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VRK1 as a synthetic lethal target in VRK2-methylated cancers of the nervous system

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Abstract

Collateral lethality occurs when loss of a gene/protein renders cancer cells dependent on its remaining paralog. Combining genome-scale CRISPR/Cas9 loss-of-function screens coupled with RNA-sequencing in over 900 cancer cell lines, we found that cancers of nervous system lineage, including adult and pediatric gliomas and neuroblastomas, required the nuclear kinase Vaccinia-Related Kinase 1 (VRK1) for their survival in vivo. VRK1 dependency was inversely correlated with expression of its paralog VRK2. VRK2 knockout (KO) sensitized cells to VRK1 loss, and conversely, VRK2 overexpression increased cell fitness in the setting of VRK1 loss. DNA methylation of the VRK2 promoter was associated with low VRK2 expression in human neuroblastomas, and adult and pediatric gliomas. Mechanistically, depletion of VRK1 reduced Barrier-to-Autointegration Factor (BAF) phosphorylation during mitosis, resulting in DNA damage and apoptosis. Together, these studies identify VRK1 as a synthetic lethal target in VRK2 promoter-methylated adult and pediatric gliomas and neuroblastomas.
Introduction

Tumors of the nervous system constitute some of the most devastating malignancies in both adult and pediatric patients (1). Tumors arising in both the central and peripheral nervous system (CNS and PNS, respectively) often exhibit an aggressive clinical course and are refractory to currently available systemic therapy (2, 3).

Glioblastoma is the most common primary brain tumor in adults and is characterized by poor prognosis and low cure rates (4). Diffuse midline gliomas (DMG) that harbor histone 3 lysine27-to-methionine mutations ("pediatric diffuse midline glioma, H3 K27-altered") occur in children with a peak incidence of 6 to 9 years of age (5). Due to their infiltrative growth pattern, these gliomas are unresectable and uniformly fatal. Neuroblastoma (NB) is the most common extracranial solid tumor malignancy in childhood, commonly originating in the adrenal medulla or paraspinal ganglia (2). Standard of care treatment schemes for these tumors remains cytotoxic radio-chemotherapy and surgery, and patient prognosis has not substantially improved over the last decade (3, 6, 7). Thus, there is an urgent need for the identification of novel, targetable biomarkers in these tumor entities to translate into improved patient outcomes.

The implementation of CRISPR/Cas9 screening technologies has facilitated systematic studies to identify novel therapeutic targets and biomarkers of response across many cancers. Such dependency maps have unveiled a number of gene targets beyond known oncogenic drivers and hold the potential for tumor-specific, personalized therapy (8). Integration of genome-scale functional studies with genome-wide transcriptomics or epigenomics allows for correlative connections between gene dependencies and cancer transcriptional landscapes.
By comprehensively integrating genome-scale, loss-of-function genetic screens, RNA-sequencing and analysis of DNA methylation patterns, we identified the nuclear, serine-threonine kinase, Vaccinia-Related Kinase 1 (VRK1), as a highly selective dependency in adult and pediatric CNS and PNS tumors that exhibit low expression of the VRK1 paralog, VRK2.

Results

VRK1 is a selective dependency in adult and pediatric glioma and neuroblastoma

The Cancer Dependency Map includes CRISPR-Cas9 loss-of-function screens performed in over 900 cell lines representing 25 different cancer lineages(9). Using this dataset, we found that VRK1 is a strong genetic dependency in the adult glioma (p = 2x10^{-12}; Student’s t-Test; n = 61) and pediatric neuroblastoma (p = 3x10^{-8}; Student’s t-Test; n = 20) lineages (Fig. 1A). Indeed, VRK1 was the gene with the highest differential dependency in central nervous system (CNS) and peripheral nervous system (PNS) lineages as compared to all other tumors (Fig. 1B).

We validated that VRK1 is required for cell proliferation using CRISPR/Cas9 knockout (KO). Three of the four VRK1 sgRNAs that were included in the Cancer Dependency Map led to robust VRK1 KO and proliferation defects in NB, DMG, and GBM cultures (Fig. 1C, Supplementary Fig. S1A-C). VRK1 KO resulted in a significant decrease in cell fitness approximately 8 days following viral transduction in neuroblastoma NB-1 cells (Fig. 1D), as well as in a larger panel of neuroblastoma and GBM cell models (Fig. 1E). In addition, the Cancer Dependency Map includes a pediatric glioma model (KNS42), which also demonstrated strong VRK1 dependency. We therefore tested primary, pediatric H3K27M DMG neurosphere models and confirmed that VRK1 single-gene KO significantly decreased cell viability (Fig. 1E). To determine whether the reduced viability was due to apoptosis, we
performed live-cell experiments with a CASP3/7 cleavage reporter in the LN443 GBM cell line and found significantly higher CASP3/7 activity after VRK1 KO (Fig. 1F, Supplementary Fig. S1D). In an orthogonal approach, we found significant induction of apoptosis in NB and DMG models following VRK1 KO, as assessed by Annexin-V / PI staining (Fig. 1G, Supplementary Fig. S1E). We failed to observe significantly altered cell-cycle profiles in response to VRK1 KO in GBM or DMG models, and only a small increase of cells in G2/M phase in NB cells (Supplementary Fig. S2A-D). Taken together, these observations demonstrate that VRK1 is a robust dependency in tumors of nervous system lineages, and VRK1 KO results in apoptotic cell death.

**VRK2 expression is a biomarker for VRK1 dependency**

To identify genes or pathways that predict VRK1 dependency, we correlated gene expression from the Cancer Cell Line Encyclopedia (CCLE) with VRK1 dependency and found that it is most strongly correlated with the loss of expression of its paralog VRK2 (Pearson Correlation = 0.37, q < 10^-25) (Fig. 2A). VRK2 has two known functional isoforms, VRK2A and VRK2B. VRK2A contains a C-terminal domain that anchors the protein to the endoplasmic reticulum, while alternative splicing of the VRK2B isoform results in the loss of the C-terminal domain and primarily nuclear localization. We evaluated whether the VRK1 dependency could be explained by isoform-specific expression. We observed that expression of the VRK2A and VRK2B isoforms strongly correlate with each other (Supplementary Fig. 3A) and that VRK2A represents the majority of total VRK2 transcripts in tumor cell lines (Supplementary Fig. 3B). The correlation of VRK2 isoform expression with the VRK1 dependency showed that both isoforms predict VRK1 dependency, although the magnitude of difference in VRK2A expression more clearly discerns the VRK2^low^ population (Supplementary Figs. 3C-D).
We next used Celligner, which integrates RNA-sequencing data from TCGA, Treehouse, and TARGET human tumor sequencing studies to identify whether low VRK2 expression correlates with nervous system lineage cancers (10). We found that CNS and PNS tumors exhibit the lowest expression of VRK2 across all tumor lineages, while VRK1 expression was slightly reduced in CNS/PNS tumors as compared to all other tumor lineages (Supplementary Fig. S4A, B). Within NB, we found that VRK2 was not differentially expressed among high MYCN expressing tumors and was slightly elevated in NB tumors enriched for a mesenchymal (MES) gene expression program. However, we note that VRK2 was still repressed as compared to all other tumor lineages (Supplementary Fig. S4C). We also found that in primary DMG models, VRK2 is not expressed significantly (Supplementary Fig. S4D). These observations suggest that VRK2 is expressed at low levels in human cancers of the nervous system, mirroring the Cancer Cell Line Encyclopedia (CCLE) data.

