NF1* knockdown, cells expressing the *KRAS* oncogene potently bypassed RA induced growth arrest (Figure 2E).

To investigate the role of endogenous KRAS in the RA resistance caused by *NF1* loss, we performed an epistasis analysis. SH-SY5Y cells were simultaneously infected with shRNAs against *KRAS* and *NF1*. Suppression of *KRAS* (Figure 2H) in combination with *NF1* impaired the occurrence of RA resistant cells driven by *NF1* knockdown, whereas cells co-infected with sh*NF1* and pRS were highly resistant to RA (Figure 2G). This indicates that KRAS is critically required to mediate the effects of *NF1* knockdown on the RA response.

NF1 loss inhibits transcriptional response to RA

RA exerts its biological effects largely through gene regulation. We therefore addressed whether suppression of *NF1* interferes with the transcriptional response to RA. ShRNAs targeting *NF1* or *GFP* were co-transfected with a reporter gene containing a consensus Retinoic Acid Response Element (RARE) linked to luciferase (RARE-Luc). Both shRNAs against *NF1* inhibited the reporter gene activation by RA (Figure 3A). Moreover, *NF1* knockdown also suppressed expression of *bona fide* endogenous RA target genes, *RARβ*, *CRABP2* and *TGM2* (Figure 3B–D). Similarly, exogenous expression of $KRAS^{V12}$ also repressed RA dependent target gene activation. Similar results were obtained in other neuroblastoma cell lines (data not shown). These results further suggest that hyperactive RAS signaling downstream of NF1 blocks the transcriptional response to RA in neuroblastoma cells. Transcriptional induction of the RET tyrosine kinase, a gene associated with neuroblastoma differentiation (Bunone et al., 1995), was consistently blocked in sh*NF1* and $KRAS^{V12}$ expressing cells, indicating that *NF1* suppression not only overrides proliferation arrest, but also differentiation induced by RA (Figure 3E).

NF1 loss downregulates the RAR/RXR co-activator ZNF423

Since co-factors are critical determinants of RAR/RXR transcriptional activity, we asked whether modulation of a co-factor(s) might play a role in the RA resistance caused by *NF1* knockdown. Global gene expression analysis in *NF1* knockdown cells identified 196 and 167 genes that were greater than 2-fold up- or down-regulated (Table S1). Among these genes, we found the RAR/RXR co-activator ZNF423 to be significantly repressed in sh*NF1* expressing cells. We have previously shown that ZNF423 physically interacts with RAR/RXR heterodimers and binds to RA responsive promoters (Huang et al., 2009). Suppression of ZNF423 caused RA resistance in multiple neuroblastoma cell lines and reconstitution of ZNF423 levels in *ZNF423*^{low} neuroblastoma cell lines restored their response to RA. Thus, repression of ZNF423 by loss of *NF1* could play an important role in mediating RA resistance. We confirmed down-regulation of *ZNF423* mRNA and protein levels in sh*NF1* expressing cells by qRT-PCR and Western blotting (Figure 4A, B). A reduction in *ZNF423* expression by knockdown of *NF1* was also seen in additional neuroblastoma cell lines (Figure S2A–D).

To address whether suppression of *ZNF423* is causal for the RA resistance of *NF1* knockdown cells, we re-expressed ZNF423 at physiological levels in sh*NF1* expressing SH-SY5Y cells (Figure 4C, D). Reconstitution of ZNF423 was capable of partially restoring responsiveness to RA in otherwise insensitive *NF1* knockdown cells (Figure 4C). In line with our previous study, *ZNF423* expression caused hypersensitivity to RA along with a moderate inhibition of proliferation (Fig. 4C). *ZNF423* reconstitution in *NF1* knockdown cells also restored expression of *bona fide* RA target genes, such as *TGM2* and *CRABP2* (Figure 4E, F). Our results demonstrate that ZNF423 is a major, but possibly not the only, component downstream of NF1 in the control of RA sensitivity in neuroblastoma cells.

ZNF423 predicts RA responsiveness

As *NF1* loss causes resistance to RA, at least in part, by suppression of *ZNF423*, our data raise the possibility that *ZNF423* levels might predict sensitivity of neuroblastomas to RA. To begin to address this, we explored the predictive value of *ZNF423* expression for RA responsiveness in a panel of 26 different neuroblastoma cell lines. We investigated the sensitivity to RA (100nM) by long-term colony formation assays. The response to RA was determined by the relative growth of treated versus untreated cultures. *ZNF423* mRNA levels were measured by qRT-PCR in the absence of RA (Figure 4G and S2E, F). The response to RA ranged from highly sensitive to fully resistant. High expression of *ZNF423* was significantly associated with sensitivity to RA (Spearman correlation p=0.01). The range of *ZNF423* expression within the cell line panel was comparable to the regulation observed by knockdown of *NF1* in SH-SY5Y and other neuroblastoma cell lines (Figure 4A, S2E). This underscores the functional relevance of *ZNF423* suppression by knockdown of *NF1*.

NF1 expression predicts outcome of neuroblastoma

We have shown previously that *ZNF423* expression is prognostic for event-free survival of neuroblastoma patients (Huang et al., 2009). Given the functional connection between *NF1* and *ZNF423*, we investigated a possible prognostic significance of *NF1* expression in neuroblastoma by microarray analysis in three independent patient cohorts. The first cohort consists of 88 neuroblastoma patients from the Academic Medical Center (AMC) in Amsterdam, Netherlands (Huang et al., 2009). We used this cohort as a “training set” to generate an optimal cut-off value of *NF1* expression by a Kaplan scanning approach (see experimental procedures for details). Patients were then classified into *NF1* low or high based on their *NF1* levels in the primary tumors using this cut-off value and progression-free survival was assessed by Kaplan-Meier analysis. The *NF1*^{high} subgroup had a significantly

better outcome than the *NF1*^{low} subgroup (Figure 5A). Similar results were obtained with median expression (p=0.028) or average expression (p=0.017) as cut-off values for *NF1* expression. Importantly, *NF1* retains prognostic significance in combination with any of the four clinically used prognostic markers: *MYCN* amplification, deletion of chromosomal band 1p36 (LOH 1p36), age (<18 months) and stage in a multivariate analysis (Table S2).

