Quantitative Proteomic Analysis of Cellular Protein Modulation upon Inhibition of the NEDD8-Activating Enzyme by MLN4924

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Cullin-RING ubiquitin ligases (CRLs) are responsible for the ubiquitination of many cellular proteins, thereby targeting them for proteasomal degradation. In most cases, the substrates of the CRLs have not been identified, although many of those that are known have cancer relevance. MLN4924, an investigational small molecule that is a potent and selective inhibitor of the Nedd8-activating enzyme (NAE), is currently being explored in Phase I clinical trials. Inhibition of Nedd8-activating enzyme by MLN4924 prevents the conjugation of cullin proteins with NEDD8, resulting in inactivation of the entire family of CRLs. We have performed stable isotope labeling with amino acids in cell culture analysis of A375 melanoma cells treated with MLN4924 to identify new CRL substrates, confidently identifying and quantitating 5122–6012 proteins per time point. Proteins such as MLX, EID1, KLF5, ORC6L, MAGEA6, MORF4L2, MRFPAP1, MORF4L1, and TAX1BP1 are rapidly stabilized by MLN4924, suggesting that they are novel CRL substrates. Proteins up-regulated at later times were also identified and siRNA against their corresponding genes were used to evaluate their influence on MLN4924-induced cell death. Thirty-eight proteins were identified as being particularly important for the cytotoxicity of MLN4924. Strikingly, these proteins had roles in cell cycle, DNA damage repair, and ubiquitin transfer. Therefore, the combination of RNAi with stable isotope labeling with amino acids in cell culture provides a paradigm for understanding the mechanism of action of novel agents affecting the ubiquitin proteasome system and a path to identifying mechanistic biomarkers.

MLN4924 is an investigational small molecule inhibitor of the NEDD8-activating enzyme (NAE) (1) that is currently being explored in Phase I clinical trials. MLN4924 has been shown to be a selective inhibitor of NAE, inhibiting ~9% of bulk protein turnover in cells without impacting protein synthesis (1). Inhibition of NAE leads to the stabilization of a minor subset of proteasome-degraded proteins, namely those ubiquitinated in a cullin-RING ligase (CRL) dependent fashion (1). Many of the proteins targeted by cullins are known to have cancer relevance (2–4). In particular, the stabilization of Cdt1 leads to DNA rereplication and accumulation of cells in S-phase and this effect has been shown to be especially important for cell death by MLN4924 in most cancer cell lines studied (1, 5, 6), although stabilization of IκB plays a role in some settings (7). Rereplication leads to the activation of DNA damage repair processes, including ATR and ATM. However, it is likely that additional proteins affecting the sensitivity of cancer cells are stabilized by MLN4924. Such proteins may include NFE2L2 (Nrf2), p21, p27, cyclin E1, cyclin D1, Emi1, and Orc1, all of which are previously characterized CRL substrates (6). The identification of proteins that are stabilized by MLN4924 and the impact they have on cell death could provide important insights into the mechanism of cell death, inform the clinical utility of MLN4924, and identify possible pharmacodynamic and predictive biomarkers. It would also expand our understanding of the biological roles of the cullins.

The NEDD8-activating enzyme transfers the small ubiquitin-like protein NEDD8 onto Ubc12 in an ATP-dependent fashion, which then transfers NEDD8 onto one of seven cullins (8). Cullins are subunits within the CRL family of ubiquitin E3 ligases. Neddylation of the cullin allows the associated ubiquitin E2 enzyme to polyubiquitinate its substrate, thereby targeting it to the proteasome for degradation (9). Additional proteins modified by NEDD8 have been proposed (10, 11), as have proteins that associate with NEDD8 (12, 13). The dynamics of the cullin interactome following inhibition of NAE by MLN4924 has recently been extensively studied (14). Proteomic experiments designed to identify ubiquitinylated proteins have primarily used epitope-tagged ubiquitin (15–22) or ubiquitin affinity methods (23–27). However, because NAE inhibition blocks the ubiquitination of a minor subset of proteins...
teasome substrates, approaches relying on changes in global ubiquitination are unlikely to sufficiently enrich NAE-dependent changes.

Recently, major strides in the identification and quantification of proteins by mass spectrometry have been achieved by improvements in methodology and instrumentation. Stable isotope labeling with amino acids in cell culture (SILAC) has emerged as a particularly promising approach to quantitate protein abundance. A number of recent studies that provide a global quantitation of proteins from cell extracts have identified between 3880 and 5619 proteins (28–35). Therefore, such an approach might provide a means to detect changes in protein levels caused by MLN4924 treatment of cells.

