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Title

- Dynamic multi-OMICs of glioblastoma reveal sensitivity to neddylation inhibition dependent on nuclear PTEN and DNA replication pathways
- Nuclear PTEN mediates MLN4924 sensitivity in GBM

Authors

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Summary

Neddylation inhibition kills glioma cells by DNA damage and re-replication events which are dependent on wild-type nuclear PTEN.

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25 Abstract

26 Finding and matching drugs to treat subsets of cancers remains a daunting challenge of
 27 precision medicine. Neddylation inhibition, affecting posttranslational protein function and
 28 turnover, is a promising therapeutic approach to cancer including glioblastoma (GBM). We
 29 report vulnerability to neddylation inhibition by MLN4924 in a subset of GBM preclinical
 30 models and identify mechanisms underlying this differential response. MLN4924 treatment
 31 of GBM cells with intact *PTEN* die due to DNA damage and re-replication events. Loss of
 32 *PTEN* drives resistance to MLN4924. Time-course transcriptomics elevates PTEN
 33 signaling, DNA replication, and chromatin instability pathways as significant differentiators
 34 between MLN4924-sensitive and resistant models. Shotgun proteomics corroborates these
 35 findings and also identifies elevated TOP2A in resistant models. TOP2A inhibitors
 36 combined with MLN4924 prove synergistic. We show that *PTEN* status serves as both a
 37 novel biomarker for MLN4924 response in GBM and reveals a vulnerability to TOP2A
 38 inhibitors in combination with MLN4924.

40 MAIN TEXT

42 Introduction

43 Glioblastoma (GBM) is the most common malignant (46.6%) and most lethal (5.5% five-
 44 year survival rate) CNS tumor (1). The current standard of care for GBM is maximal
 45 resection of the tumor tissue, followed by radiation therapy concomitant with temozolomide
 46 (TMZ), and, lastly, adjuvant TMZ (2). Although TMZ remains the main chemotherapy drug
 47 for GBM, its overall survival benefit is relatively modest (~2.5 months). Other more recent
 48 advances in GBM therapies include the use of tumor treating fields and novel approaches
 49 to immunotherapy (3, 4). However, both require further investigation to solidify their place

in GBM's standard of care, and the most aggressive primary lethal brain tumor still calls for novel therapeutic development.

Analogous to ubiquitination in reaction scheme and enzyme classes used, neddylation is a post-translational modification pathway that conjugates NEDD8 to target protein substrates (5). NEDD8 is first activated by its E1 ligase NEDD8 activating enzyme (NAE), then transferred to its E2 ligase Ubc12, and, via NEDD8 E3 ligases, is conjugated to its target substrate(s) (5, 6). MLN4924 (also known as Pevonedistat) is a small molecule inhibitor that binds to NAE and creates a covalent NEDD8-MLN4924 adduct (7). In doing so, it blocks further enzymatic interactions and subsequent NAE activity (7). Although many different neddylation targets have been investigated in recent years, the most commonly studied neddylation targets are the cullin subunits of cullin-RING ligases (CRLs) (5, 6). These CRLs are E3 ligases in the similar but separate ubiquitination pathway. Covalent modification of the cullin subunit by NEDD8 is required for CRL ubiquitin ligase activity (5, 8, 9). Rather than inhibiting the entire proteasome, inhibiting a specific E3 ligase will only prevent the degradation of the proteins whose ubiquitination is dependent on that specific E3 ligase (10). Through this mechanism, MLN4924 inhibits NAE, the neddylation pathway, and the degradation of CRL substrates (5, 10). While proteasome inhibitors (e.g., bortezomib) have been clinically effective in treating cancers, adverse effects such as peripheral neuropathy and frequently acquired resistance indicate room for improvement within the treatment strategy of targeting protein turnover (11).

MLN4924 has proven to be effective in multiple types of cancers, including GBM, but the determinants of vulnerability are not fully understood (12). Hua *et al.* demonstrated

that the activation of the neddylation pathway correlates with glioma grade and overall survival in GBM (12). Inhibition of neddylation using MLN4924 results in the accumulation of well-known CRL substrates and in growth arrest of GBM cells *in vitro* and *in vivo* (12). One of these well studied CRL substrates is CDT1, a DNA licensing factor that recruits the MCM2-7 complex to initiate DNA replication in dividing cells (5, 13, 14). CDT1 is tightly regulated by two CRLs (SCF/CRL1 & CRL4); MLN4924 blocks neddylation of these CRLs, stabilizes CDT-1, and consequently induces DNA re-replication, DNA damage, and cell death in various cancer models (13-16). High throughput techniques have identified DNA damage repair (DDR) and chromatin dynamics pathways as key mediators of response to re-replication stress (17, 18). Specifically, Garcia *et al.* elegantly demonstrated that the depletion of DNA damage repair machineries, perhaps surprisingly, reduces the efficacy of MLN4924 alone or in combination with other chemotherapy agents (18). While these factors may begin to elucidate the effects of MLN4924 on cancer cells, the mechanisms driving the differential vulnerability to this treatment need to be evaluated in detail for precision cancer care.

This study aims to explain some of the factors underlying differential sensitivity of GBM to MLN4924. Here, we show that the efficacy of neddylation inhibition in GBM is dependent on status of the phosphatase and tensin homolog (PTEN) gene, a commonly mutated and/or deleted tumor suppressor in cancer, including in approximately 35% of GBM cases (19). PTEN is well known for antagonizing the PI3K/AKT pathway. While we found that PTEN-status dependent resistance is independent of its phosphatase functions, including the PI3K/AKT signaling axis, we did find it contingent on PTEN's ability to bind chromatin. Following MLN4924 treatment, time-course transcriptomic and proteomic analyses revealed that this response was due to PTEN's non-canonical roles in

the nucleus and that sensitivity to MLN4924 is associated with enrichment of DNA replication and chromatin dynamics pathways. Transcriptomics and proteomic analyses also identified vulnerabilities that were targetable with novel drug combinations. Our results reveal that targeting the neddylation pathway not only serves as a promising therapy for GBM but also exemplifies the broader potential of precision medicine in the treatment of cancer.

06 Results

07

08 MLN4924 sensitivity in a subset of GBM designates the neddylation pathway as a therapeutic 09 target

10 MLN4924 has been shown to suppress tumor growth in diverse cancer and GBM models
11 by stabilizing pro-apoptotic factors and cell cycle checkpoint proteins among other
12 substrates (5, 10, 12). Across 860 established cancer cell lines in The Cancer Therapeutics
13 Response Portal (CTRP), MLN4924 demonstrated a broad range of efficacy across all
14 cancers (20). The lower quartile of models had an AUC of 10.8 or less indicating at least
15 170 cell lines were sensitive *in vitro* (Figure 1A). Based on AUC values from the CTRP,
16 we selected two sensitive and two non-sensitive established GBM cell lines (LN18, GB1,
17 M059K, and SNU1105, respectively) for subsequent experimental investigation to
18 determine mechanisms governing this differential response. The results of a drug dose
19 response (DDR) assay validated LN18 and GB1 as sensitive ($IC_{50} = 0.195 \mu M$ and $IC_{50} =$
20 $0.279 \mu M$, respectively) and M059K and SNU1105 as non-sensitive ($IC_{50} = 5.50 \mu M$ and
21 $IC_{50} = 20.9 \mu M$, respectively) (Figure 1B). Differential vulnerability to MLN4924 was
22 further substantiated by the significant differences between the ability of LN18 and
23 SNU1105 to form colonies in the presence of 0.1 and 0.5 μM MLN4924 (Figure 1C).
24 Differential vulnerability to MLN4924 in PDX models was evident in GBM43 ($IC_{50} = 0.797$
25 μM) and GBM39 ($IC_{50} = 13.1 \mu M$) (Figure 1D). The differential sensitivity to neddylation
26 inhibition was not observed with proteasomal inhibition by bortezomib in either established
27 or PDX models (Figure 1E, 1F). Viability of *TERT*-immortalized normal human astrocytes'
28 (NHA-hTERT) was not significantly affected by MLN4924 at doses as high as 100 μM

(Figure 1G), whereas bortezomib treatment of NHA-*hTERT* had an IC₅₀ of 5.81 nM which suggests lower toxicity of MLN4924 compared to bortezomib (Figure 1G).

