Synthetic Lethal Targeting of Superoxide Dismutase 1 Selectively Kills 
*RAD54B*-deficient Colorectal Cancer Cells

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ABSTRACT

Synthetic lethality is a rational approach to identify candidate drug targets for selective killing of cancer cells harboring somatic mutations that cause chromosome instability (CIN). To identify a set of the most highly connected synthetic lethal partner genes in yeast for subsequent testing in mammalian cells, we used the entire set of 692 yeast CIN genes to query the genome-wide synthetic lethal datasets. Hierarchical clustering revealed a highly connected set of synthetic lethal partners of yeast genes whose human orthologs are somatically mutated in colorectal cancer. Testing of a small matrix of synthetic lethal gene pairs in mammalian cells suggested that members of a pathway that remove reactive oxygen species that cause DNA damage would be excellent candidates for further testing. We show that the synthetic lethal interaction between budding yeast rad54 and sod1 is conserved within a human colorectal cancer context. Specifically, we demonstrate RAD54B-deficient cells are selectively killed relative to controls via siRNA-based silencing and chemical inhibition, and further demonstrate that this interaction is conserved in an unrelated cell type. We further show that the DNA double strand breaks, resulting from increased reactive oxygen species following SOD1 inhibition, persist within the RAD54B-deficient cells and result in apoptosis. Collectively, these data identify SOD1 as a novel candidate cancer drug target and suggest that SOD1 inhibition may have broad-spectrum applicability in a variety of tumor types exhibiting RAD54B-deficiencies.
INTRODUCTION

Understanding the pathogenic origins of colorectal cancer (CRC) is critical to develop the next generation of therapeutic strategies and targets. Chromosome instability (CIN) is characterized by increased frequencies of numerical and structural chromosomal aberrations (LENGAUER et al. 1998), and is prevalent within a large fraction of tumor types. CIN not only drives tumorigenesis (LENGAUER et al. 1998) but is associated with poor prognosis (GAO et al. 2007; HEILIG et al. 2010) and the acquisition of multidrug resistance (LEE et al. 2011). CIN has been studied in CRC where it is an early event in the pathogenesis of the disease (SHIH et al. 2001) and is found in up to 85% of sporadic tumors (RAJAGOPALAN et al. 2004). Although the somatic gene mutations that drive CIN remain largely unknown, it is clear that no single gene is responsible for the CIN phenotype observed in CRCs. Rather, the entire mutational spectrum that underlies CIN is accounted for by a set of genes, with each individual gene typically representing <4% of the entire spectrum (RAJAGOPALAN et al. 2004; WANG et al. 2004; BARBER et al. 2008; CANCER GENOME ATLAS 2012). Gene re-sequencing efforts have identified several candidates involved in chromosome segregation, DNA replication, and DNA repair that are somatically mutated or deleted in a subset of sporadic CRCs exhibiting CIN (WANG et al. 2004; SJOBLOM et al. 2006; BARBER et al. 2008; CANCER GENOME ATLAS 2012). CIN therefore represents a defining characteristic that distinguishes cancerous from normal cells and it is within this feature, where we believe that potential exists to identify novel therapeutic targets capable of selectively killing cancer cells.

In 1997, Hartwell and colleagues (HARTWELL et al. 1997) posited that cancer cells harboring specific somatic mutations may be selectively killed by targeting or inhibiting a second unlinked gene target through a synthetic lethal (SL) paradigm. Synthetic lethality refers
to the lethal combination of two independently viable mutations and is well studied in model organisms such as the budding yeast. Indeed, several extensive screens have been performed in yeast (TONG et al. 2001; PAN et al. 2006) with the collective goal of generating a comprehensive list of SL interactors for all known yeast genes (i.e. non-essential and essential). Through cross-species approaches, these yeast datasets can be exploited to identify candidate SL interactors of yeast CIN genes whose human orthologs are somatically mutated in CRC. Once validated in mammalian cells, these SL interactors will represent novel candidate drug targets. Recently, we showed that a SL interaction first observed in yeast (rad54 rad27) was conserved in a human CRC context (RAD54B FEN1, respectively) (McMANUS et al. 2009). We showed that RAD54B-deficient CRC cells, and not isogenic controls, were selectively killed following FEN1 silencing and thus validated FEN1 as a novel candidate drug target. Since this initial work, many additional studies in various model systems have been performed that suggest that >50% of SL interactions among selected subsets of CIN genes observed in budding yeast are conserved in other model organisms (TARAILO et al. 2007; DIXON et al. 2008; MCLELLAN et al. 2009).

To identify novel candidate therapeutic targets, we significantly expanded our initial cross-species candidate approach to uncover conserved SL interactors of CIN genes. Using the 692 yeast CIN genes (YUEN et al. 2007; STIRLING et al. 2011) and publicly available yeast datasets, we assembled all known SL interactors to date of the yeast CIN gene set. Hierarchical clustering identified several data-rich regions including one that harbored an abundance of SL interactors of yeast CIN genes whose human orthologs are somatically mutated in CRC. Preliminary direct tests performed in human cells suggested that members of a pathway required to remove reactive oxygen species (ROS), would be excellent candidates for further study, and specifically focused our attention on superoxide dismutase 1 (SOD1). Here we show that RAD54B-deficient cells and
not isogenic controls are selectively killed following either SOD1 silencing or chemical inhibition. By expanding this work into an unrelated cell type, we show that the $RAD54B \ SOD1$ SL interaction is evolutionarily conserved and independent of cell type. To address the mechanism of killing we show that the DNA damage resulting from the increase in ROS following SOD1 inhibition, persists within the $RAD54B$-deficient cells, and is associated with apoptosis. Thus, our data strongly suggest that SOD1 is a novel candidate therapeutic target for CRCs and perhaps other tumor types harboring $RAD54B$ defects.

