Class I Histone Deacetylase HDAC1 and WRN RECQ Helicase Contribute Additively to Protect Replication Forks upon Hydroxyurea-Induced Arrest

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Running title: WRN and HDAC1 facilitate replication fork recovery

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ABSTRACT

The WRN helicase/exonuclease is mutated in Werner syndrome of genomic instability and premature aging. WRN-depleted fibroblasts, while remaining largely viable, have a reduced capacity to maintain replication forks active during a transient hydroxyurea-induced arrest. A strand exchange protein RAD51 is also required for replication fork maintenance, and here we show that recruitment of RAD51 to stalled forks is reduced in the absence of WRN. We performed a siRNA screen for genes that are required for viability of WRN-depleted cells after hydroxyurea treatment, and identified HDAC1, a member of the class I histone deacetylase family. One of the functions of HDAC1, which it performs together with a close homolog HDAC2, is deacetylation of new histone H4 deposited at replication forks. We show that HDAC1 depletion exacerbates defects in fork reactivation and progression after hydroxyurea treatment observed in WRN- or RAD51-deficient cells. The additive WRN, HDAC1 loss-of-function phenotype is also observed with a catalytic mutant of HDAC1, however, it does not correlate with changes in histone H4 deacetylation at replication forks. On the other hand, inhibition of histone deacetylation by an inhibitor specific to HDACs 1-3, CI-994, correlates with increased processing of newly-synthesized DNA strands in hydroxyurea-stalled forks. WRN co-precipitates with HDAC1 and HDAC2. Taken together, our findings indicate that WRN interacts with HDACs 1 and 2 to facilitate activity of stalled replication forks under conditions of replication stress.

INTRODUCTION

Replication stress, defined as disturbances to normal progression rate, density, or distribution of replication forks, is a major driver of genomic instability and carcinogenesis (1-3). Replication stress caused by fluctuations in cellular pools of NTPs and dNTPs is highly relevant to the understanding of the mechanisms of oncogene-driven mutagenesis and chemosensitivity (ibid.). Hydroxyurea (HU), a ribonucleotide reductase inhibitor, depletes dNTP pools in a dose-dependent manner to cause a reversible global reduction in replication fork progression rate. Slowing or stalling of forks in HU and subsequent reactivation of normal fork progression after HU are highly regulated processes, which protect forks from inactivation and ensure faithful and complete replication of the genome. This includes
preserving the ability of forks to resume DNA synthesis after conditions normalize, as well as preventing excessive truncation of nascent DNA strands at the fork, and involves coordinated activities of many proteins, including checkpoint effectors and mediators, exonucleases, helicases, ATPases, low fidelity DNA polymerases, and proteins of homologous recombination machinery (4,5). Nonetheless, prolonged stalling eventually leads to development of double strand breaks (DSBs), and activation of the DNA damage response (DDR) (6-8).

We and others have shown that the human RECQ helicases WRN and BLM are among the proteins that are important for normal progression of replication forks, as well as for the recovery of stalled forks after a transient HU arrest (9-15). Mutations in the \textit{BLM} (16), and \textit{WRN} (17) genes cause, respectively, Bloom syndrome (BS) and Werner syndrome (WS), two heritable human genomic instability disorders characterized by developmental abnormalities (BLM) and premature aging (WRN), respectively (18,19). Both syndromes are also associated with increased predisposition to specific types of cancer (20,21). BLM and WRN are caretaker genes that maintain genomic stability through their roles in replication, repair, and telomere homeostasis (for review, see (6,22-24)).

In order to delineate unique versus overlapping functions of WRN and BLM, we have previously depleted WRN and/or BLM in SV40-transformed human fibroblasts and shown that these fibroblasts exhibit comparable defects in reactivation of replication forks after an HU-induced arrest (15). Despite comparable fork reactivation defects, WRN-depleted fibroblasts showed less HU cytotoxicity than BLM-depleted cells (25), enabling us to look for additional genes that may modify cytotoxicity induced by HU in WRN-deficient cells. Identification of such genes may provide novel insight into the mechanisms of resistance to replication stress, as well as differences in the roles of WRN and BLM in the cell.

We thus conducted a siRNA screen for genes that were synthetic lethal with WRN deficiency in HU-treated human fibroblasts. We found that depletion of class I histone deacetylases HDAC1 or HDAC2 confers such a phenotype. Following up on this finding we show that in WRN-depleted but not in WRN-proficient fibroblasts HDAC1 is needed for efficient fork reactivation after HU. Moreover, we demonstrate co-immunoprecipitation of WRN with HDAC1 and HDAC2. Lastly, inhibition of deacetylase activity of HDAC1 and HDAC2 by a small molecule CI-994, leads to enhanced nascent strand processing at stalled forks. Based on the analysis of histone acetylation at stalled and moving forks, and WRN, HDAC1, and RAD51 recruitment to forks, we propose new roles of histone deacetylases during the replication challenged with dNTP pool fluctuations. Our results highlight the importance of chromatin environment in mitigating disruptions to replication.

**EXPERIMENTAL PROCEDURES**

**Cells and culture**

The SV40-transformed human fibroblast GM639 fibroblast cell line and its pNeoA derivative GM639cc1 have been described before (14,15,25,26). WV1 is an SV40-transformed Werner syndrome patient-derived fibroblast line that does not express any WRN protein (26). MCF10a spontaneously immortalized mammary epithelial and UW289.B1 \textit{brca1-/-} (27) ovarian cancer cell lines were a gift of Drs. Piri Welch and Elizabeth Swisher (University of Washington). Embryonic rhabdomyosarcoma cell line RD was obtained from ATCC (ATCC CCL-136). Primary human fibroblast line HFF4 was described by us previously (28).

GM639cc1, RD, and HFF4 were grown in Dulbecco Modified Minimal Essential Medium (DMEM) supplemented with L-glutamine, sodium pyruvate, 10% fetal bovine serum (Hyclone, Ogden, UT) and antibiotics, and MCF10a were grown in MGEM media (Lonza) supplemented with Single Quots (Lonza), 1% fetal bovine serum and antibiotics. UW289.B1 cells were grown in 1:1 mixture of RPMI and MEM supplemented with 3% FBS. All cell lines were kept in a humidified 5% CO\textsubscript{2}, 37°C incubator.

**Drugs and other reagents**

Stock solution of 5-iododeoxyuridine (IdU) was at 2.5mM in PBS, 5-chlorodeoxyuridine (CldU) was at 10mM in PBS, and 5-ethynyldeoxyuridine (EdU) was at 10mM in DMSO, and hydroxyurea (HU) was at 1M in PBS. IdU, CldU, and HU were purchased from Sigma-Aldrich, and EdU from Life...
Technologies and Sigma-Aldrich. IdU and CldU were used at a concentration of 50μM and EdU was used at 10μM. Stock solution of CI-994 (LC Laboratories) was made at 10mM in DMSO, and used at 3-4μM unless specified otherwise. All reagent stocks were stored at -20°C.

**High throughput screen**

We screened a library of siRNAs listed in Supplementary Table S1 at the University of Washington Quellos Screening Core. WRN-depleted and mock-depleted GM639cc1 fibroblasts were generated for this screen by infection with a pLKO.1-based WRN shRNA construct (14,15) or empty pLKO.1, respectively. Infected cells were selected by resistance to puromycin, and depletion was verified by Western blotting. siRNA transfection conditions were optimized prior to the screen using universal siRNA control as a transfection toxicity readout and siRNA to an essential gene, KIF11, as a transfection efficiency readout. Of interest, WRN-depleted cells were less sensitive to the non-specific toxicity associated with transfection. Conditions were adjusted to keep transfection-associated toxicity in the two cell lines at the same level (Figure S1A). Cells were then plated for transfection into 384-well dishes and the next day each cell line was transfected with library siRNAs (one gene per well in triplicate for treatment and control arms, four siRNAs per gene), as well as universal siRNA control and Kif11 siRNA controls per each plate. The next day, wells in the treatment arm were incubated with 2mM hydroxyurea for 6 hrs. Cell number and metabolism were evaluated after three days using CellTiter Glo dye (Promega) per manufacturer’s instructions, and plates were scanned to quantify signal.

**RNAi-mediated depletion of WRN and RAD51**

Short hairpin (sh) RNA constructs for depletion of WRN and RAD51 are described (14,15,25). Scrambled shRNA is 5’-CTCCATATCGAACAGTTGG-3’. siRNAs against HDAC1, HDAC2, WRN, and a Negative Control non-targeting siRNA were purchased from Qiagen (HDAC1-5 SI02634149, HDAC1-6 SI02663472, HDAC2-1 SI00434952, HDAC2-3 SI00434966, WRN-6 SI02663759). AllStars Cell death control siRNA (Qiagen) was used as transfection efficiency control. TET-inducible shRNA construct against WRN expresses the same hairpin as the previously described shRNA construct (14).

