Published in final edited form as:

Sci Transl Med. 2012 January 4; 4(115): 115ra3. doi:10.1126/scitranslmed.3002977.

Paracrine Signaling Through MYCN Enhances Tumor-Vascular Interactions in Neuroblastoma

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Abstract

Neuroblastoma, a tumor of peripheral neural crest origin, numbers among the most common childhood cancers. Both amplification of the proto-oncogene *MYCN* and increased neoangiogenesis mark high-risk disease. Because angiogenesis is regulated by phosphatidylinositol 3-kinase (PI3K), we tested a clinical PI3K inhibitor, NVP-BEZ235, in

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SUPPLEMENTARY MATERIAL

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Fig. S1. NVP-BEZ235 has little effect on normal vasculature, although cessation of NVP-BEZ235 was followed by tumor relapse.

Fig. S2. NVP-BEZ235 blocks phosphorylation of Akt, mTOR, and GSK3 β and destabilizes MYCN.

Fig. S3. *MYCN*^{T58A} prolongs MYCN protein half-life and confers resistance to NVP-BEZ235.

Fig. S4. MYCN induces transcription of VEGF and does not affect VEGF stability.

Fig. S5. VEGFA levels correlate with expression of MYCN in vivo.

Fig. S6. MYCN knockdown reduces VEGF level.

Fig. S7. A role for MYCN in recruitment of endothelial cells.

Fig. S8. HUWE1 knockdown stabilizes MYCN protein.

Fig. S9. HUWE1 knockdown blocks the efficacy of NVP-BEZ235.

Fig. S10. Minimal effect of HUWE1 knockdown in MYCN nonamplified neuroblastoma.

Author contributions: Y.H.C. designed the project, performed most of the experiments, analyzed the data, and wrote the manuscript. W.C.G. provided the original primary human neuroblastoma tissues, generated full-length human *MYCN*^{WT} and *MYCN*^{T58A} cells, and contributed to the writing of the manuscript. M.I. and C.S.H. contributed to Fig. 1B. M.G. contributed to fig. S7. A.P. provided SFNB-05, SFNB-06, and SFNB-07 primary cells and contributed to Fig. 1A. M.I., S.Y., and E.C. maintained the transgenic mouse colony. K.K.M. helped to write the manuscript. G.K. reviewed all pathology. W.A.W. conceived and initiated the study, analyzed the data, supervised the overall project, and wrote the manuscript. All authors had the opportunity to discuss the results and comment on the manuscript.

Competing interests: The authors declare that they have no competing interests.

MYCN-dependent neuroblastoma. NVP-BEZ235 decreased angiogenesis and improved survival in both primary human (highly pretreated recurrent *MYCN*-amplified orthotopic xenograft) and transgenic mouse models for *MYCN*-driven neuroblastoma. Using both gain- and loss-of-function approaches, we demonstrated that the anti-angiogenic efficacy of NVP-BEZ235 depended critically on *MYCN* in vitro and in vivo. Thus, clinical PI3K/mammalian target of rapamycin inhibitors drove degradation of *MYCN* in tumor cells, with secondary paracrine blockade of angiogenesis. Our data demonstrated significantly improved survival in treated animals and suggest that NVP-BEZ235 should be tested in children with high-risk, *MYCN*-amplified neuroblastoma.

INTRODUCTION

Neuroblastoma, a tumor of peripheral neural crest origin, is the most common extracranial solid tumor of childhood. *MYCN*, a *Myc* family proto-oncogene, is amplified in 25% of tumors and is a genetic marker for treatment failure (1). We previously generated a model for high-risk neuroblastoma by directing expression of a *MYCN* transgene to the peripheral neural crest of genetically engineered mice (GEM) under control of the rat tyrosine hydroxylase (TH) promoter (2). Murine tumors closely recapitulate high-risk human neuroblastoma both histologically and genetically and, like their human counterparts, are highly vascular (3).

In human neuroblastoma, amplification of *MYCN* correlates with increased vascular density and poor survival (4). A direct role for *MYCN* in regulating angiogenesis has not yet been demonstrated, although a number of basic observations suggest such a role for *c-myc*, a homolog of *MYCN*. Embryos from *c-myc* knockout mice show defective vasculogenesis, with tumors derived from *c-myc* knockout embryonic stem cells showing poor vascularization (5). The vascular endothelial growth factor (VEGF) family has emerged as a key regulator of angiogenesis in cancers including neuroblastoma, correlating with unfavorable histology and aggressive behavior (6, 7). A *c-myc* transgene targeted to the epidermis of transgenic mice led to skin tumors, with angiogenesis in these lesions dependent on VEGF (8). *MYCN* may similarly regulate angiogenesis, because *MYCN* can indirectly induce VEGF in cultured neuroblastoma cells (9, 10).

Because both *c-myc* and *MYCN* contribute to the regulation of VEGF and angiogenesis, can clinical small-molecule inhibitors that block *MYCN* or *c-myc* be used to block angiogenesis? Like *c-myc*, *MYCN* is stabilized by activation of phosphatidylinositol 3-kinase (PI3K) (11), with blockade of PI3K and mTOR (mammalian target of rapamycin) leading to decreased secretion of VEGF and decreased levels of *MYCN* protein (9, 12, 13). Although these observations suggest that inhibitors of PI3K might block angiogenesis in *MYCN*-driven neuroblastoma, the design and interpretation of clear experiments are complicated by the fact that inhibitors of PI3K and mTOR can block angiogenesis directly [through inhibition of PI3K in endothelial cells (14)] and by blocking fibroblasts and immune components of the tumor microenvironment, all of which contribute to angiogenic signaling [reviewed in (7)].

Here, we evaluated a role for PI3K blockade as a translational therapeutic approach to inhibit tumor-vascular interactions in neuroblastoma, incorporating multiple genetic strategies to distinguish direct effects of these interventions on vascular cells from paracrine effects of tumor cells on endothelial cells. Our studies demonstrate that *MYCN* is a critical target for PI3K inhibitors, because blockade of *MYCN* contributed prominently to the antiangiogenic effects of this class of compounds.

