Common Variation at \textit{BARD1} Results in the Expression of an Oncogenic Isoform That Influences Neuroblastoma Susceptibility and Oncogenicity

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\textbf{Abstract}

The mechanisms underlying genetic susceptibility at loci discovered by genome-wide association study (GWAS) approaches in human cancer remain largely undefined. In this study, we characterized the high-risk neuroblastoma association at the BRCA1-related locus, \textit{BARD1}, showing that disease-associated variations correlate with increased expression of the oncogenically activated isoform, \textit{BARD1b}. In neuroblastoma cells, silencing of \textit{BARD1b} showed genotype-specific cytotoxic effects, including decreased substrate-adherence, anchorage-independence, and foci growth. In established murine fibroblasts, overexpression of \textit{BARD1b} was sufficient for neoplastic transformation. \textit{BARD1b} stabilized the Aurora family of kinases in neuroblastoma cells, suggesting both a mechanism for the observed effect and a potential therapeutic strategy. Together, our findings identify \textit{BARD1b} as an oncogenic driver of high-risk neuroblastoma tumorigenesis, and more generally, they illustrate how robust GWAS signals offer genomic landmarks to identify molecular mechanisms involved in both tumor initiation and malignant progression. The interaction of \textit{BARD1b} with the Aurora family of kinases lends strong support to the ongoing work to develop Aurora kinase inhibitors for clinically aggressive neuroblastoma.

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\textbf{Introduction}

Genome-wide association studies (GWAS) have proven to be a powerful tool to identify susceptibility variants in complex disease, including cancer. However, the molecular mechanisms driving a majority of these signals remain undefined (1). In addition, GWAS efforts in cancer have largely focused on tumor initiation and to date have not generally explored the role of implicated genes on tumor progression or clinical phenotype.

Neuroblastoma is a pediatric malignancy that arises from the developing sympathetic nervous system (2). It is the most frequently diagnosed neoplasm during infancy and accounts for up to 10% of childhood cancer mortality (3). Most neuroblastomas occur sporadically and approximately 50% of patients present with metastatic disease with survival rates below 50% despite dose-intensive chemoradiotherapy (2, 4). The genetic basis of neuroblastoma has recently come into focus, as we have discovered \textit{ALK} as the major familial neu-roblastoma gene and have used our single-nucleotide polymorphism (SNP)-based GWAS to identify multiple genomic loci highly associated with sporadic neuroblastoma (5–10). These GWAS findings collectively provide the first evidence that common polymorphisms work in an additive fashion to influence neuroblastoma initiation. Moreover, a majority of these alleles are associated with specific neuroblastoma clinical subsets, strongly suggesting that the ultimate disease phenotype may be determined, at least in part, by initiating events.

One of the most significant and robustly replicated association signals enriched in the high-risk subset of neuroblastomas resides at the BRCA1-associated RING domain 1 gene (\textit{BARD1}) locus at chromosome 2q35 (6, 10). \textit{BARD1} has classically been thought of as a tumor suppressor because it...
dimerizes with BRCA1 via their respective RING domains (11, 12). However, recent evidence suggests that increased expression of alternatively spliced BARD1 isoforms may be integral in driving cancer progression, independent of BRCA1 (11, 13–16). Here, we sought to explore the potential mechanisms of common genetic variation at the BARD1 locus in causing neuroblastoma and determine the extent to which BARD1 may also be involved in driving and maintaining neuroblastoma oncogenesis in established tumors.

Materials and Methods

Research samples and genotyping
Panel of fetal tissues and sympathetic ganglia RNA was obtained as previously described (7). Neuroblastoma tumor and constitutional samples were acquired from the Children’s Oncology Group. All genomic DNA was genotyped as previously described (7, 8, 10). Lymphoblastoid cell line RNA was isolated from Epstein-Barr Virus-transformed lymphocytes. Additional details can be found in Supplementary Methods.

Cell culture
Neuroblastoma cell lines (described in Supplementary Methods), RPE-1-hTERT cells, and NIH-3T3 cells were regularly passaged in RPMI and routinely mycoplasma-tested and genotyped (AmpFISTR Identifier Kit; Applied Biosystems) to verify identity. HeLa cells were grown in Dulbecco’s Modified Eagle Medium (DMEM). Additional details can be found in Supplementary Methods.