We then validated the prognostic value of *NF1* expression in an independent cohort of 102 neuroblastoma patients with metastatic tumors lacking *MYCN* amplification from the Childrens Hospital Los Angeles (CHLA), for which progression-free survival data are known (Asgharzadeh et al., 2006). Since the same microarray platform was used, the *NF1* expression from both datasets was normalized and the cut-off value used in AMC series was applied to the validation cohort. Again, high level of *NF1* expression correlated with longer progression-free survival and in return low *NF1* expression was associated with poor outcome (Figure 5C). When we combined the expression status of *NF1* and *ZNF423*, patients classified as *NF1*^{low}/*ZNF423*^{low} had the poorest disease outcome, whereas the *NF1*^{high}/*ZNF423*^{high} groups had the best outcome (Figure 5B, D). High expression of either *NF1* or *ZNF423* alone was correlated with an intermediate progression free survival. As the CHLA validation cohort was restricted to a subset of high-risk tumors without *MYCN* amplification, we analyzed a third independent data set that covers all stages including *MYCN* amplified tumors (Oberthuer et al., 2006). These gene expression data were generated on a different microarray platform. Therefore, we randomly divided the entire cohort into a training (n=125) and validation set (n=126) and cut-off values for *NF1* and *ZNF423* were determined as described above in the training set and then applied to the validation set. Again, low expression of *NF1* and *ZNF423* correlated with poor outcome (Figure 5E, and data not shown). When we combined *NF1* and *ZNF423* expression, patients classified as *NF1*^{low}/*ZNF423*^{low} had the poorest disease outcome, whereas the *NF1*^{high}/*ZNF423*^{high} group had the best outcome (Figure 5F). Prognostic significance of *NF1* and the combined *NF1/ZNF423* expression status was independent of *MYCN* amplification in a multivariate analysis (Table S2). Differences in progression-free survival were consistent and statistically significant in all three patient cohorts. Moreover, the *ZNF423* low group was significantly enriched for *NF1* low tumors (Wilcoxon test, p<0.001 in all three cohorts). This is in line with the functional interaction between *NF1* and *ZNF423* that we observed in cell lines. In summary, the combined expression status of the two functionally connected genes, *NF1* and *ZNF423*, is a powerful prognostic marker in neuroblastoma.

NF1 mutations in neuroblastoma

Most of the patients with neurofibromatosis type 1 harbor mutations of the *NF1* gene, ranging from single point mutations to larger deletions (Messiaen et al., 2000; Wimmer et al., 2006; Zatkova et al., 2004). *NF1* mutations and deletions were also found in 23% of glioblastomas, a highly aggressive adulthood brain tumor (TCGA_Network, 2008). A role for *NF1* in the pathogenesis of neuroblastoma has been discussed based on cell line observations, but never studied systematically in a large cohort of primary neuroblastomas (The et al., 1993).

In a panel of 25 neuroblastoma cell lines and we found loss of NF1 protein expression in approximately one third (Figure 6A). Absence of NF1 protein was significantly associated with resistance to RA and also lower expression of *ZNF423* (Figure S3). An overview of genomic defects of *NF1* was obtained by a comprehensive SNP analysis (Illumina 657K platform) in 20 neuroblastoma cell lines (Figure 6B). We detected homozygous micro-deletions in SJ-NB10 (NB90-9) and SK-N-FI cells, deleting part of *NF1* or *NF1* and a neighboring gene (Figure S4A, B). The cell line GI-ME-N showed a 3.6 Mb hemizygous deletion encompassing *NF1*, while the second *NF1* allele had a micro-deletion. AMC106 and SK-N-AS cells showed hemizygous deletions of the *NF1* region of 3.7 and 5.4 Mb. Five

more cell lines (NMB, N206, SK-N-BE, UHGNP and SJ-NB8) showed loss of heterozygosity encompassing the *NF1* region. Only 10/20 cell lines had two apparently normal *NF1* alleles.

These results urged us to screen for mutations of *NF1* in the AMC patient cohort that covers a representative spectrum of neuroblastoma and ganglioneuroma cases including different disease stages, ages and *MYCN* amplifications. Sequencing of the full coding region in cDNA identified two mutations, which were both confirmed by sequencing of the genomic exons (Figure 6C). One neuroblastoma carried a 5 base pair deletion in exon 34 inducing a frame shift. A ganglioneuroma was detected with a missense mutation in exon 15, which is also known from neurofibromatosis type 1 patients (Griffiths et al., 2007). Re-analysis of the clinical records revealed that the patient in our series was indeed diagnosed with neurofibromatosis type 1. Sequencing of the exonic DNA of this tumor revealed that besides the mutated allele, also a normal NF1 allele was present. However, at the cDNA level, only expression of the mutant allele was observed, suggesting epigenetic silencing of the normal *NF1* allele (Figure S4C). Finally, we performed a SNP analysis of 50 neuroblastoma patients from the AMC series. This identified one tumor with a 390 kb heterozygous deletion covering *NF1*, as well as two tumors with translocations involving chromosome 17 with breakpoints in *NF1*, resulting in gain of distal chromosome 17q starting in *NF1* (Figure 6C). We conclude that *NF1* aberrations are detected in primary neuroblastomas, but at lower frequency than in neuroblastoma cell lines.

Restoration of RA responsiveness

RA maintenance therapy has a modest, but significant, benefit for high-risk neuroblastoma patients. Our results suggest that tumors with activated RAS signaling e.g. by *NF1* loss/mutation and low expression of *ZNF423* would respond poorly to RA. We reasoned that inhibitors of downstream components of RAS could potentially restore sensitivity to RA in these tumors. To address this concept, we first dissected the contribution of the major RAS downstream effector pathways by expressing respective active alleles. $BRAF^{V600E}$ and MEK-DD conferred resistance to RA, in contrast to $PIK3CA^{H1047R}$, $RALA^{Q75L}$ and $RALB^{Q72L}$ (Figure 7A). Noteworthy, the activation of AKT by $PIK3CA^{H1047R}$ was comparable to $KRAS^{V12}$ (Figure 7B). Even expression of highly active myristylated AKT failed to potently rescue from RA mediated growth arrest. Further, only $KRAS^{V12}$, $BRAF^{V600E}$ and MEK-DD blocked RA target gene activation (Figure 7C). Therefore, the RAF-MEK cascade is the predominant pathway responsible for resistance to RA by *NF1* loss in neuroblastoma cells.