RESULTS

A dual PI3K/mTOR inhibitor enhances survival and suppresses tumor burden in GEM and orthotopic xenograft models of *MYCN*-driven neuroblastoma

To clarify whether *MYCN* amplification sensitizes tumor cells to dual inhibitors of PI3K and mTOR, we treated a panel of *MYCN*-amplified and nonamplified human neuroblastoma–derived cell lines with the PI3K/mTOR inhibitor NVP-BEZ235 (Fig. 1A). NVP-BEZ235 was most active in *MYCN*-amplified lines, suggesting *MYCN* amplification as an indicator of sensitivity to NVP-BEZ235.

We next used mice transgenic for TH-*MYCN* as a platform to test the efficacy of PI3K/mTOR inhibition in vivo. Upon detection of tumors by palpation (mean age, 60 days), tumor-bearing mice transgenic for TH-*MYCN* were treated with daily oral gavage of NVP-BEZ235 (35 mg/kg) or vehicle for 28 days (Fig. 1B). Notably, vehicle-treated animals showed substantial mortality starting 21 days after initiating treatment, with complete mortality by 30 days. In contrast, animals receiving active agent remained alive throughout treatment, relapsing only after discontinuation of drug (arrow in Fig. 1B). From the time of tumor detection to killing, drug-treated mice survived an average of ~1.5-fold longer than animals receiving vehicle alone.

We validated this in vivo result by orthotopic transplantation of a highly pretreated recurrent *MYCN*-amplified primary human tumor (SFNB-06) into the kidney capsule of nude mice. Mice were treated at 3 days after transplant with daily oral gavage of NVP-BEZ235 (35 mg/kg) or vehicle for 28 days (arrow in Fig. 1C). NVP-BEZ235 treatment again significantly improved overall survival (Fig. 1C).

We also demonstrated that NVP-BEZ235 significantly inhibited tumor burden in mice transgenic for TH-*MYCN* and in mice carrying orthotopic xenografts of SFNB-06 primary tumors. Tumor volume was measured in mice at the time of initial palpation (before treatment) and again after daily treatment with vehicle or NVP-BEZ235 (14 days). Tumor burden was calculated by comparing the initial volume to the end volume in treatment and control groups (Fig. 1, D and E). Full necropsies revealed no abnormalities apart from those documented in tumors.

A dual PI3K/mTOR inhibitor suppresses tumor proliferation and angiogenesis

Because NVP-BEZ235 has a known role in blocking angiogenesis (15), we collected TH-*MYCN* tumors (from Fig. 1D) and SFNB-06 orthotopic xenografts (from Fig. 1E) and analyzed vascular complexity. NVP-BEZ235 induced a significant reduction in vascular density within tumors in both models, as indicated by CD31 endothelial cell staining (Fig. 2, A and E and J and N).

Hematoxylin and eosin (H&E) staining showed reduced vascular density in the NVP-BEZ235 treatment arms in both models (Fig. 2, D and H and M and Q). Animals treated with NVP-BEZ235 also showed decreased perivascular cell coverage [α -SMA (smooth muscle actin) staining] in both TH-*MYCN* and human orthotopic mouse models (Fig. 2, B, F, and I and K, O, and R, respectively). Further, NVP-BEZ235 affected tumor cells directly, leading to decreased proliferation in both TH-*MYCN* and SFNB-06 tumors (Fig. 2, C, G, and I and L, P, and R, respectively). NVP-BEZ235 had no appreciable effect on established retinal vasculature in control animals, consistent with a therapeutic index for PI3K/mTOR inhibition on tumor-associated vessels, relative to normal, established vasculature (fig. S1, A and B). Rapid revascularization followed drug withdrawal, concomitant with tumor growth and overall mortality (fig. S1, C and D). These data show that NVP-BEZ235 treatment

reversibly reduced vascularity and inhibited proliferation of *MYCN*-driven neuroblastoma tumors in vivo, and that continuous treatment is required to maintain the tumor inhibition.

Inhibition of PI3K/mTOR leads to decreased levels of MYCN protein

We next analyzed PI3K and mTOR signaling in tumor extracts from mice treated for 7 or 14 days. NVP-BEZ235 treatment of both mouse and human neuroblastoma was associated with reduced levels of MYCN protein, correlating with reduced phosphorylation of the PI3K targets Akt and glycogen synthase kinase 3 β (GSK3 β) and of the mTOR target p-rpS6 (phosphorylated ribosomal protein S6) (Fig. 3, A and B, and fig. S2, A to C). After 14 days of treatment, levels of MYCN mRNA were not significantly changed (Fig. 3, C and D), consistent with NVP-BEZ235 blocking MYCN posttranscriptionally.

MYCN in tumor cells contributes to paracrine signaling between tumor and endothelial cells: Genetic studies

To test whether PI3K-driven stabilization of MYCN contributed functionally to the tumor vascular microenvironment, we transduced SHEP human neuroblastoma cells (which have no detectable MYCN) with retroviral vectors expressing wild-type murine *MYCN* (*MYCN*^{WT}) or a phosphomutant of *MYCN* (*MYCN*^{T58A}) that is resistant to inhibitors of PI3K and mTOR (11, 12, 16). We also transduced SHEP with control vector expressing green fluorescent protein (GFP); these cells responded similarly to parental SHEP shown in Fig. 1A (fig. S3A). We first confirmed that *MYCN*^{T58A} protein was stabilized by cycloheximide pulse-chase assay with NVP-BEZ235 (Fig. 4A and fig. S3B) compared with *MYCN*^{WT} control (fig. S3D). *MYCN*^{T58A} protein conferred partial resistance to treatment with NVP-BEZ235 even in the setting of effective inhibition of downstream Akt and rpS6 phosphorylation (Fig. 4B and fig. S3C). The ability of NVP-BEZ235 to block proliferation and viability was accordingly blunted in *MYCN*^{T58A} cells compared with *MYCN*^{WT} cells (Fig. 4C and fig. S3, F to H).