Transcriptional repression is enforced through epigenetic regulation, including methylation of CpG dinucleotides at gene promoters (11). We observed that low levels of VRK2 RNA expression was associated with VRK2 promoter CpG methylation as determined by reduced representation bisulfite sequencing microarrays and was enriched in the CNS and PNS lineages (Fig. 2B). We confirmed, by bisulfite-sequencing, widespread CpG methylation at the VRK2 promoter in a panel of DMG, GBM, and NB cell lines that exhibited low VRK2 expression (Supplementary Fig. S5A). Cell lines that expressed higher levels of VRK2 did not exhibit a similar pattern of CpG methylation. In addition, we found a strong association of CpG island probe methylation with VRK2 expression when we analyzed a cohort of GBM patient tumors (Supplementary Fig. S5B). Gene expression data from healthy neural tissue also demonstrated low VRK2 expression relative to other tissues, suggesting VRK2 promoter methylation may be specific to the neural lineage (Supplementary Fig. S5C).
Based on the link between VRK2 methylation and expression, we next wanted to evaluate whether VRK2 methylation can be detected in primary human tumors. Using methylation array data from the TCGA low-grade and high-grade gliomas dataset, we identified robust VRK2 promoter methylation across subtypes that occurred more frequently in tumors that exhibit IDH mutations, MGMT methylation, the G-CIMP methylator phenotype or lower grade (Fig. 2C). In a separate dataset of over 1000 pediatric high-grade glioma including DMG, we found VRK2 promoter methylation in subsets of histone 3 wild-type and H3K27M tumors but most highly associated with the histone H3 G34R mutation (Fig. 2D). We conclude that VRK2 promoter methylation is observable among cancer subtypes and is a predictor for VRK2 expression and thus a VRK1 dependency.

To verify the synthetic lethal relationship between VRK1 and VRK2 experimentally, we focused on a panel of 4 GBM cell lines with heterogeneous expression of VRK2 (Supplementary Fig. S6A, B). Consistent with our observations in the Cancer Dependency Map, we found greater VRK1 dependency in the two VRK2low cell lines (LNZ308, LN443) than in the two VRK2high cell lines (GAMG, SF172) (Fig. 2E).

We then directly tested whether modulation of VRK2 expression altered the response to VRK1 KO. To create an isogenic experimental model, we deleted VRK2 in the VRK2high SF172 GBM cell line and then introduced either a control or VRK1 sgRNA. We found that VRK2 KO sensitized SF172 to subsequent VRK1 KO (Fig. 2F, Supplementary Fig. S6B, C). In contrast, ectopic overexpression of wild-type VRK1, insensitive to VRK1 sgRNAs via synonymous mutations, but not kinase-inactive (12) VRK1K179E rescued VRK1 KO (Fig. 2G-H, Supplementary Fig. S6D-G). Similarly, VRK2 overexpression in VRK2low GBM lines and primary DMG neurospheres rescued VRK1 dependency, which required VRK2 kinase activity, as expression of the kinase-inactive VRK2K168E mutant did not rescue VRK1 KO-induced cell death (Fig. 2G-I, Supplementary Fig. S6D-H). Overexpression of either VRK2A or VRK2B isoforms, and not their kinase-inactive forms,
rescued VRK1 knockout, suggesting that either isoform suffices to substitute for the loss of VRK1 function (Supplementary Fig. 6I). In summary, VRK1-dependent cell lines require VRK1 kinase activity for survival. Furthermore, VRK2 can act as a surrogate kinase for VRK1, providing a mechanistic explanation for the observed VRK1 dependency in CNS and PNS tumors with low VRK2 expression levels.

Global phospho-proteomics link VRK1 loss to DNA damage and nuclear membrane substrates.

Given the requirement of VRK1 in CNS/PNS tumors, we sought to understand the immediate effects of VRK1 loss. To answer these questions, we designed a degradable VRK1 construct using the dTAG system(13), providing the ability to rapidly deplete exogenous dTAG-VRK1 from cells. Cells were transduced with VRK1 fused with a C-terminal FKBP12$^{F36V}$ domain (dTAG-VRK1), which can be rapidly degraded with a small molecule (dTAG$^V$-1) in a VHL-dependent manner (Fig. 3A). Exogenous expression of dTAG-VRK1 rescued growth defects in the CRISPR KO of endogenous VRK1 in LN443 (GBM), NB-1 (NB) and Kelly (NB) cells, signifying that the fusion protein itself had no effect on canonical VRK1 function (Fig. 3B-C, Supplementary Fig. S7A). However, addition of dTAG$^V$-1 and subsequent degradation of dTAG-VRK1 resulted in significantly reduced cell viability in VRK1 dependent cell lines, establishing a functional system to rigorously examine mechanisms underlying VRK1 dependency (Fig. 3D, Supplementary Fig. S7B).

To identify downstream effectors/pathways of VRK1 kinase, we performed quantitative, phospho-proteomics using the dTAG-VRK1 degrader system (Fig. 3E). dTAG-VRK1-NB-1 cells were treated with dTAG$^V$-1 to identify early phosphorylation changes following degradation of VRK1. Following cell lysis, isobaric, tandem mass tagging (TMT) allowed for de-convolution of pooled samples, and relative quantitation among the samples.
Phosphorylated peptides were enriched using immobilized metal affinity columns (IMAC) and analyzed by mass spectrometry. We performed kinase set enrichment analysis (KSEA) of phospho-peptide dynamics following either 4 or 8 hours of acute dTAG-VRK1 degradation(14). We found that substrates of cell-cycle and mitotic kinases (CDK1 and AURKA) were down-regulated, while substrates of DNA damage response kinases (ATM and WEE1) were up-regulated (Fig. 3F). A total of 208 phospho-proteins were down-regulated at both the 4h and 8h time-points. Gene set enrichment analysis (GSEA) of these overlapping proteins revealed an enrichment of proteins associated with the nuclear envelope and spindle assembly (Fig. 3G). Notably, members of the inner nuclear membrane, LEM-domain family of proteins, including LEMD3, EMD, and TMPO, showed at least one phosphorylation site that was significantly reduced upon VRK1 degradation (Supplementary Fig. S8). Overall, these observations suggest a critical role of VRK1-regulated pathways in mitosis, nuclear envelope and chromatin homeostasis, as well as DNA damage, in CNS and PNS cell models.

**VRK1 and VRK2 loss leads to post-mitotic nuclear membrane deficits and DNA damage**

Phospho-proteomic analysis following VRK1 degradation strongly suggested that VRK1 loss alters the phosphorylation of protein substrates in the nuclear membrane. To visualize nuclear membrane dynamics following VRK1 degradation, we transduced cells with GFP-labelled nuclear lamina-associated proteins: BAF and Emerin. In addition, we also stained cells with anti-LaminB1/2 antibody. Twenty-four hours following degradation of dTAG-VRK1, the nuclear membrane of LN443 GBM cells became misshapen with the formation of lobes and ruffling as well as chromatin bridging between nuclei (Fig. 4A, B). In concordance with reduced cell viability, KO of both VRK1 and VRK2 in VRK2 high cells (SF172) increased irregular nuclei compared to individual kinase KO alone (Fig. 4C, Supplementary Fig. S9A).
The nuclear envelope protein BAF serves to tether chromatin to proteins in the inner nuclear membrane and is a substrate of both VRK1 and VRK2 individually on the Serine-4 (S4) residue(15, 16). During mitosis, phosphorylation of BAF on Serine-4, and subsequent nuclear lamina-DNA un-tethering, is required for mitotic chromosome segregation, as well as post-mitotic nuclear envelope re-assembly(16). BAF(S4) phosphorylation was not detectable in our phospho-proteomic analysis; however, the observed reduced phosphorylation of LEM-domain proteins that bind to BAF (Supplementary Fig. S8) led us to hypothesize that the altered nuclear envelope dynamics observed upon VRK1 KO was due to decreased phosphorylation of BAF. Indeed, dTAGV-1-mediated degradation of exogenous VRK1 in dTAG-VRK1-NB-1 cells and CRISPR KO of VRK1 in the VRK2low DMG neurosphere models BT869Luci and SU-DIPGXIIIP*Luci strongly decreased levels of phosphorylated BAF (S4) but not total BAF (Fig. 4D, Supplementary Fig. S9B). We tested whether ectopic overexpression of the non-phosphorylatable mutant BAFS4A would mimic VRK1/2 loss, and indeed, following doxycycline-induced expression, we observed similar nuclear bridges and distorted nuclear envelope morphology (Fig. 4E, Supplementary Fig. S9C). We also found that doxycycline-induced ectopic overexpression of BAFWT resulted in the same phenotype, perhaps by saturating the phosphorylation capacity of VRK1/2, leading to a shift in the pool of BAF towards its unphosphorylated form (Fig. 4E, Supplementary Fig. S9C). In contrast, overexpression of the phospho-mimetic mutant BAFS4D had no effect on nuclear morphology (Fig. 4E, Supplementary Fig. S9C), suggesting that the Serine-4 phosphorylation site plays a crucial role in VRK1 kinase dependency. Using live-cell imaging, we followed BAF dynamics after dTAG-VRK1 degradation in LN443 GBM cells (Fig. 4F). We observed the same nuclear envelope ruffling and bridging in cells immediately following mitosis. We also found similar phenotypes in NB-1 NB cells, where nuclear membrane ruffling predominated (Supplementary Fig. S9D). Taken together, these findings indicate that BAF Serine-4
phosphorylation by VRK1 is essential for CNS and PNS tumor cells to maintain the integrity of nuclear envelope structure and function.