Inducible shRNA and siRNAs were used in HDAC+ and HDAC1 KO RD cells because in these lines lentiviral infection combined with immediate onset of WRN RNAi triggered cessation of cell division by day 8-10 post-infection. In HDAC1 KO RD cells transduced with HDAC1 gene constructs, siRNA were used because they achieved a more rapid depletion of the protein than a combination of transduction, followed by a recovery period and then by induction of RNAi by addition of doxycycline to the cells.

**CRISPR-Cas9-mediated gene deletion**

Gene knockouts were performed in the RD embryonic rhabdomyosarcoma cell line. The HDAC1 knockout clone (#17) used in Figures 4, 5C, and 8D was generated by transduction of a pLenti-CRISPRv1 lentiviral vector (29) expressing Cas9 and a single HDAC1 gRNA, followed by puromycin selection and isolation of individual clones. The knockout was sequence- and Western blot verified. The wild type isogenic control for this clone is parent RD line expressing GFP. For HDAC1 or HDAC2 deletion RD cell lines used in Figures 5D-F, two gRNAs per gene were used to enhance targeting efficiency. The gRNA sequences were GGACTGTCCAGTATTCGA, and GGCTCAGACTCCCTATCT for HDAC1 and GGAATACTTTCCTGGCAC and GGTCATGCGGATTCTATG for HDAC2. These were cloned into a modified pLenti-CRISPRv1 (Addgene # 49535) viral vector containing TagRFPt in place of Cas9 (29). Cas9 was expressed from a separate modified pLenti-CRISPRv1 lacking a gRNA cassette (29). Virus particles were generated using standard protocols and used for transduction of RD cells. Transduced mass cultures were selected with puromycin, and >80% efficient knockout of HDAC1 or HDAC2 was verified by Western blotting. The isogenic wild type control for these cells is the parent RD line expressing only Cas9. Individual clones were subsequently derived from these mass cultures and Western blot verified. These clones (#2, 4, and 13) were used in the results shown in Figures 6, 7, and their supporting (not shown) data.

**HDAC1 mutant construction and expression**
A plasmid with a HDAC1 ORF bearing a C-terminal flag-tag and expressed under the control of the CMV promoter was a gift from Eric Verdin (Addgene # 13820). A His to Ala mutation (CAT to GCT) at position 141 of the protein sequence was introduced using the NEB base changer site-directed mutagenesis protocol. Expression of wild type and mutant HDAC1-flag and their deacetylase activity were checked in transient transfections. For stable expression, HDAC1 ORFs were cloned into a 2A linked emGFP lentiviral expression vector (pLenti-EFS-T2A-emGFP). Virus particles were prepared using standard protocols and used for transduction of HDAC1 KO RD cells. Transduction efficiency and expression of HDAC1 proteins was verified by flow cytometry of GFP+ cells and Western blotting with the α-HDAC1 antibody, respectively. Transduced cells were subsequently purified by flow cytometric cell sorting based on GFP expression.

Deacetylation activity of wild type and mutant HDAC1 was determined in vitro using Histone Deacetylase Activity kit (Active motif), according to the manufacturer’s recommendations.

Western blotting

Western blotting of WRN was done as described (14,25). Antibodies were as follows: mouse antibodies α-WRN 195C Cat. No. W0393 (Sigma), α-HDAC1 Cat. No. 5356 (Cell Signaling), α-HDAC2 Cat. No. 5113 (Cell Signaling), α-PhosphoH2AX (Ser139) Cat. No.05-636 (Millipore), α-PCNA Cat. No. sc-56 (Santa Cruz), α-Nucleolin Cat. No. 396400 (Life Technologies), α-RAD51 Cat. No.05-530 (Millipore), α-CHK1 Cat. No. sc-8408 (Santa Cruz); α-Flag Cat. No. MA1-91878-BTIN (Thermo Fisher); and a rabbit antibody α-H4K12ac, Cat. No. 39165 (Active Motif).

All proteins were visualized on Western blots by ECL (ThermoScientific) and quantified using Storm Phosphorimager (Molecular Dynamics) or FluorChem Imager (Alpha Inotech). For presentation, images were saved in TIFF format, adjusted for brightness/contrast and cropped using Adobe Photoshop or CorelPhotoPaint, then assembled into figures in CorelDraw. Image brightness/contrast adjustments were made to the whole Western blot images. In some cases lane order was changed and extra lanes were deleted.

Microchannel fabrication, DNA fiber stretching and replication track analysis.

These procedures were done as described (14,28,30). Microscopy of stretched DNAs was performed on the Zeiss Axiocam microscope with a 40x objective, and images were captured with the Zeiss Axiocam HRm camera. Lengths of tracks were measured in raw merged images (jpeg) using Zeiss AxiomVision software. Fluorochromes were Texas Red for EdU, Alexa594 for CldU, and Alexa488 for IdU. Details of statistical analysis are described in Figure Legends.

iPOND and niPOND.

Immunoprecipitation of nascent DNA (iPOND) was performed as described (31), with modifications detailed in (28). Briefly, aprotinin and leupeptin were added to permeabilization buffer (0.25% triton X100 in PBS) to a final concentration of 1μg/ml ea. The buffer used for sonication and lysis of cells was 50mM Tris HCl pH8.0; 0.5%SDS; 0.1% Na dioxycholate; 0.25% TritonX100; 1μg/ml aprotinin, 1μg/ml leupeptin. niPOND (native iPOND) was performed as described in (32). All steps were performed with ice-cold reagents. Briefly, nuclei were isolated in nuclei extraction buffer (20mM HEPES pH7.2, 50mM NaCl, 3mM MgCl2, 300mM sucrose, 0.5% NP40 Substitute (Sigma-Aldrich), protease inhibitor cocktail (Roche)), washed in PBS, subjected to Click-iT reaction, and extracted twice with B1 buffer (25mM NaCl, 2mM EDTA, 50mM Tris-HCl pH8.0, 1% NP40 Substitute, protease inhibitors) by incubating for 15 min on ice followed by sonication at 20W for 10 sec in a cup-horn sonicator (Misonix) and pelleting. This step enriched for chromatin-associated proteins. Lysates were generated by sonication in B1 buffer for 6 min total in 30 sec on, 30 sec off pulses at 40W setting. Lysates were cleared by 16,100xg centrifugation for 10 min, then diluted 1:1 with B2 buffer (150mM NaCl, 2mM EDTA, 50mM Tris-HCl pH8.0, 0.5% NP40 Substitute, protease inhibitors). EdU+ DNA and associated proteins were captured by overnight incubation with streptavidin-agarose beads (Novagen).

To measure EdU amounts in lysates, serial 1:2 dilutions of each lysate in PBS (typically from...
0.2 to 0.02 μl of lysate in 0.5 μl total volume), were loaded onto a nitrocellulose membrane, dried for 1-2 hrs, blocked in 5% BSA in TBST, and incubated overnight with 1:150 dilution of HRP-conjugated α-biotin antibody Cat. No. 7075 (Cell Signaling) in 5% BSA, TBST overnight at 4°C, then washed and subjected to ECL and quantified using FluorChem Imager. Values obtained for 2-3 serial dilutions that were within linear range of signal were averaged. EdU was also measured in some pulldown samples by the same procedure: 1 μl of pulldown sample was diluted in 50 μl of PBS, then loaded as two-fold dilution series after volume-adjusting to 0.5 μl. Normalizing results by EdU levels in starting lysates or in pulldowns demonstrated similar results.

Immunoprecipitation
Immunoprecipitation was performed with nuclear extracts prepared as described above for niPOND except extraction of soluble proteins from permeabilized nuclei was performed only once and typically did not include mild sonication prior to high-speed centrifugation. Chromatin lysates were incubated for 2-3 hrs with α-HDAC1 (Cat. No. 5356, Cell Signaling) antibody used at 1:400 dilution, or α-HDAC2 (Cat. No. 5113, Cell Signaling) antibody used at 1:200 dilution, and with protein G Dynabeads (Invitrogen). Immunoprecipitates were washed 4 times with niPOND B2 buffer and analyzed in SDS PAGE gels.

RESULTS
High-throughput siRNA screen identifies HDAC1 and HDAC2 as genes that enhance HU cytotoxicity in a WRN-deficient background

We assembled a siRNA library targeting 320 DNA damage response, repair and replication genes (Supplementary Table S1), where each gene was targeted by a mixture of 4 siRNAs. Cell lines used in transfection were GM639cc1 SV40 fibroblasts that were either mock-depleted with an empty lentiviral vector (controls), or depleted of ≥75% of WRN by the same vector expressing shRNA against WRN (14,15). Cells were plated in 384-well plates, transfected with the library of siRNAs, and allowed to grow for 3 days, with or without a 6-hr treatment with 2 mM hydroxyurea (HU) on Day 1 of outgrowth. Cell growth and viability was evaluated in a CellTiter Glo luminescence assay and growth values were normalized to the average of mock-transfected (“Mock”) controls for each cell line and condition (Figure 1). Two-tailed p values were calculated in pairwise comparisons of untreated vs. treated normalized growth for each gene, and only the measurements with p values ≤0.05 were considered. ATR and CHEK1 siRNAs were controls that were expected to show HU-dependent cell lethality. Of note, CHEK1 siRNA suppressed growth in untreated WRN-depleted but not control cells (Figure 1B). This is consistent with the idea that WRN-depleted cells experience constitutive replication stress that requires ongoing CHK1-mediated checkpoint surveillance.

