Identification of Mixed Lineage Leukemia 1 (MLL1) Protein as a Coactivator of Heat Shock Factor 1 (HSF1) Protein in Response to Heat Shock Protein 90 (HSP90) Inhibition*

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Background: The efficacy of HSP90 inhibitors may be limited by HSF1-mediated feedback mechanisms. Results: MLL1 regulates HSF1-target genes upon HSP90 inhibition, and MLL1 depletion shows a striking combination effect in human cancer. Conclusion: MLL1 functions as a coactivator of HSF1 upon HSP90 inhibition. Significance: This is the first report of MLL1 as a coactivator of HSF1 upon HSP90 inhibition.

Heat shock protein 90 (HSP90) inhibition inhibits cancer cell proliferation through depleting client oncoproteins and shutting down multiple oncogenic pathways. Therefore, it is an attractive strategy for targeting human cancers. Several HSP90 inhibitors, including AUY922 and STA9090, show promising effects in clinical trials. However, the efficacy of HSP90 inhibitors may be limited by heat shock factor 1 (HSF1)-mediated feedback mechanisms. Here, we identify, through an siRNA screen, that the histone H3 lysine 4 methyltransferase MLL1 functions as a coactivator of HSF1 in response to HSP90 inhibition. MLL1 is recruited to the promoters of HSF1 target genes and regulates their expression in response to HSP90 inhibition. In addition, a striking combination effect is observed when MLL1 depletion is combined with HSP90 inhibition in various human cancer cell lines and tumor models. Thus, targeting MLL1 may block a HSF1-mediated feedback mechanism induced by HSP90 inhibition and provide a new avenue to enhance HSP90 inhibitor activity in human cancers.

Molecular chaperone proteins assist in the folding of nascent polypeptides and promote the correct assembly or disassembly of protein complexes (1, 2). The molecular chaperone heat shock protein 90 (HSP90) is involved in stabilizing and activating many proteins with potentially oncogenic functions (“oncoproteins”), such as tyrosine kinases (2–4), steroid hormone receptors (5), and AKT (6), and is required for the survival of cancer cells dependent on these oncoproteins (7, 8). Since tanezumycin (17-AAG), the first HSP90 inhibitor, entered clinical trials in 1999, 13 different HSP90 inhibitors have undergone clinical evaluation in cancer patients (8, 9). Among them, AUY922 and STA9090 are novel, non-geldanamycin-derivative HSP90 inhibitors that have shown significant antitumor activity in a wide range of cancer cell lines, primary tumor cells, and animal cancer models (10). These effects have also been observed in clinical trials. AUY922 has shown activity in a phase II study in patients with anaplastic lymphoma kinase rearranged and epidermal growth factor receptor mutated non-small cell lung cancer (10).

However, the efficacy of HSP90 inhibitors might be limited by cell stress responses induced by HSP90 inhibition (11, 12). We have shown recently that the induction of an HSF1-dependent heat shock response plays a critical role in limiting the efficacy of HSP90 inhibitors (13). As such, heat shock factor 1 (HSF1) knockdown, combined with HSP90 inhibitors, leads to a striking inhibitory effect on tumor growth both in vitro and in vivo (13). In addition, dual targeting of the HSF1-mediated genes heat shock cognate protein 70 (HSC70) and heat shock protein 72 (HSP72) have been shown to enhance the efficacy of HSP90 inhibitors and induce tumor-specific apoptosis (14). Previous work suggests that coregulatory complexes play an important role in regulating HSF1-mediated transcriptional activity under heat shock (15, 16). For example, coexpression of RE1 silencing transcription factor (CoREST), an integral component of a histone deacetylase complex including the histone lysine-specific demethylase (LSD1), is bound to the HSP70 gene promoter under basal conditions. CoREST recruitment is decreased during the heat shock response, suggesting its role in repressing HSF1-dependent transcriptional activation of HSP70 gene expression (15, 16). However, it is presently unclear how HSF1 may interact with coregulatory mechanisms to elicit such changes in epigenetic status and affect transcription.

We performed a large scale siRNA screen to discover coregulators whose activities may be required for HSF1-mediated transcriptional activity induced by HSP90 inhibition and iden-
tified mixed lineage leukemia 1 (MLL1) as a coactivator of HSF1. MLL1 is a SET domain containing histone H3K4\(^3\) methyltransferase and involved in chromatin remodeling and transcriptional regulation (17–25). In this study, we show that MLL1 regulates HSF1-dependent transcriptional activation upon HSP90 inhibition and that MLL1 depletion combines with HSP90 inhibition to increase cell death in various cell lines and robustly affect growth in a mouse xenograft model. These studies suggest that MLL1 is an important coactivator for HSF1 and that its inhibition may further enhance the activity of HSP90 inhibitors.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—A375, A2058, and HCT116 cells were obtained from the ATCC. MLL1\(^{−/−}\) MEFs were from the laboratory of Jay L. Hess (University of Michigan). Men1\(^{−/−}\) MEFs were from the laboratory of Xiaxin Hua (University of Pennsylvania). All cell lines were maintained in Dulbecco’s modified Eagle’s medium, McCoy’s 5a medium, or advanced RPMI 1640 medium (Invitrogen) with 10% FBS (Invitrogen). Infected cell lines were maintained under 1 μg/ml puromycin (MP Biomedicals) for selection.