BARD1 CNV breakpoint cloning
Primers were designed with Primer 3. Genomic regions from constitutional DNA were PCR amplified and TA cloned into a pCR2.1-TOPO vector (Invitrogen) and sequenced. Primer sequences that span the BARD1 5’ copy number variant (CNV) can be found in Supplementary Methods.

Real-time quantitative PCR validation of DNA copy number
Primers and probes were designed and synthesized; PCR reactions were set up; and DNA copy number calculated as previously described (7).

Fetal ganglia and neuroblastoma cell PCR and BARD1 isoform sequencing
Total RNA from a panel of fetal sympathetic ganglia and neuroblastoma cell lines and tumors were prepared and PCR amplified as previously described (7, 9) using the following primers BARD1 F1: 5’-ATGGAACCGGATGGTC-3’ and BARD1 R1: 5’-CAGCTGTCAAGAGGAAGCAAC-3’ located in BARD1 exons 1 and 11, respectively. PCR products were TA cloned into a pCR2.1-TOPO vector (Invitrogen) and sequenced.

Real-time quantitative RT-PCR for BARD1β expression
Primers and probes to assess BARD1β expression were designed with Primer Express 3.0 (Applied Biosystems, ABI) targeting the exon 1/4 boundary. Sequences can be found in the Supplementary Methods. Total lymphoblastoid cell line and neuroblastoma cell line RNA was prepared, PCR amplified, and normalized to endogenous controls (TaqMan assays, ABI) as previously described (7). Quantitative PCR for BARD1 exons 2 and 3 and BARD1 exons 10 and 11 was carried out similarly.

Affymetrix Human Exon 1.0 ST expression analysis
Total RNA from 251 primary neuroblastoma tumors was analyzed with the Human Exon 1.0 ST (HuEx) exon expression array (Affymetrix). Data from core probe set regions were normalized and summarized by the robust multichip analysis (RMA) method (Affymetrix APT tools). Heat maps were generated with matrix2png software. Microarray data are available at the NCI TARGET data matrix (17).

Neuroblastoma tumor tissue microarray
The neuroblastoma tumor tissue microarray was constructed as previously described (18, 19). Immunohistochemical staining with antibodies against BARD1 exon 3 (PVC) and exon 4 (WFS) was conducted as previously described (13). Each tumor was evaluated for staining percentage and intensity (0/none to 3/ intense) and a staining score was calculated (intensity x % cells: 0–300).

BARD1β short interfering RNA knockdown in neuroblastoma cell lines
Neuroblastoma cells were plated in triplicate in a Real-time Excelligence system (F Hoffman-La Roche) and growth was monitored continuously as previously described (9). BARD1β targeting short interfering RNA (siRNA) (sequence: 3’-CUUCUGCGUGAUUGUUGAUU-5’) was designed by the siDESIGN Center (Dharmacon) to target the exon 1/4 boundary of BARD1β and synthesized with ON-TARGET-Selectivity Enhanced modifications. Cell transfection, growth inhibition calculation, and mRNA knockdown analysis were conducted as before (9, 10, 20) and described in detail in the Supplementary Methods.

Foci formation assay
NLF or Nb-Ebc1 neuroblastoma cells transfected with either BARD1β or NTC siRNA were plated in 100-mm² dishes and grown for 3 to 5 weeks, fixed, stained with 0.4% crystal violet, and photographed.

Soft agar tumorigenicity assay
NLF or Kelly neuroblastoma cells transfected with BARD1β or NTC siRNA were plated in soft agar in triplicate as previously described (21). Colonies were counted on the resulting JPEG images.

Apoptosis assay
NLF and Nb-Ebc1 neuroblastoma cells were transfected with BARD1β siRNA and caspase activities were measured with the Caspase-Glo 3/7 Assay (Promega) and quantified relative to NTC siRNA-transfected cells and further normalized to the cell number with the CellTiter-Glo Luminescent Cell Viability Assay (Promega) conducted in parallel. BARD1-transfected NIH-3T3 cells were plated in DMEM with 1% FBS and analyzed similarly.
Immunoblotting

Protein lysates from neuroblastoma cell lines and RPE1 cells (50 μg) were immunoblotted as previously described (9, 20). Antibodies targeting BARD1 exon 4 (1:500, BL518; Bethyl Laboratories, A300-263A), BARD1 p25 (1:250) targeting the peptide sequence, MVAVPGTVAPRC, encoded in alternative open reading frame (ORF) of exon 1 of BARD1β (14), actin (1:2,000, I-19; sc-16146), p53 (Cell Signaling, 2524), phospho-p53 (serine 15; Cell Signaling, 9284), Aurora kinase A (Cell Signaling, 4718), and Aurora kinase B (BD Biosciences, 611082) antibodies were used.