Next, we added increasing concentrations of the MEK inhibitor U0126 with or without RA to SH-SY5Y cells expressing either sh*GFP* or sh*NF1* (Figure 7D). Addition of U0126 restored sensitivity to RA in *NF1* knockdown cells that were otherwise resistant. Application of U0126 affected cell growth only moderately in the absence of RA. Importantly, MEK inhibition partially restored *ZNF423* expression and subsequently RA target gene expression in *NF1* knockdown cells and also other $NF1^{low}$ neuroblastoma cell lines (Figure 7E–G and S5). Thus, the combination of RA and a MEK inhibitor might be a valid strategy for the maintenance therapy of high-risk neuroblastoma, in particular for tumors with low or mutated *NF1*.

Discussion

We identify here an unexpected role for the tumor suppressor *NF1* in RA signaling through an unbiased large-scale RNAi screen in neuroblastoma cells. The NF1 protein is known to antagonize activation of RAS proteins, but it has also been implicated in other pathways such as cAMP/PKA signaling (Tong et al., 2002). In this study, we provide several lines of

evidence that NF1 controls the response to RA in neuroblastoma through the RAS-MEK signaling cascade. We identify here a mechanistic connection between NF1-RAS-MEK and RA signaling by showing that NF1-RAS-MEK cascade suppresses expression of the RAR/RXR co-activator ZNF423 (Figure 4A, B and 7B). We have shown recently that ZNF423 is critically required for the response to RA in neuroblastoma (Huang et al., 2009). Restoration of *ZNF423* expression in *NF1*^{low} neuroblastomas partially restored their responsiveness to RA, underscoring the relevance of the regulation of *ZNF423* by NF1. Furthermore, we find that inhibition of the MEK kinase, a downstream effector of RAS signaling, with a small molecule inhibitor restored *ZNF423* expression in *NF1* knockdown cells and restored both RA target gene expression and sensitivity to RA. These experiments place *ZNF423* downstream of the NF1-RAS-MEK signaling cascade and provide a rationale for the combination of RA with MEK inhibitors in the clinic to induce differentiation, apoptosis and growth arrest in *NF1*^{low} tumors, a phenotype seen in over half of primary neuroblastomas.

Other studies have also suggested a role for MAPK signaling in regulation of responses to RA in different cellular systems (Antonyak et al., 2003; Bruck et al., 2009; Delaune et al., 2008; Gianni et al., 2006; Kosa et al., 1995; Quinlan et al., 2008; Verheijen et al., 1999). These data indicate that the effects of MAPK signaling on RA responses can be heterogeneous. This may be explained by cell type specific regulatory mechanisms. Indeed, RARs can use a multitude of co-activators, some of which are cell type specific, including ZNF423. Moreover, there is evidence that short-term responses to MAPK signaling are quite distinct from the long-term effects we studied here. For example, p38MAPK activates RAR signaling by phosphorylation of RARα by MSK1, a p38MAPK activated kinase, but also inhibited RAR signaling later by phosphorylation and degradation of the RARα coactivator SRC3 (Gianni et al., 2006). However, a role for the p38MAPK pathway in RA-mediated long-term growth arrest in neuroblastoma has not been established.

Since RA plays a central role in neuronal differentiation and given that *Rarα*-knockout mice exhibit neural crest defects (McCaffery et al., 2003), we reasoned that *NF1* expression could potentially impact the clinical course of neuroblastoma and even act as a tumor suppressor in human neuroblastoma. In three independent gene expression data sets of 316 neuroblastoma patients in total, we found that low expression of *NF1* (*NF1*^{low}), in particular in combination with *ZNF423*^{low}, correlated with a very poor outcome (Figure 5). Tumors having both *NF1*^{low} and *ZNF423*^{low} constitute a clinically significant subgroup. In contrast, high expression of both was associated with the best prognosis. *NF1*^{low} tumors were significantly enriched in the *ZNF423*^{low} subgroup, suggesting that hyperactive RAS-MEK signaling also contributes to suppression of *ZNF423* in primary neuroblastomas. Proteasomal degradation of NF1, as observed in glioblastoma, could also account for active RAS signaling and suppression of *ZNF423* (McGillicuddy et al., 2009). As *RAS* mutations occur rarely if ever in primary neuroblastoma, other negative or positive regulators of RAS signaling could be involved as well. In addition, control of ZNF423 level by independent signaling pathways could antagonize or synergize with RAS signaling.

Germ line mutations in *NF1* are linked to neurofibromatosis type 1 and somatic mutations and deletions are frequent in glioblastoma (Messiaen et al., 2000; TCGA_Network, 2008). The data demonstrating the poor prognosis of patients with NF1^{low} expression sparked our interest in asking if NF1 could also be lost by mutation in neuroblastoma. We found two sequence mutations and three genomic aberrations (microdeletion, 17q duplication) in the *NF1* gene out of 83 analyzed tumors. Based on a few incidental case reports, a predisposition of neurofibromatosis type 1 patients for neuroblastoma has been proposed (Martinsson et al., 1997; Origone et al., 2003). Indeed, the identified H553R missense mutation in NF1 proved to be a neurofibromatosis type 1 associated germ line mutation. The germ line status is unknown for the other patient having a five base pair deletion in *NF1*.

Remarkably, this patient was diagnosed as stage 2 disease, which in general has an excellent prognosis. Nevertheless, this patient was the only one of the 23 stage 1/2 neuroblastoma patients in the AMC cohort that died of disease.

The mutation rate of *NF1* in neuroblastoma was lower than reported for glioblastoma. It is reasonable to expect fewer mutations in an embryonal tumor like neuroblastoma than in a highly genomic unstable and largely p53 deficient adulthood malignancy. In addition, low- and intermediate-risk neuroblastomas (~50% of the AMC cohort) have a very good disease outcome and since low expression of NF1 correlated with poor outcome, we speculate that *NF1* mutations are more prevalent in high-risk disease. However, larger cohorts will be required to investigate this hypothesis.

In conclusion, we found genomic aberrations of the *NF1* gene in 6% (5/83) of primary neuroblastomas, supporting a direct role for *NF1* in the pathogenesis of the disease, at least in a subset of cases. Noteworthy, activating mutations in the protein-tyrosine phosphatase *PTPN11* (*SHP2*), an enhancer of RAS signaling, have been previously described in 3.4% (3/89) of neuroblastomas (Bentires-Alj et al., 2004). Dominant mutations in *PTPN11* cause the developmental disorder Noonan syndrome and possibly predispose to neuroblastoma (Cotton and Williams, 1995; Tartaglia et al., 2001). Together with our functional, mutational and survival studies on *NF1*, it further substantiates a role for aberrant RAS signaling in the development of neuroblastoma, even though *RAS* mutations are rarely, if ever, found in this type of cancer (Moley et al., 1991).