We identified 4 classes of growth-suppressing knockdown phenotypes resulting from siRNA targeting: 1) constitutive, 2) WRN-dependent, 3) HU-dependent, and 4) WRN and HU-dependent. In the current study we were interested in the candidate genes identified in the category (4), and specifically in those that, when silenced by siRNAs, suppressed growth only mildly in the absence of HU (by less than 40% in WRN-depleted or control cells). Among these, we focused on the genes that, when silenced together with HU treatment, caused a greater than 2-fold growth reduction only in WRN-depleted cells. No candidates from the library showed strong (over 80%) growth inhibition in WRN-depleted cells while leaving control cells unaffected (Figure 1A). Of the genes that displayed the most dramatic HU- and WRN-dependent reduction of growth at least some (TOPBP1, EME1) proved to be HU-dependent but not WRN-dependent growth suppressors upon retesting (data not shown).

siRNA-mediated depletion of HDAC1 histone deacetylase conferred a phenotype that ranked within our criteria, causing a greater reduction of viability in HU-treated WRN-depleted cells compared to control cells (Figure 1). Notably, HDAC2, a close homolog and binding partner of HDAC1 (33,34), also exhibited some WRN- and HU-selectivity (Figure 1). We validated these phenotypes of HDAC1 and HDAC2 using two independent siRNAs against these genes in population outgrowth assays. HDAC1 siRNA HDAC1-6 and HDAC2 siRNA siHDAC2-3, suppressed growth additively with WRN-depletion and HU treatment (Figure 2A), and, respectively, depleted 70-80% of HDAC1 and 40-50% of HDAC2 (Figure 2B, C). The
strong growth suppression elicited by the moderate depletion of HDAC2 levels suggested that complete depletion of HDAC2 in the GM639ce1 WRN-deficient background may be lethal. Additionally, moderate depletion of HDAC2 by siHDAC2-3 and even by siHDAC2-1 (which depleted only 10-30% of HDAC2, Figure 2C), was associated with a compensatory increase in the HDAC1 levels (Figure 2B, 3A). These findings prompted us to focus on HDAC1, which we could deplete effectively and without concurrent changes in HDAC2 levels, as a priority for our follow-up studies.

**HDAC1 is important for replication fork activity in WRN-depleted cells**

One of the functions of HDAC1 (and HDAC2) is to remove acetyl groups on de novo synthesized histones incorporated into chromatin during DNA replication, notably acetylons on histone H4 Lysine residues 5 and 12, i.e. H4K5ac and H4K12ac (35). Both HDAC1 and 2 were detected at a replication fork (36). Fork progression rates are reduced in the absence or upon inhibition of both HDACs (35), although no phenotype specific to individual HDAC or to recovery after HU treatment has been reported yet. To address the interplay between HDAC1 and WRN during normal and HU-perturbed DNA replication, we performed microfluidic-assisted Replication Track Analysis (maRTA) (30) on GM639ce1 fibroblasts depleted of WRN and/or HDAC1. In accordance with standard approaches (15,30), reactivation of replication forks after a 6-hour arrest by HU was measured by counting forks that resumed DNA synthesis within the first 30 minutes after removal of HU (i.e. incorporated both 1st and 2nd label) versus those that failed to reactivate (i.e. incorporated only the 1st label, Figure 3B). Also, progression of replication forks before/during or after HU was assessed by measuring lengths of 1st and 2nd label segments in two-label tracks. We previously showed that the fraction of reactivating forks drops in cells depleted of 80% or more of the WRN protein (15). In addition, we showed that in WRN-depleted cells reactivated forks progress slower than in WRN-proficient cells (14,15). We will subsequently use the term fork recovery to describe both of these phenotypes of WRN loss.

When we co-depleted HDAC1 and WRN, fork reactivation was additively impaired (Figure 3C). In control cells HDAC1 depletion had minimal to no effect on percentage of fork reactivation (Figure 3C). Also, depletion of WRN or HDAC1 each reduced progression of forks in the absence of HU as well as after HU treatment, as evidenced by shorter track lengths, and notably, double depletion had an additive negative effect (Figure 3D). In all cases, comparison of progression through 1st versus 2nd labelling periods for each ongoing fork revealed that it was reduced similarly, that is, the ratios of 2nd to 1st label segment lengths remained virtually unchanged for all cells and averaged close to 1 in untreated cells (Figure 3E) and below 0.5 in HU-treated cells (data not shown). Together, the results in Figure 3D, E are more consistent with a uniform fork rate reduction in HDAC1- and/or WRN-depleted cells rather than increased premature termination. Lastly, the additive fork reactivation phenotype was reproduced in a cell line of a different (epithelial) lineage, MCF10a (Figure 3F, G). Overall, the data suggest an additive reduction of fork recovery resulting from WRN and HDAC1 depletion, which is consistent with reduced cell growth post-HU treatment of these co-depleted cells.

**WRN and HDAC1 are present on newly replicated DNA**

To assess whether HDAC1 and WRN associate with replication forks or newly replicated DNA, we performed native iPOND (niPOND, or native Immune Precipitation Of Nascent DNA). niPOND (32) pulls down proteins associated with EdU-labeled DNA via Click-IT-mediated conjugation of biotin-azide to EdU in permeabilized cells, followed by lysis and incubation with streptavidin beads (Figure 4A). Unlike regular iPOND (31), niPOND does not employ formaldehyde-crosslinking of proteins to DNA, facilitating recovery of large proteins, particularly as large as WRN (~167kD). As these experiments require large starting cell numbers, we used a WRN null, patient-derived SV40 fibroblast line WV1 and RD rhabdomyosarcoma lines with either a CRISPR-Cas9-mediated HDAC1 knockout (KO) or a GFP-expressing empty vector (WT, Figure 4B). RD HDAC1 KO clones grew more slowly that the control (for the clone shown in Figure 4, the average population doubling was 0.64±0.33/day vs. 0.75±0.26/day in
the control). Also, we found lower levels of WRN expression in two independently derived HDAC1 KO clones compared to HDAC1 wild type controls (Figure 4B and data not shown), which may indicate effects of HDAC1 on the WRN gene expression.

Using niPOND with nuclear lysates enriched in chromatin-bound proteins (see Materials and Methods), we confirmed previous observations (36,37) that HDAC1 was pulled down with EdU-labeled genomic DNA that corresponds to replication forks (Figure 4C). We also detected the presence of H4K12ac, which corresponds to the acetylated histone H4 newly deposited at replication forks (Figure 4C, E). We demonstrated association of WRN with replication forks (Figure 4C). Identification of WRN in EdU pull-downs was further confirmed by using a WRN null fibroblast line WV1 (Figure 4D).

The levels of PCNA and H4K12ac on EdU+ DNA decreased after a 2 hr-long chase following EdU pulse, indicating that the DNA replication machinery has moved away from the EdU-labeled DNA segments and the nascent chromatin matured (Figure 4E, F). By contrast, the association of HDAC1 and WRN with EdU+ DNA was more long-lived, lasting at least 2.5 hours after EdU pulse (Figure 4F).

WRN recruitment to EdU+ DNA was observed in WT and HDAC1 KO cells (Figure 4F). This result was also observed in another, independently derived HDAC1 KO clone (data not shown). Apparent lower abundance of WRN on EdU+ DNA paralleled its lower expression in HDAC1 KO RD cells. Interestingly, in the WRN null line recruitment of HDAC1 to new DNA was reduced (though not eliminated) compared to the non-isogenic WRN+ counterpart (Figure 4D). This may be explained in part by the lower S-phase fraction in WRN null cells compared to WRN+ cells, however, a WRN-specific effect cannot be ruled out.

Incubation of HDAC1 KO or control cells with HU for 6 (Figure 4G) or 12 hrs (data not shown) after the EdU pulse, did not lead to dramatic changes in WRN or HDAC2 association with EdU+ DNA. As expected, HU reduced both total nuclear (chromatin-associated) and nascent DNA-associated PCNA (Figure 4G). Overall, these results demonstrate that both HDAC1 and WRN can be found on newly replicated DNA, WRN association with DNA is not abolished in the absence of HDAC1, and vice versa. However, in at least one WRN null, patient-derived fibroblast line we see a reduction in HDAC1 associated with new DNA.