**siRNA Screening**—The A375 cell line with an integrated HSP70 promoter-driven luciferase reporter (A375-HSP70-pro-LUC) activated by HSP90 inhibitor treatment was established. To perform a high-throughput siRNA screen, the full siRNA library was stamped out in 384 well plates as well as HSF1 siRNA as a positive and negative control. RNAiMax was added to each well and incubated further. Then, cancer cells with the HSP70 promoter-driven luciferase reporter were plated and incubated for 72 h, and AUY922 was added and incubated for 6 h. Finally, Bright-Glo (BG) was added to measure the luminescence of the HSP70 reporter. In the second-round screen, siRNA screen data were analyzed by both BG and CellTiter-Glo (CTG) assays. The latter measured overall cell viability. Data were normalized and exported to a spotfire file for viewing. An average by siRNA replicate was calculated for each assay. Following this, differences between the BG and CTG scores for each siRNA average were taken. These differences were averaged for each gene ID and then sorted by Δ (the greatest difference between BG and CTG should then be the strongest hits because the top hits that affected BG signal without affecting CTG were searched). The follow-up assays, such as the HSP70 luciferase assay and the endogenous HSP70 gene expression assay after knockdown of hits selected from the above screen, were performed. siRNA reagents to knock down each individual hit were from Dharmacron (Lafayette, CO). For each transfection, 30 pmol of siRNAs were transfected into cells using RNAiMax (Invitrogen) with 2.5 ml of growth medium according to the protocol of the manufacturer. Knockdown efficiency was examined after 72 h by RT-PCR.

**Plasmid and Short Hairpin RNA Constructs**—pCXN2 plasmids expressing full-length MLL1 or MLL1 with deletion of its SET (catalytic) domain (MLL1ΔSET) were obtained from Dr. Jay Hess (University of Michigan). Control shRNA (GGATAATTGTTAGTATGATG), MLL1 shRNA#1 (GCACTGT-TAAACATCCCCT), and MLL1 shRNA#2 (CGCCTAAAGCAGCTCTCATTT), were cloned into the inducible pkLO-Tet-On puromycin vector (26, 27).

**Lentivirus and Infection**—Lentiviral supernatants were generated according to our protocol established previously (26, 27). A total of 100 μl of lentivirus was used to infect 300,000 cancer cells in a 6-well plate in 8 μg/ml Polybrene (Chemicon). The medium was replaced, and, after 24 h, cells were selected by 1 μg/ml puromycin (MP Biomedicals) and expanded. Induction of shRNA was obtained by addition of 100 ng/ml doxycycline (Clontech) to the medium.

**RNA Extraction and Quantitative Reverse Transcription PCR**—Total RNA was isolated using the RNeasy mini kit (Qiagen). ABI TaqMan gene expression assays included HSP70, BAG3, HSP27, ZFAND2A, HMOX1, HSF1, and MLL1. VICMGB primers/probe sets (Applied Biosystems) were used in each reaction to coamplify β2-microglobulin transcripts as a normalization control. Quantification of relative mRNA expression levels was determined and normalized to β2-microglobulin expression. All experiments were performed in triplicate. Relative mRNA expression was calculated using the formula 2\(^{−(CT \text{ of sample} − CT \text{ of β2-microglobulin})}\), where CT (cycle count) is the threshold cycle value. The means ± S.D. of three replicates were normalized to the corresponding values of the internal control gene β2-microglobulin.

**Chromatin Immunoprecipitation Assay**—A ChIP assay was carried out according to the protocol of the manufacturer (catalog no. 17-295, Upstate Biotechnology Inc., Lake Placid, NY). Immune complexes were prepared using anti-HSF1 antibody (Cell Signaling Technology, catalog no. 4356) antibody, anti-MLL1 antibody (Bethyl Laboratories, catalog no. A300-086A), anti-H3K4me2 antibody (Thermo Scientific, catalog no. MA511196), and anti-H3K4me3 antibody (Thermo Scientific, catalog no. MA511199). The supernatant of the immunoprecipitation reaction carried out in the absence of antibody served as the total input DNA control. Quantitative PCR was carried out with 10 μl of each sample using the following primers: HSP70 promoter, 5’-GGCGAAAACCCCTGGAATTTCCCGA-3’ and 5’-AGCCTTGGGACAACGGGAG-3’; BAG3 promoter, 5’-GTCCCTCCTTACAAGGGA-3’ and 5’-CAAATGCTA-TGTGAAACCTG-3’; and MEIS1 promoter, 5’-CGGGCT-TGATCCCAAATTATTCA-3’ and 5’-CACACAAAGCAGGCTAG-3’. The percent input method was used to analyze ChIP quantitative PCR data. Signals obtained from the ChIP were divided by signals obtained from an input sample. The input sample represents the amount of chromatin used in the ChIP. Typically, 1% of starting chromatin is used as the input. Values (ratios of ChIP to corresponding inputs) are the mean ± S.E. of two independent experiments.

