Novel DNA Damage Checkpoints Mediating Cell Death Induced by the NEDD8-Activating Enzyme Inhibitor MLN4924

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Abstract

MLN4924 is an investigational small-molecule inhibitor of the NEDD8-activating enzyme (NAE) in phase I clinical trials. NAE inhibition prevents the ubiquitination and proteasomal degradation of substrates for cullin-RING ubiquitin E3 ligases that support cancer pathophysiology, but the genetic determinants conferring sensitivity to NAE inhibition are unknown. To address this gap in knowledge, we conducted a genome-wide siRNA screen to identify genes and pathways that affect the lethality of MLN4924 in melanoma cells. Of the 154 genes identified, approximately one-half interfered with components of the cell cycle, apoptotic machinery, ubiquitin system, and DNA damage response pathways. In particular, genes involved in DNA replication, p53, BRCA1/BRCA2, transcrip- tion-coupled repair, and base excision repair seemed to be important for MLN4924 lethality. In contrast, genes within the G2-M checkpoint affected sensitivity to MLN4924 in colon cancer cells. Cell-cycle analysis in melanoma cells by flow cytometry following RNAi-mediated silencing showed that MLN4924 prevented the transition of cells from S–G2 phase after induction of rereplication stress. Our analysis suggested an important role for the p21-dependent intra-S-phase checkpoint and extensive rereplication, whereas the ATR-dependent intra-S-phase checkpoint seemed to play a less dominant role. Unexpectedly, induction of the p21-dependent intra-S-phase checkpoint seemed to be independent of both Cdt1 stabilization and ATR signaling. Collectively, these data enhance our understanding of the mechanisms by which inhibition of NEDD8-dependent ubiquitination causes cell death, informing clinical development of MLN4924. Cancer Res; 73(1); 225–34. ©2012 AACR.

Introduction

MLN4924 is an investigational small-molecule inhibitor of the NEDD8-activating enzyme (NAE; ref. 1). MLN4924 has shown antitumor activity in multiple xenograft models of human cancer at well-tolerated doses (1–3). MLN4924 is currently being explored in phase I clinical trials, where some clinical activity has been observed in solid tumor and hematologic malignancies (4). Therefore, a more complete understanding of the impact of NAE inhibition on cancer cell biology is warranted.

NAE catalyzes the activation and transfer of NEDD8 onto ubiquitin E3 ligases that support cancer pathophysiology. The cullin-RING ubiquitin E3 ligases (CRLs) have been shown to conjugate proteins with Lys48-linked polyubiquitin chains (8, 9) thereby promoting the degradation of a relatively small subset of proteasomal substrates (1). CRLs have been shown to monoubiquitinate some proteins, especially histones (9). Recently, the function of Cul1-Rbx1-Skp1 (CRL1) has been expanded to include Lys63-linked polyubiquitin chain formation (10). Cdt1 stabilization, and ATR signaling. Collectively, these data enhance our understanding of the mechanisms by which inhibition of NEDD8-dependent ubiquitination causes cell death, informing clinical development of MLN4924. Cancer Res; 73(1); 225–34. ©2012 AACR.

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Initial studies to define the mechanism of action of MLN4924 have shown that blocking of CRL1-Skp2 and Cul4-Rbx1-Ddb1 (CRL4)-Cdt2 function results in Cdt1 stabilization, which leads to DNA rereplication and accumulation of cells in S-phase, thereby promoting cell death in most cancer cell lines studied (1, 11, 12), although stabilization of 1 kB via inhibition of CRL1-TRCP plays a role in some settings (2). To prevent genomic instability, initiation of DNA replication is tightly controlled by a well orchestrated process that involves the origin recognition complex (ORC), Cdc6, Cdt1, geminin, the Mcm2–7 complex, Mcm10, Cdc4, and the GINS complex (13–17). Following DNA replication initiated by the protein kinases Cdk2 and Dbf4-Cdc7, Cdt1 is degraded, whereas Cdc6 is exported from the nucleus, thereby preventing repriming of the origin. Degradation of Cdt1 is controlled in a Cdk2-dependent manner by CRL1-Skp2 and in a PCNA-dependent manner by CRL4-Cdt2 (18–21). Cdt2 is also known as denticless (DTL).