**MATERIALS AND METHODS**

**Network Generation and Testing:**

For gene clustering, all known negative genetic, synthetic lethal and synthetic growth defects (collectively referred to in the text as SL) involving the 692 yeast CIN genes were identified in BIOGRID (version 3.1.71). Interacting genes were sorted based on their total number of SL interactions regardless of interaction strength. It was not possible to perform statistical analyses to prioritize and select candidates as the strengths of the negative genetic interactions are typically qualitative measurements and experimental conditions are expected to differ significantly between the assays and the laboratories in which the experiments were performed. The top ~500 yeast genes were clustered with the 692 CIN genes using Cluster and viewed using Java TreeView. To directly test SL interactions in HCT116 cells, we used RNAi and previously established protocols (van Pel et al. 2013). For siRNA-mediated knockdown, cells were seeded in 6-well dishes 24h prior to transfection with 50nM of single or double siRNA depending on the interaction tested. The next day, cells were detached, counted, and reseeded at low density in 96-well (6 replicates) plates. After 5 days, cells were paraformaldehyde fixed, nuclei were counterstained with Hoechst 33342 and enumerated.
Cell Culture:

HCT116 (CRC) cells were purchased from ATCC and grown in McCoy’s 5A medium (HyClone) supplemented with 10% FBS. RAD54B-deficient HCT116 cells were generously provided by Dr. K. Miyagawa (Hiroshima University, Japan). Immortalized (telomerase) BJ normal human skin fibroblasts, hTERT, were generously provided by Dr. C.P. Case (Cogan et al. 2010) (University of Bristol) and were grown in DMEM (HyClone) containing 10% FBS. Cell lines were authenticated on the basis of recovery, viability, growth, morphology and spectral karyotyping. RAD54B expression status was confirmed by Western blots prior to their use in experiments. All cells were grown in a 37º humidified incubator containing 5% CO2.

Gene Silencing:

Cells were transiently transfected with siRNA duplexes using RNAiMax (Invitrogen) as detailed elsewhere (McManus et al. 2009). ON-TARGETplus siRNA duplexes targeting SOD1, RAD54B, GAPDH and PLK1 were purchased (Dharmacon) and were employed as either individual siRNAs (100nM total) or pools that consisted of four different siRNAs (25nM each or 100nM total) targeting the same gene. The efficiency of gene silencing was confirmed by Western blot analyses.

Western Blot Analyses:

Western blots were performed as detailed elsewhere (McManus et al. 2009) and blotted with RAD54B (Abcam ab83311; 1:1,000), SOD1 (Abcam ab13498; 1:3,000), and α-tubulin (Abcam ab7291; 1:10,000) antibodies. Protein knockdown efficiencies were evaluated through semi-quantitative analyses of scanned blots using ImageJ (Gel Analyzer Tool). All semi-quantitative data were normalized to corresponding α-tubulin loading control for each lane.
Direct SL Test:

High content microscopy was used to evaluate the SL interactions a minimum of three times as detailed elsewhere (McManus et al. 2009). Briefly, 8,000 RAD54B-proficient or RAD54B-deficient HCT116, or 4,000 hTERT cells were seeded into 96-well optical plates. Cells were transfected in sextuplet (i.e. 6-wells/plate) with either individual or pooled siRNAs targeting RAD54B, SOD1 and controls (GAPDH and PLK1). GAPDH serves as a negative control (McManus et al. 2009), while PLK1 is a positive control for cell death independent of any SL interaction (Spankuch-Schmitt et al. 2002), and also serves as an indicator of transfection efficiency. Cells were supplemented with 150µl of normal growth medium 24h post-transfection, and permitted to grow for an additional 3 days (HCT116) or 4 days (hTERT), prior to paraformaldehyde fixation. Nuclei were counterstained with Hoechst 33342 (Sigma; 0.25µM) and imaged. Nine central non-overlapping images were acquired per well using a 20x plan apochromat dry lens (numerical aperture = 0.8) on a Zeiss AxioObserver Z1 equipped with an LED Colibri light source and AxioVision v4.8 software. Images were analyzed using the Assaybuilder (Physiology Analyzer) within the AxioVision software. The total number of cells (Hoechst-positive nuclei) in each well was determined. All data were imported into Prism v5.0 (GraphPad), normalized to GAPDH-silenced controls, and statistical analyses were performed.

Dose Response Curves:

Dose response curves were generated using a dilution series for ATTM and 2ME2. Asynchronous cells (8,000) were seeded into 96-well optical plates, and following attachment the growth medium was supplemented with DMSO (vehicle control), ATTM or 2ME2. Cells were permitted to grow for 3-days, fixed, counterstained with Hoechst, imaged and analyzed as above. All data were imported into Prism v5.0 (GraphPad), normalized to DMSO-treated...
controls, and EC\textsubscript{50} values were determined. The EC\textsubscript{50} values calculated for ATTM (4.2\,\mu M) and 2ME2 (7.3\,\mu M) in \textit{RAD54B}-deficient cells were employed in all subsequent experiments.