**Cell Viability Assay**—Cell viability at the starting (day\(^0\)) and ending (day\(^{d\text{end}}\)) days of compound treatment was determined by measuring cellular ATP content using the CTG luminescence assay (Promega). CTG reagent was added to each well, and luminescence was recorded on an Envision plate reader (PerkinElmer Life Sciences). Luminescence values were used to
calculate the inhibition of cell viability relative to DMSO-treated cells (0% inhibition). Half-maximal inhibitory concentration (IC$_{50}$) and median lethal dosage (LD$_{50}$) were also calculated. The results of CTG at day$^0$ and day$_{end}$ were collected. The LD$_{50}$ curve was plotted by using the value of (CTG at day$^0$ – CTG at day$_{end}$) / CTG at day$^0$ at a different drug dosage.
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Microarray Data Profiling and Analysis—RNA was isolated using the Qiagen RNeasy mini kit. Generation of labeled cDNA and hybridization to HG-U133 Plus2 arrays (Affymetrix) were performed as described previously (28). Two-sample regulated Student’s t tests were performed using Limma Package in R (citing limma), and the resulting statistics scores were used further for a gene set enrichment analysis (29, 30).

All expression arrays were summarized by robust multi-array average with Michigan Custom CDF. A differential expression analysis was performed by Limma Package in R. Heat maps were generated in the space of top differential genes with hierarchical clustering, and top differential genes were used to generate heat map hierarchical clustering and Java Treeview (31). The heat map compares subject-level gene expression under each condition, and each column represents one biologic sample. Gene expression was normalized for each row. Expression profiles for a subset of genes up-regulated by AUY922 are shown as a heat map. The heat map compares subject-level gene expression under each condition. Gene expression is normalized for each row. Lower levels of expression are represented in shades of blue and higher expression in yellow.

Pathway Enrichment Scores—For the candidate signature (32), two-tailed Fisher’s exact test was used to determine whether probe sets representing genes in those signatures were under- or overrepresented in the set of probe sets that were up- or down-regulated at least 1.5-fold compared with expressed but nondifferentially expressed probe sets, with a nominal p value of 0.05 or less compared with all probe sets. For an unbiased approach, pathways derived from Gene Ontology terms and transcription factor networks were analyzed for overrepresentation via one-tailed interpolated Fisher’s exact test using genes that varied 1.5-fold or more with a nominal p value of 0.05 or less compared with all genes represented on the array. A Benjamini-Hochberg correction was then applied to these p values (33).

Flow Cytometry—Cells were collected from a 6-well plate, washed with PBS, and stained with 7AAD (BD Biosciences) and Annexin V (Life Technology) for 30 minutes. After staining, the cells were stained once with PBS and subjected to FACS analysis. Early apoptotic cells were 7AAD−Annexin V−, and late-stage apoptotic or dead cells were 7AAD+Annexin V+.

Western Blotting—Cells were lysed in radioimmune precipitation assay buffer (Boston Bioproducts) containing halotransfase and phosphatase inhibitor mixture (Thermo Scientific). Lysates were spun at 16,000 × g at 4 °C for 30 min and normalized for protein concentration. Western blotting was performed as follows. Total tumor lysates were separated by SDS-PAGE and electrotransferred to nitrocellulose membranes (Invitrogen). Membranes were blocked in PBS plus 0.1% (v/v) Tween 20 (PBS-T) and 4% (w/v) nonfat dry milk (Bio-Rad) for 1 h on a shaker at room temperature. Primary antibodies were added to the blocking solution at 1:500 (MLL1, Abcam, catalog no. 32402) and 1:10,000 (GAPDH, Cell Signaling Technology, catalog no. 2118S) dilutions and incubated and rocked overnight at 4 °C. Immunoblots were washed three times, 5 min each with PBS-T, and the secondary antibody was added at a 1:10,000 dilution into PBS-T milk for 1 h on a shaker at room temperature. After several washes, ECL reactions were performed according to the recommendations of the manufacturer (SuperSignal West Dura extended duration substrate, Thermo Scientific).