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MLN4924 is a small-molecule inhibitor of the NEDD8-activating enzyme (NAE) in phase I clinical trials. NAE inhibition prevents the ubiquitination and proteasomal degradation of substrates for cullin-RING ubiquitin E3 ligases that support cancer pathophysiology. The cullin-RING ubiquitin E3 ligases (CRLs) have been shown to conjugate proteins with Lys48-linked polyubiquitin chains (8, 9) thereby promoting the degradation of a relatively small subset of proteasomal substrates (1). CRLs have been shown to monoubiquitinate some proteins, especially histones (9). Recently, the function of Cul1-Rbx1-Skp1 (CRL1) has been expanded to include Lys63-linked polyubiquitin chain formation (10). Initial studies to define the mechanism of action of MLN4924 have shown that blocking of CRL1-Skp2 and Cul4-Rbx1-Ddb1 (CRL4)-Cdt2 function results in Cdt1 stabilization, which leads to DNA rereplication and accumulation of cells in S-phase, thereby promoting cell death in most cancer cell lines studied (1, 11, 12), although stabilization of 1 kB via inhibition of CRL1-TRCP plays a role in some settings (2). To prevent genomic instability, initiation of DNA replication is tightly controlled by a well orchestrated process that involves the origin recognition complex (ORC), Cdc6, Cdt1, geminin, the Mcm2–7 complex, Mcm10, Cdc4, and the GINS complex (13–17). Following DNA replication initiated by the protein kinases Cdk2 and Dbf4-Cdc7, Cdt1 is degraded, whereas Cdc6 is exported from the nucleus, thereby preventing repriming of the origin. Degradation of Cdt1 is controlled in a Cdk2-dependent manner by CRL1-Skp2 and in a PCNA-dependent manner by CRL4-Cdt2 (18–21). Cdt2 is also known as denticless (DTL).
DNA rereplication results from the multiple refiring of the origins of replication during DNA synthesis, generally resulting in partial polyploidy (16). Overexpression of CDT1 or CDC6 or deletion of GMNN, encoding an inhibitor of Cdt1, can induce DNA rereplication in some tumor cell lines, although checkpoint control is activated in normal cells and in other tumor cell lines (22, 23).

Several studies have shown that cullin-dependent ubiquitination along with the COP9 signalosome, which is responsible for deneddylation of the cullins, have additional roles in DNA damage response pathways. These include global genome and transcription-coupled nucleotide excision repair, histone modification, and regulation of checkpoints via degradation of p21, p27, p53, Cdc25A, Wee1, clasin, and Fanconi anemia, complementation group M (FANCM) (9, 24–27). Therefore, it is likely that inhibition of NAE by MLN4924 impacts multiple DNA damage response pathways beyond those involving Cdt1 stabilization.

Therefore, to ascertain the relative importance of these potentially diverse mechanisms, we have conducted a genome-wide siRNA screen to evaluate the genetics of sensitivity to MLN4924 in the A375 human melanoma-derived cell line. RNA interference (RNAi) screens provide an important avenue for understanding the sensitivity of tumor cells to drugs and drug candidates (28–30).

This screen identified 154 genes whose knockdown had significant effects on MLN4924-induced cell death. Collectively, the data suggest that NAE inhibition by MLN4924 primarily causes cell death by dysregulating components of the cell cycle, apoptotic machinery, the ubiquitin system, and the DNA damage response, including the p53 pathway, BRCA1/BRCA2 complex, transcription-coupled, and base excision repair. In particular, we show that activation of the p53 pathway is independent of Cdt1 stabilization and ATR activation, showing that MLN4924 affects cell survival by the stabilization of proteins beyond Cdt1.

Materials and Methods

Additional experimental details are contained within the Supplementary Materials and Methods.