**Chemical SL Test:**

Chemical SL tests were performed in an analogous fashion to the direct SL tests detailed above. However, ATTM and 2ME2 were employed in place of the si\textit{SOD1} duplexes, while DMSO served as the vehicle control. Following a 3-day incubation period, cells were fixed and analyzed as above.

**Real-time Cell Analyses:**

Real-Time Cell Analyses (i.e. growth curves) were performed in quadruplicate using an RTCA-DP (Acea Biosciences, Inc.). The RTCA-DP system employs microelectrodes at the bottom of each well to measure increases or decreases in electrical impedance (termed cell index) that reflect increases or decreases in cell numbers, respectively (SOLLY et al. 2004). Briefly, 8,000 cells were seeded into each well of an E-Plate and growth was monitored in real-time every 10 minutes at 37°. DMSO, ATTM and 2ME2 were supplemented into the medium when cell indices attained ~20-25\% of their untreated maximum values (~24h post-seeding) and growth was monitored for 3 additional days. All data were imported into Prism and proliferation defects were calculated as \( PD = 1 - \frac{\text{Cell Index}_{\text{Drug}}}{\text{Cell Index}_{\text{DMSO}}} \times 100 \) for each cell line and treatment.

**Colony Forming Assay:**

10,000 cells/well were seeded in soft-agar into a 6-well plate in triplicate. Colonies were grown at 37°C with media containing DMSO, ATTM or 2ME2 replaced every 2-days. Following 28-days, cells were stained with 0.1\% crystal violet and imaged using a 14-megapixel, charged-
coupled device digital camera. Total number of colonies/well was quantified using ImageJ (Analyze Particles and Measurement).

Detection of ROS:

ROS were detected by employing Image-iT LIVE green ROS detection kit (Molecular probes) by following the manufacturers protocol with slight modifications to suit HCT116 cells. Briefly, RAD54B-proficient and RAD54B-deficient HCT116 cells were seeded onto sterile coverslips in each well of a 6-well plate and were allowed to grow at 37ºC for 24 hours. Semi-confluent cells on coverslips were treated with DMSO, ATTM or 2ME2 for 6 hours. Following treatment, the cells were washed in warm HBSS (37º) and were subsequently bathed in carboxy-H2DCFDA containing growth medium for 10 minutes at 37ºC in the dark. Subsequently, cells were washed 3 times in warm HBSS as before and coverslips were mounted onto glass slides in warm HBSS and cells were immediately imaged. Cells were counterstained with Hoechst 33342, by supplementing 1µM Hoechst 33342 into the growth medium for 5 minutes before the initial wash with HBSS, after carboxy-H2DCFDA treatment. Cells were imaged on an AxioImager 2 (Zeiss) equipped with an AxioCam HR charged coupled device (CCD) camera (Zeiss) and a 63× oil immersion plan-apochromat lens (1.4 numerical aperture), using identical exposure times for each condition as previously described (McMANUS and HENDZEL 2005). The experiment was repeated an additional 2 times.

Quantitative Imaging Microscopy:

The presence of DNA double strand breaks was evaluated using an established quantitative indirect immunofluorescence-based approach (McMANUS and HENDZEL 2005). Briefly, 8,000 asynchronous cells were seeded into a 96-well plate and permitted to attach. Cells were treated with DMSO, ATTM, or 2ME2 for 6h, rinsed twice with PBS and allowed to recover
for 36h in drug-free media. Cells were fixed, permeablized, labeled with either γ-H2AX (Abcam ab18311 at 1:2,000) or 53BP1 (Abcam ab21083 at 1:200) antibodies and imaged using identical exposure times for each condition as described (McManus and Hendzel 2005). The mean γ-H2AX and 53BP1 total signal intensities were determined for each nucleus and normalized to the DAPI total signal intensity of the corresponding nucleus to account for differences in DNA content (McManus and Hendzel 2003). The induction of apoptosis was evaluated by examining the abundance of cleaved Caspase 3 (Abcam ab13847 at 1:2,000). Cells were seeded as above and treated with DMSO, ATTM or 2ME2 for 12h. Staurosporine (1µM) was used as a positive control as it is an inducer of apoptosis. Cells were fixed, labeled, imaged and analyzed as above. To account for differences in cell numbers, the cleaved Caspase 3 total signal intensity was determined for each nucleus and normalized to the corresponding DAPI signal intensity.