Because NVP-BEZ235 destabilized MYCN protein in tumor cells and blocked neuroblastoma angiogenesis in vivo, we hypothesized that MYCN may drive paracrine signaling between tumors and vasculature. To determine whether blockade of MYCN in tumor cells contributed to paracrine signaling between tumor and vascular cells, we next cocultured human umbilical vein endothelial cells (HUVECs) with *MYCN*^{WT} or *MYCN*^{T58A} tumor cells. HUVECs were plated in the top of a Boyden chamber, with neuroblastoma cells plated at the bottom. Migration of HUVECs was proportional to the level of MYCN in cocultured neuroblastoma cells, with minimal migration in *MYCN*-negative cells (fig. S3E), modest migration in *MYCN*^{WT} cells, and further HUVEC migration in *MYCN*^{T58A} cells (fig. S3H). *MYCN*^{T58A} cells were relatively resistant to NVP-BEZ235, with sustained migration of HUVECs, compared to limited migration of HUVECs cocultured with *MYCN*^{WT} cells (Fig. 4C and fig. S3H). These findings, along with control HUVEC migration in SHEP vector–GFP control cells (fig. S3E), are consistent with a component of HUVEC migration being regulated directly by PI3K in endothelial cells (17–19). In addition, our genetic experiments demonstrated a significant difference in HUVEC migration, comparing *MYCN*^{T58A} and *MYCN*^{WT} cells, suggesting that a substantial fraction of HUVEC migration is driven by *MYCN*-dependent signaling between tumor and HUVECs (Fig. 4C and fig. S3H).

In light of the known importance of VEGF in angiogenesis, we analyzed the effects of NVP-BEZ235 on VEGF production and secretion. VEGF protein was increased in cells transduced with *MYCN*^{T58A} compared to *MYCN*^{WT}, and was nearly absent in negative controlSHEP cells (fig. S4A). Treatment with NVP-BEZ235 reduced secretion of VEGF by 30% in *MYCN*^{WT} cells compared with 10% reduction in *MYCN*^{T58A} cells (Fig. 4C).

Analysis by real-time quantitative reverse transcription–polymerase chain reaction (qRT-PCR) showed that NVP-BEZ235 treatment minimally affected levels of VEGF mRNA in *MYCN*^{T58A} cells while substantially reducing levels of VEGF mRNA in *MYCN*^{WT} cells, consistent with transcriptional regulation of VEGF downstream of MYCN (fig. S4B). In cycloheximide pulse-chase assay, we further verified that MYCN did not regulate VEGF at the posttranscription level, because neither *MYCN*^{T58A} nor treatment with NVP-BEZ235 affected the half-life of VEGF protein (fig. S4C). To extend this result, we analyzed treated tumors from mice transgenic for TH-*MYCN* and from orthotopic xenografts of SFNB-06, verifying that NVP-BEZ235 treatment was associated with decreased levels of both VEGF protein and VEGF mRNA in vivo (fig. S5, A and B).

Knockdown of MYCN substantiates a role in paracrine signaling

To further evaluate a role for MYCN in paracrine signaling between tumor and vascular cells, we next analyzed *MYCN*-amplified Kelly neuroblastoma cells, comparing the effect of NVP-BEZ235 with short hairpin RNA (shRNA) directed against MYCN. Knockdown of MYCN resulted in decreased abundance of MYCN protein, with potency equivalent to or increased compared with NVP-BEZ235 treatment, and cooperative in combination (Fig. 4, D and E, and fig. S6A). Proliferation and viability of tumor cells decreased proportionately with decreasing levels of MYCN protein in cells treated with shRNA against MYCN, NVP-BEZ235, or in response to both interventions, resulting in a cooperative response (Fig. 4F). MYCN knockdown and treatment with NVP-BEZ235 also attenuated levels of VEGF secretion and VEGF mRNA (Fig. 4F and fig. S6B). Reduced MYCN protein in tumor cells also resulted in subsequent inhibition of migration in cocultured HUVECs (Fig. 4, E and F). These data validate a role for MYCN in directing paracrine signaling from tumor cells to endothelial cells in neuroblastoma.

A doxycycline-inducible allele verifies a role for MYCN in tumor-vascular interactions

To further clarify *MYCN*-dependent versus *MYCN*-independent roles of NVP-BEZ235 on both tumor and endothelial cells, we used SHEP-TET21/N, a human neuroblastoma cell line in which transcription of *MYCN* can be toggled “off” or “on” by the addition of doxycycline to the medium (20) (fig. S7A). As expected, NVP-BEZ235 treatment destabilized MYCN in TET21/N cells (fig. S7B). Doxycycline led to reduction of MYCN with significantly decreased proliferation and viability compared to MYCN on cells (fig. S7C). NVP-BEZ235 treatment reduced proliferation and viability in *MYCN* on cells to levels comparable to that of *MYCN* off cells, with no significant effect on proliferation and a modest effect on viability in *MYCN* off cells (fig. S7C).

Doxycycline-regulated suppression of *MYCN* transcription also inhibited VEGF secretion by 70%, compared to levels in *MYCN* on cells, and attenuated migration of cocultured HUVECs (fig. S7C). We excluded an off-target effect of doxycycline on viability by treating HUVECs with doxycycline directly (fig. S7D). NVP-BEZ235 treatment of *MYCN* on cells also reduced VEGF secretion and HUVEC migration to baseline levels. Similar treatment of *MYCN* off cells showed no significant effect on secretion of VEGF and modest effects on migration (fig. S7C). These data again showed that MYCN promoted angiogenesis by augmenting paracrine crosstalk between tumor cells and endothelial cells in the microenvironment. Targeting MYCN at the mRNA level (through addition of doxycycline in TET21/N cells or by transducing shRNA in Kelly cells) or at the protein level (using NVP-BEZ235) abrogated this MYCN-dependent paracrine signaling and contributed to angiogenic collapse.

Impairing E3 ligase–dependent degradation of MYCN in tumor cells promotes resistance to NVP-BEZ235

To further evaluate the importance of MYCN degradation to the action of NVP-BEZ235, we used shRNA in *MYCN*-amplified Kelly cells to knock down HUWE1 (Fig. 5A), an ubiquitin ligase known to degrade T58-phosphorylated MYCN protein (21, 22). MYCN protein accumulated in cells depleted for HUWE1, with no clear changes noted in abundance of p-Akt (phosphorylated Akt) or p-rpS6 in response to shHUWE1 (Fig. 5A). HUWE1 knockdown also prolonged the half-life of MYCN protein (Fig. 5C and fig. S8A) and mediated resistance to NVP-BEZ235–induced degradation of MYCN with two different shRNA constructs compared to shRNA control cells (Fig. 5B and figs. S8B and S9A).