Herein, we detail our global quantitation by SILAC of proteins within A375 melanoma cells treated with MLN4924 or aphidicolin, an inhibitor of S-phase. We identified 7689 proteins with two or more unique peptides in at least one sample. One hundred and thirty proteins were confidently up-regulated by MLN4924 by 1.8-fold or greater; 29 of 30 proteins evaluated by Western blotting were confirmed. Many of the proteins identified as being up-regulated by MLN4924 were near detection limits of the mass spectrometer. Furthermore, a larger set of 606 up-regulated proteins were identified by relaxing the selection criteria. Their impact on MLN4924-sensitive biology was then assessed by evaluating their genetic interaction with MLN4924 on cell viability by RNA interference. Thirty-eight of these stabilized proteins gave significant genetic interactions with MLN4924, half of which were confidently up-regulated. Four of 16 proteins identified with less confident mass spectrometric data but with demonstrated genetic interactions were confirmed to be up-regulated at least 1.8-fold by Western blot analysis. Strikingly, MLX, EID1, KLF5, ORC6L, MageA6, MORF4L2, MRFAP1, MORF4L1, and TAX1BP1 were stabilized within 4 h, suggesting that these may be novel CRL substrates. These results suggest that the sensitivity of SILAC experiments using modern analytical techniques has now made near proteome-wide analysis of ubiquitin-proteasome system achievable.

**EXPERIMENTAL PROCEDURES**

**SILAC Sample Preparation—**A375 cells were grown for 11 days with three passages in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin and containing either 0.5 mm each of L-Lysine-2HCl and L-Arginine-HCl or 15C415N4 L-Lysine-2HCl and 15C415N4 L-Arginine-HCl. All reagents for isotope metabolic labeling of the cells were from Pierce (Rockford, IL) except 2HCl and 13C6 (Carlsbad, CA). A sample of 1.5 mm fluoride, 25 mM orthophosphate, 50 mM sodium orthovanadate, 10 mM iodoacetamide, 1,10-phenanthroline monohydrate and 500 U/ml benzonase nuclease HC. Lysates were combined such that for each time point, drug-treated heavy metabolically labeled cells were mixed 1:1 by cell count with vehicle-treated light metabolically labeled cells, and vice versa. Lysates were stored frozen overnight at −80 °C. Following thawing of the sample, the soluble fraction was prepared by centrifugation of the extracts at 14,000 rpm for 10 min at 4 °C.

**Sample Preparation for Mass Spectrometry—**Samples were mixed with 2 x Laemmli SDS sample buffer containing alkylation reagent iodoacetamide (final concentration 50 mm). After 30 min of incubation at room temperature in the dark, the lysates were fractionated on a large format Protean xi (BioRad, Hercules, CA) tri-glycine SDS-PAGE gel (8–16%). The gel was sliced into 70–80 sections and trypsin in-gel digestion of the gel slices was performed (36). Dried protein digests were reconstituted in a solution containing 1% formic acid and 2% acetonitrile (v/v) for liquid chromatography/tandem MS (LC/MS/MS) analysis.

**Mass Spectrometry and Data Analysis—**Reconstituted protein digests were analyzed on a LC/MS/MS system comprised of an Eksigent NanoLC Ultra 2D-Plus liquid chromatography system, a NanoLC AS-2 autosampler (Eksigent, Dublin, CA), and a LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). A 15 cm PicoFrit column (75 μm inner diameter) packed with ProteoPep II C18 packing material (New Objective, Woburn, MA) was used for online peptide separation. A linear gradient of 2–40% B (89.9% acetonitrile and 0.1% formic acid with A as 0.1% formic acid) was used to elute peptides over 85 min. Eluted peptides were sprayed into the mass spectrometer through a Digital PicoView nanospray ion source (New Objective, Woburn, MA). MS1 scans were acquired using an AGC target of 2 x 106 for the Orbitrap, and a resolution of 100,000 at 400 amu. Each MS1 scan was followed by 10 data-dependent collision-induced dissociation MS/MS scans in the iontrap.