**RESULTS**

**Global SL Data from Yeast can Predict Highly Connected Interactions in Humans with Therapeutic Potential:**

To identify potential SL interactors that are highly connected to CIN genes that are somatically mutated in CRC (e.g. RAD54B), we used the yeast interaction data available in BioGRID. We queried the database for all known SL interactions for the entire set of 692 yeast CIN genes (Yuen et al. 2007; Stirling et al. 2011). A total of 4,146 interactors were identified that were subsequently prioritized based on the total number of interactions with the query CIN genes. Two-dimensional hierarchical clustering was performed between the CIN genes and the top ~500 SL target genes (i.e. those with ≥22 interactions; a threshold chosen arbitrarily) (Fig. 1A; left). Several data rich-regions were identified including one that harbored an abundance of SL interactions with DNA repair genes (Fig. 1A; right) that was subsequently filtered based on
their connectivity within gene networks (Fig. 1A; bottom). This most highly connected set of data held several DNA repair CIN genes (Table S1), including sgs1 (human BLM) and rad54 (human RAD54B). The SL interactors of these CIN genes include many well-known DNA replication and repair factors such as the yeast Mre11/Rad50/Xrs2 complex (human Mre11A/Rad51/NBS1). In addition, the data-rich region also included all three members of a highly conserved pathway required to remove superoxide radicals (i.e. ROS), yeast tsa1, ccs1 and sod1, whose human orthologs are PRDX2, CCS, and SOD1, respectively (Fig. 1A; bottom).

A focused mini-network was selected for preliminary testing in HCT116 (CRC) cells (Fig. 1B) and using RNAi-based approaches, and gene knockout cell lines with relevant controls, we determined that 53% (8/15) of the SL interactions tested appear to be conserved, including the RAD54B SOD1 (yeast rad54 sod1) and RAD54B PRDX2 (yeast rad54 tsa1) SL interactions (Fig. 1C). SOD1 was specifically selected for subsequent testing as it encodes an enzyme that is highly conserved between yeast and humans (>70% similarity), its role in the removal of superoxide radicals is well established in both organisms, and SOD1 inhibitors have already been developed some of which are currently being evaluated for their ability to prevent angiogenesis in cancer (JUAREZ et al. 2006; DONATE et al. 2008; LOWNDES et al. 2009). Furthermore, since SOD1 is dispensable in both yeast (CHANG et al. 1991) and mice (KONDO et al. 1997), it is likely that targeting SOD1 in a CRC context will have minimal effects on normal cells.

**The Budding Yeast Rad54 Sod1 SL Interaction is Evolutionarily Conserved in Humans:**

We specifically chose to evaluate a potential SL interaction between SOD1 and RAD54B because RAD54B is a chromosome stability gene (MCMANUS et al. 2009) involved in homologous recombination repair (MIYAGAWA et al. 2002) that is somatically mutated in CRC (CANCER GENOME ATLAS 2012) and many additional tumor types (FORBES et al. 2011) (Table
S1). We first evaluated the silencing efficiencies of SOD1 siRNAs (Fig. 2A) and selected the two most efficient silencers (siSOD1-2 and -3). Next, we employed our established siRNA-based approach using isogenic *RAD54B*-proficient (control) and *RAD54B*-deficient HCT116 cells (McManus et al. 2009) to validate the *RAD54B* SOD1 SL interaction. As predicted, there was a visually striking decrease in the total number of *RAD54B*-deficient cells relative to controls (Table S2) following SOD1 silencing (Fig. 2B), that were deemed statistically significant relative to controls (Fig. 2C). Further inspection of the images revealed that a few remaining *RAD54B*-deficient cells exhibited hallmarks of cytotoxicity (i.e. apoptosis) including chromatin condensation, nuclear blebbing and fragmentation (Fig. 2B, arrowheads) that were not apparent within the controls, while immunofluorescent labeling with a mitosis-specific marker (anti-phosphorylated serine 10 of histone H3) confirmed the cells were not arrested in G2/M phase (data not shown).

Although the above data support an evolutionarily conserved SL interaction, it remained possible that the putative *RAD54B* SOD1 interaction occurs due to a *de novo* background mutation that arose during the generation of the *RAD54B*-deficient cells. Consequently, we confirmed our ability to silence RAD54B and SOD1 (Fig. S1A, Fig. 2A) and performed dual siRNA experiments in *RAD54B*-proficient parental cells. As anticipated, the simultaneous silencing of *RAD54B* and *SOD1* resulted in a larger decrease in the relative percentage of cells than when either RAD54B or SOD1 are silenced alone (Fig. 2D), or what is expected as calculated by a multiplicative model (Table S3). To extend our findings beyond a CRC context, similar dual siRNA tests were performed in a karyotypically stable, immortalized (telomerase) fibroblast cell line, hTERT (Fig. S1B, S1C, S2 and Table S4). As above, the simultaneous silencing of *RAD54B* and *SOD1* resulted in fewer cells than silencing either alone (Fig. 2E), and
a decrease in relative percentage of cells beyond that expected using a multiplicative model (Table S5). Collectively, these data suggest that the \( \text{RAD54B SOD1} \) SL interaction is evolutionarily conserved and is independent of cell type.

**RAD54B-deficient Cells Hypersensitive to ATTM and 2ME2:**

Next, two chemical compounds were evaluated for their ability to phenocopy the RAD54B-specific killing identified above; ammonium tetrathiomolybdate (ATTM) is a SOD1 inhibitor that chelates Cu\(^{2+}\), an essential co-factor required for its activity (JUAREZ et al. 2006), and 2-methoxyestradiol (2ME2), which does not inhibit SOD1 *per se*, but rather induces the formation of ROS including superoxide radicals and thus mimics SOD1 inhibition (HUANG et al. 2000; KACHADOURIAN et al. 2001). Standard dose response curves (Fig. 3A) revealed that \( \text{RAD54B} \)-deficient cells were >10-fold more sensitive to ATTM (EC\(_{50} = 4.2 \mu \text{M}) and 2ME2 (7.3 \mu \text{M}) treatments relative to controls (67.7 \mu \text{M} and 75.1 \mu \text{M}, respectively). Western blots confirmed that the drug sensitivities were not due to changes in SOD1 abundance following treatments (Fig. S3).