BARD1 immunoprecipitation

Protein lysates (400 μg) from NLF cells were immunoprecipitated with BARD1 exon 4 (BL518, Bethyl Laboratories, Inc.; A300-263A) and BARD1 p25 (targeting BARD1β) antibodies as previously described (14). Immunoprecipitation conditions are further described in the Supplementary Methods.

BARD1 forced overexpression in NIH-3T3 cells

NIH-3T3 cells were transfected with BARD1β pcDNA3 or FL-BARD1 pcDNA3 (a kind gift from Makiko Tsuzuki; construct synthesis further described in the Supplementary Methods) with the TransIt-3T3 Transfection Kit (Mirus Bio LLC), bulk selected, plated in triplicate in 1% FBS, and growth was monitored as above (9).

Results

Genomic characterization of the BARD1 locus

The neuroblastoma-associated SNPs at chromosome 2q35 cover a 113-kb genomic region (Human Genome Build 36.3; Fig. 1A). One SNP upstream of BARD1, rs10498025, is in modest linkage disequilibrium (LD) to the intronic SNPs (r² = 0.49–0.55). However, the other 2 SNPs (rs2592232 and rs10498026) are in weaker LD with these SNPs (r² = 0.28–0.39 and 0.14–0.41, respectively; Fig. 1A) but have a similar degree of association with neuroblastoma, suggesting that more than one disease-contributing variant may exist at 2q35 (10).

We first sought evidence for CNVs at this locus that may be in LD to these SNPs and contribute to neuroblastoma susceptibility. First, we analyzed data generated from a high-resolution oligonucleotide array (22), which suggested the presence of a CNV upstream of BARD1. We sequenced this CNV breakpoint from the constitutional DNA of a neuroblastoma subject, identifying a 2-kb deletion (Fig. 1A and B), which is in strong LD with the neuroblastoma-associated SNP rs10498026 (r² = 0.64; 8 other associated SNPs r² = 0.12–0.23 [Supplementary Table S1]). We next directly examined whether this CNV was associated with neuroblastoma by quantitative PCR in an independent case series (6) and in blue are additional SNPs identified in an expanded case-control series (10). The LD plot was constructed with Haploview software with HapMap CEU data (Release 22). B, chromatogram showing 2 kb deletion 5' upstream of BARD1 (human Genome Build 36.3). C, histogram showing association of CNV with high-risk neuroblastoma (% = nondeleted allele, the risk allele), kb, kilobases.

for the effect of the most significant SNP, rs7587476. These data suggest that this CNV represents an independent association signal with modest effect size and that more than one neuroblastoma causal variant exists at the BARD1 locus.

BARD1 SNP and CNV variations are associated with differential BARD1 isofrom expression

Given the genomic location of the neuroblastoma-associated common variations (Fig. 1A) along with the growing evidence that SNPs may influence gene splicing (23), we next sought to fully characterize the BARD1 isoforms present in both normal and neuroblastoma cells. With reverse transcriptase (RT)-PCR primers spanning the BARD1 coding region, we found multiple alternatively spliced isoforms in all cells (Fig. 2A). To identify the exon structure of these isoforms, we first cloned and sequenced cDNA from fetal sympathetic ganglia tissue (20–24 weeks of gestational age, N = 3), identifying full-length (FL)-BARD1 and 15 unique BARD1 isoforms (Fig. 2B). Nine of these isoforms are novel, 9 maintain an ORF, and 4 (FL, B, p, and η) may use an alternative translational start
BARD1β in Neuroblastoma