Raw MS data were processed using a combination of MaxQuant (version 1.0.13.13) (32) and Mascot (version 2.2.03, Matrix Science, London, UK). MaxQuant was used for postacquisition precursor m/z calibration, MS/MS spectrum peak picking, SILAC peptide ratio calculation, as well as protein grouping and quantitation. Database searching was done by using Mascot. For peptide/protein identification, the following modifications were included: carbamidomethylation (Cys, fixed), oxidation (Met, variable), N-acetylation (protein, variable), and pyro (Gln, variable). Trypsin specificity was set to exclude cleavages between Lys-Pro and Arg-Pro. Up to two missed cleavages and three labeled SILAC residues (Lys and Arg) per peptide were allowed. The MS/MS data were searched against a canted database combining the International Protein Index (IPI) human database (version 3.63, 84,118 sequences), the reversed sequences of all sequences in the IPI human database, and 262 commonly observed contaminates. The global false discovery rate for both peptides and proteins were set to 0.01. The posterior error probability threshold for peptide identification was set to 1 (no filtering). Minimum peptide length was six residues. Mass tolerances for precursor ions and product ions were 7 ppm (after calibration by MaxQuant) and 0.5 Da, respectively. For protein quantitation, three modifications were included, carbamidomethylation (Cys), oxidation (Met), and pyro (Gln), along with all unmodified peptides. Requantify function was enabled to capture SILAC pairs that were missed in the initial SILAC pair identification.

**siRNA Screening—**A375 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Three hundred cells/well were reverse transfected with 15 nM siRNA (siGENOME SMARTpool, Dharmacon) in OptiMem (Invitrogen) using 20 nL/well DharmaFECT 4 transfection reagent (Dharmacon, Lafayette, CO). Transfection occurred in 384-well CELLCOAT poly-D-lysine coated, black, clear-bottom plates (Greiner, Longwood, FL). Following 48 h incubation, 250 nM
MLN4924, 650 nM MLN4924, or 0.27% DMSO was added. After an additional 48 h incubation, viability was assayed using ATPtite (Perkin Elmer) and luminescence was measured using a LeadSeeker plate reader (GE Healthcare). SMARTpool deconvolution was performed identically, except that individual duplexes were transfected at 8 nM.

Gene Expression Profiling—A375 cells were seeded on 6-well plates (Falcon #353046) with 5 x 10⁵ cells/well (2 ml media) at 37 °C overnight. Following treatment with 650 nM MLN4924 or vehicle (0.065% DMSO) for the times indicated, A375 cells were harvested by trypsinization and centrifugation and stored in 350 μl RLT (Qiagen #79216) and 2-mercaptoethanol at −80 °C. Total RNA was extracted using MagMAX kits (Ambion, Austin, TX; #AM1839) on KingFisher Magnetic Particle Processors (Thermo Scientific). Labeled antisense RNA (cRNA) was synthesized by using the MessageAmp™ Premier RNA Amplification Kit (Ambion #AM1792). The resulting cRNA was then evaluated on Affymetrix Human Genome U133 Plus 2.0 arrays. All procedures were done according to manufacturers’ protocols and specifications. The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE30531 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE30531). Triplicate results were normalized by RMA and scored for significance by 2-way ANOVA for drug-time interaction with Benjamini-Hochberg multiple test correction (FDR) p < 0.05 being judged as significant. Sum of differences between MLN4924 and vehicle treatment were used to rank effect sizes.

Western Blot Analysis—A375 cells were maintained at 37 °C in a humidified atmosphere of 6% CO₂:94% air using DMEM medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen).

Following treatment with 650 nM MLN4924 or vehicle (0.065% DMSO) for the times indicated, cells were rinsed once with phosphate-buffered saline (PBS) and then harvested by scraping and centrifugation.

Cell pellets were lysed in RIPA buffer containing 1 x protease inhibitor mixture (Calbiochem, #539131), 50 mM sodium fluoride, 50 mM sodium orthovanadate, 25 mM β-glycerophosphate, and benzamidine, followed by freezing at −80 °C overnight and thawing at 4 °C with periodic vortexing. Insoluble material was removed by centrifugation (Eppendorf refrigerated microfuge, 14,000 rpm for 20 min).

Protein concentration was determined by Bradford Assay (Pierce) and quantitated by OD at 600 nm.

Cell samples were adjusted with RIPA buffer and normalized with tubulin (Millipore, #AB5564). Protein concentration was determined by Bradford assay and quantitative immunoblots (Odyssey Infrared Imager, LI-COR Biosciences). Protein expression levels were quantified using Odyssey Quant software (version 1.0.13.13) and Mascot (version 2.2) (32). After removing the common contaminant proteins and reverse protein hits, the number of proteins identified in each sample is given in Table I, whereas complete SILAC data is given in supplemental Tables S1A and S1B. On average, more than 6000 proteins were identified for individual samples with two or more unique peptides. Fig. 1A and supplemental Fig. S1 show the protein ratio distributions (plotted against total peptide MS signal intensities of each protein) for the 24-hour MLN4924 treated samples, averaging the two labeling directions. The use of both labeling directions, although doubling the time of analysis, provides an effective way of filtering out false identification of up- or down-regulated proteins. To assess the effectiveness of the fractionation, we tallied the numbers of fractions from which individual proteins have been detected. As shown in Fig. 1B, more than 72% of all identified proteins have been detected in three or fewer fractions. The median of all peptide ratios for a given protein is used as the protein ratio.