Tumor Xenografts—Mice were maintained and handled in accordance with Novartis Biomedical Research Animal Care and Use Committee protocols and regulations. A375 cells with Tet-inducible shRNA against MLL1 were cultured in DMEM supplemented with 10% Tet-approved FBS. Mice (6–8 weeks old, n = 8) were inoculated subcutaneously with 1 × 106 cells in the right dorsal axillary region. Tumor volume was measured by calipering in two dimensions and calculated as (length × width²) / 2. Tumor weight was estimated with the assumption that 1 mg is equivalent to 1 mm³ of tumor volume. Drug treatment started 11 days after implant when the average tumor volume was 200 mm³. Animals received vehicle (5% dextrose, 10 ml/kg, orally, once a week) or NVP-HSP990 (10 mg/kg, orally, once a week) for the duration of the study. At termination of the study, tumor tissues were excised and snap-frozen in liquid nitrogen for immunoblot analyses of biomarkers. Data are expressed as mean ± S.E., and differences are considered statistically significant at p < 0.05 by Student’s t test.

RESULTS

An siRNA Screen Identifies MLL1 as a Potential Coactivator of HSF1-mediated Gene Expression in Response to HSP90 Inhibition—To identify transcriptional modulators of HSF1 in response to HSP90 inhibition, we first generated a derivative of

FIGURE 1. An siRNA screen identifies MLL1 as a coregulator of HSF1 in response to HSP90 inhibition. A, HSP70-driven luciferase reporter in response to AUY922 treatment. A375-HSP70.pro-LUC cells were treated with AUY922 for 6 h and then harvested for the luciferase assay. B, schematic of the siRNA screening experiment design. C, list of 18 genes selected from the second round of siRNA screening. D, HSP70 LUC reporter activities after knockdown of individual hits. A375-HSP70.pro-LUC cells were treated with four mixed siRNAs per gene for 2 days, followed by AUY922 treatment for 6 h, and then harvested for the luciferase assay. The relative HSP70 LUC reporter activities were normalized to the control. Values are the relative values to the normalized activity from NTC siRNA (bar 1, set as 1) and are expressed as the mean ± S.D. of triplicates from one representative experiment. E, relative HSP70 mRNA expression after knockdown of individual hits. The mRNA expression of HSP70 was measured by real-time PCR upon gene knockdown under AUY922 treatment conditions. The relative gene expression was normalized to the control. F, HSP70-LUC reporter activities after knockdown of individual H3K4 methyltransferase. A375-HSP70.pro-LUC cells were treated with four mixed siRNAs for each individual H3K4 methyltransferase for 2 days, followed by AUY922 treatment for 6 h, and then harvested for the luciferase assay. Values are the relative values to normalized activity from NTC siRNA (bar 1, set as 1) and are expressed as the mean ± S.D. of triplicates from one representative experiment. G, HSP70-LUC reporter activities were normalized to the control. H, mRNA expression of HSP70, BAG3, and HSF1. A375 cells were treated with four mixed siRNAs for each gene for 2 days, followed by AUY922 treatment for 6 h, and then harvested for mRNA isolation. Real-time PCR analysis was performed to measure the expression of HSP70, BAG3, and HSF1. The relative gene expression was normalized to the control. I, mRNA and protein expression of MLL1 upon MLL1 knockdown. shNTC- or shMLL1-transduced stable cell lines were treated with doxycycline (Dox) for 3 days, cell pellets were collected, and real-time PCR and Western blotting were performed. *, p < 0.05; **, p < 0.01.
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**A**

- **MLL1**
- **HSF1**
- **HSP70**

- **BAG3**
- **HSP27**
- **ZFAND2A**

- Relative mRNA levels
- DMSO vs. AU922
- shNTC, shMLL1#1, shMLL1#2
- Dox

**B**

- **HSP70 promoter**
- **BAG3 promoter**
- **MES1 promoter**

- % Input
- DMSO vs. AU922
- shHSF1, Beads, anti-HSF1

**C**

- **HSP70 promoter**
- **BAG3 promoter**
- **MES1 promoter**

- % Input
- DMSO vs. AU922
- shMLL1, Beads, anti-MLL1

**D**

- **BAG3 promoter**

- % Input
- DMSO vs. AU922
- shMLL1, Beads, H3K4 Me2, H3K4 Me3

**E**

- Western blot
- shMLL1
- MLL1, HSP70, GAPDH
- DMSO, 100nM, 200nM AU922 (6h)

**F**

- Western blot
- Immunoprecipitation (IP)
- Input, IgG α-HSF1
- AUY922 (100nM)
- MLL1, HSF1