Our data predict that low levels of *ZNF423* and *NF1* will be associated with poor response to RA-based therapies in the clinic. To begin to address this, we asked if we could observe a correlation between *ZNF423* levels and RA responsiveness in a panel of 26 neuroblastoma cell lines. We reasoned that loss of *NF1* causes suppression of *ZNF423* expression (Figure 4A, B) and hence that *ZNF423* levels would also report *NF1* status in neuroblastoma cell lines. Indeed, we found that high expression of *ZNF423* significantly correlated with sensitivity to RA (Figure 4E). The range of *ZNF423* expression in the cell line panel corresponds well to the 3 to 4-fold repression of *ZNF423* caused by loss of *NF1*.

RA is used as a maintenance therapy with some success for high-risk neuroblastoma and levels of *NF1* and/or *ZNF423* may well account for at least a fraction of non-responders in the clinic. Our finding that MEK inhibitors can restore responsiveness to RA in vitro in $NF1^{low}$ cells suggests a potential strategy to improve the therapeutic efficacy of retinoids in the treatment of neuroblastoma. As highly selective MEK inhibitors are currently evaluated in clinical trials, their combination with RA could be an attractive therapeutic approach.

Most, if not all, established predictive biomarkers for targeted therapies affect the target itself or key downstream components of the signaling pathway targeted by the drug. For example, *KRAS* mutations confer resistance to EGFR targeted therapy in colon cancer and *PIK3CA* mutations or lack of *PTEN* expression correlate with poor response to trastuzumab in HER2-positive breast cancer (Berns et al., 2007; Karapetis et al., 2008). Our results reveal a new paradigm for predictive biomarkers by demonstrating that aberrant activity of lateral signaling pathways can determine the response to a targeted therapeutic without mutations in any of the core components of the pathway itself. Our data highlight the importance of mapping crosstalk between signaling pathways to predict responses to targeted therapies in cancer. Unbiased genetic screens are powerful tools to identify these genetic interactions and the present case even suggests a therapeutic strategy to restore drug response in resistant patients.

EXPERIMENTAL PROCEDURES

shRNA Barcode Screen

The NKI shRNA library and the barcode screen are as described (Berns et al., 2004; Brummelkamp et al., 2006). Additional details can be found at www.screeninc.nki.nl.

Cell Proliferation Assays

Single cell suspensions were seeded into 6-well plates ($1–2 \times 10^4$ cells/well) and cultured both in the absence and presence of RA. At the endpoints of colony formation assays, cells were fixed, stained with crystal violet and photographed. All knockdown and overexpression experiments were done by retroviral infection. For the experiments to measure RA responsiveness in the panel of 26 neuroblastoma cell lines, each line was subjected to long-term colony formation assays in triplicates in the absence or presence of 100nM RA. Treated and untreated dishes of each cell line were harvested at the same time. Crystal violet stain was then extracted using 10% acetic acid and quantified at OD 590nm. The growth curves were performed according to the standard 3T3 protocol. All relevant assays were performed independently at least three times.

Patient Samples

For all studies presented, the expression data were obtained using Affymetrix micro-array analyses on the untreated primary tumor samples at the time of diagnosis. The patient samples for both cohorts and the definition of an event (Progression-free survival) for both cohorts are as described (Asgharzadeh et al., 2006; Huang et al., 2009). Written informed consent was obtained from patients' parents or guardians in accordance with institutional review board policies and procedures for research dealing with tumor specimen and clinical information. The institutional review board at Childrens Hospital Los Angeles (CHLA) and the medical-ethics committee of the Academic Medical Center (AMC) in Amsterdam approved the study.

Statistical Analysis

To determine the optimal value to set as a cut-off for *NF1* expression in the AMC cohort, kaplan scanning was used as described for the cut-off for *ZNF423* (Huang et al., 2009). The cut-off value was validated using a second independent set of 102 CHLA patients (Asgharzadeh et al., 2006). See supplemental experimental procedures for more details. Progression-free survival was measured for all outcome analysis presented in this study using the same *NF1* or/and *ZNF423* cut-off values.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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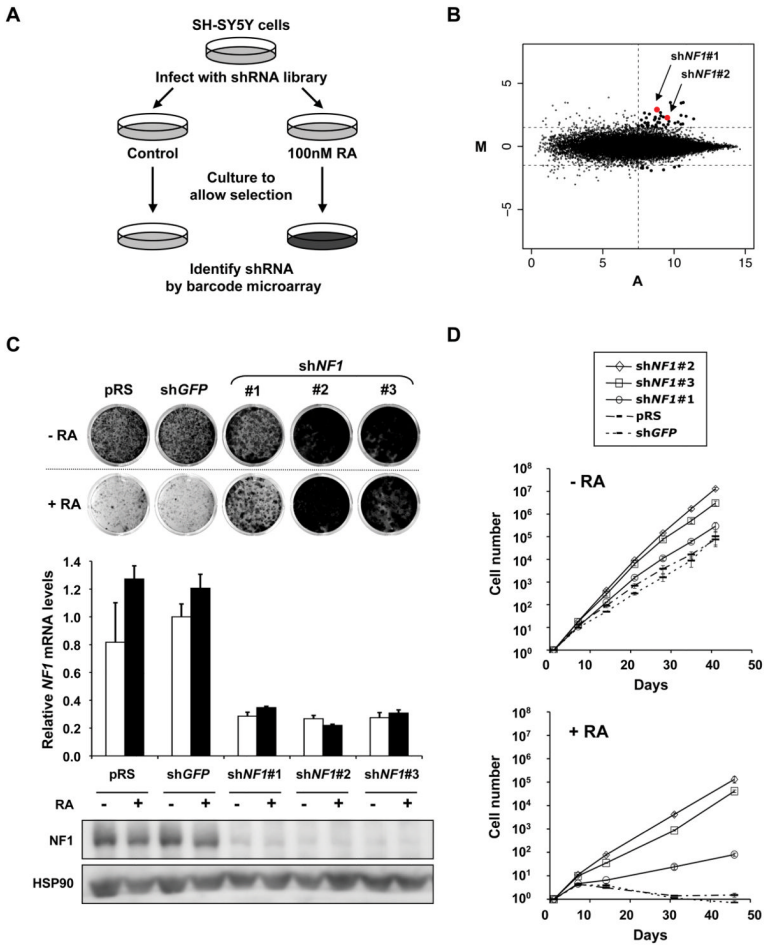

Figure 1. A genome-wide RNAi screen identifies *NF1* as a critical determinant of RA sensitivity in neuroblastoma cells

(A) Schematic outline of the RA resistance barcode screen performed in SH-SY5Y cells. Human shRNA library polyclonal virus was produced to infect SH-SY5Y cells, which were then left untreated (control) or treated with 100 nM all-trans retinoic acid (RA). After 4 weeks of selection, shRNA inserts from both populations were recovered, labeled and hybridized to DNA.