Where ID = the LI-COR intensity with vehicle treatment = d at time = t; IΔ is LI-COR intensity with vehicle treatment at time = 2 h and ID is LI-COR intensity with MLN4924 treatment at time = t. For supplemental Fig. S6, Δ was used instead of Δ.

RESULTS

SILAC Analysis of MLN4924-Induced Protein Stabilization—As an inhibitor of the NAE, the primary effect of MLN4924 is the stabilization of proteins rapidly turned over by the CRLs. In order to obtain a more complete understanding of the effects of this inhibition, A375 melanoma cells were prepared for SILAC analysis by growth in heavy (13C₆,15N₄-lysine and 13C₆,15N₄-arginine) or light media for 11 days (3 passages, ~7 doublings). In preliminary experiments, A375 cell growth was monitored in the presence of the SILAC medium supplemented with the heavy or light amino acids, each at a concentration of 100 mg/mL, and found to be essentially identical to that observed in normal growth medium (DMEM) which contains 146 mg/L L-lysine-HCl and 84 mg/L L-arginine-HCl. The heavy-labeled cells were treated with either 650 nM MLN4924 (LC₇₀ at 48 h) for 1, 4, or 24 h or 1 μg/ml aphidicolin, a DNA polymerase α inhibitor used as an S-phase arrest control, for 24 h. In parallel, light-labeled cells were treated with vehicle (0.05% DMSO). The experiment was repeated with light-labeled cells treated with MLN4924 or aphidicolin and heavy-labeled cells treated with vehicle. Cells were lysed in RIPA buffer and paired samples from each of the labelings were then mixed 1:1.

The soluble lysates were fractionated by SDS-PAGE, followed by in-gel tryptic digestion and LC/MS/MS analysis on an LTQ-Orbitrap Velos mass spectrometer. For the eight SILAC samples, a total of 584 runs of LC/MS/MS were collected. The data were analyzed using a combination of MaxQuant (version 1.0.13.13) and Mascot (version 2.2) (32). After removing the common contaminant proteins and reverse protein hits, the number of proteins identified in each sample is given in Table I, whereas complete SILAC data is given in supplemental Tables S1A and S1B.

On average, more than 6000 proteins were identified for individual samples with two or more unique peptides. Fig. 1A and supplemental Fig. S1 show the protein ratio distributions (plotted against total peptide MS signal intensities of each protein) for the 24-hour MLN4924 treated samples, averaging the two labeling directions. The use of both labeling directions, although doubling the time of analysis, provides an effective way of filtering out false identification of up- or down-regulated proteins. To assess the effectiveness of the fractionation, we tallied the numbers of fractions from which individual proteins have been detected. As shown in Fig. 1B, more than 72% of all identified proteins have been detected in three or fewer fractions. The median of all peptide ratios for a given protein is used as the protein ratio.

Importantly, from a list of 462 genes with known roles in DNA replication and damage response, 336 proteins (73%)...
protein represents one or a group of IPI entries. The column for Summary of protein identification and quantitation for individual samples. For protein identification, the global FDR was set at 1%. An identified indication that the inclusion of additional parameters does not need to include additional criteria (supplemental Results, supplemental Table S1). Also, from a list of 549 genes with published association with cullins, especially substrate recognition subunits and substrates, 228 proteins (42%) were detected in at least one sample (supplemental Table S1C).

Characterization of High Confidence Up-regulated Proteins—Receiver operating characteristic curve analysis provides a method of evaluating the diagnostic accuracy of multiple tests for a single outcome (37), in this case stabilization of known cullin-associated proteins. Receiver operating characteristic curve analysis applied to the prediction of stabilization of a known set of cullin substrates demonstrated that using thresholded normalized ratios for both heavy and light labelings of cells treated with MLN4924 for 24 h effectively captures the true positives from the SILAC data without the need to include additional criteria (supplemental Results, supplemental Fig. S2, S3, supplemental Table S2). Even though our receiver operating characteristic curve analysis indicated that the inclusion of additional parameters does not increase the power of the metric, we applied more stringent criteria for the validation of the experiment, namely up-regulation of more than 1.8-fold in both labelings at 24 h with a ratio significance (B) value of less than 0.05 and 2 or more unique peptides (“stringent criteria”). Under these stringent criteria, 120 proteins were found to be up-regulated (Table II), of which 34 were known cullin-associated proteins. Cullins were also found; the SILAC experiment is capturing changes in their neddylation status rather than total protein changes (supplemental Fig. S4). By evaluating which peptides were extracted from which slice of the gel (supplemental Table S3), the changes in mobility because of loss of neddylation for cullins 1–5, UBE2M (Ubc12), UBA3, and NAE1 can be observed (supplemental Fig. S5). The loss of NEDD8 from these proteins accounts for most of the profile of NEDD8 across the gel slices (supplemental Fig. S5); three more NEDD8 peptides were detected at 14–17 kDa. Finally, 27 proteins were up-regulated at 1 or 4 h, using the similarly stringent criteria (supplemental Table S4), including 10 proteins that did not pass the criteria at 24 h.