We next investigated whether stabilization of MYCN (in response to HUWE1 knockdown) could antagonize the effects of NVP-BEZ235 on tumor cells. Knockdown of HUWE1 and subsequent stabilization of MYCN partially attenuated the effects of NVP-BEZ235 on proliferation and viability of tumor cells (~30% reduction) compared to similar treatment of tumor cells transduced with shRNA control (>60% reduction; Fig. 5D and fig. S9, B to D). Further, as control, knockdown of HUWE1 in *MYCN* nonamplified cells led to insignificant differences in viability and proliferation, comparing NVP-BEZ235 and vehicle (fig. S10, A and B). We conclude that stabilizing MYCN in neuroblastoma cells attenuates the effects of NVP-BEZ235 on proliferation and survival.

Impairing E3 ligase–dependent degradation of MYCN in tumor cells partially rescues the antiangiogenic effects of NVP-BEZ235

Next, we assessed whether preventing HUWE1-dependent destabilization of MYCN could similarly attenuate the paracrine effects of NVP-BEZ235 on angiogenesis. We therefore cocultured HUVECs with either HUWE1 knockdown or shRNA control tumor cells in the presence of either NVP-BEZ235 or vehicle. In the absence of NVP-BEZ235, both secretion of VEGF and migration of HUVECs were similar in HUWE1 knockdown compared with shRNA control cells (Fig. 5D). In treated cells, NVP-BEZ235 caused only 25% reduction in levels of VEGF in HUWE1 knockdown cells compared with 60% in shRNA control (Fig. 5D). The efficacy of NVP-BEZ235 in blocking migration was significantly reduced in HUVECs cocultured with HUWE1 knockdown cells (55% reduction) compared with HUVECs cocultured with control cells (75% reduction; Fig. 5D and fig. S9E). Thus, stabilization of MYCN in tumor cells caused resistance to NVP-BEZ235, leading to decreased secretion of VEGF and decreased recruitment of endothelial cells. We conclude that MYCN represents a critical target of NVP-BEZ235 in tumor cells, resulting in reduced paracrine signaling to cocultured HUVECs.

To extend these results, we transplanted both control and HUWE1 knockdown Kelly cells into the renal capsules of nude mice for a cohort study. Two weeks after transplantation, mice were treated with either vehicle or NVP-BEZ235 (35 mg/kg by daily oral gavage for 14 days). The efficacy of NVP-BEZ235 in blocking tumor growth was markedly attenuated by knockdown of HUWE1 (Fig. 6, A and B; 95% reduction combining NVP-BEZ235 with shRNA control versus 50% reduction combining NVP-BEZ235 with HUWE1 shRNA). These data demonstrated that attenuating the efficacy of NVP-BEZ235 against MYCN markedly reduced the activity of this drug against tumor cells.

Next, we evaluated the angiogenic microenvironment to determine whether HUWE1 knockdown in tumor cells affected response to NVP-BEZ235 in vascular cells. In response to vehicle, both shRNA control and HUWE1 knockdown tumors were highly vascularized (Fig. 6, C and E and O and Q, quantified in S), with tumor cells showing prominent proliferation (Fig. 6, G, I, and S). NVP-BEZ235 effectively blocked both formation of

angiogenic blood vessels and proliferation of tumor cells in control mice (Fig. 6, D and H and P and S). The efficacy of NVP-BEZ235 in HUWE1 knockdown tumors was blunted markedly, with retention of both vascular networks and proliferating tumor cells (Fig. 6, F, J, R, and S). These data suggest that stabilization of MYCN in tumor cells in vivo reduced the potency of NVP-BEZ235 against both neuroblastoma tumors and vascular elements in the tumor microenvironment. We conclude that MYCN protein represents a critical target for the efficacy of NVP-BEZ235 in neuroblastoma tumors driven by *MYCN*, and that destabilization of MYCN in tumor cells contributes prominently to the antiangiogenic efficacy of NVP-BEZ235.

DISCUSSION

Amplification of *MYCN* is a genetic marker for high-risk neuroblastoma (1). The correlation of *MYCN* amplification with vascularity (4) and the demonstration that withdrawal of MYC in tumors driven by a MYC-ER (estrogen receptor) fusion protein led to angiogenic collapse (8) collectively suggest MYCN as a potential target. Yet, the therapeutic blockade of MYCN and other transcription factors remains generally challenging. We previously demonstrated that tool compound inhibitors of PI3K/mTOR can destabilize MYCN and can block proliferation of tumors driven by TH-*MYCN* (12). Others have validated these findings and, in addition, demonstrated that these tool compound inhibitors of PI3K/mTOR can block VEGF (9, 13). These observations suggest that PI3K-driven blockade of MYCN (using clinical inhibitors) could affect angiogenesis inhibition.

Historically, translational therapeutic studies of cancer have been performed in xenograft models. Although such models provide important insights, immunodeficient mice do not recapitulate the tumor micro-environment interaction seen in human disease. To study the intricacies of these interactions, we therefore demonstrated that the clinical agent NVP-BEZ235 drove angiogenic collapse in both orthotopic xenograft and transgenic models for *MYCN*-driven neuroblastoma in vivo. We also studied xenografts of two parental *MYCN* nonamplified cell lines and one nonamplified human primary tumor, observing no growth in vivo by 3 months. Similar difficulties in achieving successful xenografts of *MYCN* nonamplified tumors have been noted by others (23). Thus, we limit our in vivo conclusions in this study to *MYCN*-driven disease.

The functional importance of MYCN as a target of NVP-BEZ235 is not fully clarified by our initial in vivo studies, however, because inhibitors of PI3K/mTOR can block endothelial cells directly (14) and can block tumor-associated pericytes, immune cells, and possibly extracellular matrix in the tumor microenvironment, all of which contribute to angiogenesis [reviewed in (7)]. Therefore, to clarify whether inhibition of MYCN in tumor cells contributed prominently to angiogenic collapse, we cocultured endothelial cells with tumor cells using PI3K-resistant alleles (*MYCN*^{T58A}), shRNA against amplified *MYCN* in human neuroblastoma cells, a doxycycline-repressible allele of *MYCN*, and shRNA against the ubiquitin ligase HUWE1. These experiments collectively demonstrate that MYCN is an indicator of relative sensitivity to PI3K/mTOR inhibitors and that blockade of MYCN is critical to the antiangiogenic effects of PI3K/mTOR inhibitors in neuroblastoma. Of course, these data do not exclude MYCN-independent effects of PI3K/mTOR inhibitors, because transcriptional and/or translational effects of this class of drugs have been shown in other cancers [reviewed in (24)]. Nevertheless, although overexpression of *MYCN* accelerated the growth of nonamplified neuroblastoma cells, it also sensitized these cells to NVP-BEZ235 (fig. S3, A and G). These observations are consistent with findings that ectopic expression of *MYCN* can sensitize cells to combination chemotherapy and targeted therapy using TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), doxorubicin, ZVAD, or PI3K inhibitors (25–27).