WRN co-immunoprecipitates with HDAC1 and HDAC2

To further investigate the interaction between WRN and HDAC1/2 we performed immunoprecipitations (IP) from nuclear extracts of GM639cc1 and WV1 fibroblasts or RD cells (Figure 5). With an HDAC1 antibody but not an isotype-matched HA control antibody, we were able to pull down most of the HDAC1 present in lysates together with 0.5-2% of WRN (Figure 5A, B). Precipitation of WRN by the HDAC1 antibody was reduced by 50% but not eliminated in HDAC1 KO cells (Figure 5C). This residual WRN may co-precipitate with the highly homologous HDAC2, which was also detectable in HDAC1 antibody pulldowns from HDAC1 KO cells (Figure 5C). To address this further, we generated mass cultures of RD cells newly transduced with CRISPR-Cas9 constructs expressing two gRNAs each against HDAC1 or HDAC2 to facilitate deletion of these genes. These cultures were puromycin-selected to eliminate untransduced cells, but we did not propagate individual clones, thus pre-empting positive selection of cells with in-frame HDAC mutations. Western blotting with HDAC1 and HDAC2 antibodies showed that expression of HDAC1 or HDAC2 was reduced by more than 80% in these cultures (Figure 5D), making them suitable for immunoprecipitation studies. Notably, as with RNAi against these HDACs (Figure 3A), HDAC1 expression increased substantially upon reduction of HDAC2 expression, suggesting a compensatory feedback. This response was reciprocal, albeit to a lesser degree, as HDAC2 went up only by 36% in cells with reduced HDAC1.

We next performed IPs with HDAC1 or HDAC2 antibodies from nuclear extracts of these HDAC1 KO or HDAC2 KO cell cultures. As before, precipitation of WRN was reduced by about one-half if HDAC1 level in the extract was reduced (Figure 5E). In contrast, when HDAC2 level was down, precipitation of WRN was substantially reduced (by 80%, Figure 5F). These results suggest that WRN may physically associate with HDAC2, and via HDAC2, with HDAC1.
Effect of HDAC1 on fork recovery is modified by an H141A mutation that reduces its deacetylase activity

As mentioned above, HDAC1 and 2 deacetylate histones incorporated de novo into nascent DNA, inviting a hypothesis that histone hyperacetylation at the fork may be responsible for the negative effect of HDAC1 deficiency on fork activity. However, our study (Figure 3A) and previous work (35,38) indicate that depletion of HDAC1 alone is not sufficient to change histone H4 acetylation levels in the cell due to likely redundancy with HDAC2 and HDAC3. We therefore asked whether deacetylase activity of HDAC1 is important for its cooperation with WRN at forks.

We introduced a H141A mutation into a flag-tagged HDAC1 (39), a change that was shown to inhibit deacetylase activity of the protein while preserving its associations with such proteins as RBBP4 and mSin3A (40). The defect in the HDAC1 H141A deacetylase activity was confirmed in vitro using α-Flag antibody immunoprecipitates of the wild type and mutant proteins transiently expressed in HDAC1 KO cells (Figure 6A). The genes were subsequently cloned into pLenti-EFS-T2A-emGFP vector and transduced into HDAC1 KO RD clones. HDAC1 expression in GFP+ cells was verified by Western blotting. No increase in whole cell levels of H4K12ac was observed, as before (compare Figure 6C and Figure 3A). Notably, at least two HDAC1 KO clones downregulated expression of more than one isolate of HDAC1 H141A transgene over time, compared to the wild type HDAC1 (data not shown and Figure 6D), suggesting a possible toxicity of the mutant protein. We used siRNA (Figure 6B, D) in order to rapidly deplete WRN in these cells within a 2-week window in which the levels of at least some HDAC1 H141A isolates were comparable to the wild type protein (see Materials and Methods for more detail). In separate experiments, WRN was also depleted in parental HDAC1 KO and HDAC1+ control cells.

All cell lines showed only minor differences in cell cycle distribution and proliferation index on the day of maRTA assays (data not shown). Using our labelling and HU treatment protocol (Figure 7A), we found that WRN depletion had only a minor effect on fork reactivation in HDAC1+ cells and no effect in two untransduced HDAC1 KO clones (data not shown). Also, WRN depletion did not reduce fraction of reactivated forks in HDAC1 KO cells transduced with wild type HDAC1 versus HDAC1 H141A or empty vector (Figure 7B). However, in all cases tested, WRN depletion elicited its phenotype of slowed fork progression, and this phenotype was exacerbated when combined with HDAC1 deficiency. Moreover, the HDAC1 H141A mutant showed a phenotype distinct from and more severe than HDAC1 KO.

In particular (Figure 7C), depletion of WRN reduced fork progression without HU in vector control compared to HDAC1-expressing cells. Also, WRN depletion slowed fork progression after HU in vector control and HDAC1 H141A but not in HDAC1 WT-expressing HDAC1 KO cells. Further insight was gained by analysing consistency of progression of each individual ongoing fork by plotting ratios of lengths of its 1st and 2nd label segments. In untreated cells, only the HDAC1 H141A mutant demonstrated excessive shortening of 2nd label segments versus 1st label segments (Figure 7C, D), suggesting premature fork termination. Furthermore, in HU-treated cells HDAC1 H141A mutant showed discordant impact on fork progression before/during HU (1st label) versus after HU (2nd label), which was significantly exacerbated by WRN depletion (Figure 7E). Overall, these findings indicate that HDAC1 H141A deacetylase activity mutant displays a replication phenotype, and that WRN depleted, HDAC1 H141A-expressing cells show an additive defect in fork progression upon HU treatment.

HDACs 1-3 inhibition and histone H4 hyperacetylation at forks do not mimic the effect of HDAC1 deletion on fork recovery

A complementary approach to address the impact of hyperacetylated histones on replication fork activity is to chemically inhibit more than one HDAC in order to achieve hyperacetylation and assess its effect on forks. The small molecule CI-994 inhibits HDACs 1, 2, and 3 (41), and we demonstrated its dose-dependent effect on whole cell levels of HDAC1 and 2 substrate, Lysine 12-acetylated histone H4 (H4K12ac) as a readout (35,36) (Figure 3A). We next wanted to demonstrate effect of CI-994 on fork-associated H4K12ac, and employed iPOND analysis of
proteins associated with nascent EdU-labeled DNA captured by formaldehyde crosslinking prior to cell lysis and precipitation of EdU+ DNA (31).

Protein levels at forks were quantified relative to the amounts of EdU in input samples (Figure 8A-C). As seen previously (36), HU arrest resulted in reduction of H4K12ac at forks compared to no-HU controls (Figure 8A, B). Addition of CI-994 increased the level of H4K12ac globally and at ongoing and HU-stalled forks (Figure 8A, B). Importantly, both in control and WRN-depleted cells, CI-994 treatment led to a comparable, approximately 4-fold increase in H4K12ac levels at forks in HU (Figure 8A, C), indicating effective inhibition of histone deacetylase activity by CI-994. At the same time, iPOND analysis of RD wild type and HDAC1 KO cells showed that H4K12ac levels on EdU+ DNA appeared virtually the same in replicating cells and were not higher in HDAC1 KO compared to WT cells in HU arrest (Figure 8D).

Having demonstrated significant increase in fork-associated acetylated histone H4 in CI-994-treated cells, we asked whether this affected the ability of forks to reactivate upon release from HU (Figure 8E-H). In contrast to the additive deficiency elicited when HDAC1 was co-depleted with WRN in GM639cc1 fibroblasts (Figure 3), CI-994 treatment of the same cells resulted only in a minor decrease in fork reactivation in HU-arrested WRN-depleted cells that was not statistically significant (Figure 8F). Lack of effect of CI-994 on fork reactivation was also reproduced in an epithelial cell line MCF10a (data not shown). CI-994 suppressed fork progression rates in fibroblasts (Figure 8G), however, forks in CI-994-treated, WRN-depleted fibroblasts did not show evidence of premature termination or excessive slowing, that we observed for HDAC1 H141A mutant (compare Figure 8H and 7D,E). As expected, in WRN-proficient cells CI-994 had virtually no effect on fork reactivation (data not shown). Lastly, consistent with these data, CI-994 did not synergize with HU in suppressing growth of WRN-depleted or control cells (data not shown).

These results demonstrate that inhibition of HDAC1-3 deacetylase activity does not affect replication fork reactivation and progression in the same way as observed in cells with a knock down or H141A mutation of HDAC1. Also, hyperacetylation at stalled replication forks, at least at the levels achieved with CI-994, does not impede fork reactivation.

WRN affects recruitment of RAD51 to stalled forks in a pathway parallel to HDAC1

Our previous studies (15) and findings by Su et al (42) suggest that WRN may affect fork recovery by functioning upstream of RAD51. HDAC1 (and 2) are thought to affect recruitment of RAD51 in DSB repair (43). Thus we asked whether WRN modulates association of RAD51 with stalled forks, and whether HDAC1 may affect RAD51 function independently of WRN.