In addition to observing altered protein phosphorylation at a number of proteins in the nuclear envelope, we also noted an enrichment for substrates of the DNA damage pathways (i.e., substrates of ATM and WEE1) (Fig. 3F). Therefore, we performed imaging of DNA damage response foci. At 7 days following KO of VRK1, we found an increased number of phospho-H2AX foci (S139), phospho-ATR (S428), and phospho-DNAPK (S2056), representing induction of both non-homologous end-joining and homologous recombination pathways of DNA double-strand break repair (Fig. 5A). Corroborating potentiated apoptosis induction, concomitant KO of VRK1 and VRK2 increased DNA damage foci (phospho-H2AX) in VRK2^{high} GBM cells (Fig. 5B) and in two NB cell lines after degradation of dTAG-VRK1 (Fig. 5C).

**VRK1 is a dependency in tumor models in vivo**

To evaluate VRK1 dependency in vivo, we utilized a tamoxifen-inducible CRISPR-Cas9 system(17). Plasmids expressing Cas9, Cre-ERT2, and the pLenti_Switch-ON guide plasmid targeting VRK1 were transduced into LN443 or SF295 GBM cell lines. The “Switch-ON” plasmid has CRISPR guide expression suppressed with a LoxP-STOP-LoxP site. Upon tamoxifen treatment, Cre recombinase is induced which removes the transcriptional stop, and allows expression of the guide RNA. We first validated VRK1 KO efficiency in vitro and observed decreased viability after VRK1 depletion (Fig. 6A). This cell line (SF295; Cas9; CreERT2; pLenti_Switch-ON_sgVRK1) was subsequently injected into flanks of NSG mice (Fig. 6B). Once the xenografts reached ~200 mm³, VRK1 KO in tumor cells was induced by intraperitoneal administration of tamoxifen. VRK1 KO resulted in virtually complete and durable tumor remission in all mice (n=10 tumors) 10-20 days following tamoxifen treatment, whereas tumors in vehicle-treated controls continued exponential growth (Fig. 6C). We harvested a
subset of tumors 7 days following treatment with tamoxifen or vehicle control, stained for phospho-H2AX (S139) and found evidence of increased DNA damage in tumors in which we induced VRK1 depletion by tamoxifen treatment (Fig. 6D). To evaluate the VRK1 dependency in vivo for NB, we introduced doxycycline-inducible, VRK1-specific sgRNAs (Supplementary Fig. S10A) into the Kelly NB cell line and confirmed a robust anti-proliferative effect in vitro (Supplementary Fig. S10B). Tumor cells were subsequently injected into the rear flank of mice and randomized into groups receiving vehicle or doxycycline. We found that VRK1 knockout repressed tumor growth (Supplementary Fig. S10C-D).

To extend these findings to patient-derived models, we generated intracranial xenografts of patient-derived DMG neurospheres that express ZsGreen-Luciferase, Cas9, and a doxycycline-inducible guide vector targeting control or VRK1. Cells were stereotactically injected into the striatum of NSG mice. We induced VRK1 deletion by treating these animals with doxycycline. 30 days post injection, we observed decreased luciferase signal in sgVRK1 mice as compared to sgCtrl (p=0.08) (Fig. 6E-F). Decreased tumor growth corresponded to increased survival of mice with VRK1 KO neurospheres (p=0.1) (Fig. 6G). Taken together, we observed in three independent models and cancer lineages that VRK1 depletion leads to tumor repression in vivo, suggesting that VRK1 is a potential therapeutic target in VRK2 promoter-methylated adult and pediatric gliomas and neuroblastomas.
Discussion

Synthetic lethal interactions are a potential source of new biomarker-linked targeted cancer therapy. Specifically, synthetic lethal interactions may involve tumor-specific down-regulation of a gene or pathway, resulting in sensitivity to inhibition of another gene or pathway. The success of PARP inhibitors in multiple cancers with homologous recombination pathway deficiency provides evidence that this approach can lead to clinical benefit(18, 19). Specifically, \textit{BRCA1/2} mutations in breast cancer result in dependency on the non-homologous end-joining DNA repair pathway that is exploited by PARP inhibitors such as olaparib(20).

Gene paralogs are potentially promising sources of synthetic lethal interactions as they usually exhibit strong sequence homology and functional redundancy. For example, alpha-enolase (\textit{ENO1})-deleted GBMs are sensitive to KO of its paralog gamma-enolase (\textit{ENO2}), blocking glycolysis(21). Loss-of-function \textit{ARID1A}-mutant cancers are sensitive to \textit{ARID1B} KO, causing destabilization of the SWI/SNF chromatin remodeling complex(22). Synthetic lethality in the context of paralogs can occur by epigenetic mechanisms as well. For example, in \textit{NXT2}-methylated NB cell lines, \textit{NXT1} is required to facilitate stability of the essential RNA-exporting protein NXF1(23). Targeting paralogs holds the promise of an increased therapeutic ratio as one interaction partner may be a silenced tumor suppressor or may be co-silenced with other tumor suppressors but not affected in normal tissues. Here, we discovered that tumors with low VRK2 expression are dependent on its paralog, VRK1. IDH-mutant gliomas, with their hyper-methylated phenotype, also exhibit high VRK2 gene methylation. In fact, VRK2 promoter methylation is highly enriched in tumors of the CNS and PNS lineages. During development, differential gene methylation is involved in neuronal cell-fate determination, neuronal plasticity, and memory formation(24). Such lineage-specific, differential methylation may lead to other synthetic lethal vulnerabilities in cancer.
The VRK family of atypical Serine-Threonine kinases was initially discovered for their homology with vaccinia virus B1 kinase, which is required for viral replication(25). The family branches early from the kinase evolutionary tree and consists of the functional kinases VRK1 and VRK2 and a pseudokinase VRK3(12). Clinically, VRK1 expression has been associated with high grade and poor prognosis in patients with glioma(26), whereas VRK2 expression is correlated with improved survival in high-grade astrocytoma(27). In the physiological context, VRK1 localizes to the nucleus where it is thought to phosphorylate substrates involved in DNA damage response (e.g., histone H2AX) and mitosis (e.g., BAF)(12). Previous work showed that VRK1 is the primary kinase that phosphorylates BAF during mitosis(16). BAF phosphorylation removes its association with chromatin and LEM-domain containing proteins of the nuclear envelope, such as Emerin. The paralog, VRK2, exists as two main isoforms: VRK2A and VRK2B. VRK2B, which is expressed at lower levels, does not have the C-terminal membrane anchor and so is expressed both in the cytoplasm and the nucleus(28). It has been shown to share substrates with VRK1, namely p53(28). VRK2A has also been shown to phosphorylate BAF, similar to VRK1, and modulates the association of BAF with the nuclear membrane in mitosis(15). Unlike VRK1, which localizes to the nucleoplasm, VRK2A associates with A-type Lamins of the nuclear envelope. Birendra et al. hypothesized that VRK1 may modulate BAF phosphorylation in the nucleoplasm, while VRK2A modulates BAF at the nuclear envelope(15). This difference in localization may explain the only partial rescue of VRK1 loss by VRK2A that we observed (Fig. 2H, I). Further, while our data show a robust connection between VRK2 expression and VRK1 dependency, some tumor lineages, like Ewing sarcoma, demonstrated high VRK2 expression and a strong dependency on VRK1, suggesting that VRK1 may also be required for other tumor cell functions in particular contexts. Future work is needed to understand the VRK1 dependency in VRK2high models.

