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HORMAD1 is a negative prognostic indicator in lung adenocarcinoma and specifies resistance to oxidative and genotoxic stress

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Running Title: HORMAD1 promotes DNA damage repair and survival in NSCLC.

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Abstract

Cancer Testis Antigens (CTAs) are expressed in testis and/or placenta and anomalously activated in a variety of tumors. The mechanistic contribution of CTAs to neoplastic phenotypes remains largely unknown. Using a chemigenomics approach, we find that the CTA, HORMAD1, correlates with resistance to piericidin A in NSCLC. Resistance is due to a reductive intracellular environment that attenuates the accumulation of free radicals. In human lung adenocarcinoma (LUAD) tumors, patients expressing high HORMAD1 exhibit elevated mutation burden and reduced survival. Differential expression profiling revealed that HORMAD1 tumors are enriched for genes essential for homologous recombination (HR). Mechanistic studies find that HORMAD1 promotes RAD51-filament formation, but not DNA resection, during HR. Accordingly, HORMAD1 loss enhances sensitivity to gamma-irradiation and PARP inhibition. Furthermore, HORMAD1 depletion significantly reduces tumor growth *in vivo*. These results suggest that HORMAD1 expression specifies a novel subtype of LUAD, which has adapted to mitigate DNA damage. In this setting, HORMAD1 could represent a direct intervention strategy to enhance sensitivity to DNA damage agents and/or an immunotherapeutic target in patients.

Significance: Using a chemigenomics approach, this study demonstrates that anomalous expression of the cancer-testes antigen, HORMAD1, specifies resistance to oxidative stress and promotes homologous recombination to support tumor cell survival in NSCLC.

Introduction

Cancer Testis Antigens (CTAs) are genes defined by their expression pattern, which is normally found in testis or placenta, but activated in nearly every tumor type. Given that the testis is immune-privileged, the expression of these proteins has long been prized for their immunotherapeutic potential. More recently, a number of reports from our lab and others indicate that these anomalously expressed proteins are not merely bystanders in the tumor cell regulatory environment, but can be engaged to promote neoplastic phenotypes. Specifically, CTAs are reported to promote mitotic fidelity, degrade tumor suppressor proteins, confer tolerance to DNA damage, and reprogram transcriptional networks (1-8). These reports suggest the provocative hypothesis that CTAs may represent direct intervention targets with an extraordinarily broad therapeutic window.

A cohort of CTAs are essential for recombination of homologous chromosomes during meiosis. These include components of the synaptonemal complex (SYCE1 and SYCP1), the meiotic topoisomerase that catalyzes DNA double-strand breaks (SPO11), as well as multiple proteins that mediate homologue alignment and recombination (HORMAD1, HORMAD2, TEX15) (9-18). Mice lacking these genes are healthy, but infertile (11,17-21). In all cases, spermatocytes arrest during Prophase I due to defective synapse formation or an inability to undergo recombination and chromosome segregation (11,17-21). In some cases, females are also infertile due to defects in chromosome segregation and quality control (15,16,22). Expression of each of these genes has been reported in human cancer, but scant information exists regarding the tumorigenic function of meiotic CTAs and whether they have any context selectivity (23-26).

Through a large-scale effort to identify anti-tumor natural products collected from marine-derived bacteria, we have identified NSCLC cell lines that are resistant to the electron transport chain (ETC) Complex I (NADH dehydrogenase) inhibitor, piericidin A (PA).

Strikingly, we found that this resistance correlates with expression of the CTA, HORMAD1. We find that the basis for PA resistance is a highly reductive environment that prevents accumulation of H_20_2. Mechanistic follow-up studies indicate that HORMAD1 is not complicit in this resistance, but is essential for remediating DNA double-strand breaks (DSB). This data suggests that HORMAD1 expression specifies a subtype of NSCLC that has evolved cellular mechanisms to both avoid and mitigate excessive DNA damage.

Materials and Methods

Natural product fraction library. The natural product fraction library contains extracts from 300 marine-derived bacterial strains and 20 marine invertebrates. Fermentation of each bacterial strain gave rise to a total of 20 natural product fractions/strain (Nomenclature: SNX-###-F1 through SNX-###-F20, where F1 is the most polar and F20 the least polar). All natural product fractions in the library are standardized to 10 mg/mL in DMSO. See Supplementary Methods for details of library and fraction generation and piericidin A purification.

Natural Product Fraction Viability Screening. Each of the 4358 natural products fractions was screened at 4 doses (0.18 µg/mL, 0.55 µg/mL, 1.65 µg/mL and 5 µg/mL) across 26 cell lines. Activity scores at each dose were used as a response vector, resulting in 17,432 total input response vectors. Activity scores were calculated as the percent cell death relative to control (0 = no death; -100 = 100% cell death) and the equation is: -1*[100-(value/DSMO)*100]. Dose response curves for piericidin A were performed at 12 half-log doses with concentration ranging from 50 µM to 50 pM in triplicate in two independent runs for each cell line.

Elastic Net Analysis. To discover RNAseq expression features predictive of response to piericidin A, an elastic net analysis was employed as previously described using a publically

available RNAseq dataset (27,28). ED_{50} values were used as a response vector. Elastic net parameters were fit with a 5-fold cross validation analysis.