(B) Analysis of the relative abundance of the recovered shRNA cassettes from RA barcode experiment. Averaged data from three independent experiments were normalized and 2log transformed. Among the 44 top shRNA candidates (M>1.5 and A>7.5), two independent sh*NF1* vectors (in red) were identified.

(C) Validation of independent shRNAs targeting *NF1*. The functional phenotypes of non-overlapping sh*NF1* vectors are indicated by the colony formation assay in 100 nM RA. The pRS vector and sh*GFP* were used as controls. The cells were fixed, stained and photographed after 14 days (untreated) or 21 days (RA treatment). The knockdown ability of each of the shRNAs was measured by examining the *NF1* mRNA levels by qRT-PCR and the NF1 protein levels by western blotting. Error bars denote standard deviation (SD). See also supplemental figure S1.

(D) RA resistance by *NF1* RNAi was dependent on RA signaling. Proliferation curves according to the 3T3 protocol of SH-SY5Y cells expressing sh*NF1* vectors, sh*GFP* or pRS, in the absence and presence of RA (100 nM). Error bars denote SD.

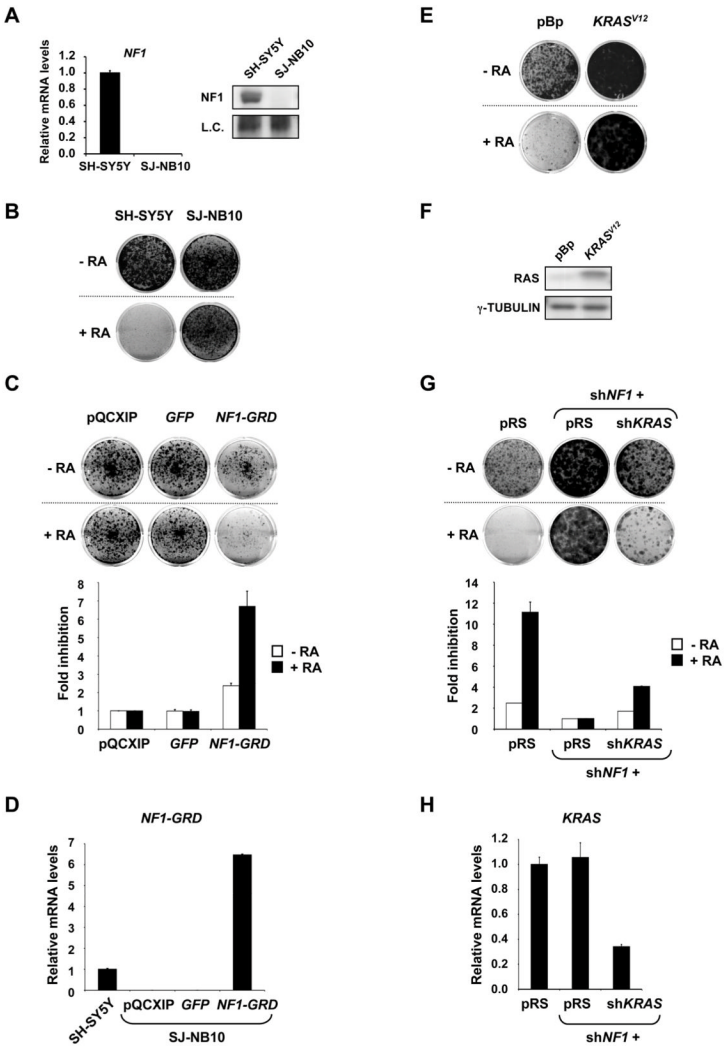

Figure 2. RAS signaling linked to RA response in neuroblastoma cells

(A) NF1 deficiency in SJ-NB10 (NB90-9) cells. NF1 mRNA and protein were undetectable by qRT-PCR and western blotting. SH-SY5Y cells were included as a positive control. Error bars denote SD.

(B) SJ-NB10 cells are insensitive to RA. SJ-NB10 and the control SH-SY5Y Cells were grown in the absence or presence of RA (100 nM). Treated and untreated dishes of each cell line were fixed, stained and photographed at the same time. SH-SY5Y cells were harvested after 14 days and SJ-NB10 cells after 28 days.

(C) Enforced-expression of NF1-GRD in SJ-NB10 cells leads to growth inhibition in the absence of exogenous RA and enhanced responses to RA. Cells expressing pQCXIP-vector, -*GFP* or -*NF1-GRD* were grown in the absence or presence of RA (100 nM) for 28 days, after which cells were fixed, stained and photographed. Relative cell growth was then measured by crystal violet quantification. Fold inhibition values were normalized to cells infected with pQCXIP vector. Error bars denote SD.

(D) Relative *NF1-GRD* mRNA levels in SH-SY5Y cells and SJ-NB10 cells expressing pQCXIP-vector, -*GFP* or -*NF1-GRD*. Error bars denote SD.

(E) Ectopic-expression of $KRAS^{V12}$ in SH-SY5Y cells leads to RA resistance. Cells expressing pBABE-vector or -$KRAS^{V12}$ were grown in the absence or presence of RA (100

nM). The cells were fixed, stained and photographed after 14 days (untreated) or 21 days (RA treatment).

(F) The RAS protein expression levels in SH-SY5Y cells expressing pBABE-vector or -KRAS^{V12}.

(G) Endogenous KRAS is required for the RA resistance driven by *NF1* knockdown. SH-SY5Y cells expressing pRS, sh*NF1* plus pRS or sh*NF1* plus sh*KRAS* were grown in the absence or presence of RA (1 μM). The cells were fixed, stained and photographed after 14 days (untreated) or 21 days (RA treatment). Relative cell growth was then measured by crystal violet quantification. Fold inhibition values were normalized to cells infected with shNF1 plus pRS, in the absence or presence of RA. Error bars denote SD.

(H) The *KRAS* mRNA levels in SH-SY5Y cells expressing pRS, sh*NF1* plus pRS or sh*NF1* plus sh*KRAS*. Error bars denote SD.