Figure 2. Multiple BARD1 isoforms are expressed in fetal sympathetic ganglia and neuroblastoma cells. A, representative PCR with primers in BARD1 exons 1 and 11, showing multiple BARD1 splice variants. B, exon structure of BARD1 variants identified in fetal sympathetic ganglia. BARD1 isoforms also expressed in neuroblastoma cells indicated with an asterisk. C, exon structure of additional BARD1 variants identified in neuroblastoma cells. BARD1 functional domains are shown at top. Exon 1 alternative start site indicated with arrow in B. LCL, lymphoblastoid cell line.

site located upstream to the native AUG in exon 1 (Fig. 2B; refs. 11, 13, 16). We also cloned BARD1 variants from 4 neuroblastoma cell lines (SKNAS, NB-Ebc1, SH-SY5Y, and NGP) and found that FL-BARD1 and 6 of these BARD1 isoforms (β, γ, ρ, φ, δ, η) were also expressed in neuroblastoma cells (Fig. 2A and B), suggesting a possible mechanistic role in not only tumor initiation but also malignant progression. Ten additional BARD1 isoforms were cloned only from neuroblastoma cells, 6 of which are novel (Fig. 2C).

Considering that BARD1 was identified as a neuroblastoma susceptibility gene via a GWAS approach, we next investigated how neuroblastoma-associated common variants correlated with BARD1 isoform expression. First, we examined the relationship between disease-associated SNP variation and BARD1 exon expression by using the SNP Express database that profiles BARD1 exon expression in peripheral blood mononuclear cells (24). A majority of the neuroblastoma-associated SNP alleles correlated with differential expression of at least one BARD1 exon (P<0.05). Most notably, 6 of the 9 neuroblastoma-associated SNPs were significantly correlated with decreased expression of BARD1 exons 2 and 3 (P = 8.9 × 10⁻⁴ – 0.03), an expression pattern that is consistent with the putative oncogenic BARD1β isoform that splices out these 2 exons (14, 16). The 2q35 SNPs that lack association with neuroblastoma (rs3754546, rs7591615, and rs4234006) did not show a statistically significant similar relationship for exon 2 (P = 0.07, 0.10, and 0.54, respectively) or exon 3 (P = 0.42, 0.28, and 0.66, respectively).

We next sought to directly determine the relationship of BARD1β expression to the neuroblastoma-associated common SNP and CNV alleles. First, we analyzed the expression of the BARD1β isoform in lymphoblastoid cell lines (N = 33) and found that lymphoblastoid cell lines with 2 copies of the nondeleted allele of the BARD1 5′ CNV (risk allele) had a significantly higher expression of BARD1β versus samples with
0 or 1 copy of this allele (Fig. 3A). This relationship was not seen with TaqMan probes targeting the BARD1 exon 10/11 or exon 2/3 boundaries, suggesting that this common variation is directly associated with upregulation of BARD1β. In addition, a trend toward increased expression of BARD1β in lymphoblastoid cell lines harboring a homozygous risk allele genotype (GG) at SNP rs6435862 was found but did not reach statistical significance. The BARD1β isoform was also found to be ubiquitously expressed in a panel of fetal tissues, including 8 fetal sympathetic ganglia from a wide range of gestational ages (19–36 weeks; Supplementary Fig. S1).

This correlation between neuroblastoma common risk alleles at the BARD1 locus with BARD1β expression was also found in primary neuroblastoma cells as we detected significantly increased BARD1β expression in neuroblastoma cells harboring a homozygous risk allele genotype (GG) at SNP rs6435862 (Fig. 3B). However, the complexity of the neuroblastoma genome with frequent chromosome 2q gain and hyperdiploidy prevented the determination of an accurate 5' BARD1 CNV copy number state precluding a similar CNV and BARD1β expression analysis in this sample set.

Finally, we analyzed BARD1 isoform expression in a set of neuroblastoma primary tumors with both an Affymetrix HuEx array (N = 251) and a nonoverlapping neuroblastoma tumor tissue microarray (N = 119; Fig. 3C–F, Supplementary Table S4). We first explored differential BARD1 exon

![Figure 3](image-url)
expression at the RNA level, finding higher exon 4 expression than exon 3 expression, a pattern consistent with BARD1β expression (Fig. 3C). Probe sets within exon 2 of the BARD1 gene showed global poor hybridization kinetics, so these data were not considered. We also used antibodies raised against isoform-specific epitopes to examine BARD1β protein expression in neuroblastoma primary tumors (14). As shown in Fig. 3D and E and Supplementary Table S4, the majority of diagnostic human neuroblastomas also showed immunohistochemical staining patterns consistent with BARD1β isoform expression. These array data correlated well with direct TaqMan measurement of BARD1β expression (Fig. 3F). Taken together, these results suggest that developing nervous tissues and a majority of neuroblastoma cells express the BARD1β isoform and that its expression correlates with neuroblastoma-associated common SNP and CNV variation at chromosome 2q35.