Genomic characterization and authentication of cell lines

A375 and HCT-116 were received from the American Type Culture Collection (ATCC) in July 2006 and June 2009, respectively, and passaged twice before freezing. Genomic DNA was isolated from 2 million cells under vendor-specified protocols. Five-hundred micrograms of DNA per sample was then amplified, labeled, and processed on the Affymetrix SNP-6 whole genome array platform on August 22, 2011 and evaluated using Partek Genomics Suite for copy number variation analysis. Spearman correlation was determined for each cell line against an ATCC-authenticated stock vial received at Millennium (A375 ATCC CRL-1619 and HCT-116 ATCC CCL-247). Consistent regions of amplification and deletions were found between the cell lines of the same origin and correlation on genotypes were greater than 0.995. Cell lines were passaged for fewer than 8 weeks following resuscitation.

Genome-wide screen and hit deconvolution

A375 melanoma cells (300 cells/well) were reverse transfected in duplicate on 384-well poly-lysine (PDL)-coated black, clear bottom plates (CELLCOAT, Greiner) using 15 nmol/L siRNA oligos (siGENOME SMARTpool, Dharmacon) and 28 nL/well DharmaFECT 4 reagent (DH4, Dharmacon) in 64 µL Opti-MEM (Life Technologies). After 48 hours of knockdown, cells were treated with 0, 250, or 650 nmol/L MLN4924, in the continued presence of siRNA oligos, and incubated for a further 48 hours, after which viability was assessed with ATPlite reagent according to the manufacturer’s instructions (Perkin-Elmer). Luminescence was measured using a LEAD-seeker imaging system (GE Healthcare). Viability interactions of SMARTpools with MLN4924 were scored according to Bliss independence (BI) assumptions that enable siRNAs to be ranked based on magnitude of effect (31). BI and Rescue scores allow for the assignment of synthetic lethal (BI < 0), epistatic (BI > 0, Rescue > 0), or suppressor (BI > 0, Rescue < 0) phenotypes to the RNAi results.

SMARTpool hits were deconvoluted in sextuplicate under identical conditions, except that 8 nmol/L individual oligo was used. Calculation of false discovery rates (FDR) was achieved using a random permutation of deconvoluted data, as outlined in Supplementary Materials and Methods.

For HCT-116 cell transfections, changes included the use of 450 cells per well, transfection with Lipofectamine RNAiMax (Life Technologies) at 20 nL/well, and McCoy5A growth medium (Life Technologies). After 48 hours of knockdown, HCT-116 cells were treated with 0, 91, or 116 nmol/L MLN4924 for an additional 48 hours before assaying cell viability by ATPlite. MLN4924 will be made available to qualified researchers once a standard Materials Transfer Agreement has been executed.

Flow cytometry

A375 cells were reverse transfected on 96-well PDL-coated black, clear-bottom plates (CELLCOAT, Greiner) using 15 nmol/L siRNA oligos (siGENOME SMARTpool, Dharmacon) and 84 nL/well DharmaFECT 4 reagent (DH4, Dharmacon) in Opti-MEM (Life Technologies). Forty-eight hours after transfection, cells were treated in triplicate with 500 nmol/L MLN4924 or vehicle [0.005% dimethyl sulfoxide (DMSO)] and incubated for 22 hours. Cells were pulsed with 10 µmol/L 5-bromo-2-deoxyuridine (BrdUrd, Life Technologies) for 30 minutes, trypsinized, fixed, and denatured. Cells were stained with undiluted fluorescein isothiocyanate (FITC)-conjugated anti-BrdUrd (BD Pharmingen), washed, and stained with 1 mg/mL propidium iodide (Life Technologies). Flow cytometry was conducted using a Becton Dickinson FACSCanto II with High Throughput Sampler. Flow cytometry data were analyzed as outlined in Supplementary Materials and Methods.

cDNA synthesis and TaqMan Gene Expression assays for qPCR

The cDNA to be used for the quantitative PCR (qPCR) reactions was generated using ABI TaqMan Gene Expression
Cells-to-CT kit (Applied Biosystems #AM1729) according to manufacturer’s protocols and was conducted on Biomek FX Liquid Handling System. TaqMan Gene Expression Assays kits were purchased from Applied Biosystems and data were generated with an ABI7900HT system.