Together, our observations are consistent with a synthetic lethal interaction of VRK1 and VRK2 (Fig. 7). In VRK2high tumors where the VRK2 promoter is unmethylated, both VRK1 and VRK2
may phosphorylate BAF during mitosis to mediate nuclear envelope dis-assembly. However, in VRK2\textsuperscript{low} tumors, loss of VRK1 prevents BAF phosphorylation during mitosis. Thus, our data suggest that VRK1 depletion results in retained association of nuclear envelope fragments with mitotic chromosomes, leading to aberrant nuclear envelope re-assembly, nuclear bridging between daughter cells, and ultimately to DNA damage and apoptotic cell death. VRK2\textsuperscript{low} tumors may also be sensitized to DNA damaging effects, independently of VRK1 loss-of-function(29). Further research is warranted to investigate the potential for VRK1 inhibitor and DNA-damaging agent combinations in VRK2\textsuperscript{low} tumors.

Small molecule kinase inhibitors have been investigated for their potential differential effect on VRK1 versus VRK2 activity(30, 31). Vázquez-Cedeira et al. noted that, based on amino-acid sequence and protein structural differences from other kinases, both VRK1 and VRK2 are predicted to have low promiscuity and be relatively insensitive to extant kinase inhibitors(30). They further showed that in a small-molecule library screen of 20 kinase inhibitors, few molecules decreased VRK1 or VRK2 kinase activity even at high concentrations (100 µM). The compounds that did inhibit kinase activity did so with ATP concentrations three orders of magnitude lower than intracellular levels, which the authors noted may limit in vivo use. Recently, a small molecule, based on an aminopyridine scaffold, was developed that showed potent activity against VRK1 in vitro (IC\textsubscript{50} = 150 nM)(31). However, this compound did not significantly decrease viability in cell culture(31). Potent kinase inhibitors that show differential effect against VRK1 versus VRK2 do not yet exist. A degrader strategy, as modeled in this current study, may represent an alternate approach to targeting VRK1 as a growing number of small-molecule degraders (e.g. PROTACs, molecular glues, etc.) targeting specific proteins are undergoing clinical trials in diverse cancers(32).

For VRK1 inhibition to be a viable therapy option, a significant therapeutic ratio is required where normal tissues are spared while cancer cells are targeted. The existence of human genetic
variants and mouse transgenic models allow for an approximation of potential on-target toxicities. A rare germ-line mutation in VRK1 (R358X) results in lack of VRK1 protein production, and manifests in pediatric patients as spinal muscular atrophy with pontocerebellar hypoplasia (SMA-PCH)(33). Although VRK2 is expressed in most tissues, it has low expression in normal brain tissue, especially the cerebellum, which may explain the CNS phenotype of mutant VRK1. Partial KO of Vrk1 by gene-trapping resulted in a slight reduction in brain size, mild motor dysfunction, and male infertility in mice(34, 35). These findings suggest that side effects of VRK1 inhibition may be tolerated in adults.

In summary, by integrating genome-wide, loss-of-function genetic screens with RNA-sequencing and DNA methylation, we identified VRK1 as a selective vulnerability in CNS and PNS cancers with low VRK2 expression. Taken together, these studies suggest that targeting VRK1 in cancers that harbor promoter-methylated VRK2 is a potential therapeutic strategy.

Methods

Cell Culture

Neuroblastoma (NB-1, Kelly) and GBM (LN443, SF172, GAMG, LNZ308) cell lines were collected from the Cancer Cell Line Encyclopedia and Cancer Dependency Map projects and obtained from the Broad Institute. The cell lines that express pLX_311-Cas9 were generated by Project Achilles (https://depmap.org/portal/achilles)(20). SK-N-BE(2)C were purchased from ATCC. LAN-1 was kindly gifted by Rani George at DFCI. SK-N-BE(2)C, LAN-1, and GBM cell lines were grown in 10% DMEM supplemented with glutamine, penicillin and streptomycin, and incubated at 37°C in 5% CO2. Kelly and NB-1 were grown in RPMI-1640 supplemented with 10% FBS and glutamine, penicillin, and streptomycin, and incubated at 37°C in 5% CO2. Cell lines identities were validated by STR profiling and tested negative for mycoplasma with
MycoAlert Mycoplasma Detection Kit (Lonza, Cat#LT07-418) prior to experimental use. Cell lines used in this study are summarized in Supplementary Table 1.

**Neurosphere Culture**

Patient-derived H3K27M and H3WT-glioma neurosphere lines were established at Dana-Farber Cancer Institute (BT869/BT869Luci; available from the DFCI Center for Patient Derived Models), and Hospital Sant Joan de Deu Barcelona (HSJD-DIPG007, HSJD-GBM001) as previously described (36–38). Neurosphere lines SU-DIPGXIIILuci, SU-DIPGXIIIP*Luci, SU-DIPGXV, SU-pcGBM2, and SU-DIPG48 were a kind gift from Michelle Monje at Stanford University. H3K27M-glioma cells were grown as neurospheres in tumor stem media (TSM) base (38) supplemented with B27 minus vitamin A (Thermo Fisher Scientific), human growth factors (EGF, FGF, PDGF-AA, PDGF-BB [Shenandoah Biotechnology]) and heparin (Stemcell Technologies) in ultra-low attachment flasks. Indicated cell models expressing luciferase were generated as previously described (39). Neurosphere cultures were dissociated for passaging using Accutase cell detachment solution (Stemcell Technologies) for 3-5 minutes at 37°C. All neurosphere models were authenticated by high resolution short tandem repeat (STR) profiling (Molecular Diagnostics Core, Dana-Farber Cancer Institute). Whole exome or whole genome sequencing was conducted on neurosphere models to obtain copy number alterations.

**Public Data Sets**

Log2(TPM) + 1 RNA-sequencing, CERES gene dependency scores, and DNA methylation array data were downloaded from the Dependency Map portal (https://depmap.org/portal/, CCLE expression: 21Q3)(40). Density plots displaying the distribution of CERES scores per tumor lineage were generated with ggridges software in R (v4.0.3). Projection of VRK2 or VRK1 expression for tumor lineages from TCGA/TARGET/TREEHOUSE tumor datasets was generated using UMAP projection plots available in the Celligner alignment portal (available

**Lentiviral production**

Lentiviral production was conducted using HEK293T cells, as described on the Broad Institute Genetic Perturbation Platform (GPP) web portal: (https://portals.broadinstitute.org/gpp/public/). Briefly, high-titer lentivirus was produced by transfection of HEK293T cells with the lentiviral vector, psPAX2 (Addgene #12260) and vsvg (Addgene #8454) with Lipofectamine 2000 (Life Technologies Cat#11668027). Viral supernatant was collected 48 hours after transfection and filtered with 0.2 micron filter. Cells were transduced with virus in the presence of 5 μg / mL polybrene and selected with blasticidin (5 μg / mL) or puromycin (1 μg / mL) according to appropriate selection agent. dTAG-HA-VRK1-expressing cell lines were derived by first expressing stable dTAG-HA-VRK1 prior to infection with sgVRK1#2.