Cell lines and chemicals. All NSCLC cell lines were obtained from John Minna (UT Southwestern) between 2004 and 2015 and were curated as previously described (29). Cells were cultured in RPMI medium supplemented with 5% FBS at 37°C and 5% CO2 and were not passaged more than 25 times post thawing. Cells were most recently authenticated between 2016 and 2017 using short tandem repeat profiling and periodically evaluated for mycoplasma contamination by DAPI stain for extra-nuclear DNA within one year of use. Chemicals (and manufacturer): piericidin A (Enzo), rotenone (Sigma-Aldrich), 6-aminonicotinamide (Alfa Aesar), 5-aza-2′deoxycytidine (MP Biomedicals).

Immunoblotting. Immunoblotting was performed as previously described (5). Antibodies used: HORMAD1 (HPA037850, 1:1000, Sigma-Aldrich), Actin (sc-8432, 1:1000, Santa Cruz) GADPH (G8795, 1:5000, Sigma-Aldrich).

Immunohistochemistry. NSCLC tumor microarrays were stained using a Leica Bond Max automated stainer (Leica Biosystems, Nussloch, Gmbh). Tissue sections were deparaffinized and rehydrated following the Leica Bond protocol. Antigen retrieval was performed with Bond Solution #2 (Leica Biosystems, equivalent to EDTA buffer pH 9.0) for 20 min, then HORMAD1 antibody (HPA037850, 1:500, Sigma-Aldrich) was employed for 15 minutes at room temperature. The primary antibody was detected using the Bond Polymer Refine Detection Kit (Leica Biosystems) with diaminobenzidine as chromogen. The slides were counterstained with hematoxylin, dehydrated and coverslipped. Nuclear expression of HORMAD1 was evaluated by pathologists using the H-score system.

Immunofluorescence. Immunoflourescence was performed as previously described (5). Antibodies used: RAD51 (398587, 1:800, Santa Cruz), RPA2 (NA19L, 1:500, Millipore). Cells

were imaged using a Zeiss LSM510 confocal microscope or Keyence Fluorescence Microscope BZ-X710.

Cell quantification assays. Cells were plated into 96-well plates (Corning, #3904) at 30-50% confluency, treated for 72 hours, trypsinized, and then quantified using a hemocytometer.

EdU incorporation assays. Cells were plated into 96-well format (Corning, #3904) at 30-50% confluency, treated for 72 hours, then exposed to EdU for 2 hours before fixing the cells in 3.7% formaldehyde. Cells were stained using the protocol for Click-iT^{TM} EdU Alexa Fluor 488 Imaging Kit (Invitrogen) and co staind with Hoechst 3342 (Invitrogen). Cells were quantified using fluorescence microscopy.

Stable cell lines. sgCTRL and sgHORMAD1 lines were generated using pLX-sgRNA and pCW-Cas9 constructs (Addgene plasmid #50662, #50661) (30).

Clonogenic cell survival assays. Cells were plated at 30-50% confluency. 48 h post plating, cells were irradiated using a Caseium^{137} source, trypsinized, and replated at various densities (escalating with dose). For olaparib assays, cells were replated directly into drug. Cells were cultured for 10-14 days, fixed and stained in a 0.5% crystal violet, 10% acetic acid, and 90% methanol solution for 10 min. A cluster of 50 cells was considered a colony. SF=(PE treated samples)/(PE of control) where SF is surviving fraction and PE is plating efficiency.

FACS analyses. For superoxide assays, cells were plated at ~50% confluency and treated 24 hours later for 1 hour. Cells were subsequently treated with 5 μM MitoSOX^{TM} (Invitrogen) in HBSS (Invitrogen) for 10 min at 37°C, 5% CO2. For H_20_2 assays, cells were plated at 30-50% confluency and treated for 72 hours. Cells were subsequently treated with 5 μM CM-H2DCFDA (Invitrogen) in HBSS for 20 minutes at 37°C, 5% CO2, washed with HBSS, then recovered in fresh medium for 15 min. Cells were immediately analyzed by flow cytometry using a BD LSR Fortessa instrument and BD FACSDiva 6.2 software. A minimum of 1.0 X

10^4 cells were analyzed per condition. FlowJo® software was used to generate flow charts and calculate KS Max difference.

NADPH/NADP+ assays. Cells were plated into 96-well tissue culture plates (Corning, #3904) and treated for 72 hours. Cells were processed according to the manufacturer's protocol for NADP/NADPH-Glo^{TM}Assay (Promega).

siRNA transfection. siRNA studies was performed as previously described (5).

Dose curves. Cells were plated into 96-well tissue culture plates (Corning, #3904) at 30-50% confluency, treated for 72 hours with drug, then analyzed using Cell-Titer Glo® (Promega). Curves were fitted and ED50s calculated as previously described (28).

Kaplan-Meier and mutation analyses. RNAseq expression values from The Cancer Genome Atlas (TCGA) provisional datasets for lung adenocarcinoma (LUAD) And lung squamous cell carcinoma (LUSC) were used to bifurcate patient tumors into high and low HORMAD1 expressing groups. Bifurcation point was made at the point which generated the highest hazard ratio. Cox regression analysis was used to obtain hazard ratios and p-values. Mutations were assessed from TCGA whole exome sequencing data. The number of mutations reflect the number of genes that are mutated.