Western blotting
A375 cells were treated with 650 nmol/L MLN4924 or vehicle (0.065% DMSO) for the times indicated. For UV irradiation, A375 cells were exposed to 30 J/m² UV and then incubated for 24 hours. To induce a G1-S-phase arrest, A375 cells were treated with 1 µg/mL aphidicolin for 24 hours. For each sample, a large batch of cells was lysed in radioimmunoprecipitation assay (RIPA) buffer and prepared for SDS-PAGE and quantitative immunoblot analysis with antisera as specified in the Supplementary Table S1A using tubulin as a normalization control so that the coefficient of variance across samples was less than 16%. This set of samples was then used to run multiple Western blots. Western blot intensity was quantified using an Odyssey Infrared Imager (LI-COR Biosciences). Full-length blots are presented in Supplementary Fig. S1. Antibody details are presented in Supplementary Table S1A.

Results

Genome-wide siRNA screen
Gene knockdown in A375 cells was conducted with 15 nmol/L Dharmacon SMARTpool siRNA oligos for 48 hours to deplete cellular proteins, followed by 48-hour treatment with 2 concentrations of MLN4924 [LC25 (250 nmol/L) and LC70 (650 nmol/L)] or a vehicle control. These concentrations of MLN4924 enabled the evaluation of either enhancement or suppression of MLN4924-induced cell death by the SMARTpools. A375 cells are highly transfecable and display an average amount of repletion following MLN4924 treatment relative to other cell lines, as measured by the accumulation of cells with a more than 4N DNA content (Supplementary Fig. S2). The genome-wide screen was conducted with 21,062 SMARTpools in duplicate (Supplementary Table S1B and Supplementary Fig. S3A). Following reconfirmation of 2239 SMARTpool hits (Supplementary Table S1C and Supplementary Fig. S3B–E), 1221 different genes were deconvoluted, with separate wells for each individual oligo at 8 nmol/L siRNA (Supplementary Table S1D and S1E and Supplementary Fig. S4).

Using permutation analysis and confirmation of gene expression (Supplementary Table S1F and S1G), 109 hits were identified with an FDR P < 0.05 based on 3 of the 4 oligos interacting with MLN4924 (O3 criteria, Table 1). An additional 45 hits were identified with an FDR 0.05 < P < 0.10 based on 3 of 4 oligos (O3’ criteria). These hits were classified according to their gene annotation (Fig. 1).

Importantly, the screen captured key aspects of biology previously shown to be important for MLN4924 mechanism of action, including genes involved in the ubiquitin pathway, DNA damage repair (DDR), cell cycle, TNF family receptors, and apoptosis. However, genes within the p53 pathway, BRCA1/BRCA2 complex, transcription-coupled, and base excision repair pathways were identified that had not previously been implicated in the mechanism of cell death induced by MLN4924. These hits represent genes whose function may be directly dysregulated by MLN4924. In interpreting RNAi screens, it is important to remember key limitations of RNAi, including transient protein knockdown, incomplete elimination of the protein, genetic redundancy, and off-target effects.

Results

Consistent with NAE being the primary target of MLN4924, the 2 hits with the greatest degree of synthetic lethality following deconvolution (Table 1) were UBA3 and NAE1, encoding the heterodimeric subunits of NAE. Depletion of either UBA3 or NAE1 by RNAi in cells therefore seems to reduce the effective concentration of MLN4924 required to achieve a critical level of inhibition of the NEDD8 pathway that results in cell death.

The hits with the greatest degree of suppression of MLN4924-induced lethality included CDT1, CASP8, and TEX10, a protein associated with the SLX4 Holliday junction resolvase (32). Depletion of CDT1 would be expected to reduce prereplication licensing, thereby preventing cell death, consistent with its scoring as a suppressor. Hits were also evaluated using 2 or 4 out of 4 oligos (O2 and O4 criteria, Supplementary Table S2A and S2B), although these criteria captured fewer hits.