MLN4924 induces re-replication, polyploidy, and DNA damage in sensitive cell lines

Inhibition of neddylation has been shown to induce re-replication and G2 arrest, and we sought to uncover whether the differential MLN4924 vulnerability was associated with differences in cell cycle progression (5, 10, 12). Using flow cytometry, we investigated whether there were cell-line dependent effects on the cell cycle following treatment with MLN4924. All cell lines had a dose-dependent increase in G2/M populations (Figure 1H), which is consistent with previous findings (5, 10, 12). In the resistant cell lines, we found significantly decreased S-phase populations at baseline and at various concentrations of MLN4924 treatment (Figure 1H). Interestingly, it has also been shown that S-phase cells are more susceptible to re-replication following MLN4924 treatment, and this was reflected in the significantly higher tetraploid (4N) populations in the sensitive models (Figure 1H) (14). Thus, while all models increased G2/M fraction, resistant GBM models have smaller S-phase populations and less re-replication following MLN4924 treatment.

Given these differences in cell cycle progression between sensitive and resistant groups, GB1 and M059K were treated with 0.5 μ M of MLN4924, and their protein lysate was collected at serial time-points to investigate differences in CRL substrate and DNA damage accumulation (Figure 1I). Accumulation of WEE1, a G2 checkpoint protein, in both cell lines is consistent with the G2 arrest seen in our and others' models (12). Multiple groups have found that knockdown of p21 increases MLN4924 efficacy; however, we found p21 accumulation in both sensitive and resistant models, and, thus, concluded that the differences in MLN4924 response were not due to p21 accumulation or lack thereof (Figure

1I) (14, 17, 18). MLN4924's main mechanism of DNA damage is re-replication from CDT-1 accumulation (14, 17, 18). Consistent with the larger fraction of tetraploid (re-replicating) population in sensitive models, we found more DNA damage — seen in elevated H2AX levels — and potentially more DNA repair machinery recruitment — seen in modest elevated levels of RAD51 and Ku70 — in the sensitive GB1 cell line (Figure 1I). Interestingly, both sensitive and resistant cell lines accumulated CDT1 post-MLN4924 treatment, despite it accumulating slightly earlier in GB1. Accordingly, other factors, such as decreased S-phase populations, must be contributing to the lack of re-replication in M059K and SNU1105.

Absence of PTEN drives resistance to MLN4924 in select GBM models

We aimed to identify any genetic markers that associated with sensitivity or resistance to MLN4924. We used the Cancer Cell Line Encyclopedia (CCLE) and the Genomics of Drug Sensitivity in Cancer (GDSC) databases to identify the largest genetic differentiators of response to MLN4924 in primary brain tumors (www.broadinstitute.org/ccle and <https://www.cancerrxgene.org/>). First, we grouped 21 GBM lines based on the median of the AUC values into sensitive and resistant groups, noting that here we took resistant to mean the inverse of sensitive and distinguished it from its usage in acquired resistance. We then compared their differences in mutation frequency of all genes between these two groups. For the 6 genes with the largest difference in mutation frequency between sensitive and resistant response to MLN4924, *PTEN* mutation status showed the largest delta, $P=0.05$, by chi-squared test (Figure 2A). While *PTEN* was mutated in nearly half of the GBM lines in the non-sensitive group, none of the sensitive group cell lines harbored *PTEN* mutations (Figure 2A). Using the same databases, we analyzed 28 different glioma cell lines (derived from all grades of glioma) and found a similar distinction between *PTEN* status

and MLN4924 response (Supplemental Figure 1). After grouping the glioma cell lines into three bins — *PTEN* null, mutated, or wild type — we found that *PTEN* null glioma lines had the highest median area under the curve (AUC) value followed by *PTEN* mutated and, then, *PTEN* wild type glioma lines (Supplemental Figure 1). The distribution of AUC values among the three groups was also noteworthy: whereas the *PTEN* mutated and wild type glioma models had broad distributions of AUC values, the cell lines in the *PTEN* null group had tightly clustered high AUC values (Supplemental Figure 1).

After identifying *PTEN* status as a potential biomarker for MLN4924 response, we turned to our four GBM models — LN18 and GB1 (sensitive) and M059K and SNU1105 (resistant) — to further query genetic determinants of response. Using cBioPortal for Cancer Genomics, we tested the top six genes that arose from the CCLE analysis (Figure 2B) (19). Of the six genes analyzed in our four established models, the only genotype that differentiated sensitive and non-sensitive groups was *PTEN* status. The resistant models, M059K and SNU1105, had a non-functional *PTEN* status via a truncation mutation and deep deletion, respectively. *PTEN* is not only commonly mutated in GBM but also regularly post-transcriptionally silenced (21), which underscores the need to validate the differential expression of PTEN at the protein level (Figure 2C).

We next tested whether the absence of PTEN had a causal role in resistance to MLN4924. In our sensitive, *PTEN*-expressing models, we examined the effects of transiently knocking down *PTEN* with siRNA on MLN4924 response. In both sensitive models, LN18 and GB1, we observed a three- to four-fold increase in IC₅₀ values following *PTEN* knockdown by siRNA (Figure 2D, E, Supplemental Figure 2C). This increase in IC₅₀ value was validated in the colorectal cancer cell line HCT116 and its isogenic pair in which *PTEN* was knocked

out (Figure 2F, Supplemental Figure 2A). To test whether the relationship between PTEN and MLN4924 response would be consistent in the reciprocal direction — i.e., expressing PTEN into a typically *PTEN*-null model — the *PTEN*-null glioma stem cell model, GSC11, was genetically engineered to re-express PTEN and was treated with MLN4924. Consistent with our previous findings, we saw an approximately four-fold decrease in the IC₅₀ value of the *PTEN* expressing GSC11 model (Figure 2G). Taken together, these results indicate that the absence of PTEN is a functional driver of resistance to neddylation inhibition by MLN4924.