Signal-to-noise (S2N) and GSEA analyses. Datasets from TCGA for provisional lung adenocarcinoma (LUAD) were used (data downloaded March 2017, 517 tumors). Patients were bifurcated based on the following criteria: HORMAD1 low: HORMAD1 FPKM <1 and <150 mutations, HORMAD1 high: HORMAD1 FPKM >300 and >500 mutations. Genes which did not have an FPKM >1 in at least one tumor sample were excluded. Data were log2 transformed and genes that did not have >2 fold change (log(a) - log(b) > 1 OR log(a) - log(b) < -1) were also excluded. S2N values were based on the following equation: (mean(A)−mean(B))/(sd(A) + sd(B)). For gene set enrichment analysis (GSEA) the top 10% of genes most differentially expressed in HORMAD1 high tumors based on S2N analysis

(251 genes) were analyzed using the Broad Institute's Molecular Signatures Database Hallmark and KEGG sets (31,32).

Xenograft experiments. All animal experiments were conducted with IACUC approval. For the 5-aza experiments, 6-8 week old female NOD.cg-PRKDC^{SCID}Il2rg^{tm1Wjl}/SzJ (NSG) mice were subcutaneously injected in the flank with 1 million cells (HCC44 and H2122) in 200 µl PBS. Once tumors reached 175 mm^3, mice were intraperitoneally injected with 2mg/kg of InSolution^{TM} 5-aza-2'-Deoxycytidine (Millipore 189826) daily for 5 days, followed by euthanization, extraction and flash frozen in liquid nitrogen. For the CRISPR experiments, 6-8 week old female Hsd:Athymic Nude-Foxn1^{nu} mice were subcutaneously injected in the flank with 2 million cells (A549 Cas9/sgCTRL or A549 Cas9/sgHORMAD1) in 100 µl PBS. Once tumors were visible, volume was measured by calipers twice per week.

Statistical analysis. Graphpad Prism (Graphpad Software) was used to perform all statistical analyses. Data were assessed by two-tailed, unpaired t-tests, Mann-Whitney or Shapiro-Wilk tests as indicated. P-values less than 0.05 were considered significant. For xenograft experiments, simple randomization was used to assign mice to control or experimental groups.

Results

Piericidin A resistance correlates with HORMAD1 expression in NSCLC. We devised a discovery pipeline to identify natural products with selective anti-tumor activity in NSCLC (Fig. 1A). A natural product fraction library with > 4000 fractions from marine-derived bacteria was screened at four doses (a total of 17,432 fraction/dose combinations) for effects on viability in each of 25 tumor-derived NSCLC lines and 1 normal, immortalized lung line that were previously annotated for gene expression, mutation-status and copy number variation. We prioritized further investigation of fractions that exhibited activity against at least one NSCLC cell line at ng/mL concentration, were not pan-toxic and exhibited a distinctive chemical

signature by LC/MS analysis. From this analysis, we identified 63 fractions that reached our criteria (Supplementary Figs. S1A-S1B). Among these fractions was SNB-051-14, which was a selective, non-polar fraction that originated from a strain of *Streptomyces variabilis* (Supplementary Fig. S1C). We cultured the *S. variabilis* strain, SNB-051, on a large scale and obtained fractions used in iterative bioassay analysis with two sensitive NSCLC cell lines (HCC44 and H2122) to identify a single, potent fraction: SNB-051-F36-H7 (see Supplementary Methods; Supplementary Fig. S1D). High resolution ESI-MS (HRMS) analysis of SNB-051-F36-H7 identified a mass/charge consistent with the molecular formula $C_{25}H_{37}NO_4$ (*m/z* [M+H] 416.2794). Further ^{1}H NMR analysis indicated that the active constituent was piericidin A (PA) (see Supplementary Methods; Supplementary Fig. S1E). As a quinone analog, PA binds to Complex I and inhibits the oxidation of ubiquinone and generation of the proton gradient essential for generation of ATP (33). PA also enhances ROS production by Complex I (33). Although Complex I inhibitors have been suggested as anti-cancer compounds, the selective toxicity observed for PA in NSCLC has not been previously observed (34,35).

We next generated a twelve-point dose response curve for PA in 26 NSCLC cell lines. A regularized linear regression algorithm was applied to determine if distinct gene expression features from whole-genome transcript profiles were predictive of PA response (28). This revealed a correlation of HORMAD1 with resistance to PA (Fig. 1B). HORMAD1 is a meiotic chromatin binding protein that is essential for synaptonemal complex formation, generation of double-strand breaks and the meiotic silencing of unsynapsed chromatin (MSUC) checkpoint (16). HORMAD1 is expressed in human cancer, which classifies it as a cancer testis antigen (CTA) (25). HORMAD1 expression is normally restricted to the testis (data accessed from GTEx Portal on 04/04/2018) (Supplementary Fig. S1F). The functional role of HORMAD1 in NSCLC has not been previously evaluated, however a report in triple negative breast cancer

indicated a possible tumorigenic function in DNA repair (1). Thus, we chose to further focus on the significance of HORMAD1 in NSCLC.