Behavior of genes in HCT-116

The deconvoluted siRNA oligos against 240 genes were also evaluated in HCT-116 cells at 91 and 116 nmol/L MLN4924 (LC90 and LC80, respectively, Supplementary Tables S1H and S1I), of which 24 (10%) scored as hits by O4 criteria following permutation of results from 240 randomly selected genes (Supplementary Table S2C). Fewer hits were identified by either O2 or O3 criteria (Supplementary Table S1I). With the exception of the TCR/BER gene set, the depletion of the DDR and cell-cycle genes that impacted A375 sensitivity of MLN4924 did not similarly impact HCT-116 sensitivity. Because HCT-116 rereplicates its DNA following treatment with MLN4924 even more substantially than does A375 (Supplementary Fig. S2), we hypothesized that genetic differences in DDR mechanisms between A375 and HCT-116 may substantially impact the response to MLN4924. Therefore, deconvoluted siRNA oligos against 317 genes with known DNA damage response roles were evaluated in HCT-116 cells at 91 nmol/L MLN4924 (Supplementary Table S1H & S1I, Run 3), of which 9 scored as hits (Supplementary Table S2C, annotated with #). Notably, 6 of these have known roles within the G2–M checkpoint, with knockdown of FBXO5 (encoding Emi1), CDK1, and PLK1 giving the most significant effects.

Cell-cycle interactions of hits with MLN4924

MLN4924 induces DNA repletion by stabilizing the replication licensing factor Cdt1 (12). To quantify the effect of MLN4924 on the cell cycle and to better characterize how MLN4924 might affect previously studied repletion checkpoints, A375 cells transfected with control siRNA (GL2) were treated with vehicle or 500 nM MLN4924 for 22 hours, pulse-labeled with BrdUrd, stained with propidium iodide, and then subjected to flow cytometry (Fig. 2A). MLN4924 resulted in changes to most phases of the cell cycle (Fig. 2B), including...
increases in the number of cells with S-phase DNA content but not incorporating BrdUrd (labeled as 2N-4N) and cells with more than 4N DNA actively incorporating BrdUrd. Of note, sub-G1 cells were not captured in this analysis due to their loss in plate washing.

We evaluated the effect of depleting 11 genes directly associated with the prereplication complex or origin firing, as well as UBA3 (Fig. 2C), treated with either vehicle (DMSO) or MLN4924. Knockdown of 9 of these genes in vehicle-treated cells resulted in an accumulation in G2-M, generally with loss of S-phase. These results suggest that depletion of key replication licensing proteins results in defective replication leading to cell-cycle arrest at a G2-M checkpoint rather than in inhibition of origin firing. Engagement of the G2-M checkpoint is consistent with the response to rereplication as previously characterized genetically.(15, 16) Following treatment by MLN4924, a loss of S-phase was again seen, although the pattern shifted, as depletion of key replication licensing proteins resulted in an accumulation of cells with 2N-4N (arrested) or more than 4N (rereplicating) DNA content, suggesting that the rereplicating cells were less able to progress to G2 in the presence of MLN4924.

Table 1. 154 Genes whose knockdown significantly affects the induction of cell death by MLN4924

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<th>mRNA Processing</th>
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<th>Ubiquitin</th>
<th>Cell Adhesion</th>
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NOTE: Oligo 3 scored set (109 genes: FDR P < 0.05, *45 genes: 0.05 < FDR P < 0.10). Genes contained within each classification of Fig. 1 ordered according to their BI score from most negative. Gray background genes are synthetic lethal hits, white background genes are epistasis hits, except for the red-lettered genes, which are suppressor hits. Bold letters indicate the 15 genes with the strongest BI scores. Human Genome Organization (HUGO) names are given for the genes.
We also evaluated the effect of knockdown of a further 86 genes that function more broadly in cell cycle and DNA damage responses for changes to the cell-cycle effects of MLN4924. In total, depletion of 69 genes significantly impacted the cell-cycle distribution following MLN4924 treatment (Fig. 3, Supplementary Fig. S5 and Supplementary Table S1). Broadly, depletion of these genes can be categorized as affecting MLN4924-induced cell-cycle changes by their most significant impact on the cell cycle, namely increase in G1 content (BRIP1-XRCC3), decrease in S-phase content (ATR-UBE2L3), accumulation of cells with 2N-4N (arrested) DNA content (CHEK2-RPA1), accumulation of cells with more than 4N (rereplicating) DNA content (ATM-XRCC2), or reduction in the number of cells with 2N-4N (arrested) DNA (DDX3X-TNFRSF10B). Notably, knockdown of 6 of the 12 genes from Fig. 2 caused a similar impact following treatment with
MLN4924 to those oligos in the third set (CHEK2-RPA1). Many of the genes whose depletion shifts cells between the arrested population and the rereplicating population are likely to be involved in activation of an S-phase checkpoint in response to rereplication.