Previous studies have established MYCN as a direct target of HUWE1 (21, 22). Therefore, to verify MYCN as a critical target in vivo, we used HUWE1 knockdown tumor cells (deficient in PI3K/mTOR-mediated MYCN proteolysis) to establish orthotopic xenografts, demonstrating HUWE1 knockdown tumors to be resistant to the antiangiogenic activity of NVP-BEZ235. Efficient knockdown of HUWE1 did not completely attenuate the efficacy of PI3K/mTOR inhibition, likely because additional ubiquitin ligases signal downstream of the PI3K/mTOR/MYCN pathway, including FBXW7, which also degrades MYCN in neuroblastoma (28, 29). FBXW7 also targets the mTOR protein (30) and is itself phosphorylated by PI3K (31). These complex interactions make FBXW7 a poor candidate for interpreting knockdown studies that also incorporate PI3K/mTOR inhibitors. It therefore remains possible that HUWE1 substrates, in addition to MYCN (32, 33), could contribute to therapy resistance observed in our HUWE1-related experiments.

Several paracrine effectors contribute to angiogenesis in neuroblastoma (34). Our observations that blockade of MYCN led to reduction of VEGF in vitro and in vivo and to decreased migration of endothelial cells implicate VEGF signaling as one effector of tumor-vascular interactions. These observations do not exclude roles for other paracrine factors and do not exclude VEGF regulation through pathways independent of MYCN. To clarify the mechanism, we showed that transcription of VEGF is regulated transcriptionally through a MYCN-dependent pathway. This finding is consistent with previous demonstration that VEGF is an indirect target of MYCN (10, 35) and that MYCN cooperates with hypoxia-inducible factor 1 α (HIF1 α) to regulate angiogenesis and progression (10, 35). Because PI3K and mTOR inhibitors can suppress HIF1 α and other effectors of VEGF (9, 10), it is likely that NVP-BEZ235 regulates VEGF levels, HIF1 α , and angiogenesis in both MYCN-dependent and MYCN-independent manners.

In overview, our studies clarify that MYCN is a critical target of NVP-BEZ235, with inhibition of PI3K and mTOR signaling effect angiogenic blockade in part through a MYCN-dependent pathway, and highlight the importance of paracrine MYCN-directed signaling between tumor cells and vascular cells in this disease. Further, our studies argue that NVP-BEZ235 should be tested in children with high-risk, *MYCN*-amplified neuroblastoma tumors.

MATERIALS AND METHODS

In vivo experiments

TH-*MYCN* mice with tumors, detected by palpation (mean age at detection was 60 ± 15 days), were treated with NVP-BEZ235 [35 mg/kg in PEG300 (polyethylene glycol, molecular weight 300)] or vehicle (PEG300), once daily by oral gavage, for 7 or 14 days. IC₅₀ (median inhibitory concentration) for NVP-BEZ235 activity was as previously described (36). Dosages for both in vitro and in vivo experiments were by recommendation from Novartis. For Western analysis and qRT-PCR, tumors were snap-frozen. For immunohistochemistry, tumors and tissues were fixed and processed as described below. For tumor burden assessment, record of tumor was collected at $t = 0$ day before treatment and again at $t = 14$ days, the last day of treatment.

Kidney capsule orthotopic models

A human *MYCN*-amplified primary neuroblastoma tumor was obtained from a child with relapsed metastatic disease of the highest grade and stage (SFNB-06). Tumor pieces (2 mm³) were implanted into the kidney capsule of nude mice. After 3 weeks, when palpable tumors were detected, mice were treated with the vehicle PEG300 or NVP-BEZ235 daily for

14 days. Tumors were collected at the last day of treatment, weight and volume were recorded, tissues were processed as below, and histology was assessed.

For orthotopic kidney capsule experiments using human cell lines, neuroblastoma cells were resuspended in 1:1 phosphate-buffered saline (PBS) + Matrigel (BD Biosciences). Nude mice at 4 to 6 weeks of age were anesthetized with isoflurane. Cells (1×10^6) were injected into the kidney capsule. Two weeks after transplantation, mice were treated for 14 days, tumor weight and volume were recorded, and histology was processed as above. All animals were handled in accordance with institutional Laboratory Animal Resource Center guidelines.

Survival studies

TH-*MYCN* mice with palpable tumors were treated with NVP-BEZ235 (35 mg/kg in PEG300) or vehicle (PEG300) once daily for 28 days. Human primary SFNB-06 mice were treated once daily for 28 days, starting 3 days after orthotopic transplantation (dosage as above). Continuation of NVP-BEZ235 beyond 28 days was associated with marked weight loss due either to repeated gavage injury to the esophagus or possibly from systemic toxicity, requiring cessation of treatment per Institutional Animal Care and Use Committee (IACUC) guidelines. All mice were monitored until euthanasia was required in accordance with institutional IACUC guidelines.

Boyden chamber migration assay

Cell migration was studied using Boyden chambers with 8- μ m pores (BD Biosciences). Transwell inserts were coated with Attachment Factor (Cascade/Invitrogen). Basal Medium 200 with fetal bovine serum (FBS) was used in all migration assays. First, neuroblastoma cells were resuspended in medium, and 1×10^5 cells (500 μ l) were plated in the bottom chamber and incubated for 3 hours, with NVP-BEZ235 or vehicle then added. After 16 hours, HUVECs (1×10^5 cells suspended in 100 μ l of assay medium) were added to the upper chamber of Transwell inserts. To control for spontaneous migration, we also plated HUVECs in the top chamber without neuroblastoma cells in the bottom. After 16 hours of coincubation, nonmigrated HUVECs in the upper surface of the membrane were scraped off. Cells migrating to the lower surface were then stained (SYTOX, Molecular Probes/Invitrogen) and imaged at 5 $\times$. Quantification of six randomly selected fields per condition was performed with ImageJ program [National Institutes of Health (NIH)], normalized to the internal control. All experiments were done in triplicate and repeated at least three times.