We examined HORMAD1 protein expression in NSCLC and found that RNA-Seq expression values above 4.0 corresponded with robust protein expression (Fig. 1C; Fig. S1G) (28). We refer to HORMAD1 mRNA expression > 4.0 as HORMAD1 positive (+) while < 4.0 are classified as HORMAD1 negative (-). Using these cutoffs, we recapitulated the differential sensitivity profile with independently synthesized PA (Fig. 1D). The endpoint assay in these experiments, CellTiter-Glo, measures cellular ATP, which is likely depleted following inhibition of the ETC by PA. Thus, we quantitated viable cells at the experimental end point. Treatment of HORMAD1(-) cells led to a greater than 50% decrease in cell number, however no such reduction was observed in the HORMAD1(+) samples (Fig. 1E). Furthermore, EdU incorporation indicated that only HORMAD1(-) cells reduced proliferation (Fig. 1F). We also measured ATP levels by CellTiter-Glo in a panel of HORMAD1(-) and (+) cells lines but observed no significant association with expression status. This data indicates that a subset of NSCLC cells are highly sensitive to PA and expression of the CTA protein, HORMAD1, correlates with resistance.

HORMAD1 expressing NSCLC exhibit elevated reductive capacity.

We next assessed superoxide ($O_2^{\cdot-}$) generation following PA exposure in both the HORMAD1(-) and HORMAD1(+) cell lines. In this setting, all NSCLC tested, irrespective of PA sensitivity, induced $O_2^{\cdot-}$ (Fig. 2A). Superoxide is subsequently converted to hydrogen peroxide (H_20_2), by superoxide dismutase (36). We assayed accumulation of H_20_2 and observed little, if any, increase in HORMAD1(+) cells following PA exposure (Fig. 2B). In contrast, HORMAD1(-) cells exhibited a robust accumulation. This data indicates that PA resistant NSCLC are not reliant on the ETC for ATP production and can mitigate the accumulation of H_20_2, which may otherwise inhibit cellular proliferation (37).

To determine whether resistance to Complex I inhibition was a general phenomenon of HORMAD1(+) NSCLC cells, we evaluated sensitivity to rotenone, another Complex I inhibitor (38-40). HORMAD1(+) and HORMAD1(-) cells exhibit similar sensitivities to rotenone, although we observed slightly enhanced sensitivity in HORMAD1(-) cells (Fig. 2C). Importantly, exposure of HORMAD1(+) cells to rotenone led to an accumulation of H_20_2 (Fig. 2D). Unlike PA, rotenone is a non-competitive inhibitor of Complex I and rotenone is more potent at inducing ROS *in vitro* (41,42). The PA resistance in HORMAD1(+) cells may reflect differences in the properties of these inhibitors that lead to differential induction of ROS.

H_20_2 is well known to induce oxidation of lipids, proteins and DNA (36). Indeed, exposure of HORMAD1(-) cells to PA led to an accumulation of the DNA-damage response indicator, 53BP1 (Supplementary Fig. S2A). Reduction of cellular H_20_2 is highly dependent upon the NADPH/NADP+ ratio, which maintains a pool of the reductant, glutathione (GSH). We measured NADPH/NADP+ ratios in HORMAD1(+) and (-) cells to monitor cellular oxidative stress. In HORMAD1(+) NSCLC cells exposed to PA, NADPH/NADP+ was unchanged, while it decreased by more than half in HORMAD1(-) cells, indicating an exhaustion of the antioxidant machinery (Fig. 2E). Glucose-6-phosphate-dehydrogenase (G6PD) is the main cellular source of NAPDH. We next co-inhibited HORMAD1(+) cells with the G6PD antagonist, 6-aminonicotinamide (6-AN) (43). This combination resulted in a decrease in NADPH/$NADP^+$ when HORMAD1+ cells were exposed to PA (Fig. 2F). Furthermore, we observed a combinatorial loss of viability in three HORMAD1(+) NSCLC cell lines (Fig. 2G). We conclude that resistance to PA in HORMAD1(+) tumors is due to an enhanced capacity to reduce ROS. Conversely, HORMAD1(-) cells may have defects in reductive pathways that enhances their sensitivity to ROS accumulation.

To determine functional consequences of HORMAD1 expression on PA resistance, we performed a series of gain and loss of function experiments. HORMAD1 was stably expressed in HORMAD1(-) NSCLC cells, however we did not observe PA resistance (Supplementary Fig. S2B). We generated HORMAD1-knockdown cell lines by stably transducing HORMAD1 specific shRNAs into four HORMAD1(+) NSCLC cell lines. We also used CRISPR/Cas9 to knockdown HORMAD1 in A549 and H358 cell lines (sgHORMAD1). In none of these settings did we observe enhanced PA sensitivity (Supplementary Figs. S2C-S2D). Based on these observations, we conclude that the observed PA resistance may be a by-product of the regulatory environment of HORMAD1 expressing cells, which has been selected to attenuate free radical damage that could otherwise prevent survival.