In order to identify which proteins are transcriptionally regulated, A375 cells were prepared for gene expression profiling, with time points of 1, 2, 4, 8, and 24 h treated with vehicle (0.27% DMSO) or 650 nm MLN4924 in triplicate (supplemental Table S5). 28 of the 120 stabilized proteins also had mRNA levels significantly increased at least twofold by MLN4924 treatment at any time point (Table II), suggesting that transcriptional regulation contributes to the regulation of these proteins. Seventeen of the 120 MLN4924-regulated proteins were also up-regulated by aphidicolin, suggesting that the regulation of these proteins is mostly because of accumulation in S-phase following treatment by MLN4924.

A number of well-documented cullin-dependent substrates are captured in Table II, including CDKN1A (p21), JUN, CCNE2 (cyclin E2), CCND1 (cyclin D1), IREB2 (irp2), WEE1, ORC1L, and CLSPN (clasin). Additional known cullin-dependent substrates were up-regulated in one of the two labelings, including NF2 (merlin), CCNE1 (cyclin E1), RASSF1, USP33 (VDU1), and CDKN1B (p27). Finally, a number of known cullin-dependent substrates were not detected or ap-

TABLE I

| Sample                               | Protein identification | Protein quantitation |
|--------------------------------------|------------------------|----------------------|
|                                       | ≥2 unique peptide | ≥1 unique peptide | ≥3 ratio count | ≥1 ratio count |
| Heavy/MLN4924_1 h                    | 6388                  | 8095                | 5851          | 7748          |
| Light/MLN4924_1 h                    | 6431                  | 8119                | 6012          | 7768          |
| Heavy/MLN4924_4 h                    | 5597                  | 7456                | 5156          | 7071          |
| Light/MLN4924_4 h                    | 5607                  | 7477                | 5122          | 7088          |
| Heavy/MLN4924_24 h                   | 6278                  | 8013                | 5817          | 7723          |
| Light/MLN4924_24 h                   | 6280                  | 7959                | 5800          | 7585          |
| Heavy/Aphidicolin_24 h               | 6313                  | 8008                | 5865          | 7700          |
| Light/Aphidicolin_24 h               | 6140                  | 7839                | 5661          | 7543          |
| All 8 samples                        | 7689                  | 9536                | N/A           | N/A           |

Fig. 1. Evaluation of protein ratios from SILAC data. A, Protein ratio distribution for the two samples at the 24 h time point. The plotted ratios are averages of ratios from the two labeling directions. The intensity on the y axis represents the sum of precursor MS intensities of all peptides for each protein. Proteins were excluded if their ratios from two labeling directions point to opposite regulations or if only one unique peptide was detected. B, Distribution of proteins based on number of fractions (gel slices) from which the proteins have been detected. The plot is based on a 24 h time point sample in which light labeled cells have been treated with DMSO and heavy labeled cells have been treated with MLN4924. All proteins were detected with 2 or more unique peptides.
peared to be unregulated, including CDC25A, CDT1, CTNN1B (β-catenin), HIF1A, MYC, NFE2L2 (Nrf2), NFkBIA (IkBα), and NFkBIB (IkBβ). Therefore, although our approach was successful in identifying a number of cullin-dependent substrates, clearly many are still being missed.

**Evaluation of Stabilized Proteins by siRNA**—In order to pursue the identification of important new CRL substrates, a balance must be sought between eliminating too many true positives using overly stringent criteria and including too many false positives by making the criteria too relaxed. The tolerance for false positives within a particular data set can often be increased by including an orthogonal data set that clarifies the interpretation of the results. Since we are interested in not only which proteins are increased following treatment by MLN4924, but also how the changes in protein levels affect sensitivity of cells to MLN4924, we evaluated the effect of knock-down of these proteins by siRNA on the ability of MLN4924 to induce cell death.