**BARD1β has the characteristics of a neuroblastoma oncogene**

To determine whether high levels of BARD1β are functionally relevant in models of high-risk neuroblastoma, we examined the consequences of disrupting BARD1β expression with a specific siRNA targeting the alternatively spliced exon 1/4 boundary of the BARD1β mRNA (Fig. 4A and B). We transfected a panel of neuroblastoma cell lines (N = 12) and BARD1β knockdown showed differential effects on cellular proliferation that correlated with both the allelic state of SNP rs6435862 and the level of BARD1β expression (Fig. 4 and Supplementary Table S5). Neuroblastoma cells harboring at least one risk allele at SNP rs6435862 (GT or GG) and the highest BARD1β expression (N = 4; mean BARD1β expression = 2.02) showed the most significant inhibition of cell proliferation upon BARD1β knockdown [% growth inhibition (range) (% GI |avg|) = 41%–86%, P |range| = 0.0002–0.002; Fig. 4C, D, and G and Supplementary Table S5). BARD1β knockdown had no effect on cellular proliferation in a majority of neuroblastoma cell lines with a homozygous major allele genotype (TT) at SNP rs6435862 and lower BARD1β expression [N = 6, mean BARD1β expression = 1.05; % GI |range| = −29%–6%, P > 0.5; Fig. 4E–G and Supplementary Table S5].

In addition, we found that cell growth was also inhibited upon BARD1β knockdown in HeLa cells (Supplementary Fig. S2A; % GI |avg| = 33%, P = 0.002), consistent with previous reports (14). However, BARD1β silencing did not affect cell growth in the nontransformed neuroblastoma control cell line, retinal pigment epithelial cells (RPE1-hTERT), despite a similar degree of BARD1β knockdown, suggesting that BARD1β only drives growth in malignant cells (Supplementary Fig. S2B and S2C; % GI |avg| = −18%).

To further investigate the oncogenicity of BARD1β in neuroblastoma, we studied how BARD1β silencing influenced growth in soft agar and clonogenic assays. Similarly, BARD1β silencing decreased growth in these assays in BARD1β-dependent cells (NLF, Nb-Ebc1; GG risk genotype at SNP rs6435862) but not BARD1β-independent cells (Kelly; TT protective genotype at SNP rs6435862; Fig. 4H–J). BARD1β knockdown in this cell line panel ranged from 23% (KCN) to nearly 100% (RPE1). Importantly, those cell lines showing growth inhibition had similar mRNA and protein knockdown to those with no phenotype (mean BARD1β mRNA knockdown = 62% and 56%, respectively, P = 0.62; and insets in Fig. 4C and E) and the level of BARD1β knockdown showed no correlation with the amount of growth inhibition (r² = 0.017).

**BARD1β blocks apoptosis in neuroblastoma cells**

Given the role of BARD1 in mediating apoptosis, we next sought to determine whether high levels of BARD1β protect neuroblastoma cells from induction of programmed cell death. First, we found significant upregulation of the activity of the apoptotic pathway effectors caspase-3 and -7 after BARD1β silencing in NLF and Nb-Ebc1 cells (Fig. 5A and B). However, this apoptosis does not appear to be due to removal of a dominant-negative effect of BARD1β on the role of FL-BARD1 in phosphorylating and stabilizing p53 in response to DNA damage (11, 25–27), as BARD1β silencing did not change cellular levels of Ser15-phosphorylated p53 (Fig. 5C). Similarly, the growth-promoting effects of BARD1β seem to be largely independent of any antagonistic functions on the BARD1/BRCA1–driven homology-directed repair (HDR) of double-stranded DNA breaks (28). Suppressing the level of PARP1 mRNA to less than 20% of baseline in these BARD1β-dependent NLF cells did not result in synthetic lethality as would be expected if a deficiency in the HDR pathway was present (28). Only a minor fraction of the growth inhibition observed in BARD1β-silenced NLF cells was seen with PARP1 silencing (25% vs. 87% for BARD1β silencing; Figs. 5D and 4C and G). Taken together, these results suggest that the increase in neuroblastoma risk observed with increased BARD1β expression and evasion of apoptosis in neuroblastoma cells does not involve BARD1β-driven defects in the HDR maintenance of genomic stability or interruption of the FL-BARD1/p53 phosphorylation interaction. However, given that total p53 levels decrease upon BARD1β silencing (Fig. 5C), this oncogenic protein may be exerting its effects by stabilization of p53 in a different manner.