Using iPOND, we demonstrated recruitment of RAD51 to HU-arrested forks, as well as accumulation of γH2AX and loss of PCNA from stalled forks (Figure 9A, also see Figure 8 for PCNA). We also determined that WRN-depleted GM639cc1 had approximately 40% less RAD51 associated with HU-arrested forks than isogenic control cells (Figure 9B).

To determine whether HDAC1 depletion required RAD51 to affect activity of stalled forks, we measured frequency of stalled fork reactivation in HDAC1- and RAD51-depleted cells (Figure 9C). Co-depletion of HDAC1 and RAD51 reduced fork reactivation after HU compared to RAD51-depleted cells with active HDAC1 (Figure 9D). In contrast, addition of CI-994 had no effect on fork reactivation in RAD51-depleted cells, though both CI-994 treatment and HDAC1 depletion reduced fork progression rates in these cells with or without HU treatment (data not shown). Thus, RAD51-depleted cells displayed the same additive phenotype with HDAC1 loss, as did WRN-depleted cells. As we had previously shown that combined loss of WRN and RAD51 was not additive with respect to fork reactivation compared to the individual effects of these genes (15), our new results suggest that HDAC1 may facilitate fork recovery after HU-induced stress via a pathway parallel to the WRN/RAD51-dependent pathway.

HDAC1-3 inhibition may modulate RAD51-mediated daughter strand truncation at stalled forks

Despite the fact that CI-994 treatment of fibroblasts did not reduce fork reactivation after HU, by iPOND we found that it reduced association of RAD51 with stalled forks upon HU-induced arrest (Figure 9E, F). Thus, we were interested to investigate what function of RAD51
at stalled forks other than reactivation may be affected by CI-994.

RAD51 is central to the pathway of protection of newly-synthesized DNA strands at HU-stalled forks uncovered by Schlacher et al (44,45), which notably does not affect the ability of forks to reactivate replication after HU. In this pathway, nascent strands can be truncated (likely, resected or digested by endonucleases) at HU-stalled forks, and this process is limited by loading of RAD51 onto DNA. Truncation of nascent DNA at stalled forks is upregulated if RAD51, BRCA1, or BRCA2 are defective (44,46). Thus we predicted that by inhibiting association of RAD51 with stalled forks, CI-994 may enhance truncation of newly-synthesized, daughter DNA strands.

Increased truncation of daughter DNA strands can be readily detected by maRTA and similar techniques as a shortening of replication tracks corresponding to forks that are stalled by HU (ibid.). In order to observe this, cells are pulsed with the 1st label followed by a high dose of HU that ensures complete stalling of forks and is added in the absence of label (e.g. for 6hrs); then cells are released from HU into the 2nd label, allowing forks to resume replication. Control cells are pulsed with 1st and 2nd labels separated by a 6-hr no-label gap (Figure 10A).

Without HU, a vast majority of 1st and 2nd label tracks are non-adjacent. With HU, a fraction of tracks have both 1st and 2nd label segments, representing forks that stalled in HU and then reactivated after HU removal; and a fraction of tracks has 1st label only, representing forks that were unable to reactivate (Figure 10A). Lengths of tracks of 1st label in HU-treated in untreated cells are compared, and in BRCA1 null cells, HU-treatment is known to elicit highly significant shortening of 1st label tracks. Specifically, both 1st label-only tracks (Figure 10B, compare lanes 1 and 2) and to an even greater extent, 1st label segments in two-label tracks (Figure 10B, compare lanes 1 and 3) were shorter in HU-treated cells compared to 1st label tracks of untreated cells. This demonstrates resection during HU arrest, and reveals that both the forks that were unable to reactivate (lane 2) and the ones able to do so (lane 3) have undergone resection. Moreover, treatment with CI-994 enhanced resection in both of these classes of forks (compare lanes 2-3 and 5-6 in Figure 10B).

We next used primary fibroblasts to determine if CI-994 can elicit a similar phenotype in a wild type background that is not sensitized to daughter strand truncation (Figure 10C). HU alone had no effect on 1st label-only tracks corresponding to inactivated forks (Figure 10C, compare lanes 1 and 2) and had only a slight, if any, effect on 1st label tracks of reactivated forks (lanes 1 and 3). However, in CI-994-treated primary fibroblasts we observed a highly significant HU-dependent shortening of 1st label tracks in both inactivated and reactivated forks (Figure 10C, lanes 4-6). This effect was in addition to the HU-independent shortening of tracks upon CI-994 treatment (Figure 10C, lanes 1 and 4), which was also observed in BRCA1 null cells (Figure 10B) and in the experiments described earlier (Figures 8, 9). Together, the results indicate that CI-994 can upregulate daughter strand truncation in HU. This is consistent with the fact that CI-994 reduces association of RAD51 with stalled forks, which would be expected to upset the balance between truncation and protection of nascent DNA strands. Moreover, the results suggest that forks that reactivate after HU actually undergo more, not less strand truncation during HU arrest than forks that fail to activate.

DISCUSSION

HDAC1 and 2 are class I histone deacetylases with roles in gene expression, cell signalling and homeostasis, as well as DNA repair and replication (33,34). HDAC1 and HDAC2 are close homologs, and frequently function as a homo- or heterodimer. Consistent with previous work (36), our study has demonstrated that HDAC1 and 2 are both present at the replication fork. We found that deficiency in HDAC1 alone can affect progression rate of replication forks. We also found that HDAC1 and HDAC2 facilitate survival of WRN-depleted human cells treated with the replication inhibitor hydroxyurea.

WRN facilitates normal fork progression as well as fork reactivation and progression after HU-induced replication arrest (12-15,47). Our results indicate that in fibroblasts and mammary epithelial cell lines, these phenotypes of WRN deficiency are exacerbated by depletion of HDAC1. We also established, in the embryonic rhabdiosarcoma cells line with a deletion of the endogenous HDAC1, that a deacetylase activity mutant HDAC1 H141A, combined with depletion...
of WRN, reduces fork progression after HU treatment more dramatically than HDAC1 null. In contrast, inhibiting HDAC deacetylase activity with CI-994, a small molecule inhibitor with the highest activity against HDACs 1-3 (41) does not affect fork reactivation and progression after HU. Moreover, unlike HDAC1 depletion (Figures 1-3), HDAC inhibition does not sensitize WRN-depleted fibroblasts to HU.

To explain the role of HDAC1 at HU-arrested forks we first turned to a known, prominent substrate of HDAC1/2 at the fork – hyperacetylated histones incorporated into newly replicated DNA. However, we observed no simple correlation between HDAC1 inactivation, histone H4 hyperacetylation, and fork reactivation after HU treatment. In fibroblasts and epithelial cells, additive defect of fork reactivation caused by co-depletion of HDAC1 and WRN was observed without any change in total level of H4K12ac; and total and fork-associated levels of H4K12ac were not higher in HDAC1 knockout or mutant clones of embryonal rhabdomyosarcoma cells. Conversely, chemical inhibition of HDACs 1-3 in WRN-depleted fibroblasts and epithelial cells had a modest or undetectable fork reactivation phenotype but led to a marked increase in H4K12ac. From these data, we conclude that, first, HDAC1 is redundant with HDAC2 and perhaps HDAC3 for histone H4 deacetylation at ongoing and stalled forks. Second, that cooperation of HDAC1 with WRN at the fork may involve histone or non-histone substrate(s) specific to HDAC1 and not HDAC2 or 3. Third, co-inhibition of HDACs 2 and 3 together with HDAC1, or the resulting hyperacetylation at the fork in and of itself, may counteract or modify the function of HDAC1. Further studies, including comparison of the effects of mutation or chemical inhibition of HDAC1 on its enzymatic and – possibly – nonenzymatic activities at forks, will be needed to fully understand the underlying mechanisms.

Association of WRN and HDAC1 with newly replicated DNA

Using native iPOND we found that HDAC1 is present at replication forks and remains on newly replicated DNA for a while after fork passage. HDAC2 behaves similarly, and associates with DNA regardless of whether or not HDAC1 is present. We also observed direct association of WRN with newly replicated DNA; and WRN, as HDACs, remains detectable after fork passage. This finding may explain why WRN was not identified as a fork-associated protein in proteomic studies that combined iPOND with mass spectrometry (48,49).

Our studies showed that a fraction of WRN co-immunoprecipitates with HDAC2 and HDAC1 (Figure 5), which would be consistent with the presence of the three proteins on nascent DNA. The functional significance and molecular details of this interaction require further investigation. WRN is detectable on DNA in the absence of HDAC1, and vice versa (Figure 4), which is not surprising, given that HDACs and WRN interact with numerous proteins, many of which can recruit them to DNA. Nevertheless, it is possible that deficiency in HDAC1 can result in reduced recruitment of co-factors associated with WRN, causing exacerbation of WRN-depleted replication fork phenotype induced by HU treatment.