**G**

- Relative fold change
- shNTC, shHSF1, shMEN1, shWDR5
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A375 cells with an integrated HSP70 promoter-driven luciferase construct (A375-HSP70.pro-LUC) and validated that this line has a robust luciferase induction upon exposure to AUY922 (Fig. 1A). Next, we performed two rounds of siRNA screens using an siRNA library containing 7000 genes (4 siRNAs/gene) (Fig. 1B). siRNAs that reduced AUY922-induced luciferase activity by 50% in the first screen were chosen for further follow-up. From this, a total of 1000 siRNAs targeting 264 genes were selected for the second-round screen. In the second-round screen, we ruled out siRNAs that reduced luciferase activity because of cell death by measuring both luciferase activity and cell viability with each siRNA upon AUY922 treatment. From this, HSF1 was identified as a positive control, and 18 genes were selected for further follow-up (luciferase activity/reduction to viability reduction >2, Fig. 1C) because cell viability was not significantly affected by knockdown of these genes (data not shown). Knockdown of IFFO1, MED6, MED21, MLL1, SMARCD3, and MED19 as well as HSF1 led to a decrease of HSP70.pro-LUC reporter activity (Fig. 1D) without appreciably affecting viability (data not shown). We then asked whether knockdown of these six genes might affect the expression of the endogenous HSF1 target gene HSP70 when induced by HSP90 inhibitor treatment. Knockdown of three genes, MED6, MED21, and MLL1, reduced endogenous HSP70 mRNA expression upon AUY922 treatment (Fig. 1E). MED6 and MED21 are chromatin remodeling factors that are generally involved in regulating RNA polymerase II-dependent transcription activity (34), and MLL1 is a known H3K4 methyltransferase involved in gene transcriptional activity, cell cycle, and tumor growth (8, 17, 23). Therefore, we focused on further validation of whether MLL1 may be a coactivator for HSF1-mediated heat shock response induced by HSP90 inhibition.

The MLL1-like family of methyltransferases includes SET1A, SET1B, MLL1, MLL2, MLL3, MLL4, MLL5, and MLL6 (35). To test whether the effect of MLL1 on HSF1-regulated activity was unique among H3K4 methyltransferases, we first examined the effects of these methyltransferases on HSP70.pro-LUC reporter activity. Knockdown of MLL1, MLL3, and SET1B, but not MLL2, MLL4, MLL5, MLL6, and SET1A, led to a reduction of HSP70-LUC reporter activity induced by HSP90 inhibition (Fig. 1F). In contrast, overexpression of MLL1 full-length, but not MLL1ΔSET, led to an increase in HSP-LUC reporter activity induced by HSP90 inhibition (Fig. 1G). To further validate those findings, we tested for effects of siRNA against MLL1, MLL3, and SET1B on the expression of the endogenous HSF1 target genes HSP70 and BAG3. Only knockdown of MLL1 and SET1B reduced the expression of HSF1 targets, whereas neither affected the expression of HSF1 itself (Fig. 1H).

These results highlight that both MLL1, and likely SET1B, are involved in HSF1-mediated transcriptional activation in response to AUY922. However, dual knockdown of both MLL1 and SET1B did not have an increased effect on HSP70 expression (data not shown), which suggests that they may have redundant functions in modulating HSF1 function. We focused further work on the validation of MLL1 as a coactivator of HSF1-regulated gene expression in response to HSP90 inhibition.