**BARD1β binds to and stabilizes Aurora kinase B in neuroblastoma cells**

The BARD1β isoform has previously been shown to bind and stabilize Aurora kinase B in HeLa cells (14). These findings coupled with the identification of Aurora kinase A as an MYCN-stabilized, high-risk phenotype-associated oncogene in neuroblastoma (2, 29) led us to investigate whether there was interaction between BARD1β and the Aurora kinases in neuroblastoma. First, we conducted co-immunoprecipitation with antibodies toward epitopes in exon 4 (BLS18), common to both FL-BARD1 and BARD1β, or to epitopes in the alternatively translated exon 1 of BARD1β (p25; ref. 14). In BARD1β-dependent NLF cells, Aurora B coprecipitated with both BARD1 antibodies (Fig. 5E, top) and Aurora A coprecipitated with the exon 4 antibody (Fig. 5E, bottom). Moreover, BARD1β silencing in NLF cells showed that levels of both Aurora A and B kinase decreased in parallel with levels of BARD1β, suggesting that this BARD1β/Aurora kinase interaction may stabilize the
latter proteins (Fig. 5C). Finally, parallel knockdown of these Aurora kinases and BARD1β in NLF cells show similar growth inhibition (Fig. 5F). Taken together, these results suggest that the oncogenic function of BARD1β in neuroblastoma, at least in part, functions through the Aurora family of kinases.

BARD1β transforms NIH-3T3 cells

We next investigated how overexpression of BARD1β and FL-BARD1 influenced the growth of nontransformed NIH-3T3 cells. NIH-3T3 cells were transfected with a BARD1β or FL-BARD1 cDNA resulting in stable clones that had a greater than 100-fold increase in BARD1 expression (Fig. 6A and B). Forced
These results con"silenced under full serum conditions (10% FBS; data not

growth inhibition was observed when the Aurora kinases were
Aurora A or B kinase were depleted (Fig. 6F). Importantly, no
down by RNA interference and partially silenced when either

oncogene and lend support to our hypothesis that BARD1
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neuroblastoma cells.

Discussion

GWAS discoveries have the potential to help decipher the
mechanistic basis of complex human disease and impact risk

overexpression of BARD1β permitted cell proliferation under
low serum (1% FBS) conditions protecting cells from apoptosis,
whereas empty pcDNA3-transfected cells underwent apopto-
sis, and FL-BARD1–transfected cells were protected from
apoptosis but unable to proliferate (Fig. 6C–E). This trans-
formed phenotype was silenced when BARD1β was knocked
down by RNA interference and partially silenced when either
Aurora A or B kinase were depleted (Fig. 6F). Importantly, no
growth inhibition was observed when the Aurora kinases were
silenced under full serum conditions (10% FBS; data not
shown). These results confirm BARD1β as a neuroblastoma
oncogene and lend support to our hypothesis that BARD1β
acts via a mechanism involving the Aurora kinase family in
neuroblastoma cells.

Figure 5. BARD1β blocks apoptosis in risk allele harboring
neuroblastoma cells and complexes with Aurora kinase B. A and B,
caspase-3 and -7 activity was significantly upregulated after
BARD1β silencing in the 2 highest

GWAS discoveries have the potential to help decipher the
mechanistic basis of complex human disease and impact risk

publication. In cancer, there is additionally the question of
whether or not susceptibility genes are also involved in tumor
progression, and moreover, whether the GWAS approach has
the potential to identify clinically relevant therapeutic targets.
We have begun to address these goals in neuroblastoma by
studying the GWAS identified, highly robust association signal at the
BARD1 locus in both tumor and host genomes.