In rhabdomyosarcoma cells association of WRN with forks was not substantially changed by addition of HU. This is consistent with our and others’ previous findings of WRN involvement both in HU-arrested and unperturbed DNA replication (9,15). Alternatively, this may reflect a unique response of these neoplastic cells to HU treatment, and in fact we observed multiple differences between RD cells and SV40-immortalized fibroblasts in normal and HU-stressed replication. On the other hand, iPOND-MS studies reported enrichment of WRN on HU-arrested or HU and ATR inhibitor-collapsed replication forks, i.e. forks that developed double strand breaks (48,49). Further dissection of cell type/cell fate-specific differences in fork metabolism and fork collapse versus protection responses will be required to understand the reasons for these discrepancies.

Lastly, we showed that in patient-derived WRN null fibroblasts less HDAC1 was bound to newly-replicated DNA compared to unrelated wild type fibroblasts. This may be a result of a long-term adaptation to growth without WRN rather than being a direct effect of WRN loss. Nevertheless, if confirmed in other cell lines, this phenotype may provide insight into the effects of WRN and HDAC1 on maintenance of heterochromatin (50,51).

Roles of WRN and RAD51 in fork reactivation
We showed that HDAC1 depletion is additive not only with WRN but also with RAD51 depletion in suppressing replication fork reactivation after HU treatment (Figure 9). We and others have previously shown that RAD51, as WRN, is important for fork reactivation after HU (15,52). Co-depleting WRN and RAD51 was epistatic for fork reactivation, suggesting that the two proteins act in the same functional pathway (15). Consistent with these results, our new data demonstrate that WRN depletion reduces the association of RAD51 with stalled forks. This reduction could reflect decreased recruitment to or increased loss of RAD51 from forks, and is consistent with the phenotype recently reported in camptothecin-treated cells where chromatin-bound RAD51 was reduced in the absence of WRN (42). However, other recent findings suggest that RAD51 may function upstream of WRN (47,53) and provide the WRN/DNA2 complex with a regressed fork as a substrate (54).

A model that can reconcile these observations is that WRN and RAD51 function in a feedback loop: regression of a fork by RAD51 generates a substrate for WRN/DNA2, which generate single-stranded (ss) DNA; binding of RAD51 to this ssDNA limits WRN/DNA2 activity. Deacetylase activity of HDACs 1 and 2 and RAD51-dependent pathway of fork protection

De-novo synthesized histones H3 and H4 that are incorporated into nascent chromatin concurrent with DNA replication are hyperacetylated, and these acetyl groups are removed as chromatin matures. Thus, replication forks typically operate in a hyperacetylated chromatin environment. Interestingly, deacetylation of new histones, at least on the lysine residues K5 and K12 of histone H4, proceeds regardless of whether replication forks are progressing or are stalled by HU ((36) and Figure 6). Therefore, stalled forks become increasingly hypoacetylated as a function of HU arrest time. We were interested to determine whether this affects the ability of forks to reactivate after HU. We found that preventing histone H4 deacetylation at stalled forks by inhibiting HDACs 1-3 with a small molecule CI-994 did not affect fork reactivation (Figure 8, 9), but reduced the association of RAD51 with stalled forks (Figure 9) and enhanced truncation of newly-synthesized daughter DNA strands (Figure 10). Importantly, this daughter strand truncation does not prevent fork reactivation, in agreement with previous findings (44,45). Taken together, the data suggest that while histone deacetylase activity is not required for replication fork reactivation, it may be important for modulating RAD51 activity to maintain the integrity of nascent strands of stalled forks.

We propose that, under normal conditions, hyperacetylated chromatin around replication forks antagonizes the binding of RAD51 and other proteins that participate in DNA strand processing to facilitate fork stability (4). Loss of acetylation associated with the prolonged fork stalling creates a more permissive environment for fork remodelling. Overriding the loss of acetylation at a stalled fork may therefore disrupt the homeostasis of fork remodelling, leading to increased strand truncation.

In conclusion, our findings provide new insights into the role of epigenetic mechanisms in preserving genomic stability. Further studies will be required to delineate the molecular mechanisms by which HDAC1 contributes to the maintenance of non-histone and histone architecture at stalled replication forks to support efficient and faithful DNA replication and cell viability.

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Conflict of interest
The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions
KK performed and analysed the experiments shown in Figures 2 through 7. MP designed CRISPR/Cas9 constructs for deletion of HDAC1 and HDAC2, generated hdac1Δ and hdac2Δ cell lines, and cloned pLenti-EFS-T2A-emGFP constructs for HDAC1 expression. PL provided technical assistance and contributed to the data analyses for Figure 10. EC supervised MP, conceived some of the experiments, performed histone deacetylase assays, and co-wrote the manuscript. RM Jr. conceived and designed the siRNA library, conceived the study and critically revised the manuscript. JS conceived and coordinated the study, designed, performed (for Figures 4, 5-7), and analysed experiments, and co-wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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The abbreviations used: HU, hydroxyurea, iPOND, immunoprecipitation of nascent DNA, niPOND, native immunoprecipitation of nacent DNA, maRTA, microfluidic-assisted replication track analysis, FACS, fluorescence activated cell sorting.

**FIGURE LEGENDS**

**Figure 1.** A High throughput screen for genes that sensitize WRN-depleted cells to HU. A) A scatter plot of growth ratio values with and without HU (HU/no HU) for WRN-depleted (Y axis) versus control, mock-depleted (X axis) cells. Note that X axis begins at the value of 0.4. Shown are only the genes for which: i) the growth difference between HU and no HU conditions in WRN-depleted cells is statistically significant (p<0.005), ii) the growth ratio values (HU/no HU) are at 0.5 or lower for WRN-depleted cells and at 0.5 or higher for controls cells. B) Normalized mean growth values (as percent of growth of mock-transfected cells) are shown for a subset of individual genes for WRN-depleted and control cells. UNI is universal (non-specific) control siRNA. Error bars are standard deviations.

**Figure 2.** Depletion of HDAC1 or HDAC2 in WRN-depleted cells exacerbates growth-suppressive effect of HU. A) Population doubling levels of WRN-depleted or control (with scrambled shRNA) GM639cc1 SV40-transformed fibroblasts that were transfected with non-specific siRNA (NS), HDAC1,
or HDAC2 siRNAs (day 0), plated in triplicates into multiwell dishes (day 1) with or without treatment with 2mM HU for 6 hrs on day 2, and allowed to grow for another 3 days. Cell counts in wells were averaged, and population doubling levels (PDLs) were determined using a formula ln(average count on day_{n+1}/average count on day_{n})/0.693. PDLs were plotted to derive linear trendlines. B-C) Western blots showing levels of HDAC1 and HDAC2 in scrambled shRNA control and WRN-depleted GM639cc1 fibroblasts transfected with non-specific (NS) siRNA or siRNAs against HDAC1 or 2. HDAC1 or 2 levels are expressed as percentages of levels seen in cells transfected with NS siRNA. PCNA is a loading control.

**Figure 3.** Co-depletion of WRN and HDAC1 additively reduces efficiency of replication fork reactivation after a transient arrest by HU. A) A Western blot comparing levels of HDAC1, HDAC2, and H4K12ac in control GM639cc1 fibroblasts transfected with NS, HDAC1 or HDAC2 siRNAs, or incubated with indicated doses of CI-994 overnight prior to cell harvest. B) A labeling scheme for maRTA includes two consecutive 30 min pulses of thymidine analogs (e.g. EdU and IdU), with or without addition of 2mM HU for 6hrs at the end of the pulse of the first label. C) Percent of ongoing forks was measured as a fraction of tracks labeled with two consecutive labels among all tracks containing the first label. Between 400 and 900 tracks were analyzed in each sample. Relative fork reactivation was determined as percent of ongoing forks in HU-treated samples normalized to percent of ongoing forks in untreated samples for each cell line. The bar graph shows average values derived from two (scrambled shRNA control) or three (WRN shRNA) experimental replicates. Error bars are standard deviation and significance was determined using a two-sided t-test (p<0.014), i.e. one asterisk indicates p<0.05. D) Lengths of 1st label (white) and 2nd label (gray) segments in two-label tracks of ongoing forks were box-plotted to evaluate fork progression. Shown is a plot of a representative set of samples with numbers of analyzed tracks (n) shown below the graph. Statistical significance was determined in Wilcoxon tests, and significant differences are marked on the graph: **, p≤5E-03, ****, p≤5E-05, etc.; all p values of the orders of magnitude at or below 5E-6 are labeled as *****. E) Ratios of 2nd to 1st label segment lengths in each ongoing fork were box-plotted to evaluate consistency of fork progression. The data are for untreated samples of the experiment shown in (D). No statistically significant differences were detected. F) Western blots of depletion of WRN and HDAC1 in MCF10a spontaneously immortalized mammary epithelial cells. G) Relative fork reactivation in MCF10a cells introduced in (F) after 6hrs of 2mM HU. Average values from two experimental replicates are plotted and error bars are standard deviations.