Extent of rereplication does not significantly correlate with cell death

Surprisingly, these cell-cycle changes (Fig. 2C, 3 and Supplementary Fig. S5) did not significantly correlate with the viability effects of genes with significant MLN4924 interactions. Genes whose knockdown resulted in the significant accumulation of cells in G1 for vehicle-treated cells were marginally negatively associated with a synthetic lethal phenotype (5 of 22, \( P = 0.18 \)). Genes whose knockdown led to increased >4N DNA content following MLN4924 treatment were marginally negatively associated with a suppressor phenotype (2 of 8, \( P = 0.14 \)). Thus, S-phase content before treatment seems to be important for MLN4924 sensitivity and increased >4N DNA following treatment increased cell death, although both effects were modest. These results suggest that the function of these genes in the response to MLN4924 may not be fully realized by their effects on the cell cycle.

Upregulation of p53 and p21 is independent of Cdt1 or ATR

To understand better the sequence of events for induction of checkpoints, we evaluated the effect of depleting CDT1, TP53, ATM, and ATR by Western blot analysis on key proteins (Fig. 4). These data showed efficient knockdown of CDT1, TP53, and ATM. As expected, knockdown of CDT1 or ATR following treatment of cells with MLN4924 significantly reduced the phosphorylation of ATR substrates, including Rpa32 and Chk1, as well as the stabilization of claspin and the phosphorylation of Rb. Surprisingly, knockdown of CDT1 or ATR had little impact on increases in p53 or p21, whereas TP53 depletion was very efficient in blocking these effects. A spliceform of Puma was also unaffected by CDT1 depletion but reduced by TP53 depletion. Therefore, unexpectedly, the upregulation of p53 and p21 seems to be independent of DNA damage induced by Cdt1 stabilization, particularly as detected by ATR signaling.
Phosphorylation of Cdc25A or events further downstream from ATR CR that checkpoints, it seems to be through the stabilization of these proteins. If MLN4924 clearly activates ATR, as previously described (12). If MLN4924 directly interferes with the establishment of the ATR-dependent checkpoint, it seems to be through the stabilization of Cdc25A or events further downstream from ATR phosphorylation.

**Activation of ATR by MLN4924 in A375 cells**

MLN4924 has been previously shown to induce the phosphorylation of ATR substrates including Chk1, Rad17, and Nbs1 in HCT-116 cells (12). However, depletion of ATR and its substrates only moderately influenced the effect of MLN4924 on cell viability and the cell cycle (Table 1 and Fig. 3). Knockdown of ATR does efficiently block phosphorylation of Rpa32 (Fig. 4). Thus, activation of ATR or a critical downstream pathway may be blocked. Figure 5A compares MLN4924 with 2 control treatments known to activate ATR, aphidicolin, and UV (34). Phosphorylation of the ATR targets Chk1, Mdm2, Nbs1, Rad17, and Rpa32 was similar between all three treatments, whereas Cdc25A, a substrate of Chk1 that is targeted for degradation by CRL1–TRCP (35), was preferentially stabilized by MLN4924. Among the other proteins previously shown to be CRL4-Cdt2 substrates (Cdt1, p21, Set8) or otherwise associated with MLN4924 activity (p-H2Ax and p53), Cdt1 and p21 stabilization most differentiated MLN4924 from the other 2 treatments. Phosphorylation of ATR substrates following MLN4924 treatment occurs in a single wave at approximately 10 hours to half-maximal phosphorylation, consistent with these being substrates of a single kinase (Fig. 5B). Therefore, MLN4924 clearly activates ATR, as previously described (12). If MLN4924 directly interferes with the establishment of the ATR-dependent checkpoint, it seems to be through the stabilization of Cdc25A or events further downstream from ATR phosphorylation.