HORMAD1 portends poor survival and high mutation burden in lung adenocarcinoma. We next evaluated the significance of HORMAD1 expressing tumors in human NSCLC cancer. HORMAD1 gene expression is present in ~50% of both lung adenocarcinoma (LUAD) and lung squamous (LUSC) tumors (Fig. 3A). In LUAD patients, the highest ~10% of HORMAD1 expressing patients had significantly reduced overall survival (Hazard Ratio (HR) = 2.059, p=0.0025; Cox Regression). However, we did not detect this correlation in LUSC (HR= 0.579, p=0.0568; Cox Regression) (Fig. 3B). Furthermore, high HORMAD1 expression also correlated with elevated mutation burden exclusively in the LUAD patient population but not in the LUSC tumors (Fig. 3C). To evaluate expression of HORMAD1 protein in human tissue samples, we developed an immunohistochemical staining protocol. Consistent with the expression data, over half of NSCLC tumor cores stained positive for HORMAD1 protein (Fig. 3A; Fig. 3D). HORMAD1 was detected primarily in the nucleus of tumor cells, consistent with possible chromatin association in tumors as in its native tissue (Fig. 3D) (16).

Activation of HORMAD1 in lung cancer cells. The transcriptional regulatory mechanisms that activate expression of CTAs in cancers remain relatively obscure. However, inhibitors of

methylation have been successfully used to induce CTA expression in patient populations to boost responses to CTA-based immunotherapy (44). To test whether HORMAD1 is also regulated by DNA methylation, we exposed NSCLC cells to 5-aza-2'-deoxycitidine (5-aza) for 72 hours. We observed a robust induction of HORMAD1 mRNA in the HORMAD1(-) cells to levels of 5-20 % of HORMAD1(+) cells. The mRNA expression was stable for up to 19 days after withdrawal of 5-aza (Fig. 4D). This increase in expression was sufficient for HORMAD1 protein accumulation as observed by immunoblot (Figs. 4A-4C). We also tested the capacity of 5-aza to induce HORMAD1 expression *in vivo*. Here, we xenografted HORMAD1(-) NSCLC cell lines into immunocompromised mice. Similar to the human clinical treatment regimen, mice were then treated once daily for 5 days with a subcutaneous dose of 5-aza (44). In both xenografted tumors we evaluated, robust induction of HORMAD1 was observed in this setting (Fig. 4E). Thus, 5-aza exposure may be sufficient to boost presentation of HORMAD1 antigenic peptides for immune-targeting. However, due to the non-specificity of demethylating agents it is difficult to conclude that 5-aza exposure leads to acquisition of phenotypes associated with the HORMAD1 subtype classification.

HORMAD1 is required for DNA repair by homologous recombination. To determine whether the HORMAD1(+) LUAD subtype contains significant molecular differences from HORMAD1(-) tumors, we applied a Signal-2-Noise (S2N) expression profiling analysis on LUAD NSCLC from the TCGA expression datasets (Fig. 5A). Upregulated genes were subjected to GSEA Hallmark analysis, which indicated a significant enrichment of cell cycle, G2/M checkpoint and mitotic genes (Fig. 5B; Supplementary Fig. S3A). GSEA KEGG pathway analysis revealed genes involved in cell cycle, meiosis and homologous recombination pathways (Fig. 5C; Supplementary Fig. S3B). By manual inspection of the top 10% of upregulated genes, we identified a number of genes associated with mitosis, indicating that HORMAD1 tumors are highly proliferative (Supplementary File 1). In addition,

we noted elevated expression of genes previously classified as CTAs, suggesting that HORMAD1 may be induced as part of a broader spermatogenic program in LUAD. We also observed activation of multiple components of the E2F1 transcriptional network that promotes DNA damage signaling (Supplementary Fig. S3A; Supplementary File 1). Significantly, we identified many of the key factors involved in homologous recombination (HR) (including BRCA1, BRCA2, RAD54L, RAD51, EME1) as well as components involved in DSB checkpoint signaling (CHEK1, CHEK2) as upregulated in HORMAD1(+) tumors (Fig. 5D, Supplementary Fig. S3B). Together, these findings suggest that HORMAD1 expression is correlated with an elevated expression of DNA damage repair proteins, particularly those involved in HR.

Given HORMAD1's functional role in meiotic homologous recombination (HR) and the upregulation of a number of DNA repair genes in HORMAD1(+) human LUAD tumors, we evaluated the ability of HORMAD1 to modulate this DNA double strand break (DSB) repair pathway. We performed clonogenic survival assays following irradiation (IR) using sgCTRL and sgHORMAD1 A549 cells. In this setting, A549 sgHORMAD1 cells exhibited enhanced radiosensitivity as compared to control cells, suggesting that HORMAD1 may be instrumental in the repair of IR-induced DSBs (Fig. 6A). We next asked whether HORMAD1 plays a direct role in HR by examining DNA end resection and recruitment of HR factors to IR-generated DSBs. The repair process is initiated by resection of the DSB end, which produces ssDNA ends that are quickly bound by the RPA protein. We did not observe a significant difference in RPA foci formation following IR in sgHORMAD1 A549 cells as compared to control, which indicates that HORMAD1 is not required for initiation of HR (Fig. 6B). The ATPase, RAD51, replaces RPA on the ssDNA and is required for strand invasion for HR to occur. Thus, RAD51 nucleofilament formation is a well-established marker to measure on-going HR. Strikingly, in sgHORMAD1 cells, we observe a significant decrease in RAD51 focus formation

at multiple time points post-irradiation (Fig. 6C). A549 cells are p53 wild-type and KRAS mutant, thus we tested additional cell lines with opposite mutations: H2126 (p53 mutant, KRAS wild-type) and H2030 (p53 mutant, KRAS mutant). Importantly, the loss of RAD51 loading following HORMAD1 depletion was recapitulated in these different genetic backgrounds (Figs. 6D-6E).