**Figure 4.** HDAC1 and WRN bind nascent DNA. A) A schematic of Immuno-Precipitation of Nascent DNA (iPOND). B) Western blot verification of loss of expression of HDAC1 in the HDAC1 knockout clone (#17) of RD cells. Nucleolin (NCL) and WRN expression are shown for reference. C) A Western blot of native iPOND (niPOND) precipitates from GM639cc1 cells labeled by EdU for 30 min, harvested, and subjected to ClickIT reaction with and without biotin-azide prior to lysis and incubation with streptavidin beads. D) Western blots of niPOND performed with GM639cc1 and WV1, WRN-deficient fibroblasts. WV1 cells were labeled with EdU for 30 or 90 min. For EdU, serial 2 or 3-fold dilutions from 0.001% to 0.015% of lysates were loaded on a dot blot and visualized with HRP-conjugated anti-biotin antibody. EdU panels show a representative dilution within a linear range of EdU signal. E) A Western blot of niPOND performed with GM639cc1 cells labeled with EdU for 30 min and harvested immediately or chased for 2hrs before harvest. F) A Western blot of niPOND performed with RD cells with intact (WT) or knocked-out (HDAC1 KO) HDAC1 gene. Cells were harvested immediately after an EdU pulse or after a 2.5hr chase. G) A Western blot of niPONDs performed with RD cells labeled with EdU for 30 min or for 15 min followed by a 6 hr arrest with 2mM HU in the presence of EdU. Panels framed in separate boxes come from independent experiments since simultaneous detection of HDAC1 and HDAC2 on one Western blot is not optimal due to their close molecular weights. More RD HDAC1 KO than WT cells were used in panels F, G, order to compensate for their lower percentage of replicating cells. Residual bands larger and smaller than expected for HDAC1 seen in the HDAC1 KO, no-HU lane may be non-specific or represent crossreactivity with HDAC2.
Figure 5. Co-immunoprecipitation of HDAC1, HDAC2, and WRN. A) A Western blot showing levels of WRN and HDAC1 in GM639cc1 cell nuclear extracts prior (input) and after (s/n) IP, and in immunoprecipitates of an α-HDAC1 or an isotype-matching α-HA antibody. B) A Western blot showing levels of WRN and HDAC1 in nuclear extracts and α-HDAC1 immunoprecipitates from GM639cc1 and WV1 (WRN-deficient) cells. C) A Western blot showing WRN and HDAC1 levels in nuclear extracts and α-HDAC1 immunoprecipitates from control (WT) and clone #17 HDAC1 knockout (HDAC1 KO) RD cells. D) A Western blot of WRN, HDAC1, HDAC2, and nucleolin (NCL, loading control) in whole-cell lysates of mass cultures of RD cells after CRISPR-Cas9-mediated knockout of HDAC1 (HDAC1 KO) or HDAC2 (HDAC2 KO). Wild type (WT) cells express Cas9 only. Expression of HDAC1 or HDAC2 is quantified as percent of the levels seen in WT control. E) A Western blot of nuclear extracts and α-HDAC1 immunoprecipitates from control (WT), HDAC1, or HDAC2 knockout RD cells introduced in (D). F) A Western blot of nuclear extracts and α-HDAC2 immunoprecipitates from control (WT) or HDAC2 knockout RD cells introduced in (D). In (E) and (F) WRN levels in IPs are normalized to inputs and shown relative to these levels in WT controls.

Figure 6. Expression and activity of H141A mutant of HDAC1. A) A bar graph comparing deacetylase activity of the wild type and H141A mutant HDAC1-flag proteins expressed from plasmids transiently transfected into HDAC1 KO RD cells (clone #17), and immunoprecipitated with α-FLAG antibody prior to the assay. Negative control is untransfected HDAC1 KO RD cells. B) A work flow diagram for generation of HDAC1 transgene-expressing, WRN-depleted HDAC1 KO cells. Shown is the fastest timeline available. C) A Western blot measuring expression of wild type and H141A mutant HDAC1-flag within 10 days of transduction with pLenti-EFS-T2A-emGFP constructs in HDAC1 KO RD cells (clone #4). D) A Western blot measuring expression of WRN and of wild type and two isolates of H141A mutant HDAC1-flag, after flow-sorting to purify HDAC1-expressing cells, their outgrowth, and siRNA transfection.

Figure 7. H141A mutant of HDAC1 shows a distinct phenotype of replication fork progression. A) A labeling scheme for maRTA. HU was used at 4mM, and 1st and 2nd labels were CldU and IdU, respectively. B) Percentages of ongoing forks in WRN-depleted untreated and HU-treated (“HU”) HDAC1 KO RD cells (clone #4) expressing the indicated HDAC1 transgenes or empty vector (vec). The bar graph shows average values derived from two experimental replicates and error bars are standard deviations. C) Lengths of 1st label (white) and 2nd label (gray) segments in two-label tracks of ongoing forks were box-plotted to evaluate fork progression. The length distribution data were derived from two experimental replicates. Statistical significance was calculated in Wilcoxon tests, and p value designations are as in Figure 3D, E) Ratios of 2nd to 1st label segment lengths in each ongoing fork in untreated (D) and HU-treated (E) cells were box-plotted to evaluate consistency of fork progression. The data used are from the set shown in (C). In C-E, the statistically significant differences are marked, with asterisks standing for p values. Designationas are as in Figure 3, and all p values of the orders of magnitude at or below 5E-06 are labeled as *****). Numbers of tracks analyzed are shown below the graph.

Figure 8. HDAC deacetylase activity inhibition by CI-994 increases the level of Lysine 12-acetylated histone H4 at ongoing and stalled forks but does not affect fork reactivation after HU. A) GM639cc1 fibroblasts were maintained overnight with and without 3µM CI-994, then labeled with EdU as indicated, with or without 2mM HU. Where indicated, CI-994 was present throughout the experiment. Regular iPOND (with formaldehyde crosslinking) was performed, and samples were analysed by Western blotting. Input lanes contain 2.5% of cell lysates. EdU panels show representative levels in inputs derived as described in Figure 4. B, C) Quantitations of two (B), four (C, control cells), and two (C, WRN-depleted cells) independent iPOND experiments similar to the one shown in (A). Levels of H4K12ac in pulldowns were normalized to the EdU levels in inputs (EdU in., averaged over at least three values falling within a linear range of EdU signals in serial dilutions on a dot blot). Normalized
level of H4K12ac in untreated controls was set as baseline and the rest of the values were expressed relative to it. Error bars are standard deviations. D) Replication fork-associated Lysine 12-acetylated histone H4 is not elevated in HDAC1 knockout RD cells. A Western blot of iPOND samples treated with 2mM HU as in (A). Input lanes contain 2% of lysates. E-H) maRTA analysis of WRN-depleted GM639cc1 fibroblasts labeled with thymidine analogs (1st label, EdU, 2nd label, IdU) and treated with 2mM HU for 6 hrs in the presence or absence of CI-994. CI-994 treatment was as in (A-C) above. Plotted values are derived as described for Figure 3C-E. In (F), two independent experiments were averaged and error bars are standard deviations. Datasets from each experiment contained 400-900 track measurements per sample. P value in a one-sided t-test equaled 0.077. The (G) panel shows distributions of lengths of 1st (white) and 2nd (gray) label segments in ongoing forks derived from two experimental replicates. Numbers of tracks analyzed are shown below the graph and significance was determined in Wilcoxon tests. **, p≤5E-03. H) Ratios of 2nd to 1st label segment lengths in each ongoing fork derived from an additional experiment with a side-by-side comparison of HDAC1-depleted vs. negative control and CI-994-treated vs. untreated cells. All samples are WRN-depleted and were treated with HU. Data were box-plotted to evaluate consistency of fork progression.

**Figure 9.** RAD51 recruitment to HU-stalled forks is reduced by depletion of WRN or inhibition of histone deacetylase activity of HDACs 1, 2, and 3. A) iPOND measurement of levels of RAD51, PCNA, and γH2AX in mock-depleted and WRN-depleted GM639cc1 fibroblasts pulse-labeled with EdU and treated with 2mM HU as indicated. B) A quantitation of two independent experiments performed as in (A). Levels of RAD51 in pulldowns were normalized to EdU levels in input samples (EdU in.) as in Figure 8B. C. Normalized RAD51 levels in HU-treated control cells were set as baseline and the rest of the values were expressed relative to it. C-D) maRTA analysis of RAD51-depleted GM639cc1 fibroblasts labeled with EdU and IdU as 1st and 2nd labels, respectively, and treated with 2mM HU as indicated. C) maRTA labeling scheme and a Western blot of RAD51 depletion in GM639cc1 cells. CHK1 is internal control. D) A bar graph of relative fork reactivation, derived as in Figure 3C from two replicate experiments with 500-800 track measurements per sample in each experiment. Significance was determined in a one-tailed t-test (p=0.035). E) iPOND measurement of RAD51 and H4K12ac recruited to replication forks in mock-depleted GM639cc1 fibroblasts treated with 3μM CI-994 overnight prior to and during the experiment. 2mM HU addition and EdU pulse-labeling are as in (A). F) A quantitation of two independent iPOND experiments performed as in (E). Quantitation and plotting are as in (B), except RAD51 levels in pulldowns were normalized to EdU levels in pulldowns (EdU pd., lanes 1 and 2) or in inputs (EdU in., lanes 3 and 4), for comparison.