PTEN-driven resistance to MLN4924 is independent of its phosphatase activity and the PI3K/AKT pathway

We evaluated whether this phenotypic shift was dependent on the loss of PTEN's phosphatase activity and consequent elevated PI3K/AKT signaling. We measured baseline and post-treatment levels of phosphorylated AKT (p-AKT) in the established sensitive and resistant GBM models. As expected, at baseline we found higher levels of p-AKT in the resistant *PTEN*-null cell lines compared to the sensitive *PTEN*-WT models (Figure 3A). Levels of p-AKT across multiple time-points following MLN4924 treatment showed no consistent changes or trends among the sensitive or resistant groups (Figure 3B), leaving open the role of the phosphatase activity in vulnerability to MLN4924. We further tested whether this *PTEN*-null resistance was a consequence of lost phosphatase activity. GSC23, a known *PTEN*-null glioma stem cell model, was engineered to express PTEN or to express PTEN with a G129R mutation (phosphatase dead) (22). Re-expression of PTEN dramatically decreased GSC23's IC₅₀ value (Figure 3C). The phosphatase dead PTEN transgene sensitized the cell line to MLN4924 treatment by a strikingly identical amount as

PTEN wild type. Taken together, these results indicate that PTEN's role in MLN4924 response is independent of its phosphatase activity and the PI3K/AKT axis.

Nuclear PTEN contributes to MLN4924 sensitivity through its ability to bind chromatin

More recent evidence has shown that PTEN has phosphatase-independent roles in the nucleus where it regulates DNA replication and repair, chromatin dynamics, chromosome stability, and many other biological processes (23, 24). Ma *et al.* recently demonstrated that the majority of nuclear PTEN is phosphorylated at tyrosine residue-240 (pY240-PTEN) and that this phosphorylation was essential for PTEN's ability to bind chromatin, recruit RAD51 to DNA, and participate in DNA damage repair (25). Mutating the phosphorylation site (Y240F PTEN) abrogates these PTEN nuclear functions, and thus we sought to uncover whether this would affect sensitivity to MLN4924. Using a set of mouse embryonic fibroblast models with WT PTEN and Y240F PTEN knocked in, we found that Y240F PTEN increased IC₅₀ of MLN4924 approximately 2.5-fold, effectively pheno-copying PTEN deletions (Figure 3D). Thus, we conclude that PTEN facilitates MLN4924 sensitivity through its ability to bind chromatin in the nucleus.

Dynamic transcriptomic analysis reveals enrichment in pathways linking PTEN nuclear functions with MLN4924 sensitivity

Because the role of PTEN in mediating vulnerability to MLN4924 was not driven by its canonical functions, we used dynamic transcriptomic and proteomic approaches to uncover other candidate mechanisms. We collected RNA from the four long term glioma cell lines after 2, 8, and 24 hours of MLN4924 treatment, then performed next generation RNA sequencing. Comparing transcriptomes from sensitive (LN18 and GB1) and resistant (M059K and SNU1105) models, differentially expressed RNA species were processed

using Ingenuity Pathway Analysis to identify biological pathways that were differentially deregulated. PTEN signaling was classified as the most downregulated pathway in the resistant cell lines post-MLN4924 treatment compared to sensitive cell lines (Figure 4A).

Given that changes in the non-canonical functions of PTEN, which manifest with loss of PTEN or blocking its Y240 phosphorylation (Figure 3D), accompany resistance to MLN4924, we investigated whether there were transcriptomic differences between sensitive and resistant models in processes related to these non-canonical events. We identified 50 different biological processes related to PTEN's nuclear functions and carried out GSVA on the four models at baseline and following treatment (Supplemental Figure 3). While the overall heat map clearly differentiated sensitive and resistant models, we narrowed the analysis to the 21 gene sets that significantly differentiated (p value < 0.05) the sensitive and resistant groups in both the long term GBM models and the additional 27 GBM models in CCLE; GSVA score of those 21 gene sets were visualized in a supervised fashion (Figure 4B). Broadly speaking, the sensitive models were enriched in processes related to cell cycle checkpoints, DNA replication, and chromosome dynamics, which is in line with many of the functions of the CRL substrates that MLN4924 targets (Figure 4B).

Low enrichment in DNA fork replication pathways can predict MLN4924 resistance in GBM

PTEN has been shown to be directly involved in DNA fork progression, protection, and processing, and these pathways were among the most significant that differentiated sensitive and resistant groups (23, 24). We aimed to test whether enrichment in replication fork processing and protection associated gene sets could predict MLN4924 response. We performed unsupervised GSVA in our models at baseline and post-treatment, and our models clustered according to their MLN4924 response status (Figure 4C(i)). Specifically,

enrichment in these gene sets was associated with sensitivity to MLN4924 while low enrichment was associated with non-sensitivity (Figure 4C(i)). We conducted the same analysis in 27 GBM cell lines from CCLE and, again, found that enrichment, or the lack thereof, of DNA fork pathways to be a significant predictor of MLN4924 response in this broader set of GBM models (Figure 4c(ii)). Interestingly, we also found differences in the dynamics of key genes involved in DNA replication and chromatin dynamics pathways at the individual transcript level as well (Figure 4D). Specifically, in our resistant models, there were increases in the transcription of *EP400* and *SMARCA1* (chromatin dynamics) and *MCM5*, *MCM6*, *RBI*, *MMS22L*, and *CCNE1* (DNA replication) that were not observed in our sensitive models (Figure 4D). Consistent with these widespread transcriptional changes, significant increases in the transcription factors *E2F1* and *MYC* were also detected in the resistant models post-treatment (Figure 4D). As master regulators of DNA replication, DNA damage, and cell cycle checkpoints, the increase in E2F1 and MYC integrate the changes in transcription observed in the resistant models following MLN4924 treatment (Figure 4D) (26). Thus, while sensitive GBM models are enriched in transcripts related to DNA replication at baseline, non-sensitive models upregulate the E2F1 and MYC transcription factors and, as a result, their targets involved in DNA replication post-treatment (Figure 4D).

Proteomic analysis validates PTEN and DNA replication pathways as key mediators of MLN4924 response

Because the inhibition of neddylation by MLN4924 directly targets protein stability and the turnover of diverse CRL substrates, we also looked towards the proteome to investigate the biological landscape of MLN4924 vulnerability. We collected protein from LN18 and SNU1105 at 0, 2, 8, and 24 hours post-MLN4924 treatment at 100 and 500 nM and carried

out shotgun proteomics analysis. Proteins were identified by querying mass spectra data against a canonical human protein database, followed by differential analysis to identify candidate markers. Given the previous findings from our genomic and transcriptomic analyses, we investigated PTEN signaling and DNA replication progression via proteomic analysis. We conducted GSVA against the human Gene Ontology Annotation (GOA) database that comprises of 7134 biological processes and pathways. PTEN gene sets from Reactome showed enrichment in LN18 and a lack thereof in SNU1105 (Figure 5A). Interestingly, of the four PTEN gene sets, “Regulation of PTEN localization” was the most differentially enriched between LN18 and SNU1105 (Figure 5A). This gene set includes E3 ligases that monoubiquitinate PTEN and facilitate its nuclear transport in a mechanism distinct from the aforementioned pY240-PTEN (27). As such, our proteomic analysis provides further evidence that there are differences in the regulation of PTEN and its localization that are distinct between GBM cell lines of different vulnerability to MLN4924.