HR-defective cell lines and tumors exhibit increased sensitivity to small molecule inhibitors of PARP1. Given] that HORMAD1 appears essential for HR, we asked whether that HORMAD1 depleted cells exhibit enhanced sensitivity to PARP1 inhibition. We performed clonogenic survival assays on sgCTRL and sgHORMAD1 A549 exposed to olaparib. HORMAD1 loss led to a dramatic decrease in tumor cell survival (Fig. 6F). Collectively, the data suggest that HORMAD1 directly regulates HR in the steps between DNA end resection and RAD51 nucleofilament formation.

We next sought to determine the significance of HORMAD1 inhibition to DNA damage inducing agents *in vivo*. We attempted to establish xenograft tumors with sgCTRL and sgHORMAD1 A549 cells. However, the sgHORMAD1 cells exhibited limited, if any, *in vivo* tumor growth (Fig. 6G). This finding indicates that HORMAD1 is required for tumor establishment and growth, a phenotype which was not observed *in vitro*.

Discussion

We have demonstrated the utility of NPF toxicity profiling to reveal unique phenotypic subtypes in NSCLC. We discovered differential sensitivities to the Complex I inhibitor, piericidin A (PA), in NSCLC. PA inhibits oxidative phosphorylation and promotes the generation of ROS. While Complex I inhibitors have previously been suggested as anti-cancer agents, the inhibition of ATP production and induction of ROS would likely lead to toxicity in normal tissues. However, we find a subset of NSCLC that exhibit enhanced sensitivity to the PA-containing crude NPF fraction, SNB-051-14, as compared to non-

transformed human bronchial epithelial cells (Fig. S1C). This suggests the possibility that a therapeutic window may exist for treatment with piericidin A, perhaps due to an acute dependence on the ETC for ATP production and/or a deficiency in oxidizing H_20_2 in a subset of NSCLC tumors.

Availability of comprehensive molecular annotations of NSCLC cell lines allowed us to connect chemical sensitivity to functional biomarkers for downstream analysis. Surprisingly, HORMAD1 expression correlated with PA resistance. In our studies, HORMAD1 does not appear to be necessary or sufficient to alter PA sensitivity. However, we discovered a novel function for HORMAD1 in DNA repair, specifically by stabilizing RAD51 filaments during HR. The requirement for HORMAD1 in HR may reflect a requirement for enhanced DNA repair for survival. The activation of oncogenes is well-known to induce the generation of DNA damage, which could pose a bottleneck to survival due to the activation of the DNA damage response pathways and subsequent apoptosis or senescence (45). Engagement of HORMAD1 could promote HR efficiency and escape from cell death. In support of this hypothesis, HORMAD1 high tumors have a significantly elevated mutation burden that may otherwise engage DNA damage checkpoints and limit survival. Thus, we propose that in the context of LUAD, HORMAD1 may be conscripted to promote efficient and accurate DNA repair, thereby conferring a selective advantage by preventing further accumulation of deleterious mutations. One source of this DNA damage could be cellular ROS, which is frequently increased in tumor cells. As the HORMAD1(+) subtype appear to reduce ROS more efficiently, we hypothesize that these cells have evolved mechanisms to both mitigate ROS and ensuing DNA damage. Significantly, a growing body of evidence indicates that DNA repair capacity is influenced by the redox state of a cell as reduced forms of certain proteins are essential for activating repair pathways (46). Future studies detailing HORMAD1's mechanism of action in HR could reveal vulnerabilities related to DNA damage machinery present in this subtype.

Moreover, the capacity of HORMAD1 to promote HR also suggests that the expression of HORMAD1 could serve as a biomarker for response to chemotherapy and/or radiosensitivity.

The association of HORMAD1 with mutation burden and poor survival exclusively in LUAD, but not LUSC, suggests that its function could be lineage dependent. Well-documented differences, particularly in sensitivity to folate metabolism inhibitors, suggest that these two histological subtypes exhibit divergent dependencies for DNA repair (47). Furthermore, a study by Watkins et al. has indicated that HORMAD1 correlates with elevated chromosomal scarring in triple negative breast cancer. However, these authors found that HORMAD1 promotes non-homologous end joining in this disease setting (1). The discrepancies in reported function may reflect selectivity with respect to where and when a meiotic CTA is activated and thus how it may be engaged to promote tumorigenesis. Significantly, study of HORMAD1's molecular mechanism of action in these different contexts could reveal novel biology associated with lineage specific preferences for DNA repair.