MLL1 Affects HSF1 Target Gene Expression and Binds to the Promoter Regions of HSF1 Target Genes in Response to HSP90 Inhibitors—Next, we asked whether MLL1 may function as a coregulator in heat shock gene expression using A375 cancer cell line with doxycycline-inducible knockdown of MLL1, and knockdown of either MLL1 or HSF1 was confirmed (Fig. 1I). To do this, we first established A375 cancer cell lines with inducible knockdown of MLL1 by two different shRNA sequences by targeting the distinct sequence of the MLL1 gene, and robust MLL1 knockdown was achieved (Fig. 2A). MLL1 knockdown did not affect HSF1 expression at the mRNA level (Fig. 2A). However, the AUY922-induced mRNA expression of the HSF1 target genes HSP70, HSP27, ZFAND2A, and BAG3 was impaired upon MLL1 knockdown, as judged by real-time PCR (Fig. 2A). Next, we asked whether MLL1 may directly regulate HSF1 target gene expression or function as a coactivator of HSF1. To this end, ChIP experiments were performed to examine whether MLL1 can bind to the HSF1 target gene promoters. ChIP data revealed that, upon AUY922 treatment, the recruitment of either HSF1 or MLL1 was increased on the promoter of the endogenous HSF1 target genes HSP70 and BAG3 and that the recruitment of either HSF1 or MLL1 was decreased when either gene was knocked down (Fig. 2, B and C). As a control, we did not observe that HSF1 and MLL1 were present on the promoter of an HSF1-unrelated target gene (MEIS1, Fig. 2, B and C). Because the MLL1 protein complex mediates the di- and trimethylation of H3K4, we then tested whether those histone
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FIGURE 3. Loss of Mll1 impairs HSF1 pathway activation in response to HSP90 inhibition. A and B, Gene Ontology pathway enrichment analysis of gene expression profile changes induced by Mll1 deletion and AUY922 treatment in MEFs. C, gene set enrichment analysis displaying the expression profiling of HSF1 pathway activation between Mll1+/+ MEFs treated with AUY922 versus Mll1−/− MEFs treated with AUY922. Enrichment plots for the HSF1 activation pathway up-regulated in each cohort are shown, with the relative gene positions indicated by straight lines (line plot) under each graph. Lines clustered to the left represent higher-ranked genes in the ranked list. The top portion of the plot shows the running of the enrichment score as the analysis walks down the ranked list. The lower portion of the plot shows where the members of the gene set (black lines) appear in the ranked list of genes (red-blue gradient). D, heat map showing the differential expression of genes regulated by AUY922 in Mll1+/+ and Mll1−/− MEFs. Expression profiles for a subset of genes up-regulated by AUY922 are shown as a heat map. The heat map compares subject-level gene expression under each condition, and each column represents each biologic repeat. Gene expression is normalized for each row. Lower levels of expression are represented in shades of blue (two-time decrease) and higher expression in yellow (two-time increase). E, the mRNA expression of Hsp70, Bag3, Zfand2a, and Hsf1 in Mll1+/+ and Mll1−/− MEFs with AUY922 treatment. Mll1+/+ or Mll1−/− MEFs were treated with or without 100 nM AUY922 for 6 h. Total RNA was collected, and real-time PCR was performed.* p < 0.05; **, p < 0.01.
markers may be regulated by MLL1 in the promoter of the HSF1 target gene BAG3. ChIP data showed that H3K4me2 and H3K4me3 were increased at the BAG3 promoter by AUY922 treatment and that the recruitments were decreased by MLL1 knockdown (Fig. 2D). Next, we asked whether the protein levels of those genes were also affected. A375 cells were treated with different doses of AUY922, and MLL1 knockdown reduced AUY922-induced HSP70 protein expression (Fig. 2E). To confirm that MLL1 and its complex physically bind to HSF1 under HSP90 inhibition, we performed coimmunoprecipitation in A375 cells. The interaction between HSF1 and MLL1 under HSP90 inhibition was observed endogenously by using nuclear
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extract from A375 cells (Fig. 2F). Functionally, we showed that knockdown of WDR5 or MEN1 can attenuate HSP90 inhibition-induced, HSF1-mediated reporter activities (Fig. 2G), which suggests that the MLL complex participates in HSF1-mediated transcription activation under stress conditions. Taken together, these results indicate that the MLL1 complex is involved in heat shock gene expression and binds to HSF1 target gene promoters in response to HSP90 inhibitor treatment in A375 cells.

MLL1 Functions as a Coactivator of HSF1 Target Gene Expression in Response to HSP90 Inhibition—To determine whether HSF1-regulated pathways were regulated globally by MLL1, we performed a microarray experiment using MLL1+/+ and MLL1−/− MEFs treated with or without AUY922. Under basal conditions, we found a number of differences between MLL1 wild-type and knockout cells, suggesting that MLL1 has pleiotropic signaling effects on multiple pathways. For example, loss of MLL1 significantly down-regulates cell cycle-regulated pathways, such as E2F1 activation (Fig. 3A), which is consistent with previous findings (36). However, a pathway enrichment analysis indicated that the “HSF1 activation” pathway was the only significantly up-regulated pathway in MLL1+/+ MEFs upon AUY922 treatment (Fig. 3B). When we compared the differential pathway enrichment scores between MLL1+/+ and MLL1−/− MEFs upon AUY922 treatment, the HSF1 activation pathway was the only significant pathway down-regulated by MLL1 deletion upon AUY922 treatment, which suggests that MLL1 knockout globally reduced the heat shock response induced by AUY922 (Fig. 3B). A gene set enrichment analysis further supports that the magnitude of the “HSF1 activation gene set” is weaker in MLL1−/− MEFs compared with MLL1+/+ MEFs upon AUY922 treatment (Fig. 3C). The heat map reveals that differential HSP90 inhibition induced the gene expression profile of MLL1+/+ MEFs and MLL1−/− MEFs upon AUY922 treatment (Fig. 3D). To further validate these findings, we examined the expression of several HSF1 target genes in MLL1+/+ MEFs and MLL1−/− MEFs treated with or without AUY922 by real-time PCR. The induction of the HSF1 target genes Hsp70, Bag3, and Zfand2a2A were impaired by loss of MLL1, whereas HSF1 expression was not affected (Fig. 3E). Taken together, these data suggest that MLL1 functions as a coactivator of global HSF1-mediated heat shock activation upon HSP90 inhibition.