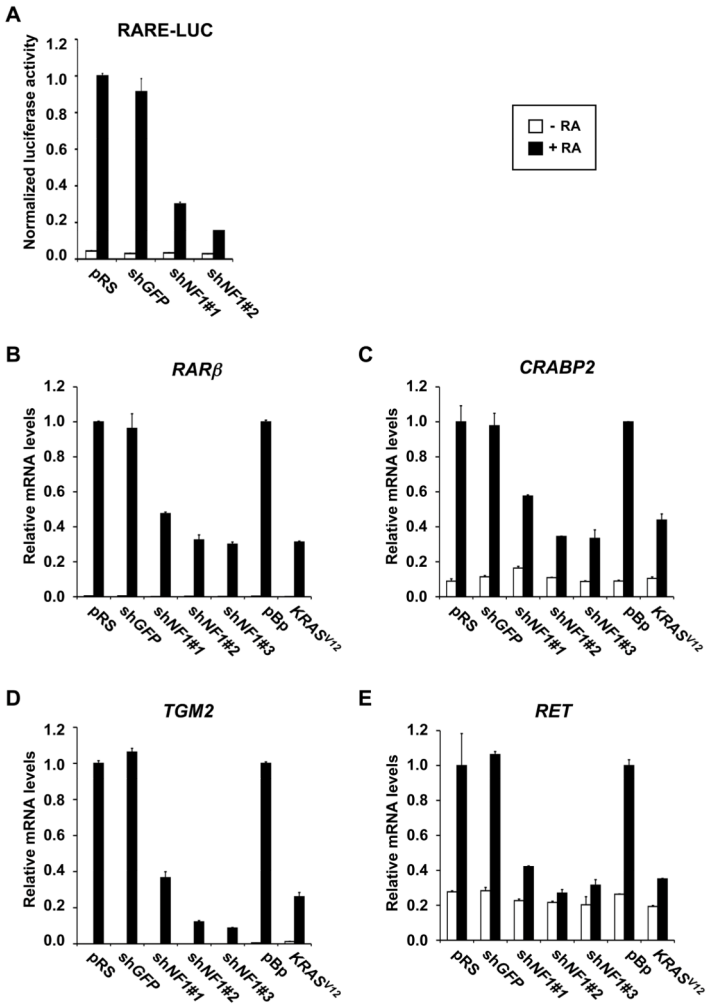

Figure 3. NF1 loss inhibits transcriptional response to RA

(A) *NF1* RNAi inhibits activation of a RARE-Luciferase (RARE-Luc) reporter gene by endogenous RAR/RXR in response to 24 hours of 100 nM RA stimulation in SH-SY5Y cells. Normalized luciferase activities shown are ratios between luciferase values and *Renilla* internal control values. Error bars denote SD.

(B to E) *NF1* knockdown or ectopic expression of $KRAS^{V12}$ suppresses transcriptional activation of endogenous RA target genes in response to RA. mRNA expression analysis of RA target genes *RARβ* (B), *CRABP2* (C), *TGM2* (D) and *RET* (E) in SH-SY5Y cells expressing controls, shRNAs targeting *NF1* or pBABE- $KRAS^{V12}$ after 100 nM RA stimulation for 7 days. Error bars denote SD.

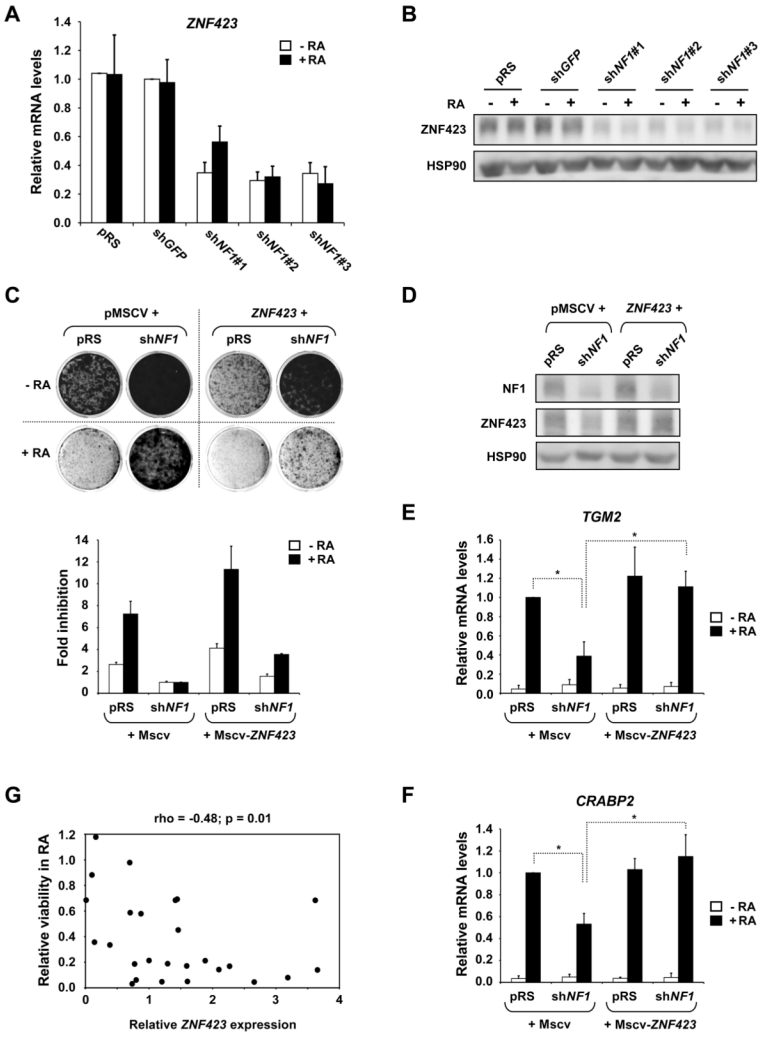

Figure 4. NF1 loss suppresses RA response by downregulating the RAR/RXR coactivator ZNF423

(A and B) *NF1* RNAi leads to down-regulation of ZNF423 mRNA and protein levels. SH-SY5Y cells expressing controls and shRNAs targeting *NF1* were grown in the absence or presence of RA (100 nM) for 7 days. A) *ZNF423* mRNA levels are suppressed in *NF1* knockdown cells. (B) ZNF423 protein levels are also reduced n *NF1* knockdown cells. See also supplemental Figure S2 and Table S1.