CTAs are well-established targets for adoptive T-cell therapies (ATC). The safety and efficacy of ATC is highly dependent on both a tumor-specific and abundant expression of the antigen target. We hypothesize that HORMAD1 may represent an ideal immunotherapeutic target for a number of reasons. First, HORMAD1 expression appears to be highly restricted to testis. Significantly, mice lacking HORMAD1 exhibit no defects aside from a loss of fertility, indicating the dispensability of HORMAD1 in adult tissues (17). Thus, HORMAD1 antigen presentation may be highly restricted to tumor cells, which is essential for the safety and specificity of antigen-based ATC. Second, our study demonstrates that exposure to 5-aza may boost HORMAD1 protein expression, particularly in the HORMAD1(-) state. Thus, this treatment may generate sufficient antigen presentation to provoke T-cell mediated engagement (48). Third, the elevated mutation burden observed in tumors expressing extremely high levels of HORMAD1 suggests an enhanced neo-antigen load. Neo-antigen

load has recently been correlated with response to both ATC and immune-checkpoint blockade (49). HORMAD1 may itself be a target for ATC in these tumors and may be able predict responsiveness to PD-1 blockade. Importantly, HORMAD1 may predict sensitivity PD-1 inhibitors in NSCLC patients that are typically classified as non-responders due to ROS1 amplification, ALK4 translocation or EGFR mutation. Prospective studies associating HORMAD1 mRNA or IHC expression with sensitivity to these agents will be important for assessing the capacity of HORMAD1 to serve as an immunotherapy biomarker. These studies will be complemented by those predicting neo-antigen load and tumor infiltrating lymphocytes (TILS) in HORMAD1 positive tumors.

Author Contributions

AWW, BAN, JBM, NWO, AJD and EAM designed experiments, analyzed data and wrote the paper. EAM, PLM, MSK and JY performed statistical analysis. BAN, JS, KG, SAL and NWO conducted experiments. IWM and BAP provided resources for immunohistochemistry and screening, respectively.

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Declaration of Interests

The authors declare no competing interests.

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Figure 1. Natural compound screen identifies correlation between HORMAD1 and piericidin A sensitivity. (**A**) Schematic for chemigenomics screen pipeline. (**B**) Heat maps of HORMAD1 expression (log2 RNAseq values) and piericidin A (ED50) in a panel of NSCLC cell lines. (**C**) Whole cell lysates of indicated cell lines were immunoblotted with indicated antibodies. (**D**) Indicated cell lines were exposed to indicated doses of piericidin A for 72 hours (h). HORMAD1 positive cell lines (50) and HORMAD1 negative cell lines (blue). Points indicate viability as measured by Cell-Titer Glo® (n=3) and bars indicate standard deviation (sd). Curves are non-linear fits to the respective data. (**E**) HORMAD1 positive cells (left) and HORMAD1 negative cells (right) were exposed to piericidin A (PA) for 72 h and quantified using a hemocytometer. Bars represent the average (n=2) ± range. (**F**) Left: Indicated cell lines were exposed to piericidin A (PA) for 72 h followed by EdU incorporation. Bars represent the mean (n=2) ± range. Right: Representative images of EdU staining (EdU=green, Hoechst=blue) of HORMAD1 positive HCC4017 and HORMAD1 negative H2172 cells.

Figure 2. HORMAD1 positive lines escape oxidative stress induced by piericidin A. (**A**) Left: HORMAD1 positive (50) and HORMAD1 negative (blue) cells exposed to piericidin A (1 μM) were stained with MitoSOX^{TM} prior to FACS analysis. Bars represent the mean KS-Max difference (compared to vehicle treated) of all cell lines and dots indicate mean values for individual cell lines (n=3). HORMAD1 positive cell lines from top to bottom: HCC4017, A549, H650, H358. HORMAD1 negative cell lines from top to bottom: HCC44, H157, H1155, H2172, H2122. P-value calculated by Mann-Whitney test (ns=non-significant). Right: Representative flow cytometry distributions for piericidin A (PA) treated HORMAD1 positive H358 (50) and HORMAD1 negative HCC44 (blue) cells. (**B**) Left: HORMAD1 positive (50) and negative (blue) cell lines exposed to piericidin A (1 μM) for 72 h and stained with CM-H2DCFDA prior to FACS analysis. Bars represent the mean KS-Max difference (compared to vehicle treated) for all cell lines and dots indicate mean values for individual cell lines (n≥2). HORMAD1 positive cell lines from top to bottom: HCC4017, H358, A549, H650. HORMAD1 negative cell lines from top to bottom: HCC193, HCC44, H2172, H157. P-value calculated by Mann-Whitney test. Right: Representative flow cytometry distributions for piericidin A (PA) treated HORMAD1 positive H358 (50) and HORMAD1 negative HCC44 (blue) cells. (**C**) Heat maps of HORMAD1 expression (log2 RNAseq values) and rotenone (ED50) in a panel of NSCLC cell lines. (**D**) H358 cells were exposed to rotenone (ROT) for 72 h and stained with CM-H2DCFDA prior to FACS analysis. (**E**) HORMAD1 positive (50) and HORMAD1 negative (blue) cell lines exposed to piericidin A (PA) or rotenone (ROT) for 72 h prior to NADPH/NADP+ ratio assay. Bars represent the mean (n≥2) ± range. (**F**) H358 cells were exposed to piericidin A (PA) (10 uM) and/or 6-aminonicotinamide (6-AN) (5 uM) for 96 h prior to NADPH/NADP+ ratio assay. Bars represent the mean (n=3) ± sd. (**G**) Indicated cell lines were exposed to piericidin A (PA) (10 uM) and/or 6-aminonicotinamide (6-AN) (5 uM) for 96 h prior to viability readout by Cell-Titer Glo®. Bars represent the mean (n=3) ± sd.