We then performed a K-mean clustering on the enrichment scores of all gene sets from the GSVA analysis, limiting the analysis to 100 clusters. We then looked into the most differentially enriched clusters and found that among them was one cluster (Figure 5b) with 217 gene sets including “Replication Fork Processing,” eight other DNA replication gene sets, 15 different DNA damage repair related gene sets, and nine different cell cycle checkpoint and regulation related gene sets (Figure 5B, Supplemental Table 1). An unsupervised heat map of 43 DNA replication pathway gene sets revealed “Replication Fork Processing” as one of the largest differentiators of response to MLN4924 (Supplemental Figure 4). Principal component analysis (PCA) of the cluster above grouped the models in a cell-line dependent fashion except for SNU1105 24 hours after treatment of 500 nM MLN4924, which began to cluster with LN18 (Figure 5B). Similar grouping is observed in

Supplemental Figure 4. Accordingly, the sensitive model is enriched in proteins required for replication progression at baseline, but the resistant model, at least transiently, upregulates these proteins and begins to resemble the sensitive model post-MLN4924 treatment.

Chromosome maintenance and dynamics pathways as differentially enriched between sensitive and resistant models

To identify biological mechanisms manifesting at the protein level, we identified the top ten positively and negatively enriched gene sets in both LN18 and SNU1105 from a total of 7134 gene sets. Seven of the ten most positively enriched gene sets in LN18 are among the ten most negatively enriched in SNU1105 (Figure 5C). Conversely, six of the top ten gene sets negatively enriched in LN18 are among the top ten positively enriched in SNU1105 (Figure 5C). Of the top 10 positively enriched in LN18 were the “establishment of protein localization to chromosome” and “establishment of protein localization to chromatin” gene sets, which is one of PTEN’s fundamental nuclear roles and is in accordance with our transcriptomic analysis. Upon closer examination, we extracted the enrichment scores for 34 different gene sets related to chromosome dynamics and clustered the samples in an unsupervised fashion (Figure 5D). The heat map clustered all models in a cell line-dependent fashion, and our sensitive and resistant models were, thus, clearly differentiated by their enrichment scores for chromosome dynamics pathways.

Models sensitive to MLN4924 are enriched in CIN gene sets

The chromosomal instability gene sets — CIN70 and CIN25 — were derived from 18 gene expression data sets and consist of the genes most associated with total functional aneuploidy (28). Perhaps paradoxically, the most positively enriched gene sets include those

that are essential for chromosomal segregation/dynamics, cell cycle checkpoints, and mitotic progression; i.e., biological processes considered essential to genomic stability (28). In consideration of the overlap between the biology underlying MLN4924 differential sensitivity and that in the chromosomal instability gene sets, we conducted GSVA using the CIN70 and CIN25 gene sets for the transcriptomic data and proteomic data on LN18 and SNU1105 (Figure 6). There were distinct differences in the enrichment of chromosomal instability gene sets between our sensitive and resistant models at the level of RNA (Figure 6A) and protein (Figure 6B): sensitive models were enriched in these gene sets relative to the resistant models. Similar to the previous findings, proteomic analysis of SNU1105 treated for 24 hours with 500 nM MLN4924 shows enrichment in chromosomal instability proteins and begins to resemble the sensitive cell lines at the level of protein (Figure 6B).

An even smaller chromosomal instability gene set (CIN4) was created by narrowing CIN70 to four genes based on their high correlation with histological grade and CIN70 signature expression (29). CIN4 consists of *AURKA*, *FOXMI*, *TOP2A*, and *TPX2*, and based on our previous findings, we examined the individual protein dynamics of the four genes (Figure 6C). The AURKA and FOXMI protein abundance signals were not detected above the noise threshold; however, TOP2A and TPX2 showed large differences between LN18 and SNU1105 in relative protein abundance both at baseline and through treatment (Figure 6C). While the sensitive LN18 model had a higher TOP2A concentration at baseline, the resistant SNU1105 showed significant increases in TOP2A abundance at eight hours after a 100 nM concentration treatment and at 24 hours after a 500 nM concentration treatment (Figure 6C). A volcano plot of SNU1105 identified TOP2A and TPX2 as two of the twelve significantly altered protein abundances after eight hours of a 100 nM MLN4924 treatment (Figure 6D). Conversely, neither TOP2A nor TPX2 were significantly altered in LN18 under these

conditions. Given the availability of many clinically active TOP2A inhibitors, we hypothesize that TOP2A may be a therapeutically advantageous target to potentiate MLN4924 in otherwise resistant GBM.

TOP2A inhibitors synergize with MLN494 in resistant models

We first determined baseline levels of TOP2A in our established models. We found that the two PTEN-null models expressed less TOP2A than the PTEN-WT models at baseline, which differentiated sensitive and resistant models accordingly (Figure 6E). We next sought to determine the effects of MLN4924 treatment on TOP2A concentration. Validating our proteomics findings, significant increases in TOP2A levels were only detected in the post-treatment lysate of MLN4924 resistant models (Figure 6F). Investigating whether this increase in TOP2A was contributing to neddylation inhibition resistance, we carried out a set of drug combination experiments with MLN4924 and two different TOP2A inhibitors: doxorubicin and etoposide. These combination studies revealed that neddylation inhibition by MLN4924 and TOP2A inhibition showed predominately additive, or even antagonistic, effects in the two sensitive models LN18 and GB1, as indicated by combination indices near or above one (Figure 6G) (30). Conversely, in the two resistant lines, there was robust synergy between MLN4924 and both TOP2A inhibitors (Figure 5G). While every combination index was less than one (indicating synergy), SNU1105, the most resistant cell line, had a combination index as low as 0.30 and required nanomolar concentrations of MLN4924 to reach 50% viability when given with low doses of either TOP2A inhibitor. Our findings show that, mechanistically, TOP2A mediates non-sensitivity to neddylation inhibition in models resistant to MLN4924.

03 Discussion

04 In this work, we establish that loss of phosphatase and tensin homolog (PTEN) drives
 05 resistance to the neddylation inhibitor MLN4924 in GBM cells. The tumor suppressive
 06 capabilities of PTEN have traditionally been explained by its antagonism of the
 07 PI3K/AKT/mTOR signaling axis, but PTEN has been implicated in a wide variety of
 08 biological processes, such as genomic stability, stem-cell renewal, the tumor
 09 microenvironment, cell migration, and DNA damage repair (23). Many of these less
 10 canonical and phosphatase independent roles are directly or indirectly mediated through
 11 events in the nucleus, including PTEN's participation in faithful DNA duplication (24).
 12 PTEN regulates DNA replication by maintaining DNA fork progression, protection, and
 13 processing through diverse mechanisms (24). Through these roles, we propose that PTEN
 14 facilitates DNA damage and cell death associated with CDT1 driven re-replication post-
 15 MLN4924 treatment.