**Discussion**

Our original interest in developing an NAE inhibitor was based on an emerging knowledge of the cullin-dependent degradation of many cancer relevant proteins, the dysregulation of which could provide a therapeutic benefit. Overall, the data obtained in our MLN4924 synthetic lethal screen align well with prior genetic studies on recombination and in that many of the genes affecting MLN4924 sensitivity are directly involved in the prereplication complex and DNA replication, including PCNA, MCM7, DT1, CDT1, TK1, RPA1, BRM2, and DHFR. Importantly, DDR mechanisms were also well represented in these viability screen results.

These DDR mechanisms included p53, BRCA1/2, transcription-coupled repair, base excision repair, and histones. Some aspects of these results could have been anticipated. For example, CRL4 has clear roles in transcription-coupled repair (36) and histone monoubiquitination (9). However, while multiple CRLs have been proposed to regulate p53 (37), the precise CRL for p53 relevant in this setting is not clear. Generally, characterization of the role of p53 and p21 in MLN4924 and recombination biology has included intra-S-phase and G2–M checkpoint control and the induction of senescence (11, 38, 39). Our data suggest that the intra-S-phase checkpoint is the most important in this setting. Finally, the roles of CRLs in regulating BRCA1/BRCA2 and base excision repair have not yet been characterized. The role of BRCA1 in recombination is understood to be promoting the G2–M checkpoint (40), thereby protecting against cell death. Contrary to these expectations, depletion of most of the BRCA1/BRCA2-associated genes, especially BARD1, resulted in suppression, suggesting a role in promoting cell death. Three BRCA-associated genes, BARD1, BRCA1, and BRIP1, significantly reduced S-phase content (Fig. 3), suggesting that the role of this complex in DNA replication and S-phase progression may account for this discordance (41). Strikingly, early steps in BRCA1 complex formation are synthetic lethal, whereas genes in later steps are suppressors, suggesting that MLN4924 may affect BRCA1 function at the transition between these 2 sets of genes, similar to the interaction between bortezomib and genes within the Myc-polyamine pathway (31).
with MLN4924 in A375 cells but not HCT-116 cells. Mismatch repair–deficient cells, including HCT-116, also fail to arrest in S-phase following ionizing radiation treatment, in contrast to mismatch repair–proficient cells (43, 44).

The evaluation of gene knockdown using orthogonal techniques generally gives a much greater sense of the biologic impact of those genes. Notably, depletion of 69 genes gave significant cell-cycle interactions with MLN4924 (Fig. 2C and 3). The combined viability and cell-cycle data inform our understanding of the phenotypic consequences of NAE inhibition as discussed below and summarized in Fig. 6.

Regulation of replication origin firing

The canonical response to rereplication stress is G2–M phase accumulation (Fig. 6, blue arrows; refs. 15, 16), consistent with the results obtained for knockdown of replication genes (Fig. 2C). Following treatment with MLN4924, the cell-cycle impact of depletion of these genes was more divergent (Fig. 6, red arrows).

Intra S-phase checkpoint activation

Of the 32 genes whose depletion resulted in a significant increase in extensive rereplication as indicated by the accumulation of more than 4N DNA content (Fig. 3), 11 were associated with p53, including TP53, ATM, MDM2, CDKN1A, SFN, RMM2B (45), FKBP3 (46), KLF5 (47), PPP1R10 (48), RAB35 (49), and RBM38 (50). Knockdown of most of these genes also resulted in the reduction of the cells with 2N-4N DNA. As we have not shown the linearity of response with our antibodies, kinetics of protein effects are only approximate.