**Single guide RNAs (sgRNAs)**

The single guide RNA (sgRNA) sequences used for the validation experiments were designed using the web-based program (CRISPick) provided by the Broad Institute GPP: (https://portals.broadinstitute.org/gppx/crispick/public). For the CRISPR-mediated gene KO (KO), annealed oligonucleotides carrying the sgRNA target sequence as well as the cloning adapters were inserted into a guide RNA-expressing vector that also expresses a puromycin-resistance gene (pXPR_003, Broad Institute GPP), the vector expressing the hygromycin-resistance gene (pXPR_016, Broad Institute GPP), or guide vectors with GFP or mCherry co-expression (LCV2_EGFP or LCV2_mCherry). LCV2_EGFP and LCV2_mCherry were gifts
from Jason Moffat (Addgene plasmid #155098 and #155096)(42). The targeting sequences for the individual sgRNAs are outlined in Table 1. For tamoxifen inducible sgRNA expression, we utilized the CRISPR-Switch system as described by Chylinski et al.(17). Guides were cloned into the vector pLenti_Switch-ON which was a gift from Ulrich Elling.

**Open Reading Frame (ORF) Constructs**

Codon-optimized, sgRNA-resistant DNA fragments encoding VRK1WT, VRK1K179E, VRK2WT were purchased from gBlock (IDT) and cloned into pDONR-221 via BP gateway cloning. VRK2K168E was generated through the QuickChange II site-directed mutagenesis kit (Agilent Technologies) using the primer: 5’- GAATATGTTTCATGGTATAGAAGCAGCAAATCTAC-3’. BAFWT and its mutants (S4A and S4D) were synthesized with Gateway-compatible AttP flanking sites (IDT) and also cloned into pDONR-221. Entry clone pENTR/D_creERt2 was a gift from Leonard Zon (Addgene plasmid # 27321)(43). VRK1WT was further cloned into PLX305(C-TAG) (Addgene #91798) and VRK1WT, VRK1K179E, VRK2WT, VRK2K168E, and creERt2 were further cloned into PLX307 (Addgene #41392) via LR Gateway cloning (LR clonase II enzyme mix, Thermo-Fisher Scientific, Cat# 11791-100). BAFWT, BAFS4A, and BAFS4D were cloned into Doxycycline-inducible expression vector PLXI403 (Addgene #41395).

Cells were transduced with virus in the presence of 5 μg / mL polybrene and selected with blasticidin (5 μg / mL) or puromycin (1 μg / mL) according to appropriate selection agent. dTAG-HA-VRK1-expressing cell lines were derived by first expressing stable dTAG-HA-VRK1 prior to infection with sgVRK1 #2.

Further information and requests for reagents should be directed and fulfilled by the lead contact William Hahn. Plasmids for the C-terminus, dTAG-VRK1, sgRNAs#1,#2, and #4, and cDNAs for VRK1WT, VRK1K179E, VRK2WT, VRK2K168E, BAFWT, BAFS4A, and BAFS4D will be made available on Addgene.
**Bisulfite sequencing**

Genomic DNA was extracted using the Qiagen DNeasy Blood and Tissue kit (Qiagen #69505). DNA was bisulfite-converted using the Qiagen Epitect Bisulfite kit (Qiagen #59104). Bisulfite-converted DNA was PCR-amplified with the EpiMark HotStart Taq (NEB #M0490) using the following primers: VRK2 TSS primer set: forward 5’- TAGGTTGTGGTATAGGAGATTTAATATT -3’, reverse 5’- AATAAAAAACTATATTACTACCTCCACCC -3’. PCR was performed at an annealing temperature of 59 degree Celsius for 40 cycles. PCR products were visualized on 2% E-Gel EX agarose gels (Fisher Scientific, Cat#G401002) for correct size and band patterning. PCR products were then column purified using the QiaQuick PCR purification kit and submitted for difficult template Sanger sequencing with Azenta with both the forward and reverse primers.

**Cell Proliferation Assay**

The viability effect of VRK1 KO in GBM cell lines and primary DMG neurosphere models was determined by the clonogenic cell proliferation assay. Briefly, cells were transduced with guide RNA sgCtrl or sgVRK1. Following 1 wk under selection, 0.5-1x10^4 cells per well were seeded in 6-well Falcon plates (Fisher Scientific, Cat #087721B) in triplicate. Media was changed every 5-7 days. After 7-10 days, cell numbers were counted using the Vi-Cell automated cell counter (Beckman Coulter, Cat # 731196).

For viability effect in neuroblastoma cell lines, 5 x 10^5 cells of either sgChr2, sgVRK1#1, sgVRK1#2, or sgVRK1#4 were plated onto 6-cm dishes. For dTAG-VRK1 cells, 5 x 10^5 cells were plated and attached 16 hours prior to incubation with either DMSO vehicle or 1 µM dTAG^V^-1. After 2-3 days, cells were detached, counted, and the number of doublings relative to the prior timepoint were calculated. Groups were replated at 5 x 10^5 cells per group, and the same steps were repeated every 2-3 days for a total of 14 days. For days in which fewer than
5 x 10^5 cells were counted, then all the cells were plated. Population doublings were calculated by the total cells compared to the number of seeded cells. Values were added to the previous time point, starting at 0 for day 0. dTAG-VRK1 cells remained in vehicle or 1 µM dTAG^V-1 for the entirety of the 14 days.

For crystal-violet staining, cells were plated in 6-well plates and stained/fixed with 2.5mg/mL solution of crystal-violet (Sigma, Cat #C3886) in 20% methanol.

**Cell-cycle and Apoptosis Assay by Flow Cytometry**

Cells were harvested, washed, and then fixed in ice-cold 70% ethanol and then re-suspended in stain buffer containing propidium iodide and RNase (BD, Cat # 550825). Apoptosis was assessed using annexin V and propidium iodide staining according to the manufacturer’s instructions (Invitrogen, Cat # 88-8005-74). Samples were analyzed on a BD LSR-II flow cytometer. Data analysis was completed using the cell-cycle analysis package in FlowJo ver.10.8.0 (Treestar).

**Western Blot**

Cell pellets were lysed with CST lysis buffer (Cat #9803) that was supplemented with phosphatase (Roche, Cat #04906845001) and protease inhibitors (Roche, Cat #11836170001) and diluted to 1 µg / µL in sample buffer.

Approximately 35 µg of whole-cell lysate protein was loaded into wells and resolved in 4-12% acrylamide gradient gels. Whole cell lysates were run with MOPS running buffer solution for high molecular weight proteins and MES running buffer solution for low molecular weight proteins. Acrylamide gels were wet-transferred onto nitrocellulose or PVDF membranes for at least 90 minutes. Primary antibodies listed in Table 2 were diluted in 3% BSA in TBS-T and incubated overnight at 4 degrees Celsius. Rabbit polyclonal anti-phospho-BAF antibody was
a gift from Dr. Robert Craigie (National Institute of Health, Bethesda, MD). Secondary LICOR goat anti-rabbit IRDye® 800 (Licor, Cat #926-32211) or goat anti-mouse IRDye®680 (Licor, Cat #926-68070) antibodies were diluted at 1:5,000 in TBS-T and incubated at room temperature for 1 hour. All membranes were imaged on LICOR Odyssey infrared imaging system at 680 and 800nm wavelengths and analyzed with ImageStudio Odyssey Lite Software (LICOR).