Cell culture and reagents

HUVECs were grown in Medium 200 and Low Serum Growth Supplement (Cascade Biologics/Gibco/Invitrogen). Neuroblastoma tumor cell lines (Kelly, IMR32, Lan-5, SK-N-BE2, SK-N-SH, SY5Y, and SHEP) were obtained from the University of California San Francisco Cell Culture Facility. Human primary lines SFNB-05, SFNB-06, and SFNB-07 were isolated from patients' neuroblastoma tumors, dissociated, and cultured in N5 with 10% serum. SHEP-TET21/N cells were from J. Shohet (Baylor University, Houston, TX). Stable SHEP *N-MYC*^{WT} and SHEP *N-MYC*^{T50A} (referred to here as *N-MYC*^{T58A}) were gifts from A. Kenney and were previously described (12). Full-length human *MYCN*^{T58A} was generated in-house, from *MYCN*^{WT}, by site-directed mutagenesis (QuikChange II kit) and cloned into pLenti6.3 lentiviral plasmid (Invitrogen). Multiclonal, stably transduced cell lines were produced by selection with blasticidin. All experiments were verified with both constructs generated in-house and by A. Kenney, and there is no functional difference between the constructs.

Neuroblastoma cells were grown in RPMI, N5, or DMEM (Dulbecco's modified Eagle's medium) with 10% FBS. In most experiments, cells were conditioned in 2% FBS for 5 hours and replaced with full medium and recombinant human insulin-like growth factor-I (20 ng/ml) (Invitrogen) for 1 hour before harvesting. NVP-BEZ235 (Novartis) was reconstituted to 10 M in dimethyl sulfoxide for in vitro or PEG300 for in vivo. IC₅₀ for NVP-BEZ235 activity was previously described (36). Dosages for both in vitro and in vivo experiments were by recommendation from Novartis.

Mission lentiviral shRNA transduction particles (pLKO.1-puro) for human MYCN, HUWE1, and shRNA control (nontargeting construct) (Sigma-Aldrich) were used according to standard Sigma lentiviral transduction protocol. Stable cell lines were selected with puromycin (2 µg/ml) for 48 hours.

Histology

For fluorescence microscopy, tissue and tumors were collected, fixed with 4% paraformaldehyde (PFA) overnight, cryoprotected with 30% sucrose, and then embedded in optimum cutting temperature (OCT) compound and frozen at -80°C. Sections (30 µm) were cut, mounted onto glass slides, blocked for 4 hours with 10% goat serum in PBST (PBS, 1% Triton X-100), and then incubated overnight at 4°C with primary antisera. Slides were washed with PBST for 1 hour, incubated for 4 hours with secondary antibodies, and mounted with Fluormount G (Southern Biotech). Images were captured with Zeiss Axiophot fluorescence microscopes. Primary antibodies are as follows: monoclonal hamster anti-mouse CD31 (1:500, Chemicon), monoclonal rat anti-mouse CD31 (1:500, Pharmingen/BD Bioscience), MYCN, VEGFA (1:500, Santa Cruz), 5% normal goat serum (Invitrogen), mouse monoclonal Cy3-conjugated α-SMA (1:500, Sigma-Aldrich), and rabbit anti-Ki67 (1:200, clone SP6, Lab Vision). Secondary antibodies are as follows: Alexa 488 goat anti-rat and goat anti-hamster immunoglobulin G (IgG) or Alexa 568 goat anti-rabbit IgG (1:200, Molecular Probes/Invitrogen) and DAPI (4',6-diamidino-2-phenylindole) nucleic acid stain (1:1000, Molecular Probes/Invitrogen). All quantitations were done with ImageJ program (NIH).

For retinal studies, eyes were collected and fixed with 4% PFA. Dissected retinas were blocked with 5% mouse serum in PBST for 3 hours and then incubated overnight at 4°C with biotinylated isolectin B4 (25 mg/ml) (Sigma) in PBLEC [1% Triton X-100, 0.1 mM CaCl₂, 0.1 mM MgCl₂, 0.1 mM MnCl₂, in PBS (pH 6.8)]. Retinas were then washed and stained with Alexa 488 streptavidin (Molecular Probes). Images of flat-mounted retinas were captured by fluorescence microscopy and quantified by ImageJ program.

For H&E analysis, 10-µm sections were cut from frozen OCT blocks, mounted onto glass slides, fixed with 4% PFA, and washed with PBS and water. Hematoxylin (Vector Labs) was applied for 2 min, and bluing agent was added for 1 min. Slides were washed three times with water and once with 70% ethanol (EtOH) for 5 min each. Eosin was added for 30 s, followed by 2× in 70% EtOH, 2× in 95% EtOH, 2× in 100% EtOH (1 to 2 min each), and dipped into xylene for 2 min. Slides were mounted with Vectashield (Vector Labs) and analyzed with light microscopy.

Immunoblotting

Lysates were collected, sonicated, and cleared as described (12). Primary antibodies were as follows: anti-MYCN, anti-VEGFA (Abcam), anti-phosphorylated S⁴⁷³ Akt, anti-Akt, rpS6, anti-rpS6, anti-phosphorylated S⁹ GSK3β and anti-GSK3β (Cell Signaling Technology), anti-actin (Santa Cruz), β-tubulin (Upstate), anti-HUWE1 (Lifespan Biosciences), and anti-Lasu1 (Bethyl Laboratories). Immunoblots were developed with horseradish peroxidase–

conjugated secondary antibodies (Calbiochem) and Enhanced Chemiluminescence Plus reagents (Amersham). Densitometry quantification was performed with ImageJ program (NIH), normalized to the total protein. All experiments were done in triplicate and repeated at least three times.

Real-time PCR analysis

Total RNA isolation was collected with TRI Reagent kit (Ambion/ABI). qRT-PCR was done with ThermoScript RT-PCR System plus Platinum Taq DNA Polymerase and Power SYBR Green PCR Master Mix (Invitrogen) using standard methodology. Output readings were collected with ABI 7900HT system. DNA primers were as follows: human *MYCN*, 5'-CGACCACAAGGCCCTCAGTA-3' (sense) and 5'-CAGCCT-TGGTGTGGAGGAG-3' (antisense); human VEGF, 5'-CCATGAAC-TTTCTGCTGTCTT-3' (sense) and 5'-ATCGCATCAGGGGCACACAG-3' (antisense); mouse VEGF, 5'-TACCTCCACCATGCCAAGTGGT-3' (sense) and 5'-AGGACGGCTTGAAGATGTAC-3' (antisense); and control mouse β -actin, 5'-GACGGCCCAGTCATCACTAATG-3' (sense) and 5'-TGCCACAGGATCCATACCC-3' (antisense). Relative expression of *MYCN* and VEGF in the sample was normalized to the expression of an internal control (β -actin).