Figure 5. Phosphorylation of ATR substrates does not show dysregulation by MLN4924, although Cdc25A is rapidly stabilized. A, MLN4924 induces the phosphorylation of multiple ATR substrates, similar to other inducers of ATR activity, including aphidicolin and UV. Only the stabilization of Cdc25A, a Chk1-regulated event, differentiates MLN4924 from the other treatments. The kinetics of these phosphorylations are compared with the changes of other proteins previously shown to be substrates of CRL4-Cdt2 (Cdt1, p21, and Set8) or regulated by MLN4924. V, vehicle; M, MLN4924. B, the data were quantified by Odyssey Infrared Imager and normalized so that the 4-hour vehicle time point was 0 and the maximally regulated time point was 1. Maximum fold effect is indicated parenthetically beside each protein name. Cdt1 and Cdc25A achieved half-maximal stabilization within 3 hours. Phosphorylation of Chk1, Mcm2, Nbs1, Rad17, and Rpa32 achieved half-maximal levels in approximately 10 hours. Stabilization of p53 and p21 as well as phosphorylation of p53 and H2Ax achieved half-maximal levels in 15 to 23 hours. As we have not shown the linearity of response with our antibodies, kinetics of protein effects are only approximate.
Figure 6. Schematic of the influence of gene depletion on rereplication stress response. The canonical response of cells to rereplication stress is indicated in this schematic by the blue arrows. Apparent changes to this response pathway induced by MLN4924 are indicated by red arrows. The role of replication origin licensing genes in these effects is inferred from the cell-cycle effects given in Fig. 2C. Roles dominant only for vehicle-treated samples are indicated parenthetically. Roles dominant only for MLN4924-treated samples are indicated with red letters. A role for CRL1-Skp2 in the degradation of Cdt1 has been previously characterized but is not evaluated here. The likely step impacted by different gene sets from Fig. 3 is also shown. Human Genome Organization (HUGO) names for the genes are used.

slow induction of p53 and p21 (Fig. 5B, ~19 hours to half-maximal levels). In addition, depletion of ATR pathway genes did not affect sensitivity of HCT-116.

These results highlight one key difference between our data and the genetic interference studies on the regulation of replication origin firing, namely that very few genes within the ATR-dependent checkpoints were found to interact with MLN4924 in the A375 viability screen. Other researchers have suggested that MLN4924 may interfere with the function of the ATR-dependent checkpoints (12, 51). Our data suggests that the p21-dependent intra-S-phase checkpoint (52) may play a more dominant role in the response of A375 cells to NAE inhibition by MLN4924. Importantly, the ability of MLN4924 to induce the p21-dependent intra-S-phase checkpoint seems to be independent of Cdt1 stabilization or signaling through ATR (Fig. 4). In addition, MLN4924 stabilizes Cdc25A (Fig. 5A), which may account for the direct interference of MLN4924 in the activation of the ATR-dependent intra-S-phase checkpoint (53). The impact of MLN4924 beyond Cdt1 stabilization shows that MLN4924 will not simply mimic CDT1 overexpression.

A genome-wide siRNA viability screen provides an unbiased way of surveying possible drug mechanisms. We have used such a screen to characterize the genetics of sensitivity of the melanoma cell line A375 to MLN4924. Although it remains to be determined precisely how NAE inhibition impacts these multiple pathways, these results together with those investigating changes in protein expression following MLN4924 treatment (33, Supplementary Table S1K) will facilitate a better mechanistic understanding of the cullin-dependent regulatory and repair processes that compromise the viability of cancer cells.

Disclosure of Potential Conflicts of Interest
G. Hather is employed by Millennium Pharmaceuticals as a Principal Biostatistician. E.S. Lightcap is employed by Millennium Pharmaceuticals as a Scientific Fellow and has ownership interest (including patents) in Millennium Pharmaceuticals. All authors were employed by Millennium Pharmaceuticals at the time of their contribution to this work. No potential conflicts of interest were disclosed by the other authors.

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References


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