**Incucyte Caspase 3/7 assay**

LN443-Cas9 cells were transduced with guide RNA sgCtrl or sgVRK1. Following 1 wk under selection, 5x10⁴ cells per well were seeded in 24-well Falcon plates (Fisher Scientific, Cat #353047). 5 mM of IncuCyte Caspase-3/7 Green Apoptosis Assay Reagent (Sartorius, cat #4440) as well as 1:500 of Nuclight Rapid Red Dye (Sartorius, cat #4717) were added to each well. The plate was transferred into the IncuCyte S3 Live-Cell Analysis System (Sartorius, cat #4647) for imaging. Phase contrast images and green/red fluorescent channel images were captured using the 10x objective magnification every four hours for a total of 48 hours. For each well, four images containing both phase contrast and green channel data were obtained.

Using the IncuCyte S3 Analysis System software, cell confluence over time was quantified along with the intensity of green (apoptosis positive) objects in mm²/well. Computer generated masks for confluence and green area, trained on a sample set of images across time points and confluency levels, were manually checked for accuracy. Each metric was averaged over the four quadrants per well. First, the green object total intensity metric for each well was divided by the confluence metric for each well, yielding a normalized measure of Caspase-3/7 activity.

**Mass Spectrometry Sample Preparation**
Samples were processed with the SL-TMT protocol and phospho-enrichment methods described previously(44). Data were acquired with Orbitrap Eclipse mass spectrometer with FAIMS and coupled to a Proxeon NanoLC-1200 UHPLC (ThermoFisher Scientific). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE(45) partner repository with the dataset identifier PXD030599.

**Mass Spectrometry Data Analysis**

A suite of in-house software tools were used for .RAW file processing and controlling peptide and protein level false discovery rates, assembling proteins from peptides, and protein quantification from peptides as previously described(46)). MS/MS spectra were searched against a Uniprot Human database with both the forward and reverse sequences. Database search criteria are as follows: tryptic with two missed cleavages, a precursor mass tolerance of 50 ppm, fragment bin tolerance of 0.02, static alkylation of cysteine (57.02146 Da), static TMT labeling of lysine residues and N-termini of peptides (304.2071 Da), variable oxidation of methionine (15.99491 Da) and variable phosphorylation on serine, threonine, and tyrosine (+79.966 Da). Phosphorylation site localization was determined using the AScore algorithm (47) using a threshold of 13 corresponding to 95% confidence in site localization. TMT reporter ion intensities were measured using a 0.003 Da window around the theoretical m/z for each reporter ion. Proteins with <100 summed signal-to-noise across all channels and <0.5 precursor isolation specificity were excluded from the final dataset.

Ratios were calculated between peptide quantitation at 4hrs post-dTAGV-1 versus 0hrs and at 8hrs post-dTAGV-1 versus 0hrs. P-values for each ratio were calculated using Student’s T-test. From the fold change values and p-values, Kinase-Substrate Enrichment Analysis (KSEA; https://casecpb.shinyapps.io/ksea/) was performed with NetworKIN score cutoff of 3(14, 48–50).
**Immunofluorescence**

The nuclear membrane and DNA damage foci were visualized by immunofluorescence using the following procedure. LN443-Cas9, SF172-Cas9, NB-1, and Kelly cells were transduced with various sgRNAs. BAF and Emerin were imaged by transducing GFP-tagged constructs. EGFP-BAF was a gift from Daniel Gerlich (Addgene plasmid #101772)(51). pLVX-EF1a-EGFP-Emerin-IRES-Hygromycin was a gift from David Andrews (Addgene plasmid #134864)(52). For the BAF experiment, inducible BAF wild-type or mutant expression vectors were transduced in LN443 cells. Following selection (~5-7days), doxycycline induction (0.5µM for 3 days), or dTAGV-1 treatment (0.5µM for 1 day), cells were seeded onto #1½ cover-glasses (Sigma, Cat #CLS285018) in 6-well Falcon plates (Fisher Scientific, Cat #087721B). The next day, cells were fixed with 4% Formaldehyde (VWR, Cat# 100503) diluted in PBS. Fixed cells were permeabilized and blocked with 0.1% Triton-X in 50% Odyssey Blocking Buffer (Licor, Cat# 927-70001) in PBS for 1hr at room temperature. The cells were then incubated with the primary antibody at the specified dilution in 0.1% Triton-X with 50% Odyssey Blocking, overnight at 4°C. After washing three times with PBS, cells were incubated with the secondary antibody at the specified dilution in 0.1% Triton-X with 50% Odyssey Blocking for one hour at room temperature. The cells were then washed three times with PBS and mounted onto glass slides with ProLong Gold anti-fade mounting media with DAPI (Life Technologies, Cat #P36941). Imaging was conducted using an Olympus IX73 inverted microscope, an Olympus DP80 CCD camera, and 20x/40x/100x objectives. Antibodies used are outlined in Table 2.

**Live-cell Imaging**

dTAG-VRK1-LN443 or dTAG-VRK1-NB-1 cells were transduced with EGFP-BAF as previously described. 2x10^4 - 5x10^4 per well were then seeded in MatTek 24-well, glass-bottom plates (Fisher Scientific NC1284979). 4 hours following dTAGV-1 addition, the plate was imaged using
the 40x objective in a Leica DMi8 Widefield microscope with automated stage, an Oko-Lab stage-top incubator, and Oko-Lab CO2/Humidity controller. 3x3 fields per well were imaged every 20min for 48hrs. Image stitching was performed using the Leica LAS X software platform. Subsequent image analysis was performed using ImageJ ver. 1.53m.

**In vivo, tamoxifen-inducible sgRNA xenografts**

This study was approved by the Institutional Animal Care and Use Committee (IACUC) of Dana-Farber Cancer Institute and performed under protocol 04-101. IACUC guidelines on the ethical use and care of animals were followed. SF295 cells constitutively expressing Cas9 were infected with tamoxifen-inducible sgRNAs targeting Chr2-2 or VRK1. 6.0e6 cells were resuspended in 1:1 vol/vol matrigel:media and subcutaneously implanted into the left and right fat pads of 6-to-8-week old female NSG (NOD-scid/IL2Rgnull) mice (Jackson Laboratory stock no. 005557). When either tumor was ~100-200 mm$^3$ mice were randomized to tamoxifen or vehicle treatment. Tamoxifen was delivered by 3 daily intraperitoneal injections of ~3mg. Tamoxifen (Sigma-Aldrich) was prepared at a stock concentration of 30mg/mL in corn oil. The control group received an equal volume of corn oil. Tumors were measured by Vernier caliper and volume was determined using the standard formula $[(\text{length} \times \text{width}^2)/2$ where length is always the larger measurement]. Animals were euthanized once they reached a humane endpoint and tumor tissue was flash frozen or formalin fixed for later protein extraction. All mice that developed tumors were included in the analysis.

7 days following treatment with tamoxifen or vehicle control, tumors were collected from a subset of xenografted mice. These were fixed in formalin and embedded in paraffin. Immunohistochemistry was performed following standard protocol, staining for phospho-H2AX (S139) (CST cat# 9718; 1:500).

**In vivo, doxycyline-inducible sgRNA xenografts**
This study was approved by the Institutional Animal Care and Use Committee (IACUC) of Dana-Farber Cancer Institute and performed under protocol 04-101. IACUC guidelines on the ethical use and care of animals were followed. Kelly cells constitutively expressing Cas9 were infected with doxycycline-inducible sgRNAs targeting Chr2-2 or VRK1. 4.0e6 cells were resuspended in 1:1 vol/vol matrigel:media and subcutaneously implanted into the left and right fat pads of 6-to-8-week old female NSG (NOD-scid/IL2Rgnull) mice (Jackson Laboratory stock no. 005557). When either tumor was ~50 mm³ mice were randomized to doxycycline-containing (625ppm) or regular diet. Tumors were measured by Vernier caliper and volume was determined using the standard formula [(length x width²)/2 where length is always the larger measurement]. Animals were euthanized once they reached a humane endpoint and tumor tissue was flash frozen or formalin fixed for later protein extraction. All mice that developed tumors were included in the analysis.