**TABLE II**

One hundred twenty high-confidence proteins up-regulated in A375 melanoma cells by MLN4924 at 24 h. SILAC analysis demonstrated that 120 proteins were up-regulated by more than 1.8-fold in both labelings at 24 h with a significance (B) p < 0.05 and 2 or more unique peptides. HUGO names are given. Proteins are ordered by the decreasing levels of up-regulation as indicated by SILAC. Proteins are classified as to whether they have previously been associated with cullins, either as substrates or subunits of cullin-RING ligase complexes, had mRNA up-regulated by MLN4924 (¶), or were separately up-regulated by aphidicolin treatment, suggesting that these proteins were predominantly up due to the accumulation of cells in S-phase. Up-regulation at 24 h by MLN4924 was confirmed for many proteins by Western blot of an independent experiment (*), as shown in Fig 2.

| Criteria | Synthetic lethal | Epistatic | Suppressor |
|----------|------------------|-----------|------------|
| >1.8X    | CDKN1A*          | SFR*      | HMOX1*     |
| 2+ peptides | FBXO44     | UBE2R2    | DTL*       |
| Both     | CCH2*            | FBXO17    | ORC1L*     |
| Labelings | CDC34*       | TK1*      | RRM2*      |
| >1.8X    | ICAM1*          | RBM38*    | INCENP*    |
| 2+ peptides | KLF5*        | PDCD2*    | CDC6*      |
| Either   | AVEN*           | CUL1*     | TAX1BP1*   |
| Labeling | LCP1            | DDX49     | USP36*     |

**TABLE III**

38 Stabilized proteins giving genetic interactions with MLN4924. Proteins are listed that had two or more oligos with either more (synthetic lethal) or less (epistatic or suppressor) cell kill than predicted by Bliss independent combination of the effects (threshold of 1.4-fold effect). HUGO names are given. Knock-down of suppressor genes resulted in less cell death than MLN4924, whereas epistatic genes had more cell death than MLN4924 but less than predicted based upon Bliss independence assumptions. Genes are in decreasing order of maximum up-regulation from MLN4924 treatment as indicated by SILAC within each group. ¶ mRNA up-regulated by MLN4924. * Regulation of protein by MLN4924 at 24 h confirmed in an independent Western blot experiment (>1.8X, except TAX1BP1 at 1.6X and CUL1 which showed deneddylation). † An independent Western blot experiment did not confirm regulation of protein by MLN4924 at 24 h.
All proteins that were up-regulated and had detection of at least two peptides in either labeling were evaluated. Six hundred and six proteins were up-regulated at least 1.8-fold following treatment by MLN4924 for 1, 4, or 24 h (supplemental Table S6A). It is anticipated that many of these proteins will be false positives. We obtained siRNA SMARTpools against 593 of the up-regulated proteins and evaluated them for interaction with MLN4924, using ATPlite as a measure of cell viability (supplemental Table S6B).

Gene knock-down in A375 cells by reverse transfection with 15 nm Dharmacon SMARTpool siRNA was allowed to proceed for 48 h to provide for adequate depletion of protein, followed by treatment for 48 h with MLN4924 (LC30 or LC75) or a vehicle control (0.27% DMSO). These drug concentrations allow for the evaluation of either enhancement or suppression of MLN4924-induced cell death by the SMARTpools, by which both activators and inhibitors of key pathways can be detected. The assays were performed in duplicate. One hundred and four SMARTpool hits were selected and rescreened in sextuplicate (supplemental Table S6C).

The scoring of the screen using Bliss independence (BI) assumptions enabled assignment of phenotypes to the siRNAs, namely, synthetic lethality, suppressor, or epistasis (38). The magnitude of the BI score indicates the relative strength of the effect on the induction of cell death.

In order to determine the likelihood that the phenotype was not because of off-target effects of the SMARTpools, the individual oligos making up each SMARTpool were further evaluated (four oligos per gene at 8 nm individual oligo). Sixty-one of the strongest interacting genes were evaluated in this way (supplemental Table S6D). Using the criteria that at least two of the four oligos had to impact MLN4924 sensitivity with a BI value of greater than 0.5 or less than −0.5 (1.4-fold), 38 proteins were identified whose increase significantly impacts MLN4924 sensitivity (Table III). Although we cannot directly determine the likelihood that two random oligos would give such an effect size from this data set, we have greater confidence in the effects of knocking down these particular proteins because the SILAC experiment had suggested that they were up-regulated by MLN4924. Importantly, half of these proteins would have been missed if we applied the more stringent proteomic criteria outlined above.