16
 17 Garcia *et al.* and others have demonstrated that the knockdown of DNA Damage Repair and
 18 chromatin dynamics pathways decrease MLN4924 efficacy (17, 18). While essential to
 19 DNA Damage Repair, these pathways are also inextricably linked to DNA replication (18).
 20 BRCA1/2, FANCD2, and RAD51 are shown to not only stabilize replication forks but
 21 also protect them from MRE11-mediated degradation (18). As such, it is proposed that
 22 this protective complex results in a higher likelihood of fork collisions and DNA damage
 23 under the CDT1 driven re-replicative state; our findings are highly consistent with this
 24 model of vulnerability (18). PTEN is essential for maintaining faithful DNA replication,
 25 especially under replicative stress, as it stabilizes RPA1 on ssDNA, maintains other key
 26 replication factors on chromatin, and participates in stalled fork recovery (23, 24). PTEN
 27 has also recently been shown to directly associate with DNA, increase chromatin

accessibility, and facilitate RAD51 loading onto double strand breaks (25). RAD51 is essential to resolving stalled DNA forks, and depletion of EP400, which encodes a protein that makes chromatin more accessible for DNA Damage Repair proteins like RAD51, completely eliminated synergy between MLN4924 and mitomycin suggesting that this similar function of PTEN is crucial for MLN4924 efficacy (18). Moreover, it has independently been shown that PTEN depletion results in premature S-phase exit and that S-phase cells are more susceptible to re-replication by MLN4924 (14). Our GBM preclinical models reaffirmed these findings, as *PTEN*-null resistant lines had significantly reduced fractions of S-phase populations and significantly less re-replication (Figure 1H). Thus, absence of PTEN not only decreases CDT1 induced re-replication but also diminishes the DNA damage associated with any re-replication (Figure 1I). From these results, we conclude PTEN loss drives resistance to MLN4924 through its nuclear roles of facilitating DNA replication.

Our analysis of transcriptional and proteomic signatures of MLN4924 response and the widespread biological programming changes following drug treatment further emphasizes the role of DNA replication pathways mediating vulnerability to the cytotoxic consequence of neddylation inhibition. The level of enrichment in DNA fork protection and processing gene sets predicted MLN4924 response to a high level of accuracy in our GBM cell models as well as the CCLE's 28 GBM models. While nearly all *PTEN* mutated GBM lines were resistant to MLN4924, not all *PTEN* WT lines were sensitive, which suggests PTEN is a necessary but insufficient factor for MLN4924 sensitivity. In our surviving resistant models, there were increases in the transcription factors E2F1 and MYC, which both regulate the transcription of proteins involved DNA replication, DNA damage, and cell cycle progression (26). Not surprisingly, there were also transcriptional increases in some of their

well-known targets, such as cyclin E, MCM complex proteins, and other genes involved in these pathways (Figure 4D). While these models are less sensitive to MLN4924, we posit that the stabilization of CDT1 and its initiation of DNA replication may induce replication stress and result in these transcriptional programming changes. Further studies are required to understand how this transcriptional response may contribute to MLN4924 resistance.

Through dynamic proteomics, we found that PTEN-null dependent resistance associated with increased stabilization of TOP2A. Fundamentally, TOP2 enzymes generate double strand breaks (DSB) in DNA, pass a DNA duplex through the induced break, and re-ligate the break (31). In doing so, TOP2 enzymes uncoil intertwined DNA strands (31). This function is crucial to relieving torsional stress from DNA supercoiling and resolving catenanes that form as a result of DNA replication (31). As such, TOP2 enzymes, especially the more stable and mitotically active TOP2A, mediate many intracellular processes including decatenation, G2 checkpoint induction, and the DNA damage response (31). Interestingly, we found that MLN4924 treatment resulted in increased TOP2A protein abundance in resistant models that is not observed in the sensitive models (Figure 6F). This increase in TOP2A likely functionally contributes to this *PTEN*-status dependent resistance, as two independent TOP2A inhibitors significantly synergized with MLN4924 in resistant models (Figures 4C, 4D). However, this synergy could come from MLN4924 increasing TOP2 poison efficacy as well. TOP2 poisons work by stabilizing a DNA-enzyme intermediate at the stage where the enzyme induces a DSB without re-ligation (31). Thus, in our resistant models, TOP2 poisons are potentially more effective following MLN4924 treatment because there is more TOP2A, DSBs, and total DNA damage. Thus, it is likely that the robust synergy in the resistant models is not only a result of TOP2 poisons increasing MLN4924's efficacy but also MLN4924 increasing TOP2 poisons' efficacy.

As a disease that has not seen lasting advances in its standard of care for decades, GBM calls for novel therapeutic approaches, and given GBM's heterogeneity, precision medicine stands out as an appealing treatment modality for this tragic disease. Phase III clinical trials for GBM have a notably high failure rate (32). One strategy to increase the success of GBM clinical trials is having better, more specific enrollment criteria: with a better understanding of whether a patient will respond to a drug based on genomic and other -OMIC markers, physicians become empowered to tailor treatments to each individual's tumor and its underlying biology. While precision medicine has become one of the most recognized treatment modalities in cancer, its use to date in treating GBM has been disappointing (33). In this work, we demonstrate that PTEN is a driver of resistance to MLN4924. Uncovering the PTEN-mediated mechanism underlying resistance to MLN4924 not only expands the knowledge of neddylation's role in cancer but may also facilitate the design of more effective clinical trials and, ultimately, arm physicians with an understanding whether a patient with GBM would benefit from including MLN4924 treatment in standard of care.

Materials and Methods

Cell Lines, Cultures, and Reagents

The cell lines LN-18 and M059K were obtained from the American Type Culture Collection (ATCC). The cell lines GB1 and SNU1105 were obtained from Japanese Collection of Research Bioresources (JCRB) Cell Bank and Korean Cell Line Bank (KCLB) respectively. Immortalized human astrocytes were kindly provided by the Pieper lab (34). All established human GBM cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM,

Hyclone) containing 10% fetal bovine serum (Biochrom AG) and 1% penicillin-streptomycin solution at 37°C with 5% CO₂. We obtained patient-derived xenograft (PDX) lines GBM39 and GBM43 from the Mayo Clinic Hospital, as described previously (35, 36). PDX lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM /F12, Hyclone) containing 20ng/mL EGF, 20ng/ml FGF, 2% B12, 1% N2, and 1% gentamycin solution at 37°C with 5% CO₂. Isogenic *PTEN* glioma stem cell (GSC) lines were cultured in the same PDX media and conditions listed above (37). Isogenic lines HCT116 *PTEN* (+/+) and HCT116 *PTEN* (-/-) were cultured in McCoy's 5a (Modified) Medium containing 10% fetal bovine serum (Biochrom AG) and 1% penicillin-streptomycin solution at 37°C with 5% CO₂ (38).

A variety of drugs were used for drug dose response and validation assays. MLN4924 (or Pevonedistat) was obtained from Selleckem (Cat# S7109). Proteasome inhibitors, bortezomib and MG132, were obtained from Sigma-Aldrich (Cat# 179324-69-7 and M869, respectively). Doxorubicin hydrochloride and etoposide from Sigma-Aldrich (Cat# D1515 and E1383, respectively). All drugs were dissolved in DMSO as 20 mM stock solutions, aliquoted, and stored at -20 degrees Celsius.

Single- and Combination- Drug Dose Response (DDR) Assay

Cells in log-phase growth were seeded in 384 well plates at 1,000-1,500 cells per well in quadruplicate, cultured for 24 hours and treated with one of the above drugs and DMSO (vehicle control) in a 12-point, 1:3 serial dilution. After 72 hours, cell viability was measured by CellTiterGlo® from Promega (Cat# G9241). Data was normalized to its vehicle control and analyzed using Prism 8.0.