**Intracranial xenografts**

This study was approved by the Institutional Animal Care and Use Committee (IACUC) of Dana-Farber Cancer Institute and performed under protocol 18-006. IACUC guidelines on the ethical use and care of animals were followed. Intracranial xenografts were established with the patient-derived neurosphere line, SU-DIPGXIIIP*, with doxycycline-inducible guides. Cells were injected stereotactically into the striatum of 6 week-old female NSG mice treated with buprenorphine 0.05mg/kg and anesthetized with isoflurane 2–3%. The skull of the mouse was exposed through a small skin incision, and a small burr hole was made using a drill at the selected stereotactic coordinates zeroed on bregma: -2.5mm X, -1mm Y and -3.0mm Z. The cells (100,000 cells in 1μL PBS per mouse) were injected using a 26-gauge Hamilton syringe. After closing the scalp with suture and staple, mice were returned to their cages placed on a warming pad and visually monitored until full recovery. The same day following the procedure, CRISPR guide expression was induced through doxycycline chow. Mice were then checked daily for signs of distress,
including seizures, weight loss, and tremors, and euthanized as they developed neurological symptoms, including head tilt, seizures, sudden weight loss, loss of balance, and/or ataxia.

Tumor growth was monitored every 1-2 weeks using the IVIS Spectrum In Vivo Imaging System (PerkinElmer). Briefly, mice were injected intra-peritoneally with 75 mg/kg D-luciferin potassium salt (Promega E1605) in sterile PBS, and anesthetized with 2% isoflurane in medical air. Serial bioluminescence images were acquired using the automated exposure setup. The peak bioluminescence signal intensity within selected regions of interest (ROI) was quantified using the Living Image Software (PerkinElmer), and expressed as photon flux (p/sec/cm²/sr). Representative planar bioluminescence images were displayed with indicated adjusted minimal and maximal thresholds.

**Statistics**

For statistical tests of significance, the statistical test and P-value are described in the respective figure legends. All t tests are two-sided unless otherwise indicated. A P-value of 0.05 was used as the cutoff for significance unless otherwise indicated. These values were calculated in GraphPad Prism (version 9.3.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com) or R version 4.0.2 and Rstudio version 1.2.5042. Error bars represent SD unless otherwise indicated. All duplicate measures were taken from distinct samples rather than repeated measures of the same sample.

**Study approval**

This study was approved by the Institutional Animal Care and Use Committee (IACUC) of Dana-Farber Cancer Institute and performed under protocols 04-101 and 18-006.
The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE(45) partner repository with the dataset identifier PXD030599.
Author contributions


ordered by experimental and draft writing contribution.
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Figure 1. VRK1 is a dependency in GBM, NB, and DMG

A. Histogram plots showing VRK1 CERES dependency scores in over 900 cell lines, representing 25 different cancer lineages from the DepMap dataset (21Q3). Compared to all other lineages cell lines in the CNS (p = 2x10^{-12}) and PNS (p = 3x10^{-18}), lineages were significantly more dependent on VRK1.

B. Differential dependency of gene KO in CNS and PNS cell lines versus all other lineages. Gene effect size is calculated as the difference in average CERES score between lineage groupings and q-value is determined by Limma eBayes methodology. The top enriched dependencies in CNS/PNS lineages are annotated.

C. VRK1 protein expression following expression of 4 different sgRNA in the NB-1 neuroblastoma cell line. The top three guides with greatest VRK1 loss were carried forward in subsequent experiments.

D. Population doubling assay following VRK1 KO with three separate guides in NB-1 cells. sgCtrl represents a non-targeting control guide (n=3; mean ± SD).

E. Single guide (sg)VRK1 KO after 14 days in cell lines representing NB (n=3), GBM (n=2), and DMG (n=2) models. (n ≥ 3; mean ± SD plotted).

F. Time-course of CASP3/7 activity, as measured by cleavage of a peptide reporter, following VRK1 KO in LN443 cells (n=3; mean ± SD). Total reporter fluorescent signal is normalized by cell confluence. Significance at each time point was determined by two-way ANOVA (treatment x time). *p < 0.05. Scale bar: 20 µm.

G. Quantification of Annexin-V positive cells following VRK1 KO with two separate guides in three cell lines representing NB and DMG lineages after 7 days. (n=3; mean ± SD; from 2 separate experiments).

*p < 0.05, **p < 0.001, ***p < 0.0001; Significance was determined by two-tailed, Student’s T-test (panel E), and one-way ANOVA with Tukey’s (panels D and G).
Figure 2. VRK1 dependency is correlated with VRK2 expression

A. Whole genome Pearson correlations between gene expression from CCLE (21Q3) and VRK1 dependency in the Depmap database (21Q3) and adjusted P-values.

B. Scatterplot showing VRK1 dependency versus VRK2 expression. Extent of VRK2 promoter methylation is indicated by dot size. Red dots represent cell lines of CNS lineage, and blue dots PNS lineage.

C. VRK2 promoter methylation status stratified by clinical characteristics across the TCGA GBM-LGG cohort. IDH: Isocitrate dehydrogenase; MGMT: O6-methylguanine-DNA methyltransferase; G-CIMP: Cytosine-phosphate-guanine (CpG) island methylator phenotype; LGG: Low Grade Glioma; GBM: Glioblastoma multiforme. Violin plots with mean (solid line) and 1st and 3rd quartiles (dashed line).

D. VRK2 promoter methylation in pediatric high-grade gliomas and DMG with wild-type histone H3 and mutant histone H3 (K27M or G34R). Data from Mackay et al., 2017 (39). Violin plots with mean (solid line) and 1st and 3rd quartiles (dashed line).

E. Cell viability following 14 days KO of VRK1 in VRK2low LNZ308 and LN443 cell lines and VRK2high GAMG and SF172 cell lines.

F. Cell viability analysis 14 days following VRK1 KO in VRK2high GBM cell line (SF172), expressing control CRISPR sgRNA or sgRNA targeting VRK2. (n=3; mean ± SD).

G. Immunoblot showing the overexpression of exogenous VRK2WT, VRK1WT, and kinase-inactive VRK1K179E in NB-1 NB cells with or without VRK1 KO.

H. Cell viability analysis for NB-1 cells in panel (G) following 14 days of VRK1 KO in VRK2WT, VRK1WT, and kinase-inactive VRK1K179E overexpressing cells. (n=3; mean ± SD).

I. Effect of VRK2WT or VRK2K168E overexpression on LN443 GBM cell viability following 14 days VRK1 KO. (n=3; mean ± SD).

*p < 0.05, **p < 0.001, ***p < 0.0001; Significance was determined by two-tailed, Student’s T-test (panel E), and one-way ANOVA with Tukey’s test (panels C,D, F, H, I)
Figure 3. Global phospho-proteomics following acute VRK1 degradation

A. Schematic of dTAG-VRK1 degrader system. The conjugated FKBP12<sup>F36V</sup> binding domain allows small-molecule (dTAG<sup>V-1</sup>) mediated recruitment of the VHL ubiquitin ligase complex, targeting exogenous VRK1 for proteasomal degradation.

B. Schematic of VRK1 degrader experiments. Exogenous dTAG-VRK1 is transduced to rescue CRISPR KO of endogenous VRK1. Exogenous dTAG-VRK1 is then under the control of the small molecule degrader (dTAG<sup>V-1</sup>) allowing for acute down-regulation.

C. Immunoblot validation of the dTAG-VRK1 degrader system in NB-1 neuroblastoma cells. Exogenous dTAG-VRK1 was degraded with dTAG<sup>V-1</sup>. Endogenous VRK1 was independently targeted with CRISPR KO. sgLacZ is a non-targeting guide control.