**Confirmation of SILAC Results by Western Blot**—To confirm the up-regulation of the proteins detected, we evaluated 30 proteins from Table II and supplemental Table S5 by Western blot quantitated using an Odyssey Infrared Imager (Fig. 2). Also included are known cullin substrates whose regulation was not evident by SILAC, including CDT1, NFE2L2 (Nrf2), CDC25A, CDKN1B (p27), CTNNB1 (β-catenin), HIF1A, and MYC. The coefficient of variance for the tubulin standard on the Western blots across the time course was less than 16%. Therefore, we used a limit of detection for up-regulation of 1.48-fold (3 standard deviation units) for Western blot analysis. SILAC suggests that 28 of these proteins are up-regulated more than 1.8-fold at 24 h meeting the stringent criteria outlined above; the other two (EID1 and CUL2) were regulated at 1 or 4 h. Western blot confirms a greater than 1.8-fold up-regulation for 25 of these (supplemental Table S7A). TK1, RRM2, and EID1 were up-regulated between 1.6- and 1.8-fold. MLX was up-regulated 1.2-fold at 24 h. Thus, >96% of the proteins that passed the stringent criteria were demonstrated by Western blot to be up-regulated at least 1.6-fold. Additionally, of 16 proteins that did not meet the stringent criteria (Table III), generally where only one labeling suggested a 1.8-fold regulation, four gave greater than 1.8-fold up-regulation at 24 h, TAX1BP1 showed 1.6-fold up-regulation at 8–12 h, and CUL1 demonstrated deneddylation. Finally, PDCD2 and ABL2 were up-regulated 1.4-fold, which may not be significant. Many lower stringency proteins confirmed (at least 25%). This result suggests that when only one labeling indicates up-regulation, there is a good probability that it is correct.

**Kinetics of Protein Stabilization**—With so many proteins whose Western blot data is substantiated by the SILAC data, we decided to evaluate the time courses for protein stabilization. It should be noted that the linearity of these antibodies has not been demonstrated under our conditions, so this kinetic characterization is only approximate. Western blots were normalized with the vehicle 2 h time point set to 0 and the maximum observed fold-regulation relative to the vehicle 2 h time point set to 1. Proteins can be classified according to when they achieve their half-maximal level (Fig. 2). These half-maxima parallel activities previously characterized to be affected by MLN4924: 6–4 h, stabilization of NFE2L2 (Nrf2) and CDT1, direct substrates of CRLs; 4–8 h, ATR phosphorylation of CHEK1; 8–12 h, stabilization of p53; and >12 h, ATM phosphorylation of H2AFX. Importantly, MLX, EID1, KLF5, ORC6L, MAGEA6, MORF4L2 (MrgX), MRFAP1 (PAM14), MORF4L1 (Mrg15), and TAX1BP1 are rapidly stabilized upon MLN4924 treatment and as such these may be novel CRL substrates. Finally, substantial post-translational modification of BRCA1 is evident in addition to its apparent stabilization. Sumoylation and ubiquitination of BRCA1 have been previously characterized (39–41).

Reduction of protein in the vehicle control lanes is apparent in a number of these blots, including CCNE2 (7.1X), DTL (4.0X), p-CHEK1 (3.0X), TP53 (2.7X), KLF5 (2.5X), TSPYL1 (2.5X), HMOX1 (2.4X), WEE1 (2.2X), CUL2 (2.2X), and H2AFX (2.2X). MYC (2.0X), KLF5 (1.8X), CTNNB1 (2.0X), JUN (1.8X) and CCND1 (1.8X). For CCNE2, DTL, p-CHEK1, KLF5, ORC6L, MAGEA6, MORF4L2 (MrgX), MRFAP1 (PAM14), MORF4L1 (Mrg15), and TAX1BP1 are rapidly stabilized upon MLN4924 treatment and as such these may be novel CRL substrates. Finally, substantial post-translational modification of BRCA1 is evident in addition to its apparent stabilization. Sumoylation and ubiquitination of BRCA1 have been previously characterized (39–41).
by SILAC for CDC6, CLSPN, CDC34, TFDP1, RRM2, RBM38, and RAD54B appear to be because of loss of protein with time in the vehicle control rather than gain with MLN4924 treatment. Two effects that could explain this loss of protein in-
ylated as cells enter S-phase, so loss of phospho-Rb1 would be consistent with cells exiting the cell cycle. p70S6K is phosphorylated by mTORC1 when growth factor activity is sufficient to support proliferation, so loss of phospho-p70S6K would be consistent with depletion of growth factors. As shown in supplemental Fig. S2, Rb1 phosphorylation in the vehicle samples instead increases 3.1-fold over the time course, so confluence cannot explain the effect. However, p70S6K phosphorylation falls 1.6-fold over the time course, parallel to the protein effects seen in supplemental Fig. S6. Thus, loss of a growth factor appears to explain the data.