For combination studies, cells were seeded in the same fashion as above. Each had a lane (four replicates) that was drugged with each single drug separately. The other three lanes were drugged with the additive drug at three different set concentrations (at a concentration ranging from IC₁₀-IC₃₀ values) and drugged with MLN4924 with the same 12-point, 1:3 serial dilution. Data was normalized to its vehicle control, and analyzed using Chou-Talalay method to determine synergy (30).

Colony Formation Assay

Cells were seeded into 6-well plates in triplicate (300-500 cells per well). 24 hours later, the media was replaced with new media with or without MLN4924, and cells were incubated at 37°C for two weeks. The plated colonies were fixed with 4% paraformaldehyde and stained with crystal violet. Colonies with more than 50 cells were counted.

Immunoblot Analysis

Immunoblot analysis and protein determination experiments were performed as previously described.(35) Briefly, cell monolayers were washed in chilled phosphate-buffered saline (PBS) containing 1 mM phenylmethylsulfonylfluoride and 1 mM sodium orthovanadate and then lysed in 2× SDS sample buffer (Cat#1610737) containing protease and phosphatase inhibitors. Protein concentrations were determined using the Pierce BCA Protein Assay kit (Cat#23225). 10-30 µg of total protein were loaded per lane and separated by SDS-PAGE. After transfer to nitrocellulose membrane (Invitrogen), the blot was blocked with either 5% nonfat-milk or 5% BSA in TBST before addition of primary antibodies and followed with peroxidase-conjugated secondary antibody (Promega). Protein bands were detected using SuperSignal Chemiluminescent Substrate (Pierce) with a UVP BioSpectrum 500 Imaging System. For time-course immunoblot analysis, some time-points were cropped to have a

consistent presentation style. This is indicated by a white line on the blot. Note: White lines do *not* indicate data from separate blots.

Blots were stained using primary antibodies to PTEN, ubiquitin, p27, topoisomerase-II- α (TOP2A), Wee1, XRCC1, CDT1, p53, PCNA, p21, yH2A.X, tH2A.X, α -Tubulin, GAPDH, p27, Ku70, PICH, and phospho-AKT (all from Cell-Signaling). All primary antibodies were used at 1:1000 dilutions, while all secondary antibodies were used at 1:2000 dilutions.

Cell Cycle Analysis

Cells treated with DMSO or MLN4924 (0.1, 0.25, or 0.5 μ M) for 24 hours were harvested and fixed in 70% ethanol at -20°C overnight, stained with FxCycleTM propidium iodide (PI)/RNase staining solution (ThermoFisher Scientific, Cat # F10797), at 37°C for 30 minutes, and then analyzed for cell-cycle profile by FACSCaliburTM (BD Bioscience). Data were analyzed with FlowJo software.

siRNA Knockdown DDR Assay

Qiagen FlexiTube siRNA was obtained for *PTEN*, *p27*, and *TOP2A* (GS5728, GS1027, and GS7153, respectively). Briefly, cells were seeded in a 6-well plate at 150,000-300,000 cells per well. Cells were reverse-transfected with siRNA. siRNA was prepared with lipofectamine RNAiMax reagent (Cat# 13778150) in Opti-Mem Reduced Serum Media (Cat# 31985062). After 48 hours, cells were lysed for immunoblot protein analysis (see Methods above).

Optimization assay for each siRNA was carried out to determine most effective knockdown siRNA and concentration. Cells were reverse transfected with 13 nM of siRNA and

AllStarNonSilencing control siRNA in a 384 well plate. 24 hours post-transfection, the DDRs were carried out in the same fashion described above under the Drug Dose Response (DDR) Assay heading. After 72 hours, cell viability was measured by CellTiterGlo®. Data was normalized to its vehicle control and analyzed using Prism 8.0.

Transcriptomic Analysis

LN18 and SNU1105 cells were seeded $0.8 - 1.2 \times 10^6$ cells per 100 mm dish, allowed to grow for 24 hours, then treated with MLN4924 at 100 nM and 500 nM or vehicle control for 0, 2, 8 and 24 h; RNA and protein were collected for transcriptomics and proteomics analysis.

For RNA, cells were collected via scraping and pelleted in conical tubes for RNA extraction. RNA was extracted using RNeasy Mini Kit (Qiagen). RNA-Seq libraries were constructed and sequenced following standard protocols (Illumina). Transcript abundance from RNA-seq data was quantified and normalized using Salmon in the unit of transcripts per million (TPM). Ingenuity Pathway Analysis (IPA) and gene set variation analysis (GSVA) were applied for pathway inquiries.

Proteomics Analysis

Sample Preparation: LN18 and SNU1105 cells were lysed by sonication (UTR200, Hielscher Ultrasound Technology) in 50 mM ammonium bicarbonate containing 8M Urea and 1X protease and phosphatase inhibitor cocktail (Pierce). Lysates were centrifuged at 4 °C for 10 minutes at 16000 g to remove cell debris and protein concentration of the lysate was measured using Pierce BCA Protein Assay Kit (Thermo Scientific). Equal amounts (10 µg) of protein extract per condition were reduced and alkylated by dithiothreitol (10 mM

final concentration) and iodoacetamide (40 mM final concentration) respectively. Samples were diluted 4-fold prior to overnight digestion at 37 °C with trypsin (Trypsin Gold, Promega) at an enzyme to substrate ratio of 1:50. Next day, samples were desalted using 1 cc Waters Sep-Pak C18 96-well plate; eluted peptides were vacuum centrifuged to dryness.

LC-MS/MS: Dried peptides (1 µg peptide per injection) were reconstituted in 2% acetonitrile, 0.1% formic acid containing 25 fmols of PierceTM Peptide Retention Time Calibration (PRTC) mixture. Mass Spectrometry data was acquired on Thermo Orbitrap Fusion Lumos mass spectrometer (ThermoFisher Scientific) interfaced with an Ultimate 3000 UHPLC system (ThermoFisher Scientific) using binary solvent system A (Water, 0.1% formic acid) and B (Acetonitrile, 0.1% formic acid). Using the direct injection mode, samples were loaded on a C18 analytical column (EasySpray ES801, 50 µm ID x 15 cm, 2 µm particle size, 100 Å pore size) kept at 45 °C and eluted at a flow rate of 300 nL/minute using the following 2 hour gradient: 2% to 19% B in 80 minutes, 19%-30% B in 20 minutes, 30%-98% B in 5 minute, plateau at 98% B for 2 minute, return to initial conditions in 1 minute and re-equilibration for 12 minutes. All MS data was acquired in data-dependent Top Speed mode with a 3 second duty cycle and the following parameters: spray voltage of 2000 V, ion transfer tube temperature of 275 °C, survey scan in the Orbitrap at a resolution of 120K, scan range of 400-1500 m/z, AGC target of 4E5 and maximum ion injection time of 50 ms. MSMS on most abundant precursors was carried out in Iontrap using High Energy Collision (HCD) dissociation (35 % NCE) with the following settings: quadrupole isolation mode enabled, isolation window at 1.6 m/z, AGC target of 5E3 with maximum ion injection time of 35 ms. Dynamic exclusion was set to 60 seconds and only precursors with a charge state of 2-7 were selected for MSMS.