MLL1 Depletion Sensitizes Mouse Embryonic Fibroblasts and Cancer Cells to AUY922—We next asked whether MLL1-dependent regulation of heat shock activation has functional consequences for cell survival in the context of pathway induction. To address this, we performed proliferation assays in MEFs and human cancer cells to examine whether MLL1 depletion, combined with AUY922, could enhance the effects of this compound. Upon AUY922 treatment, the proliferation of MLL1+/+ MEFs was inhibited, with an IC50 of around 10 nM. In contrast, the absence of MLL1 caused AUY922 treatment to have more profound effects. Although the IC50 was relatively unchanged, higher doses of AUY922 (>10 nM) caused apparent lethality, as judged by cell numbers that were less than the initial seeding density (Fig. 4A). To expand this finding, we took advantage of Men1−/− MEFs because Men1 is one component of the MII complex (37). Similar to results with MII1−/− MEFs, the induction of Hsp70 by AUY922 was impaired by loss of Men1, which suggests that Men1 is also involved in the heat shock response induced by AUY922 (Fig. 4B). Consistent with the results in MII1 null MEFs, loss of Men1 also sensitized Men1 knockout MEFs to AUY922 in a manner similar to that was observed with loss of MII1 (Fig. 4C). To exclude the possibility that loss of Men1/MLL1 may broadly sensitize MEFs to other inhibitors, we tested MEFs in response to the cell cycle kinase inhibitor Flavopiridol and the mitotic inhibitor Taxol. Both Flavopiridol and Taxol did not induce luciferase activities in A375-HSP70.pro-LUC (Fig. 4D), and real-time PCR further validated that Flavopiridol and Taxol did not induce an HSF1 mediated-cell stress response (Fig. 4E). Loss of MII1 (Fig. 4, F and G) or Men1 (Fig. 4, H and I) did not sensitize cells to those two inhibitors, suggesting that MII1/Men1 knockout has selective effects on sensitizing to heat shock pathway induction. To further understand the mechanism of the combination effect between MLL1 knockout and HSP90 inhibition, we assayed for cell death and apoptosis by FACS subsequent to staining cells with 7AAD (a marker for dead cells) and Annexin V (a marker for cell apoptosis) (38). This analysis showed that more than 80% of MII1−/− MEFs were apoptotic compared with only 30% of MII1+/+ cells after exposure to AUY922 treatment (Fig. 4F). Together, these results suggest that loss of MII1 or Men1 selectively sensitizes cells to HSP90 inhibition.

Next, we asked whether inhibition of MLL1 may also sensitize human cancer cells to HSP90 inhibition. Three cancer cell lines (A375, A2058, and HCT116) with inducible knockdown of MLL1 and HSF1 were generated, and knockdown of either MLL1 or HSF1 was confirmed (Fig. 5A) (13). Colony formation assays were used to examine whether MLL1 knockdown, together with AUY922, had a combined effect on cell proliferation. Indeed, knockdown of either MII1 or HSF1 led to increased sensitivity to AUY922 in those three cancer cell lines (Fig. 5A). In contrast, there were no obvious changes of sensi-
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FIGURE 5. Mll1 depletion sensitizes cancer cells to AUY922. A, Western blot analysis of MLL1 in A375, A2058, and HCT116 cells with or without MLL1 knockdown and cell colony formation assay of MLL1 knockdown combined with AUY922 treatment in A375, A2058, and HCT116 cells. Dox, doxycycline. B, cell colony formation assay of MLL1 knockdown with Flavopiridol or Taxol treatment in A375 cells. shNTC-, shHSF1-, or shMLL1-transduced A375 cells were seeded in a 6-well plate and treated with or without doxycycline for 5 days, followed by compound treatment for 6 days. C, cell apoptosis analysis of A375 cells treated with doxycycline for 3 days to knock down MLL1, followed by 200 nM AUY922 for 48 h. The apoptotic cells represented by 7AAD+/Annexin V+ were determined by FACS. D, Western blot analysis of A375 cells expressing the inducible shMLL1 treated with different doses of AUY922. shMLL1-transduced A375 cells were treated with or without doxycycline for 3 days and treated further with different doses of AUY922 for 48 h. P-ERK, phospho-ERK.