Having established that ATTM and 2ME2 can functionally substitute for SOD1 siRNAs and recapitulate the \( \text{RAD54B} \) SL interaction, we now wished to determine if the treatments induced cell cycle arrest or cytotoxicity. Cellular growth rates were measured in real-time where an increase (i.e. proliferation) or decrease (i.e. cytotoxicity) in cell numbers correlates with an increase or decrease in cell index, respectively (SOLLY et al. 2004; BUONTEMPO et al. 2011). Although the growth profiles for control cells were very similar (i.e. superimposable), there was a dramatic difference within the \( \text{RAD54B} \)-deficient cells treated with either ATTM or 2ME2 (Fig. 3B). In general, a large and reproducible decrease in the normalized cell indices occurred approximately 15-20h post-treatment (Fig. 3B, right) and was typically completed within 5h. The
rapid decreases in proliferation coupled with the lack of a ‘stationary’ phase argues against a cell cycle arrest. In addition to the above data, statistically significant defects in proliferation (Fig. 3C) were observed and strongly suggest that cellular cytotoxicity accounts for the decrease in cell numbers. Next, standard colony formation assays were performed in which DMSO, ATTM or 2ME2 were supplemented into the medium and colonies were permitted to grow for 28-days. As predicted, statistically significant decreases in the total number of RAD54B-deficient colonies treated with ATTM or 2ME2 occurred relative to controls (Fig. 3D). Collectively, the above data indicate that ATTM and 2ME2 selectively target RAD54B-deficient cells over short (<4-days) and long (28-day) time frames, and further support their roles as lead compounds for subsequent study.

**ATTM and 2ME2 Treatments Induce ROS, Persistent DNA Double Strand Breaks and Apoptosis in RAD54B-deficient Cells:**

ROS are chemically reactive oxygen molecules that cause oxidative stress, which frequently manifest as DNA double strand breaks (HIGUCHI 2003). In eukaryotes, this damage is normally repaired through two complementary repair pathways – non-homologous end joining, which is ‘error prone’, and homologous recombination repair, which is ‘error free’. Since SOD1 is required for the removal of superoxide radicals, we postulated that SOD1 inhibition via ATTM, or increases in ROS following 2ME2 treatment, would produce DNA double strand breaks that normally would be repaired via the two functional DNA repair pathways. However, in RAD54B-deficient cells that are defective in homologous recombination repair, we predicted that the breaks will not be accurately repaired and will underlie the cellular cytotoxicity identified above.
Using live cell fluorescence microscopy, we first demonstrated that ATTM and 2ME2 induced ROS formation in both control and RAD54B-deficient cells relative to DMSO-treated controls (Fig. 4). The prevalence and persistence of DNA double strand breaks was then evaluated at various timepoints using two surrogate markers (\(\gamma\)-H2AX and 53BP1) for DNA double strand breaks (ROGAKOU et al. 1998). Semi-quantitative imaging microscopy revealed visually apparent (Fig. S4) and statistically significant increases in DNA-normalized \(\gamma\)-H2AX and 53BP1 signal intensities following a 6h treatment in both cell lines that did not occur within the DMSO-treated controls (Fig. 5). Following washout and a 36h recovery period, the \(\gamma\)-H2AX and 53BP1 signal intensities remained elevated within the RAD54B-deficient cells but returned to basal levels within the control (Fig. 5 and Table S6). Subsequent analysis of variance (ANOVA) confirmed statistically significant differences in the mean \(\gamma\)-H2AX and 53BP1 signal intensities following the ATTM and 2ME2 treatments in both cell lines (Tables S7-S10). Tukey multi-comparison post-tests revealed highly statistically significant increases in the mean \(\gamma\)-H2AX and 53BP1 total signal intensities between the untreated (t = 0h) and ATTM or 2ME2-treated cells following washout and recovery (t = 42h) within the RAD54B-deficient cells that were not significant within the controls (Tables S11-S14).

Although the above data show that RAD54B-deficient cells are hypersensitive to ATTM and 2ME2 treatments, they do not address the underlying mechanism. Images from our siRNA-based tests (Fig. 2B) contained nuclei exhibiting classical apoptotic features such as chromatin condensation and nuclear blebbing. Consequently, we sought to determine if apoptosis was a feature that contributed to the targeted killing of the RAD54B-deficient cells treated with ATTM or 2ME2. As predicted, semi-quantitative imaging reveal a statistically significant increase in the abundance of cleaved Caspase 3 (apoptotic marker) only within the RAD54B-deficient cells.
treated with ATTM or 2ME2 relative to controls (Fig. 6). These data show that ATTM and 2ME2 treatments induce apoptosis only within the *RAD54B*-deficient cells.