(C) Re-expression of ZNF423 reverses the RA resistance driven by *NF1* knockdown. SH-SY5Y cells expressing MSCV control or MSCV-*ZNF423* were retrovirally infected with viruses containing pRS or sh*NF1*, and were grown in the absence or presence of 1 μM RA. Cells were then fixed, stained and photographed after 12 days (untreated) or 14 days (RA treatment). Relative cell growth was then measured by crystal violet quantification. Fold inhibition values were normalized to cells expressing both MSCV control and sh*NF1*, in the absence or presence of RA. Error bars denote SD.

(D) The NF1 and ZNF423 protein levels in SH-SY5Y cells described in Figure 4C. Cells were grown in the presence of 1 μM RA for 14 days.

(E and F) Re-expression of ZNF423 restores activation of the RA target genes *TGM2* and *CRABP2* in *NF1* knockdown cells. Error bars denote SD.*p<0.01 (t-test for comparison of three independent biological experiments)

(G) *ZNF423* expression and RA responsiveness in neuroblastoma cell lines. *ZNF423* mRNA levels from a panel of 26 different neuroblastoma cell lines were determined by qRT-PCR. The relative viability in 100nM RA was determined by the ratio of cell growth of treated versus untreated cultures measured by crystal violet quantification after long-term colony formation assays. A significant association between *ZNF423* expression and RA sensitivity was determined by Spearman rank correlation analysis. See also supplemental figure S2.

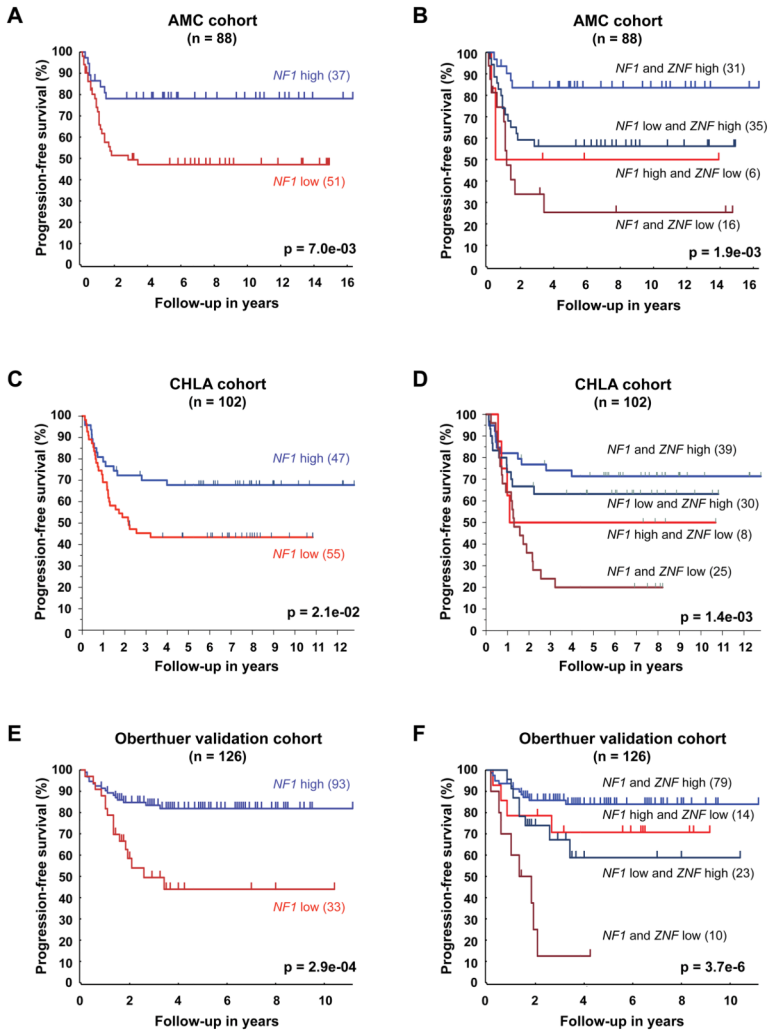

Figure 5. *NF1* expression predicts outcome of neuroblastoma and the combined expression status of *NF1* and *ZNF423* is a powerful prognostic marker

(A) Kaplan-Meier analysis of the AMC cohort (n=88) documenting increased progression-free survival (PFS) of neuroblastoma patients with tumors that have high *NF1* expression (*NF1* high) versus patients with tumors that have low *NF1* expression (*NF1* low), using the *NF1* cut-off value determined as described in the text. See also supplemental Table S2.

(B) Kaplan-Meier analysis of PFS for the AMC cohort classified by the combined expression status of *NF1* and *ZNF423*. The *NF1* cut-off value was the same as above and *ZNF423* cut-off value was as described previously (Huang et al., 2009).

(C) Kaplan-Meier analysis of PFS for a second independent set of 102 patients diagnosed with metastatic neuroblastomas lacking *MYCN* amplification from CHLA (Asgharzadeh et al., 2006). These patients were classified using the same *NF1* cut-off value determined from the AMC cohort.

(D) Kaplan-Meier analysis of PFS for the CHLA cohort classified by the combined expression status of *NF1* and *ZNF423*. The cut-off values for *NF1* and *ZNF423* were the same as above.

(E and F) Kaplan-Meier analysis of PFS for the Oberthuer cohort (validation set, n=126) classified by the expression status of *NF1* (E) and the combined expression status of *NF1*

and *ZNF423* (F). The cut-off values for *NF1* and *ZNF423* were determined in the Oberthuer training set (n=125).