Protein Identification and Statistics: Mass spectra were searched against a Human database (SwissProt/UniProt KB, 2017) using MaxQuant (v1.6.1.0) using label-free quantitation and trypsin theoretical digest. Oxidation on methionine and n-terminal acetylation were set as variable modifications, while cysteine carbamidomethyl was set as a fixed modification. The “match between runs” parameter was utilized with a minimum of one razor peptide identified per protein. Only high confidence peptide and protein identifications (FDR <0.01) were retained for analysis. Reverse and contaminants were removed. LFQ intensities were normalized using the Bioconductor DEP package in R (v3.6.3) (39). Pair-wise and time-course p-values were calculated between conditions using the Bioconductor limma package (v3.42.2) (40). Gene set variation analysis was performed using GSVA (v1.34.0) against a human gene ontology database of GO biological processes and pathways (geneontology.org, downloaded April 2019).

H2: Supplementary Materials

Fig. S1. Graph plotting MLN4924 AUC data for 28 glioma lines against their PTEN status, as either *PTEN* WT, *PTEN* mutant, or *PTEN* Null.

Fig. S2. Immunoblot analysis of *PTEN* siRNA knock -down and -out experiments.

Fig. S3. GSVA heat map of 50 different gene sets related to PTEN nuclear functions.

Fig. S4. GSVA heat map of 43 different gene sets related to DNA replication.

Table S1. Table with k-means clustering limited to 100 clusters of our proteomic GOA analysis (7,134 biological processes and pathways).

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Author contributions: SF and BT equally participated in the idea, experimental design, execution and manuscript preparation. NT and GR, RB and LH advised and performed experiments. SP and ML performed RNA-Seq data processing and analysis. PP, KG, RS from TGen proteomics core advised, executed, processed and analyzed our Mass spectrometry experiments. MB and HD equally supervised the project as senior advisors.

Competing interests: Authors have no financial interests and conflict of interest to disclose.

Data and materials availability: Accession numbers will be provided for RNA-Seq and proteomics data deposits.

A Pevonedistat (MLN4924) CCLE PanCancer

Response

- Sensitive (≤ 10.8)
- Unknown ($10.8 < \text{AUC} < 13.41$)
- Resistant (≥ 13.41)

B Neddylation Inhibition (MLN4924) DDR

Normalized Viability (%)

MLN4924 Conc (nM)

C Neddylation Inhibition (MLN4924) Clonogenic

Surviving Fraction

MLN4924 Conc (nM)

D Neddylation Inhibition (MLN4924) DDR

Normalized Viability (%)

MLN4924 Conc (nM)

E Proteasomal Inhibition (Bortezomib) DDR

Normalized Viability (%)

Bortezomib Conc (nM)

F Proteasomal Inhibition (Bortezomib) DDR

Normalized Viability (%)

Bortezomib Conc (nM)

G Normal Human Astrocytes DDR

Normalized Viability (%)

Drug Conc (nM)

H

NT 100 nM 250 nM 500 nM

GB1 Number of Events

GB1

LN18

M059K

SNU1105

Propidium iodide

I

MLN4924 500 nM

VEH 15 min 30 min 1 hr 2 hr 4 hr 8 hr 24 hr 48 hr

WEE1

Ku70

CDT1

P27

P21

γ H2AX

tH2AX

RAD51

GAPDH

22 Figures and Tables

Fig. 1. Differential response to MLN4924 is associated with S phase retention and is independent of CRL accumulation. (A) The area under the curve (AUC) data from Cancer Therapeutics Response Portal's (CTRP) MLN4924 drug screen against 860 cell lines was plotted against frequency. (B) Four established GBM cell lines (LN18, GB1, M059K, and SNU1105) were treated with MLN4924 at a twelve-step serial dilution (1:3) starting at 100 μ M for 72 hours. Cell viability was measured by CellTiterGlo® and was normalized to its vehicle control (DMSO). (C) LN18 and SNU1105 were seeded with various concentrations of MLN4924 and DMSO (0, 10, 50, 100, or 500 nM). After two weeks, respective colonies were fixed with 4% paraformaldehyde, stained with crystal violet, and counted. MLN4924 treated cells were normalized against their control. The data was analyzed and presented as surviving fractions. Graph represents $\pm$ SD, *** P <0.001 by t-test. (D) The MLN4924 drug dose response assay described in (1B) was conducted with two patient-derived xenograft (PDX) models (GBM43 and GBM39). (E) Four established GBM cell lines (LN18, GB1, M059K, and SNU1105) were treated with Bortezomib at a twelve-step serial dilution (1:3) starting at 10 μ M for 72 hours. Cell viability was measured by CellTiterGlo® and was normalized to its vehicle control (DMSO). (F) The Bortezomib drug dose response assay described in (1E) was conducted with two patient-derived xenograft (PDX) models (GBM43 and GBM39). (G) *TERT*-immortalized normal human astrocytes' (NHA-*hTERT*) were separately treated with MLN4924 and Bortezomib at a twelve-step serial dilution (1:3) starting at 100 μ M for 72 hours. Cell viability was measured by CellTiterGlo® and was normalized to its vehicle control (DMSO). (H) The sensitive (LN18 and GB1) and resistant (M059K and SNU1105) established GBM cell lines were treated with three concentrations of MLN4924 (100 nM, 250 nM, or 500 nM) or DMSO ("NT") for 48 hours, fixed with

paraformaldehyde, and analyzed via fluorescence-activated cell sorting analysis (FACS) to determine cell-cycle profile post-MLN4924 treatment. **(I)** GB1 (sensitive) and M059K (resistant) cell line lysates were collected through multiple time-points (15 min, 30 min, 1 hr, 2 hr, 4 hr, 8 hr, 24 hr, and 48 hr) following a 500 nM treatment of MLN4924. Lysate was subjected to immunoblot (IB) analysis that probed for canonical CRL substrates and DNA damage response proteins: WEE1, Ku70, CDT1, P27, P21, yH2AX, tH2AX, and RAD51. GAPDH was used as a loading control.

Figure 2

A

B

C

D

E

F

G

Fig. 2. *PTEN* deletion decreases sensitivity to MLN4924. (A) Using MLN4924 response data from the Genomics of Drug Sensitivity in Cancer (GDSC) database and the whole genome sequencing data from Cancer Cell Line Encyclopedia (CCLE), the top six most sensitive and resistant GBM cell lines (21 cell lines total) were categorized into two groups and the genes that were most differentially mutated between the two groups were identified. (B) The six most significant genetic differentiators from (1A) were analyzed in LN18, GB1, M059K, and SNU1105. (C) Treatment naïve cell line (LN18, GB1, M059K, and SNU1105) lysates were collected and analyzed for PTEN protein expression via immunoblot analysis with GAPDH as a loading control. (D-E) The *PTEN*-expressing sensitive GBM models LN18 and GB1 were reverse transfected with *PTEN* siRNA, Negative Control (AllStars), or just buffer using lipofectamine RNAiMax. (F) A MLN4924 drug dose response assay was conducted with the *PTEN* isogenic pair (*PTEN* WT and *PTEN* knock-out) of the colorectal cancer cell line model HCT116. (G) A MLN4924 drug dose response assay was conducted with the *PTEN* isogenic pair (*PTEN* null and *PTEN* knock-in) of the glioma stem cell line model GSC11.