**DISCUSSION**

CRC has a significant health burden throughout the world and thus novel therapeutic strategies and targets are needed to minimize morbidity and mortality rates associated with the disease. Here we employ a cross-species approach to identify a highly connected, evolutionarily conserved SL interactor and confirm its potential as a novel candidate therapeutic target. Using bioinformatics and publicly available yeast SL datasets, we generated a genetic interaction dataset that was subsequently used to identify CIN gene/SL partner gene candidates to interrogate in a human CRC context. Preliminary direct tests revealed several SL interactions of *RAD54B* with genes involved in the removal of ROS, including *SOD1*. Using our established siRNA-based approach (McManus et al. 2009), we show that the yeast *RAD54 SOD1* SL interaction is conserved within two independent human cell lines. We further demonstrate that ATTM and 2ME2 can functionally substitute for the SOD1 siRNAs and decrease the number of *RAD54B*-deficient cells relative to controls in both short (<4-days) and longer-term assays (28-days). Finally, we provide evidence that ATTM and 2ME2 induce the formation of ROS and DNA double strand breaks that persist within the *RAD54B*-deficient cells that are associated with significant increases in cellular cytotoxicity and apoptosis. This study identifies and validates *SOD1* as an evolutionarily conserved SL interactor of *RAD54B* and thus confirms SOD1 as a novel candidate therapeutic target. The results of this study also support ATTM and 2ME2 as lead therapeutic compounds requiring subsequent pre-clinical study. Collectively, our data support the use of cross-species approaches to uncover evolutionarily conserved SL interactors that can be exploited as novel drug targets.
Although the SL targeting of \textit{RAD54B}-deficient CRC cells through SOD1 inhibition is a novel therapeutic strategy, ATTM was originally employed by veterinarians to treat copper poisoning in livestock (Suttle 2012). In humans, ATTM has been employed to treat Wilson’s disease, a neuropsychiatric disorder that results from the accumulation of Cu$^{2+}$ within tissues (Brewer 2003), and most recently it has begun to be evaluated in various cancer contexts due to its ability to adversely impact vascularization (angiogenesis), cell motility and invasiveness (metastasis), and sensitize cancer cells to additional anti-cancer drugs (PAN et al. 2002; HENRY et al. 2006; GARTNER et al. 2009; KUMAR et al. 2010; KIM et al. 2012). Indeed, several phase I and II clinical trials are currently underway to evaluate the efficacy of various Cu$^{2+}$ chelators in a diverse array of tumor types including breast, esophageal, and prostate cancer (www.clinicaltrials.gov).

A SL therapeutic strategy represents a significant advancement over more traditional approaches that are not inherently designed to limit the therapeutic effect to cancer cells. For example, 5-FU and doxorubicin affect DNA replication while paclitaxel affects mitosis and are associated with adverse side effects including neutropenia, cardiotoxicity, and infertility. The low concentrations of ATTM and 2ME2 employed in the \textit{RAD54B SOD1} SL paradigm are purposefully selected to induce killing only within the \textit{RAD54B}-deficient cells and are therefore not expected to produce side effects within normal surrounding cells. Indeed, the data presented above strongly suggest that control cells are minimally impacted following the transient administration of ATTM and 2ME2 as they are able to tolerate the transient increase in ROS and do not display any overt signs of unrepaired DNA double strand breaks or apoptosis. In fact, we predict that in addition to invoking SL killing of \textit{RAD54B}-deficient cells, ATTM treatments may produce an additive or synergistic effect within tumors that stems from its potential role as an

Recently, it was shown in HCT116 cells that RAD54B is SL with LIG4 (Oh et al. 2013), which encodes DNA ligase IV a key protein involved in non-homologous end joining, and suggests that the homologous recombination repair and non-homologous end joining pathways can functionally compensate for each other. However, the cytotoxic effects we observe within the RAD54B-deficient cells following SOD1 silencing or ATTM or 2ME2 treatments occur within the presence of a presumably functional non-homologous end joining pathway. Although determining the exact mechanism(s) by which SOD1 silencing and ROS induction circumvent the non-homologous end joining pathway is beyond the scope of the current study, it is possible that they induce a subset of DNA breaks that can only be repaired by the homologous recombination pathway such as those that occur in S-phase as a result of collapsed replication forks. However, it is also equally possible that the damage simply overwhelms the repair capacity of the non-homologous end joining pathway, or alternatively that ROS induction induces signaling pathways that suppress non-homologous end joining. In any case, these possibilities are speculative and remain to be evaluated.