Proliferation and viability assays

Neuroblastoma cells were suspended in basal RPMI, DMEM, or N5 with 1% FBS and plated at 2000 to 3000 cells per well in 96-well tissue culture plates (Corning/Sigma-Aldrich). Cells were incubated at 37°C for 16 hours and then changed into fresh medium containing NVP-BEZ235 (1 μ M) or vehicle. To measure proliferation 24 hours after plating, we added 5-bromo-2'-deoxyuridine (BrdU) labeling solution from Cell Proliferation ELISA (enzyme-linked immunosorbent assay) (Colorimetric Kit, Roche Bioscience) and incubated the cells for another 24 hours at 37°C. BrdU incorporation was determined by chemiluminescence immunoassay. To measure viable cells, we analyzed DNA content at 48 hours after plating using CyQUANT NF assay kit (Invitrogen) according to the manufacturer's protocol. All experiments were repeated at least three times with six replicates.

VEGF ELISA

Neuroblastoma cells were split into six-well plates and treated with vehicle or NVP-BEZ235 (1 μ M). Medium was harvested from cultures at 48 hours. Medium was assayed for VEGF protein levels, measured by Human VEGF QuantiGlo ELISA Kit (R&D Systems), and determined by chemiluminescence immunoassay. All experiments were repeated at least three times with six replicates.

Cycloheximide pulse-chase assay

Cycloheximide pulse-chase studies were used to evaluate *MYCN* protein half-life in the absence of new protein synthesis. Neuroblastoma cells were split into six-well plates and treated with vehicle or NVP-BEZ235 (1 μ M) concurrently with cycloheximide (50 μ g/ml) at $t = 0$ hour. At prescribed intervals, cycloheximide (50 μ g/ml) was added in pulses of 30 min up to 6 hours. Lysates were analyzed by Western blot. Experiments were repeated at least three times with three replicates.

Statistical analysis

GraphPad Prism program version 5.01 was used for Kaplan-Meier and log-rank test (GraphPad Software Inc.). JMP program versions 8 and 9 were used for Student's t test

(SAS Corp.). All data with valued graphs are means $\pm$ SEM. A *P* value of <0.05 indicates statistical significance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank J. Chen, Q.-W. Fan, C. Cheng, J. Lau, F. Johansson-Swartzling, and K. Nguyen for useful discussions and Z. Werb, T. Vu, and G. Bergers for critical review. We thank A. Kenney for providing *N-MYC^{WT}* and *N-MYC^{T50A}* constructs, and J. Shohet for SHEP-TET21/N cells.

Funding: This work was supported by Fletcher Jones Fellowship (Y.H.C.), Burroughs Wellcome Fund (Y.H.C. and W.C.G.), NIH grants R01CA102321 (W.A.W.) and P01CA081403 (W.A.W. and K.K.M.), Alex's Lemonade Stand (W.A.W. and K.K.M.), the Katie Dougherty Foundation (W.A.W. and K.K.M.), the Samuel Waxman Cancer Research Foundation (W.A.W.), and the V-Foundation (W.A.W. and K.K.M.).

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Fig. 1.

A dual PI3K/mTOR inhibitor leads to decreased tumor burden and improved survival in GEM and primary orthotopic xenograft models of MYCN-driven neuroblastoma. **(A)** Viability and proliferation screens: MYCN-amplified cells [human primary culture (SFNB-06) and three cell lines (IMR32, Lan-5, and SK-N-BE2)] were sensitive to NVP-BEZ235 compared with nonamplified cells [two human primary cultures (SFNB-05 and SFNB-07) and cell lines (SK-N-SH, SY5Y, and SHEP)]. * $P < 0.05$, Student's t test. **(B)** Tumor-bearing TH-MYCN mice were treated with NVP-BEZ235 (35 mg/kg) or PEG300 vehicle once daily by oral gavage for 28 days. Kaplan-Meier analysis demonstrates that NVP-BEZ235 enhanced mice survival ($n = 5$ for each group; $P < 0.003$, log-rank test). Arrow indicates cessation of therapy at 28 days. **(C)** A heavily pretreated primary MYCN-amplified human tumor (SFNB-06) was established orthotopically, and mice were treated with NVP-BEZ235 or vehicle, as above, for 28 days. NVP-BEZ235 prolonged survival ($n = 10$ for each group; $P < 0.049$, log-rank test). Arrow again indicates cessation of therapy at 28 days. **(D and E)** Tumor burden: tumor-bearing TH-MYCN mice (D) and orthotopic xenografts of SFNB-06 (E) were treated with NVP-BEZ235 (35 mg/kg) or PEG300 vehicle once daily by oral gavage for 14 days. NVP-BEZ235 treatment led to decreased tumor volume and weight in both models (* $P < 0.05$, Student's t test).

Fig. 2.

A dual PI3K/mTOR inhibitor suppresses both proliferation of tumor cells and angiogenesis in neuroblastoma. (**A** to **H**) Tumors from mice transgenic for TH-*MYCN* were treated with NVP-BEZ235 (35 mg/kg) or PEG300 vehicle once daily by oral gavage, and mice were killed at 14 days of treatment. Endo-thelial cell density (CD31), pericyte density (α-SMA), proliferation of tumor cells (Ki67), and H&E staining were assessed. Scale bars, 50 μm. $n = 3$ for each arm. (**I**) Quantification of (**A**) to (**C**) and (**E**) to (**H**) using ImageJ ($*P < 0.0008$, Student's t test). (**J** to **P**) Mice carrying *MYCN*-amplified human orthotopic xenografts (SFNB-06) were treated with NVP-BEZ235 or vehicle, collected, and analyzed as in (**A**) to (**I**). (**R**) Quantification of (**J**) to (**L**) and (**N**) to (**P**) with ImageJ ($*P < 0.0008$, Student's t test). $n = 3$ for each arm. In all quantitations, vehicle arms were normalized to 100%; NVP-BEZ235 arms were graphed as percent of vehicle.

Fig. 3.