Our data suggest that the primary mechanism for increased
neuroblastoma susceptibility at the BARD1 locus is via disease-
associated common variation’s impact on differential BARD1
exon expression, leading to the preferential formation of an
oncogenic isoform, BARD1β. We show a clear correlation
between genomic haplotypes associated with neuroblastoma
and expression of BARD1β. These data are consistent with the
growing evidence that the complex regulation of gene expres-
sion involves heritable common alleles and that variation in
isoform expression influences cancer susceptibility (23, 30).

One of the major goals of this work was to determine
whether BARD1 alterations are further selected for during

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tumor progression and therefore we also studied BARD1β in neuroblastoma cells. The correlation of risk common variation with BARD1β expression also largely holds in primary neuroblastoma cells (Fig. 3B), which is quite striking considering the sheer complexity of the neuroblastoma genome (2). More importantly, however, we show that genetic manipulation of BARD1β has profound impact on neuroblastoma cell phenotype (Figs. 4–6). These data suggest that BARD1β may be an important oncogene and therapeutic target in high-risk neuroblastoma, one that would not likely have been discovered via standard genomic profiling efforts, and lend strong support to the ongoing work to develop Aurora kinase inhibitors for clinically aggressive neuroblastoma (29, 31, 32).

It is likely that additional BARD1 isoforms may also be involved in neuroblastoma tumorigenesis. For example, the β-BARD1 isoform (Fig. 2B) is expressed in highly proliferative cytotrophoblasts and stabilizes the estrogen receptor α (ESR1), a transcription factor involved in neuronal differentiation (13, 15, 33). Further exploration of the biologic implications of a subset of these isoforms in neuroblastoma is clearly needed (13, 16, 34).

Our multivariate regression analysis suggests multiple neuroblastoma causal variants at 2q35, including the BARD1 CNV, which is not surprising given the complexity of the LD block (Fig. 1A and Supplementary Table S1). However, how this CNV influences BARD1β expression has not yet been identified. We hypothesize that regulatory elements that act as either distal splicing enhancers of BARD1 or factors that increase mRNA transcription from the alternative start site in BARD1 exon 1 used to initiate BARD1β expression (Fig. 2) may be present in this CNV region. Regional resequencing and further integrated epigenetic, computational, and functional analysis will be needed to definitively identify the causal variants at 2q35 and the mechanisms by which they
enhance BARD1β expression. One strong candidate regulatory protein for control of BARD1 splicing that has binding motifs susceptible to SNP changes is the neuronal-specific, cancer-associated, splicing modifier FOX2 (30, 35, 36). Neuroblastoma-associated SNPs at 2q35 are in strong LD with multiple predicted FOX2-binding sites (36) and there are 2 FOX2-binding motifs located in and 235 bp 3' downstream to BARD1 exon 3.

Future studies will also focus on defining the mechanism by which BARD1β stabilizes the Aurora kinases. Our data suggest that BARD1β may stabilize Aurora B kinase by scaffolding this protein as previously observed in HeLa cells (14), whereas the stabilization of Aurora kinase A likely acts via a different mechanism such as via phosphorylation or stabilization of a known or unknown Aurora A scaffolding protein (37, 38). Further in vivo and cell-cycle–specific in vitro functional analysis is needed to definitively define the tumorigenic potential of BARD1β and uncover the details of how this protein interacts with the Aurora kinases in neuroblastoma cells, work that may ultimately further support BARD1 expression as a biomarker for therapeutic vulnerability to Aurora kinase inhibition.

In summary, here we have identified BARD1β as an oncogenic protein in neuroblastoma, and translational potential exists due to interaction with and stabilization of the Aurora kinases. This work shows that post-GWAS functional genomics efforts have the potential to identify both cancer susceptibility mechanisms and therapeutically relevant oncogenic vulnerabilities that may be exploitable clinically.

Disclosure of Potential Conflicts of Interest
I. Irminger-Finger has ownership interest (including patents) in BARD1AG. No potential conflicts of interest were disclosed by the other authors.

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Common Variation at \textit{BARD1} Results in the Expression of an Oncogenic Isoform That Influences Neuroblastoma Susceptibility and Oncogenicity