Figure 3. HORMAD1 indicates poor prognosis and elevated mutation burden in lung adenocarcinoma. (**A**) Heat maps of HORMAD1 mRNA expression (log2 RNAseq values) derived from TCGA data. (**B**) Kaplan-Meier survival curves from TCGA for lung adenocarcinoma (LUAD) and lung squamous (LUSC) patients. Hazard ratios and p-values calculated by Cox Regression Analysis. (**C**) Box plots indicate median and interquartile range (IQR) and bars represent min to max. P-values calculated by Mann-Whitney test. (**D**) Representative images of IHC staining and nuclear intensity scores (as scored by pathologist) for HORMAD1 in LUAD and LUSC tumor cores.

Figure 4. HORMAD1 activated by demethylation in NSCLC. (**A**) HORMAD1 negative cell lines were exposed to 5-aza (1 uM) for 48 h and mRNA expression was quantified by qPCR. Bars represent the mean (n=3) ± sd. (**B**) HORMAD1 positive cell lines were exposed to 5-aza (1 uM) for 48 h and mRNA expression was quantified by qPCR. Bars represent the mean (n=3)± sd. (**C**) Indicated cell lines were exposed to 5-aza (1 uM) for 48 h and whole cell lysates collected at 72 h and immunoblotted with indicated antibodies. (**D**) H1993 and HCC44 cells were exposed to 5-aza (1 uM) for 48 h and mRNA expression was quantified by qPCR at indicated time points. Bars represent the mean (n=2) ± range. (**E**) Top: Xenograft experiment schematic. Middle: mRNA expression from xenograft tumors quantified by qPCR. Bars represent the mean and outlined circles indicate individual tumors (n≥3). Bottom: Immunoblots with indicated antibodies for representative tumor xenografts.

Figure 5. DNA repair genes upregulated in HORMAD1 high tumors. (**A**) Signal-to-noise (S2N) analysis heat map for genes differentially expressed based on HORMAD1 mRNA expression (log2 RNAseq values) in lung adenocarcinoma (LUAD) patients. (**B**) Gene sets with significant overlap (FDR q-values <0.05) between the top 10% of genes identified by S2N (HORMAD1 high expressing tumors) and gene set enrichment analysis (GSEA) Hallmark Gene Sets. Bars indicate the –log of the p-value for overlap between the two gene sets. (**C**) Gene sets with significant overlap (FDR q-values <0.05) between the top 10% of genes identified by S2N (HORMAD1 high expressing tumors) and GSEA KEGG Gene Sets. Bars indicate the –log of the p-value for overlap between the two gene sets. (**D**) KEGG pathway schematic for homologous recombination pathway in human. Genes outlined in red are among top 10% of genes identified by S2N (HORMAD1 high expressing tumors).

Figure 6. HORMAD1 is required for homologous recombination DNA repair. (**A**) Clonogenic survival in A549 cells at doses (Gy) indicated. Each data point represents the mean (n=3) ± sd. Curves are non-linear (semi-log) fits to the respective data. Whole cell lysates were immunoblotted with indicated antibodies. (**B**) A549 cells were irradiated with 8 Gy. RPA2 foci in EdU positive cells was quantified at indicated time points (at least 60 cells per condition). Bars represent the mean (n≥2) ± range. (**C**) A549 cells were irradiated with 8 Gy. RAD51 foci in EdU positive cells was quantified at indicated time points (at least 60 cells per condition). Bars represent the mean (n=2) ± range. (**D**) H2030 cells were transfected with indicated siRNA for 72 h, fixed and stained 8 h post irradiation (8 Gy). RAD51 foci in EdU positive cells was quantified (at least 90 cells per condition). Bars represent the mean (n=4) ± sd. P-value calculated by two-tailed, unpaired t-test and normality assessed by Shapiro-Wilk test. Whole cell lysates were immunoblotted with indicated antibodies. (**E**) H2126 cells were transfected with indicated siRNA for 72 h, fixed and stained 8 h post irradiation (8 Gy). RAD51 foci in EdU positive cells was quantified (at least 45 cells per condition). Bars represent the mean (n=3) ± sd. P-value calculated by two-tailed, unpaired t-test and normality assessed by Shapiro-Wilk test. Whole cell lysates were immunoblotted with indicated antibodies. (**F**) Clonogenic survival in A549 cells at doses of olaparib indicated. Each data point represents the mean (n=3) ± sd. Curves are non-linear fits (semi-log) to the respective data. (**G**) A549 xenograft experiment. Left: Tumor volume measurements were taken by caliper on indicated days. Each data point represent the mean (n=5) ± standard error of the mean (10). Middle: Mass of excised tumors. Bars represent the mean (n=5) ± sem. P-value calculated by Mann-Whitney test. Right: Images of individual tumors (ruler indicates cm) and dashed circles indicate tumors that were not large enough to collect.