Western blot time course analysis did not confirm SILAC results for INCENP, PDCD2, AVEN, ABL2, and HSF1 as these showed only modest time dependence in either the MLN4924 or vehicle treated samples.

DISCUSSION

The major challenge to obtaining in-depth coverage of the human proteome is the range of abundance levels of different proteins in a cell, with copy numbers ranging from tens of millions (e.g. ubiquitin) to single digits. Although advanced mass spectrometers are capable of detecting low femtomoles of peptides, sensitive enough for even the least abundant proteins in cells, the presence of high abundance proteins masks the detection of low abundance proteins, because of the limited dynamic ranges of mass spectrometers (<4 orders of magnitude) and limited loading capacities of capillary HPLC columns. Sample fractionation is often performed to partially alleviate the problem. In this study, we have taken a relatively simple approach to improve our limit of detection, using large format one-dimensional SDS-PAGE gels to achieve high-resolution fractionation of proteins, followed by one-dimensional reverse phase LC/MS/MS analysis of in-gel digests of thinly excised gel bands. This fractionation approach, in combination with the use of a fast-scanning mass spectrometer (LTQ Orbitrap Velos), has resulted in the identification (1% FDR and two or more unique peptides) and quantitation (three or more peptide ratio counts) of 5122–6012 proteins for individual cell lysate samples (Table I). This depth of coverage allows us to study the regulation of many low abundance proteins.

Proteomic analysis of inhibitors of the ubiquitin-proteasome pathway offers a particularly relevant application of SILAC. The direct effect of these inhibitors is anticipated to be the stabilization of normally rapidly degraded proteins. Many of these proteins are likely to be important regulatory proteins whose stabilization either activates or blocks key pathways. Typically, in unstressed cells, these proteins are kept at very low levels. Even with stabilization, most are not anticipated to be particularly abundant. Therefore, detection limits within the mass spectrometry experiment are particularly important in this setting.

We applied stringent criteria to the data set and identified 120 proteins, including 34 known cullin-associated proteins, that were up-regulated more than 1.8-fold at 24 h (Table II).
Importantly, probable new CRL dependent substrates, namely MLX, EID1, KLF5, ORC6L, MAGEA6, MORF4L2, MRFAP1, and MORFL1, were identified and their regulation confirmed by Western blot. MORF4, a protein 95 and 77% identical to MORF4L1 and MORF4L2, respectively, was recently demonstrated to be rapidly degraded by the proteasome (42).

By combining the SILAC data with RNAi data demonstrating which of these proteins may modulate MLN4924-induced cell death, we were able to identify 38 proteins whose stabilization is likely to be biologically relevant. The resulting gene list was evaluated by Ingenuity Pathway Analysis (Fig. 3). Intriguingly, 32 of the proteins have known direct interactions with each other. The Ingenuity Pathway Analysis suggests a role for these genes in cell cycle, DNA damage repair, and ubiquitin transfer. Three proteins were added to this analysis, namely p53—in Table II but whose knock-down does not have strong interactions with MLN4924—and NFE2L2 (Nrf2) and Cdt1, which were previously shown to be stabilized by MLN4924 (1). By identifying those proteins whose stabilization modulates the sensitivity of cells to MLN4924 using both SILAC and RNAi data, we are independently able to identify a set of proteins that suggests biology very much in line with the known mechanism of action of MLN4924 (1, 5, 6).

Antibodies against 38 of the identified proteins were obtained and protein level changes evaluated over a time-course of up to 24 h. This data allowed us to classify protein kinetics into five categories: Immediate, likely direct substrates; parallel with ATR phosphorylation of CHEK1; parallel with p53 stabilization; parallel with ATM phosphorylation of H2AFX, and down in vehicle controls.

Some of the events characterized in Fig. 2 are clearly related. HMOX1 is induced by NFE2L2 (Nrf2). SFN (14–3-3 and down in vehicle controls. stabilization; parallel with ATM phosphorylation of H2AFX, parallel with ATR phosphorylation of CHEK1; parallel with p53 stabilization; modulates the sensitivity of cells to MLN4924 using both SILAC and RNAi data, we are independently able to identify a set of proteins that suggests biology very much in line with the known mechanism of action of MLN4924 (1, 5, 6).

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