D. Cell viability analysis of dTAG-VRK1-NB-1 cells following addition of either vehicle control or 0.5 µM dTAG<sup>V-1</sup>. Significance at each time point was determined by two-way ANOVA (treatment x time). *p < 0.05, **p < 0.001.

E. Schematic of the quantitative, global phospho-proteomic experiment. Samples were generated in triplicate at 4h and 8h post dTAG<sup>V-1</sup> (0.5µM) addition. Following trypsin digestion, peptides are tagged with isobaric, tandem mass tags (TMT), and then combined. Phospho-enrichment was performed using immobilized metal affinity columns (IMAC), and then run on an Orbitrap mass-spectrometer. MS2 spectra offer peptide ID’s and sample deconvolution through attached mass tag.

F. Kinase set enrichment analysis of phosphorylation site dynamics following acute degradation of exogenous VRK1. Kinase substrates of CDK1 and AURKA were significantly down-regulated following degradation (blue), while substrates of WEE1, BRSK1, and ATM were significantly up-regulated (red).

G. Top panel: Venn diagram showing number of unique proteins with a decrease in phosphorylation for at least one phosphorylation site in dTAG<sup>V-1</sup> treated samples. Bottom panel: Dot plots showing the overlap of downregulated protein phosphorylation (208 proteins) with select categories of the C5 MSigDB library. All gene sets have FDR ≤ 0.05 as determined by one-tailed Fisher’s exact test.
Figure 4. VRK1 loss is associated with nuclear envelope malformation

A. Nuclear membrane morphology in the LN443 GBM cell line following exogenous VRK1 degradation by dTAG\textsuperscript{V-1} after 1 day. White arrows point to nuclear bridges. Blue arrow points to micro-nuclei.

B. Top, left: Quantitation of irregular nuclei, by LaminB1 staining, following VRK1 degradation as seen in Fig. 4A (n=3 fields of >50 cells each; mean ± SD). Top, right: Quantitation of nuclear bridges following VRK1 degradation as seen in Fig. 4A (n=3 fields of >50 cells each; mean ± SD). Bottom, left: Quantitation of irregular nuclei following VRK1 degradation in the NB-1 NB cell line expressing GFP-BAF seen in Supplementary Fig. S9D (n=8 fields of >50 cells each; mean ± SD).

C. Quantitation of irregular nuclei, by LaminB1 staining, following KO of both VRK1 and VRK2 in SF172 as seen in Supplementary Fig. S9A. (n=4 fields of >50 cells each; mean±SD).

D. Immunoblot of phosphorylated BAF (S4) and total BAF following dTAG\textsuperscript{V-1} treatment in dTAG-VRK1-NB-1 cells (left panel) or KO of VRK1 with two independent sgRNAs in BT869Luci DMG neurospheres (right panel). Represents 2 independent experiments.

E. Left panel: Nuclear envelope morphology (GFP-Emerin) following doxycycline-induced expression of BAF mutants in LN443 GBM cell line after 3 days: wild-type (WT), S4A (non-phosphorylatable), S4D (phospho-mimetic). Right panel: Quantitation of nuclear bridging phenotype in LN443 cell lines expressing BAF mutants (n=3; mean ± SD).

F. Live-cell, time-lapse experiment showing nuclear envelope morphology following VRK1 degradation in LN443 (dTAG\textsuperscript{V-1} addition at t=0h). White arrows point to cells undergoing mitosis. Blue arrows point to chromatin bridges. Represents 2 independent experiments.

Scale bars: 20 µm. *p < 0.05, **p < 0.001, ***p < 0.0001; Significance was determined by two-tailed, Student’s T-test (panel B) and one-way ANOVA with Tukey’s test (panels C and E).
Fig. 5A

Nucleus pH2AX(S139)  Nucleus pATR(S428)  Nucleus pDNAPK(S2056)

Percentage of cells with >2 foci

Fig. 5B

SF172; Cas9; sgCtrl  SF172; Cas9; sgVRK2

ggCtrl  sgVRK1

Nucleus pH2AX(S139)

Fig. 5C

Kelly; VRK1-dTag; sgVRK1-Cas9  NB1; VRK1-dTag; sgVRK1-Cas9

dTAG-1-  dTAG-1+

Percentage of cells with >2 foci
Figure 5. VRK1 loss results in DNA damage

A. Left panel: Nuclear foci of a panel of DNA damage markers (phospho-H2AX(S139), phospho-ATR(S428), phospho-DNAPK(S2056)) following KO of VRK1 in LN443 GBM cells for 7 days. Right panel: Quantitation of percent of cells with >2 phospho-H2AX foci following VRK1 KO (n=3 fields of >50 cells each; mean ± SD).

B. Top panel: Phospho-H2AX foci following 7 days double KO combinations of sgCtrl/sgCtrl, sgCtrl/sgVRK1, sgCtrl/sgVRK2, and sgVRK1/sgVRK2. Bottom panel: Quantitation of percent of cells with >2 phospho-H2AX foci following these double-KO combinations (n=4 fields of >50 cells each; mean ± SD).

C. Top panel: Phospho-H2AX foci following VRK1 degradation with 0.5 µM dTAGV-1 in both Kelly and NB-1 NB cell lines. Bottom panel: Quantitation of percent of cells with >2 phospho-H2AX foci following dTAGV-1 addition (n=4 fields of >30 cells each; mean ± SD). Scale bars: 20 µm. *p < 0.05, **p < 0.001, ***p < 0.0001; Significance was determined by two-tailed, Student’s T-test (panel A and C), and one-way ANOVA with Tukey’s test (panel B).
Figure 6. VRK1 is a dependency in vivo

A. Left panel: Immunoblot of VRK1 following tamoxifen-induced expression of sgVRK1 in LN443 cells. Right panel: Clonogenic assay in LN443 cells 14 days following tamoxifen-induced KO of VRK1.

B. Schematic of the in vivo xenograft experiment. The SF295 GBM cell line was transduced with Cas9, Cre-ERT2, and “Switch-ON” guide plasmids and implanted in NSG mouse flanks. When the tumors reached a pre-specified size (200mm³), the mice were treated with tamoxifen. When the tumor size reached ~500mm³ or 40 days following treatment, the mice were euthanized.

C. Tumor volume measurements over time of the flank xenografts. * represents injection of tamoxifen or corn oil vehicle control.

D. Left panel: representative H&E sections of tumors taken from xenografted mice, 7 days following treatment with tamoxifen or vehicle control (Scale bar: 50µm). Sections were stained with an antibody against phospho-H2AX. Right panel: quantitation of number of phospho-H2AX positive cells per 0.5mm² in flank xenografts following tamoxifen or vehicle treatment (n=4 fields; mean ± SD) (*p < 0.05; Two-tailed, Student’s T-test).

E. Representative bio-luminescent imaging of intracranial xenografts of primary DMG neurospheres with doxycycline-inducible control vs. VRK1 targeting guides taken 30 days post doxycycline induction.

F. Quantification of bio-luminescent images from panel E (sgCtrl vs. sgVRK1, p=0.08)

G. Kaplan-Meier survival curves showing overall survival for mice injected with sgCtrl or sgVRK1 DMG neurospheres into the cranium. Significance was determined by log-rank test (sgCtrl vs. sgVRK1, p=0.10).
Figure 7. Model for mechanism of synthetic lethality between VRK1 and VRK2

A. Schematic showing proposed mechanism of synthetic lethality between VRK1 and VRK2. In VRK2 unmethylated tumors (top), VRK2 compensates for VRK1 loss in the phosphorylation of BAF during mitosis. In VRK2^low tumors (bottom), loss of VRK1 leads to retention of BAF during mitosis and the continued association of the nuclear envelope with chromatin. This leads to impaired chromosomal segregation and DNA damage, including nuclear bridging.
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Table 1. CRISPR guide sequences used
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Table 2. List of antibodies