Activity of A375 cells to Flavopiridol (Fig. 5B) or Taxol (Fig. 5B) when either MLL1 or HSF1 was depleted. To determine whether the mechanisms of the combined effect of MLL1 knockdown and AUY922 were similar to those observed with Mll1 and Men1 knockout MEFs, we examined whether MLL1 knockdown may affect cell apoptosis under HSP90 inhibitor...
treatment in A375 cells. MLL1 knockdown alone did not affect the apoptosis of A375 cells, but MLL1 knockdown markedly increased the apoptotic proportion of cancer cells upon AUY922 treatment (Fig. 5C). To show the potential functional interactions of HSP990 and MLL1 knockdown, we next test whether MLL1 knockdown attenuates MAPK signaling on the basis of our recent finding that HSF1 knockdown attenuates MAPK signaling mediated by HSP90 inhibition (13). The combination of MLL1 knockdown and HSP90 inhibitor led to a decreased level of phospho-ERK (Fig. 5D). Thus, MLL1 knockdown further enhances AUY922 activity by facilitating cell apoptosis.
Knockdown of MLL1 Sensitizes Human Cancer Cells to HSP90 Inhibition in Vivo—Finally, we tested whether the combined effect of MLL1 knockdown and HSP90 inhibitor could be observed in vivo. HSP900 is an oral HSP90 inhibitor and shows similar inhibitory effects as AUY922 on cancer cell growth (39). We examined the combined effect of MLL1 knockdown with HSP900 in an A375 xenograft mouse model. MLL1 knockdown alone slightly inhibited tumor growth, and knockdown was confirmed at the mRNA (Fig. 6A) and protein levels (Fig. 6B). HSP900 alone and the combination of MLL1 knockdown and HSP900 did not affect the body weights of xenograft mice (Fig. 6C). HSP900 alone at the maximum tolerated dosage (10 mg/kg, orally, once a week) inhibited tumor growth by 50% treated/control (T/C) (Fig. 6, D and E). Similar to results from MLL1 knockdown and HSP900 led to tumor stasis (Fig. 6, D and E).

DISCUSSION

Activation of the heat shock response is increasingly recognized as a major cause of resistance to HSP90 inhibitors (40). Consistent with this work, our previous studies indicated that the combined targeting of HSP90 and HSF1-mediated heat shock response could be an effective approach to enhance HSP90 inhibitor activities (13, 41). However, the identification of approaches to target HSF1 remains a challenge. Although progress has been made in terms of targeting HSF1, there are still many hurdles to overcome to develop effective HSF1 inhibitors (11, 40). Alternative approaches would be to develop inhibitors targeting the major HSF1 downstream effectors. For example, dual inhibition of HSC70 and HSP70 were found to mimic HSP90 inhibition and dramatically increase cancer cell sensitivity to 17-AGG (14). As such, efforts have been underway to discover and validate pharmacological inhibitors of HSP70 (42). Another way is to target a critical coactivator of HSF1 to modulate the transcription activity of HSF1 by inhibiting HSF1 target gene expression. Our study identified MLL1 as a key coregulator that is recruited to the promoters of HSF1 target genes under HSP90 inhibition. MLL1 is a H3K4-specific methyltransferase involved in activating gene transcription (18, 22, 43–46). MLL1-mediated methyltransferase activities, including the di-and trimethylation of H3K4, are also induced by HSP90 inhibition at the promoter region of HSF1-mediated genes, which is consistent with previous findings that di- and trimethylation of H3K4 recruitment are increased in heat shock motifs under heat shock conditions (47). To our knowledge, this work is the first to link altered histone methylation to HSP90 inhibitor resistance.

An important question for the clinical application of HSP90 inhibitor is to identify patients who are most likely to respond to this therapy. Some studies suggest that HSP90-dependent oncoproteins are good indicators of HSP90 inhibitors in human cancer, for example anaplastic lymphoma kinase rearranged and EGF receptor mutated non-small cell lung cancer (10). On the basis of our findings, it would be interesting to determine whether the MLL1 expression level may prove to be an additional biomarker to predict the sensitivity of the HSP90 inhibitor in human cancer patients. For example, a significant amount of acute myeloid leukemia patients harbor an MLL1 translocation, where the MLL1 SET domain is deleted, and, in this case, only one allele of MLL1 expresses the wild-type protein containing enzymatic function (18, 46, 48). It would be interesting to determine whether acute myeloid leukemia patients harboring an MLL1 translocation may be more sensitive to HSP90 inhibitors compared with acute myeloid leukemia patients without an MLL1 translocation.

Finally, we identified that depletion of MLL1 specifically sensitizes cells to HSP90 inhibitors. On one hand, HSF/MLL1-mediated heat shock activation induced by HSP90 inhibition can block the efficacy of HSP90 inhibitor in tumors. On the other hand, this induction could also protect normal tissues from some of the adverse effects of HSP90 inhibitors. It will be important to test whether systemic MLL1 inhibition provides an advantage versus increasing the toxicity of HSP90 inhibitors. Because MLL1 is an epigenetic target and its function is critically dependent on its direct interaction with MENIN and WDR5, many efforts have been made to target the interaction between MENIN and MLL1 or WDR5 and MLL1 (37, 49). Several small molecular inhibitors have been developed to block the interaction between MLL1 either with MEN1 or WDR5 (37, 49). Those compounds have been shown to be able to inhibit cell proliferation and induce both apoptosis and differentiation of leukemia cells harboring an MLL1 translocation (37, 50). It will be interesting to test such compounds (when they are developed further to a point where in vivo experiments are possible) with HSP90 inhibitors to observe the potential combined effects, which may open up a new avenue for a novel combined therapy for HSP90 inhibitors.

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