**Figure 10.** CI-994 treatment enhances nascent strand truncation. A) A labeling scheme for maRTA analysis of nascent strand truncation and examples of tracks seen in UW289.B1 BRCA1-deficient ovarian carcinoma cells. Pulses of 1st label (IdU) and 2nd label (EdU) are separated by a 6-hr interval with no label and either with or without 5mM HU. Nascent strand truncation is revealed as shortening of 1st label tracks upon incubation with HU. B) A quantitation of 1st label track lengths from two experimental replicas performed with UW289.B1 cells as depicted in (A). CI-994 was added at 3μM overnight prior to and during the experiments. Significance was measured in Wilcoxon tests, and p value designations are as in Figures 3, 7, 8. Box fill stands for: white: 1st label tracks in untreated cells, horizontal stripes: 1st label-only tracks (inactivated forks) and vertical stripes: 1st label segments of two-label tracks (reactivated forks). Note that without HU (white boxes), absolute majority of tracks are either 1st label- or 2nd label-only, although they can belong to either ongoing or inactivated forks. C) A quantitation of 1st label track lengths from two experimental replicas performed with primary human fibroblasts as depicted in (A). Designations and statistics are as in (B). CI-994 was added at 6 μM overnight prior to and during the experiments.
Figure 1

A

Fold change HU/no HU in WRN-depleted cells (0.5 or lower)

Fold change HU/no HU in control cells (0.5 or higher)

B

|            | control | WRN-depleted |
|------------|---------|--------------|
|            | no HU   | no HU        |
| Mock       | % growth| % growth     |
| UNI        | % growth| % growth     |
| HDAC1      | % growth| % growth     |
| HDAC2      | % growth| % growth     |
| ATR        | % growth| % growth     |
| CHEK1      | % growth| % growth     |
Figure 2

A Transfect (day 0) → Plate (day 1) → Treat with HU (day 2) → Measure population growth (days 3-6)

control

WRN-depleted

\[ \begin{align*}
\text{siNS} & \quad 3.5 \\
\text{siNS + HU} & \quad 3.0 \\
\text{siHDAC1-6} & \quad 2.5 \\
\text{siHDAC1-6 + HU} & \quad 2.0 \\
\end{align*} \]

Population doubling levels

Hours post-transfection

B

\begin{tabular}{c|c|c|c|c|c|c|c}
 & control & WRN-dep. & control & WRN-dep. & control & WRN-dep. \\
\hline
\text{siNS} & 100 & 100 & 100 & 100 & 100 & n/a \\
\text{siHDAC1-6} & 20 & 57 & 42 & 92 & 31 & 288 \\
\text{siHDAC2-1} & 177 & 100 & 100 & 100 & 100 & n/a \\
\end{tabular}

C

\begin{tabular}{c|c|c|c|c|c|c}
 & control & WRN-dep. & control & WRN-dep. & control & WRN-dep. \\
\hline
\text{WRN} & 100 & 57 & 100 & 92 & 100 & n/a \\
\text{HDAC1} & 20 & 57 & 42 & 92 & 31 & 288 \\
\text{PCNA} & 177 & 100 & 100 & 100 & 100 & n/a \\
\end{tabular}
Figure 3

A

B

C

D

E

F

G

HDAC1 level (%): 100 28 169 151 141
HDAC2 level (%): 100 99 87 100 154 99

C

Relative fork reactivation (% of no-HU)

siRNA: NS HDAC1-6 NS HDAC1-6

control WRN-depleted

1st label 2nd label

1st label (6hr) 2nd label

1st label +HU 2nd label after HU

D

Track lengths in ongoing forks (µm)

siRNA: NS HU HDAC1-6 HU NS HU HU

control WRN-depleted WRN-depleted

n: 269 249 280 280 364 158 398 79

E

Ratio of 2nd to 1st label segment lengths

siRNA: NS HDAC1-6 NS HDAC1-6

control WRN-depleted

MCF10a:

shRNA: Scr WRN

siRNA: NS HDAC1-6

NCL

WRN

HDAC1

G

Relative fork reactivation (% of no-HU)

siRNA: NS HDAC1-6

WRN-depleted
Figure 5

A

| Sample      | WRN | HDAC1 | IgG |
|-------------|-----|-------|-----|
| input 2%    |     |       |     |
| s/n αHDAC1  |     |       |     |
| s/h αHA     |     |       |     |
| IP αHDAC1   |     |       |     |
| IP αHA      |     |       |     |
| GM639cc1    |     |       |     |

B

| Sample      | WRN | HDAC1 | IgG |
|-------------|-----|-------|-----|
| input 2%    |     |       |     |
| IP αHDAC1   |     |       |     |
| GM639cc1    |     |       |     |
| WV1         |     |       |     |
| GM639cc1    |     |       |     |
| WV1         |     |       |     |

C

| Sample      | WRN | HDAC1 | IgG |
|-------------|-----|-------|-----|
| WT          |     |       |     |
| HDAC1 KO    |     |       |     |
| WT          |     |       |     |
| HDAC1 KO    |     |       |     |
| input 2%    |     |       |     |
| IP αHDAC1   |     |       |     |

D

| Sample      | WRN | HDAC1 | NCL | HDAC2 |
|-------------|-----|-------|-----|-------|
| control    |     |       |     |       |
| HDAC1 KO    |     |       |     |       |
| HDAC2 KO    |     |       |     |       |
| HDAC1 (%)   | 100 | 17    | 209 |
| HDAC2 (%)   | 100 | 136   | 12  |

E

| Sample      | WRN | HDAC1 | IgG |
|-------------|-----|-------|-----|
| WT          |     |       |     |
| HDAC1 KO    |     |       |     |
| HDAC2 KO    |     |       |     |
| WT          |     |       |     |
| HDAC1 KO    |     |       |     |
| HDAC2 KO    |     |       |     |
| input 2%    |     |       |     |
| IP αHDAC1   |     |       |     |

F

| Sample      | WRN | HDAC1 | HDAC2 |
|-------------|-----|-------|-------|
| WT          |     |       |       |
| HDAC2 KO    |     |       |       |
| WT          |     |       |       |
| HDAC2 KO    |     |       |       |
| input 2%    |     |       |       |
| IP αHDAC2   |     |       |       |

1x 0.6x 0.8x WRN ip/in
Figure 6

**A**

![Bar chart](http://www.jbc.org/)

- Normalized Activity
- HDAC1 WT
- HDAC1 H141A
- Neg. control

**B**

**Day 0:**
transduce cells
with lentivirally expressed
HDAC1 constructs or
empty vector

**Day 5:**
purify by FACS and plate GFP+ cells (40-70% of the starting population)

**Day 12:**
transfect cells with WRN siRNA or negative control siRNA

**Day 14:**
assay cells by maRTA

**C**

![Western Blot](http://www.jbc.org/)

- HDAC1-flag
- HDAC1
- HDAC2
- PCNA
- H4K12ac

**D**

|          | vec | HDAC1 WT | HDAC1 H141A #6 | HDAC1 H141A #7 |
|----------|-----|----------|-----------------|-----------------|
| WRN      |     |          |                 |                 |
| HDAC1-flag|    |          |                 |                 |
| PCNA     |     |          |                 |                 |
Figure 8

A

Cl-994: - - + +
HU: - - - +
PCNA
H4K12ac
EdU

B

H4K12ac p.d./EdU in.
relative units

CI-994:
HU:

C

H4K12ac p.d./EdU in.
relative units

CI-994:
HU:

D

WT HDAC1 KO
HU: - - + +

E

1st label 2nd label
30' 30'
+HU (6hr)

F

Relative fork reactivation (% of no HU)

G

Track lengths in ongoing forks (μm)

H

Ratio of 2nd to 1st label segment lengths
after HU:
siNS siHDAC1-6 CI-994
WRN-depleted

n: 524 526 352 244
n: 123 191 225 164
Class I Histone Deacetylase HDAC1 and WRN RECQ Helicase Contribute Additively to Protect Replication Forks upon Hydroxyurea-induced Arrest
Keffy Kehrli, Michael Phelps, Pavlo Lazarchuk, Eleanor Chen, Raymond Monnat, Jr. and Julia M. Sidorova

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