When ROS overwhelm the antioxidant defenses of the host, free radicals can interact with various endogenous macromolecules and alter normal cellular function. If, for example, superoxide radicals are left unchecked, they will cause DNA double strand breaks, lipid peroxidation and protein oxidation. Consequently, eukaryotes have evolved highly conserved mechanisms that are designed to minimize the impact of ROS. Of particular relevance to this study is the identification and clustering of three yeast genes tsa1, ccs1 and sod1 (Fig. 1A; bottom) and their corresponding human genes PRDX2, CCS, and SOD1, respectively, as
common SL interactors for many yeast CIN genes whose human orthologs are somatically mutated in CRC. These three genes encode products that are required to remove superoxide radicals found within the cytoplasm through a two-step process. First, SOD1 dismutates superoxide radicals into another ROS, namely H₂O₂. SOD1 activity requires Cu²⁺ as an essential co-factor, which is normally provided via the SOD1 copper chaperone, CCS (Culotta et al. 1997). Next, peroxiredoxin (PRDX2) reduces H₂O₂ into water and oxygen. Although the current study focuses on SOD1 as a novel candidate therapeutic target, we provide preliminary evidence that suggests the two remaining components of this process, PRDX2 and CCS, may also possess therapeutic potential. For example, our initial siRNA-based screen of the mini-network identified in Fig. 1A suggests that PRDX2 is a SL interactor with RAD54B and other genes somatically mutated in cancer (Fig. 1C). Furthermore, our chemical studies demonstrate that ATTM, a chemical derivative of tetrathiomolybdate (ATN-224) (Juarez et al. 2006), selectively kills RAD54B-deficient cells (Fig. 3). Although ATTM is a SOD1 inhibitor, it functions by chelating Cu²⁺, an essential co-factor of SOD1. Consequently, it is plausible that the bioavailability of Cu²⁺ decreases following ATTM treatment, thus compromising the function of CCS as a copper chaperone for SOD1. Thus, targeting CCS chaperone activity may be of therapeutic benefit. In either case, validating and confirming PRDX2 and CCS as novel candidate targets will require subsequent study and goes beyond the scope of the current study.

The pharmacologic targeting of SOD1 has implications beyond the CRC-specific context described here. Although RAD54B is somatically mutated in ~3.3% of sporadic CRCs (Table S1) (Cancer Genome Atlas 2012), it is also somatically mutated and/or deleted in lymphoma, kidney, prostate, brain, and breast cancers (Hiramoto et al. 1999; Forbes et al. 2011; Cerami et al. 2012). In light of our findings that the RAD54B SOD1 SL interaction is conserved within
an immortalized fibroblast cell line (hTERT) (Fig. 2D), SOD1 inhibition may be an effective therapeutic strategy to target and kill additional tumor types harboring RAD54B defects. It should also be noted that SOD1 inhibition might have broad-spectrum applicability beyond the RAD54B-specific paradigm presented here, as it is a member of the highly connected subset of SL partner genes described in this study. In yeast, sod1 is SL with at least 41 different genes, and many like rad54, are involved in homologous recombination repair (Fig. 1A). These genes, if conserved within a cancer context, may identify additional genetic defects that could be leveraged for therapeutic targeting. Indeed, our preliminary tests (Fig. 1B) suggest this may be the case as a putative SL interaction was observed with BLM, a homologous recombination repair enzyme. BLM is somatically mutated in ~4.2% of CRCs as well as numerous other tumor types including breast, lung, prostate and ovarian (Table S1), and the relevance of therapeutically targeting SOD1 within these tumors warrants further study. In conclusion, we have presented data that rationalizes the use of cross-species approaches to assist in the identification of novel therapeutic targets designed to exploit somatic mutations causing CIN, for more selective and broader-spectrum targeting of cancer.

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FIGURE LEGENDS

Figure 1: Analysis of yeast CIN gene SL interactions identify highly connected gene pairs.

(A) 2D hierarchical gene clustering performed on 692 yeast CIN genes and their top 500 genetic interactors (left). A “data-rich” area of the clusterogram was used to identify a smaller (right) and highly focused network (bottom) of candidate interactions. Yeast sod1 is a SL interactor with genes whose human orthologs are mutated or deleted in CRC (red asterisks; Table S1). Yeast sod1 clusters with its copper chaperone, ccs1 (human CCS), and another ROS detoxifying enzyme tsa1 (human PRDX2). (B) A mini SL network comprising the human orthologs of yeast CIN genes (blue circles) that are somatically mutated in CRC, and SL interactors evaluated in human cells (green circles). Putative conserved SL interactions are identified with a blue line, while those specific to yeast are shown in grey, and the single interaction identified only in HCT116 cells shown in green. Human gene names are indicated with the corresponding yeast gene listed within brackets. (C) A graphical depiction of a putative SL interaction observed between human RAD54B and PRDX2. Shown are the relative percentages of cells remaining ± Standard Deviation, following siRNA treatments (x-axis). Note the enhanced killing (i.e. lower percentage of cells remaining) in the RAD54B-deficient cells treated with siPRDX2 relative to controls.

Figure 2: RAD54B and SOD1 are Synthetic Lethal Interactors in Human Cells.

(A) Western blot depicting diminished SOD1 expression following siRNA-mediated knockdown in HCT116 cells; α-tubulin is a loading control. Semi-quantitative analysis was performed and the normalized SOD1 expression levels relative to siGAPDH (1.00) are shown. (B) Representative images depicting the qualitative decrease in cell numbers (i.e. DAPI-stained nuclei) following SOD1 silencing of RAD54B-deficient cells relative to controls. Arrowheads
identify nuclei exhibiting hallmarks of cellular cytotoxicity. Scale bars represent 25µm. (C) Graphical depiction for the relative percentage of control and RAD54B-deficient cells (± standard deviation [SD]) following treatment with various siRNAs (x-axis); statistical significance is indicated (*** = p-value <0.0001; * = p-value <0.05; NS = not significant). Graphical depiction of the SL interaction observed following the simultaneous silencing of RAD54B and SOD1 in (D) RAD54B-proficient HCT116 and (E) hTERT cells. Depicted are the mean normalized percentages (± SD) for the individual silencing of either RAD54B (black squares) or SOD1 (white triangles), and the expected value (grey circles) calculated for the dual combined siRNAs calculated using a multiplicative model. Black circles identify the actual observed values for the simultaneous dual silencing.