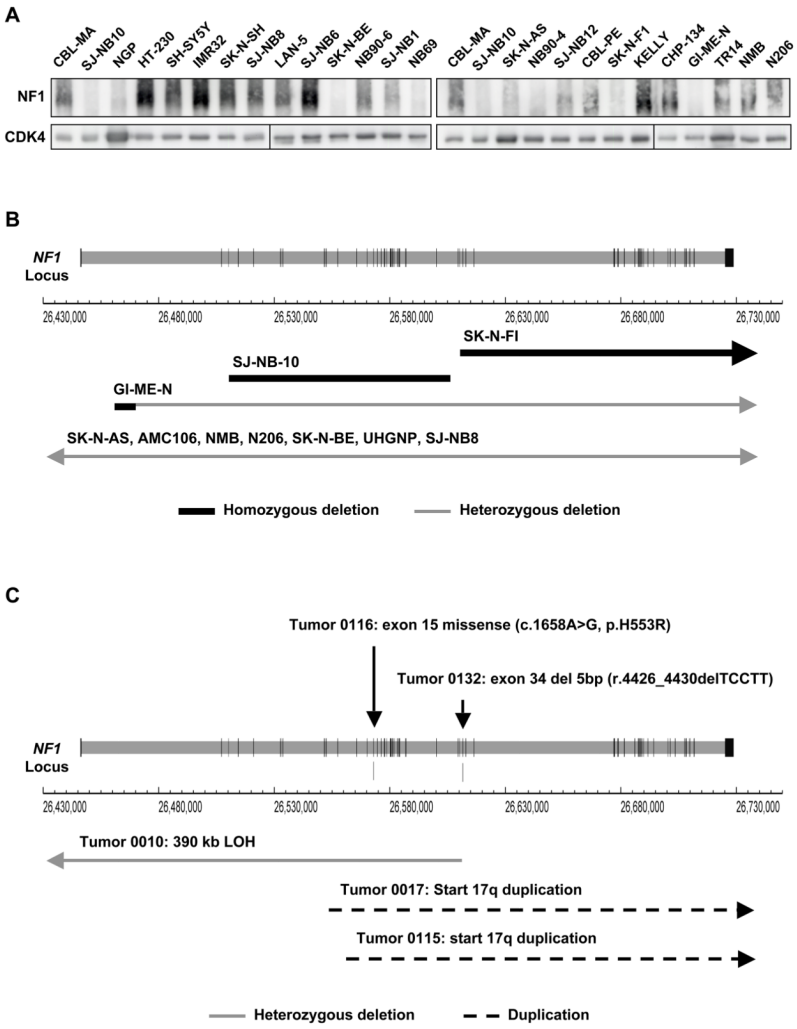

Figure 6. *NF1* mutations in neuroblastoma

(A) NF1 protein levels in a panel of 25 neuroblastoma cell lines as determined by Western blotting. See also supplemental figure S3.

(B) Schematic representation of genomic aberrations in the *NF1* gene in neuroblastoma cell lines. Vertical lines in the *NF1* locus correspond to exons and horizontal arrows depict regions of hemizygous or homozygous deletions. See also supplemental figure S4.

(C) Schematic representation of genomic aberrations in the *NF1* gene in primary neuroblastomas. Vertical arrows indicate the position of the identified *NF1* mutations. Horizontal arrows depict regions of hemizygous or homozygous deletion and dashed lines indicate the regions affected by the duplication of 17q. See also supplemental Figure S4.

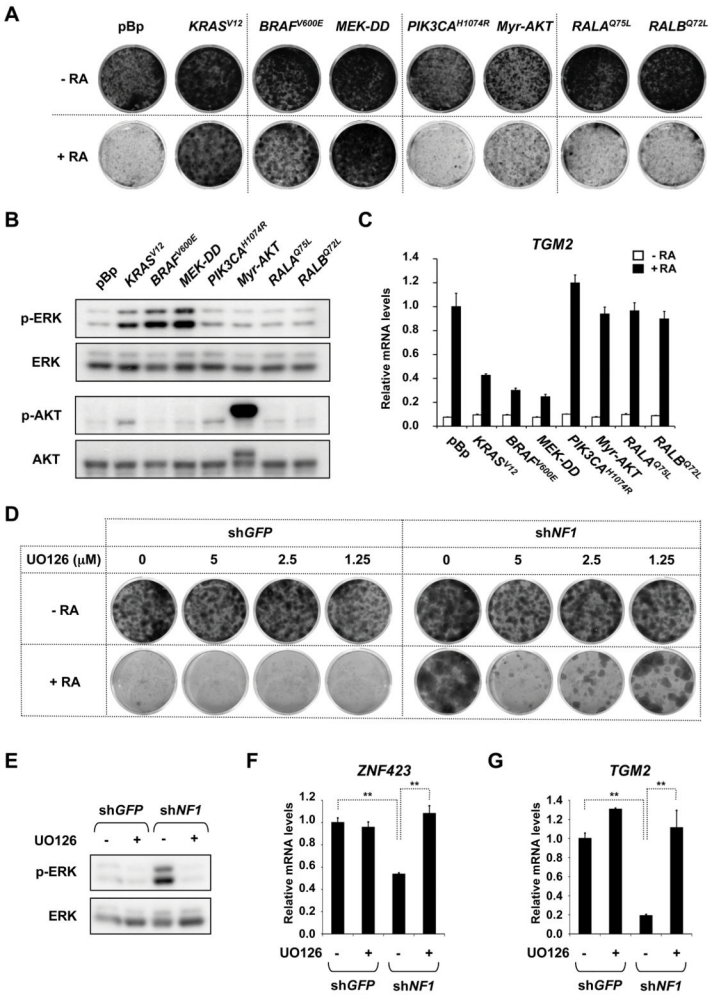

Figure 7. Restoration of RA responsiveness

(A) Constitutively active RAF ($BRAF^{V600E}$) and MEK (MEK-DD, $MEK1^{S218D,S222D}$) recapitulate resistance to RA caused by $KRAS^{V12}$. SH-SY5Y cells expressing pBabe vector control or the indicated active alleles of RAS effector pathways were cultured for 12 and 20 days in the absence or presence of 1μM RA and then fixed, stained and photographed.

(B) Level of phosphorylated ERK and AKT in the SH-SY5Y cells described in figure 7A.

(C) Activation of the RA target gene TGM2 in SH-SY5Y cells described in figure 7A. Cells were grown for 14 days in the absence or presence of 1μM RA.

(D) MEK inhibition restores RA sensitivity in *NF1* knockdown cells. SH-SY5Y cells expressing sh*GFP* control or sh*NF1* were grown in the absence or presence of 1 μM RA for 13 and 18 days. Cells were additionally treated with or without the MEK inhibitor U0126 at various concentrations. Cells were then fixed, stained and photographed.

(E to G) Inhibition of MEK signaling and re-expression of *ZNF423* and *TGM2* in *NF1* knockdown cells treated with MEK inhibitor U0126. SH-SY5Y cells expressing sh*GFP* control or sh*NF1* grown in the presence of 1 μM RA for 9 days and then additionally treated with or without U0126 for 2 days. Levels of phosphorylated ERK were detected by Western blotting (E). *ZNF423 and TGM2* mRNA expression was determined by qRT-PCR. Error bars denote SD. **p<0.001 (t-test for comparison of three biological independent experiments). See also supplemental figure S5.