Figure 3

Fig. 3. Response to MLN4924 is independent of PTEN lipid phosphatase activity but dependent of phosphorylation of PTEN Tyrosine 240. (A) Treatment naïve cell line (LN18, GB1, M059K, and SNU1105) lysates were collected and analyzed for phosphorylated AKT (pAKT) protein expression at baseline via immunoblot analysis with GAPDH as a loading control.* presented GAPDH is for pAKT blot. GAPDH for AKT blot is also similar (Supplementary Fig 2D). (B) The established models were treated with 500 nM of MLN4924, and their lysates were collected at various time-points (two, eight, 24, and 48). Their pAKT protein expression through MLN4924 treatment was analyzed via immunoblot analysis with GAPDH as a loading control. Extra time-points were cropped for consistency. This is indicated by a white line on the blot. Note: White lines do *not* indicate data from separate blots. Relative quantification to GAPDH is shown. (C) A MLN4924 drug dose response assay as described in (1B) was conducted with the *PTEN* isogenic trio (*PTEN* null, *PTEN* WT knock-in, and PTEN G129A — phosphatase dead PTEN — knock-in) of the glioma stem cell line model GSC23. (D) A MLN4924 drug dose response assay as described in (1B) was conducted with the *PTEN* isogenic pair (PTEN WT knock-in and PTEN Y240F — PTEN incapable of binding chromatin — knock-in) of the mouse embryonic fibroblast model MEF.

A

B

Fig. 4. Transcriptomics shows enrichment of PTEN nuclear function pathways in the MLN4924 sensitive cells. (A) RNA from the established GBM models was collected after 2, 8, and 24 hours of 100 and 500 nM MLN4924 treatment and next generation RNA sequencing was executed. Unbiased Ingenuity Pathway Analysis (IPA) was used to identify the most differentially deregulated pathways following MLN4924 treatment between sensitive (LN18 and GB1) and resistant models (M059K and SNU1105). (B) From a group of 50 gene sets that were related to PTEN's nuclear roles, supervised gene set enrichment analysis (GSEA) was carried out with 21 different gene sets that significantly differentiated the sensitive and resistant models. (C) Three different DNA replication fork gene sets (Replication fork protection, Replication fork protection complex, and Replication fork progression) significantly differentiated sensitive and resistant models from the four long term GBM cell lines (i) (pre and post-treated) and from the 21 GBM models from GDSC and CCLE (ii). Unsupervised GSVA was performed and clustered the models based on MLN4924 sensitivity in all three data sets. (D) Using the RNA-seq data, individual normalized RNA dynamics of transcripts related to DNA replication, DNA damage response, and chromatin dynamics were plotted from the four established models.

Figure 5

A

B

C

D

Fig. 5. Proteomic analysis reveals enrichment of chromosome dynamics pathways in response to MLN4924. (A) Protein was collected from LN18 and SNU1105 at 0, 2, 8, and 24 hours post-MLN4924 treatment at 100 and 500 nM. Gene set variation analysis revealed differential enrichment of PTEN gene sets between LN18 and SNU cell lines. (B) Enrichment score distribution of one of the most differentially enriched clusters between sensitive and resistant models (Table S1). This cluster includes the GO Replication Fork Processing gene set (red). Principal component analysis of this cluster verifies the samples cluster in a cell-line dependent fashion. (C) Unsupervised clustering of the ten most positively and negatively enriched gene sets in LN18 and SNU1105. The majority of positively enriched gene sets in LN18 were also the most negatively enriched gene sets in SNU1105 and *vice versa*. (D) Unsupervised clustering of enrichment scores for 34 gene sets related to chromosome dynamics shows these gene sets differentiate the samples in a cell line dependent fashion.

Figure 6

A

B

C

D

Accession	protein	Log2	pVal
P29279	CTGF	2.61	0.00205
P11388	TOP2A	2.19	0.0264
P53814-6	SMTN	1.93	0.00265
O00622	CYR61	1.31	0.0423
Q9NQW6	ANLN	1.22	2.51E-04
P04062-4	GBA	1.22	0.0149
Q9ULW0	TPX2	1.17	0.0148
Q9UNW1	MINPP1	1.07	0.00960
P46013	MKI67	1.00	0.00746
Q75410	TACC1	1.00	0.0490
Q58FF8	HSP90AB2P	-1.19	0.00295
P05114	HMGN1	-1.48	0.0272

E

G

H

Drug Combination	CI	Note
LN18		
Doxorubicin		
20 nM	2.34	Antagonism
40 nM	1.33	Antagonism
Etoposide		
300 nM	1.3	Slight Antagonism
600 nM	0.91	Nearly Additive
GB1		
Doxorubicin		
20 nM	1.28	Slight Antagonism
40 nM	1.25	Slight Antagonism
Etoposide		
300 nM	0.94	Nearly Additive
600 nM	0.96	Nearly Additive
M059K		
Doxorubicin		
20 nM	0.87	Slight Synergism
40 nM	0.88	Slight Synergism
Etoposide		
300 nM	0.83	Moderate Synergism
600 nM	0.48	Synergism
SNU1105		
Doxorubicin		
20 nM	0.54	Synergism
40 nM	0.45	Synergism
Etoposide		
300 nM	0.82	Moderate Synergism
600 nM	0.30	Strong Synergism

Fig. 6. Targeting Topoisomerase II synergizes with MLN4924 in the resistant models. (A-B) Supervised clustering, based on MLN4924 sensitivity, of enrichment scores from GSVA with CIN70 and CIN25 gene sets using the RNA-sequencing and mass spectrometry normalized LFQ intensity data. (C) TOP2A normalized protein intensities from mass spectrometry data were plotted for LN18 and SNU1105 at each time-point and MLN4924 concentration. (D) Protein log₂(fold-change) and -log₁₀(p-value) at eight hours after 100 nM treatment of MLN4924 treatment, compared to time 0 in SNU1105. Proteins with a log₂(fold-change) >±1 and -log₁₀(p-value)>1.301 (green) were considered significantly differentially abundant. Among these significant proteins were two CIN4 genes (*TOP2A* and *TPX2*). (E) Cell line (LN18, GB1, M059K, and SNU1105) lysates were collected and analyzed for TOP2A protein expression at baseline via immunoblot analysis with GAPDH as a loading control. Relative quantification to GAPDH is shown. (F) The established models were treated with 500 nM of MLN4924, and their lysates were collected at various time-points (two, eight, 24, and 48). Their TOP2A protein expression through MLN4924 treatment was analyzed via immunoblot analysis with GAPDH as a loading control. (G) Four established GBM cell lines (LN18, GB1, M059K, and SNU1105) were treated with individually treated with MLN4924 and two clinically available TOP2A inhibitors at a twelve-step serial dilution (1:3) starting at 100 μM for 72 hours. Models were also treated with two different set concentrations of the TOP2A inhibitors and MLN4924 in a serial-dilution fashion. Cell viability was measured by CellTiterGlo® and was normalized to its vehicle control (DMSO). Cell viability was measured by CellTiterGlo® and was normalized to its vehicle control (DMSO). Data was analyzed via isobologram and combination index analysis.