Figure 3: RAD54B-deficient Cells are Selectively Killed Following ATTM and 2ME2 Treatment.

(A) Dose response curves for cells treated with varying concentrations of ATTM (left) and 2ME2 (right). Data are normalized to the respective DMSO-treated controls. (B) Real-time growth curves for control (left) and RAD54B-deficient (right) cells treated with DMSO, ATTM or 2ME2. Chemical compounds were added 20h post seeding (arrow). (C) Graph depicting an increase in the mean proliferation defects (± SD) for RAD54B-deficient cells treated with either ATTM or 2ME2 (x-axis), relative to controls. Student’s t-tests identified highly statistically significant increases in mean proliferation defects (*** = p-value <0.0001) for the RAD54B-deficient treated cells. (D) Graphs depicting the relative number (± SD) of colonies formed in soft agar for cells treated with DMSO, ATTM or 2ME2 for 28-days (*** = p-value <0.0001).
Figure 4: ATTM and 2ME2 Treatments Induce ROS Production.

(A) Representative images depicting the abundance of ROS as reflected by qualitative changes in the global abundance of fluorescein total signal intensity (green) in \textit{RAD54B}-proficient and \textit{RAD54B}-deficient cells treated with DMSO, ATTM or 2ME2. All images were acquired using the identical exposure times and thus changes in fluorescence intensities reflect changes in the abundance of ROS in these cells. Images were acquired following 6h incubation with the chemicals. Note that both \textit{RAD54B}-proficient and \textit{RAD54B}-deficient cells show an increase in ROS after treatment with ATTM or 2ME2. Nuclei (blue) are counterstained with Hoechst. Scale bar represents 10\(\mu\)m. (B) Graphical depiction of the changes in normalized ROS total signal intensity following DMSO, ATTM, and 2ME2 treatments (***) = p-value <0.0001).

Figure 5: DNA Double Strand Breaks Persist in \textit{RAD54B}-deficient Cells Following ATTM and 2ME2 Treatment.

(A) Graphs depicting the changes in normalized \(\gamma\)-H2AX total signal intensities in cells treated with DMSO, ATTM or 2ME2. Semi-quantitative imaging microscopy was performed on cells prior to treatment (t = 0h), following a 6h treatment (t = 6h), and following a 36h recovery following washout (t = 42h) of drug (***) = p-value <0.0001). (B) Graphs depicting the semi-quantitative changes in normalized 53BP1 total signal intensities following DMSO, ATTM and 2ME2 treatments. Graphs are labeled as indicated in (A).

Figure 6: ATTM and 2ME2 Selectively Induce Apoptosis in \textit{RAD54B}-Deficient Cells.

(A) Representative images depicting the abundance of apoptosis as reflected by qualitative increases in cleaved Caspase 3 in cells treated with DMSO, ATTM or 2ME2. Staurosporine induces apoptosis and is included as a positive control. All images were acquired with identical exposure times and thus changes in fluorescence intensities identify changes in the global
abundance of the cleaved Caspase 3 (apoptosis). Images were acquired immediately prior to
treatment (t = 0h) and following a 12h treatment (t = 12h). The nuclei and cleaved Caspase 3 are
pseudocolored red and green, respectively, within the merged images. Note the striking increase
in cleaved Caspase 3 signal intensities within RAD54B-deficient cells treated with ATTM or
2ME2 relative to controls. Scale bar represents 30µm. (B) Graphs depicting the semi-quantitative
changes in the normalized cleaved Caspase 3 total signal intensities following DMSO, ATTM
and 2ME2 treatments (NS = not significant; *** = p-value <0.0001). Quantitative imaging
microscopy was performed on cells prior to treatment (t = 0h) and following a 12h treatment (t =
12h).
Figure 1

A. 692 CIN genes

B. RAD54B (rad54)

C. Percentage of Cells Relative to siGAPDH

RAD54B-Proficient

RAD54B-Deficient

siGAPDH  siPRDX2
Figure 2

A. 

B. RAD54B-Proficient  RAD54B-Deficient
+siSOD1-2  +siSOD1-2

C. 

D. HCT116

E. hTERT

Percentage of Cells Relative to siGAPDH

Percentage of Cells Relative to siGAPDH

Percentage of Cells Relative to siGAPDH

Percentage of Cells Relative to siGAPDH

Figure 4

A.  

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B.  

![Graph](Graph.png)
Figure 5

A.  

B.  

Normalized γ-H2AX Total Signal Intensity

Normalized 53BP1 Total Signal Intensity

Un-treated  DMSO  ATTM  2ME2

t=0h  t=6h  t=42h  (36h recovery)

RAD54B-Proficient  RAD54B-Deficient

***  ***  ***  ***

***  ***  ***  ***

(36h recovery)
Figure 6

A. 

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<td>t = 12h</td>
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B. 

![Graph showing normalized cleaved caspase 3 intensity](#)

- **RAD54B-Proficient**
- **RAD54B-Deficient**

**t = 0h**

**t = 12h**