NVP-BEZ235 blocks PI3K/mTOR and destabilizes MYCN in vivo. **(A)** Tumor-bearing mice transgenic for TH-MYCN were treated daily with NVP-BEZ235 or vehicle for 7 or 14 days (same dosage as in Fig. 2). Western analysis demonstrates decreased levels of MYCN protein in tumor lysates from animals treated with NVP-BEZ235 in comparison with control. **(B)** Western analysis of lysates from human orthotopic tumors in Fig. 1, I to O, confirms expression of MYCN in primary tumor and in vehicle-treated orthotopic xenografts, with decreased levels of MYCN protein in lysates from two different xenografts, treated with NVP-BEZ235 for 14 days. **(C)** Real-time qRT-PCR: NVP-BEZ235 (7 and 14 days) had no significant effect on levels of MYCN mRNA in the TH-MYCN [from (A)]. $P = 0.56$ for 7 days, $P = 0.75$ for 14 days. **(D)** Real-time qRT-PCR: primary human SFNB-06 tumors treated with vehicle or NVP-BEZ235 for 14 days [from (B)]. $P = 0.62$, Student's t test. Vehicle arms were normalized to 100%. NVP-BEZ235 arms were graphed as percent of vehicle.

Fig. 4. MYCN drives paracrine signaling between tumor vascular cells. (A to C) SHEP cells were stably transduced with *MYCN^{WT}* or *MYCN^{T58A}*. (A) Cycloheximide (CHX) pulse-chase assay: cycloheximide (50 μ g/ml) with NVP-BEZ235. *MYCN^{T58A}* protein showed a prolonged half-life. (B) Western blot: *MYCN^{T58A}* protein was resistant to NVP-BEZ235. (C) Vehicle-treated *MYCN^{WT}* and *MYCN^{T58A}* were normalized to 100%. NVP-BEZ235–treated arms were graphed as percent change of vehicle (raw data in fig. S3, F to H). Proliferation and viability assays: *MYCN^{T58A}* conferred resistance to NVP-BEZ235 versus *MYCN^{WT}* (* $P < 0.026$; ** $P < 0.05$). VEGF ELISA: NVP-BEZ235 suppressed levels of VEGF in *MYCN^{WT}* cells, with blunting response in *MYCN^{T58A}* cells (*** $P < 0.002$). HUVEC migration: HUVECs (upper chamber) with *MYCN^{WT}* or *MYCN^{T58A}* cells (bottom chamber). NVP-BEZ235 reduced migration was blunted in *MYCN^{T58A}* cocultures (**** $P < 0.0001$).

0.008). (D to F) *MYCN*-amplified Kelly cells were transduced with shRNA MYCN (Sh-MYCN) and shRNA control vector (Sh-Control). (D and E) Western blot: Sh-MYCN (#3) was more efficient than NVP-BEZ235, without altering p-Akt/p-rpS6. (F) Proliferation and viability: Sh-MYCN recapitulated the effects of NVP-BEZ235 (* $P < 0.001$; ** $P < 0.007$, Sh-MYCN versus shRNA vehicle; # $P < 0.04$, Sh-MYCN versus NVP-BEZ235). VEGF ELISA: Sh-MYCN and NVP-BEZ235 similarly reduced VEGF (** $P < 0.005$, Sh-MYCN versus shRNA control; ## $P < 0.04$, Sh-MYCN versus NVP-BEZ235). HUVEC migration: Sh-MYCN and NVP-BEZ235 blocked migration (**** $P < 0.002$, Sh-MYCN versus shRNA control; + $P < 0.0001$, all assays with Sh-MYCN + NVP-BEZ235 versus Sh-Control + vehicle).

Fig. 5.

Stabilization of MYCN protein blocks the efficacy of NVP-BEZ235. (A) Kelly *MYCN*-amplified neuroblastoma cells were stably transduced with three different HUWE1 shRNAs or shRNA control. Western analysis demonstrates that shRNA in lane 2 (Sh-1) knocked down HUWE1 without affecting the PI3K target p-Akt or mTOR target p-rpS6. Sh-1 was used for subsequent experiments. (B) Western blot: HUWE1 knockdown promoted partial resistance to NVP-BEZ235 (lane 4 versus lane 2). (C) Cycloheximide pulse-chase assay: cycloheximide was added over a 2-hour time course. HUWE1 knockdown promoted longer half-life of MYCN versus control. (D) Vehicle-treated arms from both Sh-Control and HUWE1 knockdown lines were normalized to 100%, and NVP-BEZ235-treated arms were graphed as percent change compared to vehicle-treated (original graphs in fig. S9, C to E). Proliferation and viability: with NVP-BEZ235, HUWE1 knockdown cells were resistant versus Sh-Control cells (* $P < 0.003$; ** $P < 0.003$). VEGF ELISA: NVP-BEZ235 blocks VEGF in Sh-Control, with significant resistance mediated by HUWE1 knockdown (** $P < 0.03$). HUVEC migration: HUWE1 or Sh-Control cells (lower chamber) with HUVECs (upper chamber). With NVP-BEZ235 treatment, HUWE1 knockdown cells were significantly resistant versus Sh-Control cells (**** $P < 0.003$).

Fig. 6. Tumors derived from HUWE1 knockdown cells were resistant to treatment with NVP-BEZ235 in vivo. (A to S) HUWE1 knockdown (Sh-1) or shRNA control Kelly lines were transplanted orthotopically into renal capsules of nude mice. Daily treatment with NVP-BEZ235 (35 mg/kg) or vehicle was initiated on day 14. Tumors were collected for analysis after 2 weeks of treatment. $n = 5$ for each arm. Graphs were normalized as in Fig. 4C. (A) Tumor burden (weight and volume): NVP-BEZ235 blocked shRNA control tumors, whereas HUWE1 knockdown tumors showed significant growth despite treatment with NVP-BEZ235 ($*P < 0.003$, Student's t test, for difference). (B) Representative images of whole tumors under each condition. White ovals indicate kidney in each sample. Scale bar, 5 mm. (C to R) Histological analysis: CD31 (endothelial cells), Ki67 (proliferating cells), DAPI (nuclei), and H&E. Scale bars, 50 μ m. (S) Quantification of data from (C) to (N). Vascular density (CD31) and proliferation (Ki67) in HUWE1 knockdown tumors were resistant to treatment with NVP-BEZ235 relative to shRNA control tumors ($**P < 0.003$, Student's t test). $n = 3$ mice in each arm. Three representative images per mouse were quantified using ImageJ.