

HUMAN DNA HELICASE B IN REPLICATION FORK SURVEILLANCE
AND REPLICATION STRESS RECOVERY

By

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To my dear family,

Cenap, Neşe and Sanem Güler

&

Mert Karakaş

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LIST OF ABBREVIATIONS

9-1-1	Rad9-Rad1-Hus1
Aph	Aphidicolin
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related protein
ATRIP	ATR-interacting protein
ATP	Adenosine triphosphate
β ME	β Mercaptoethanol
BLM	Bloom's syndrome helicase
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumine
Cdk	cyclin-dependent kinase
ChIP	Chromatin Immunoprecipitation
Chk1	Checkpoint protein 1
Chk2	Checkpoint protein 2
Chr	Chromatin
CPT	Camptothecin
Cyto	Cytosol
DBD	DNA binding domain
DMEM	Dulbecco-modified Eagle serum
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase

dNTP	deoxyribonucleoside triphosphate
ds-DNA	Double-stranded DNA
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
Exo1	Exonuclease 1
FBS	Fetal bovine serum
GFP	Green fluorescent protein
GST	Glutathione-S-transferase
HDHB	Human DNA helicase B
HR	Homologous recombination
HU	Hydroxyurea
IP	Immunoprecipitation
IPTG	isopropyl thiogalactoside
ITC	Isothermal calorimetry
LB	Luria-Broth
MCM	Minichromosome maintenance
MMR	Mismatch repair
MNase	Micrococcal nuclease
MRN	Mre11/Rad50/Nbs1
MutB	Walker B mutant HDHB
NMR	Nuclear magnetic resonance
ORC	Origin recognition complex

PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PIKK	Phosphoinositide 3-kinase related kinase
PMSF	Phenylmethylsulfonyl fluoride
Pol-prim	Polymerase α -primase
pre-RC	Pre-replication complex
PSLD	Phosphorylation-dependent subcellular localization domain
RFC	Replication factor C
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RPA	Replication protein A
SCE	Sister chromatid exchange
SDS	Sodium dodecyl sulfate
shRNA	Small hairpin RNA
siRNA	Small interfering RNA
SSA	Single-strand annealing
SSB	Single-stranded DNA binding protein
ssDNA	Single-stranded DNA
TBE	Tris borate EDTA
TopBP1	Topoisomerase II binding protein 1
UV	Ultraviolet
WCE	Whole cell extract

WRN Werner's syndrome helicase

CHAPTER I

INTRODUCTION

Overview

Proliferating cells need to ensure correct and complete duplication of their genome. Perturbation of this process is detrimental to multicellular organisms, as evidenced by diseases linked to DNA replication and repair defects such as cancer. A detailed understanding of this process is important to combat cancer and can potentially facilitate novel therapeutic approaches.

Faithful genome duplication is a highly regulated process that requires coordination between DNA replication and repair events. A dynamic protein machine called the replisome is central to replication with high fidelity. Additional proteins get recruited when replication forks encounter impediments in the template in the form of tightly bound proteins or DNA lesions. Altogether, this highly dynamic replication machinery enables the cell to deal with various kinds of replication stress to subsequently preserve genomic integrity. However, our understanding of the machinery responsible for genomic integrity is far from complete.

Human DNA helicase B (HDHB) is a protein component of the DNA replication and repair machinery. Well conserved among vertebrates, HDHB shares sequence similarity to homologous recombination proteins, RecD and T4 dda, and it is implicated in chromosomal DNA replication in both mouse and human. HDHB and its mouse homolog display primosome activity but the biological significance of this activity

remains to be determined. Previous work from the Fanning lab demonstrated that treatment with DNA damaging agents leads to HDHB accumulation on chromatin, implicating HDHB in DNA damage response. Furthermore, HDHB silencing leads to decreased homologous recombination. Nevertheless, a detailed understanding of HDHB involvement in DNA damage response is not available. My Ph.D. thesis research aimed to gain mechanistic insight into HDHB recruitment to damage sites and HDHB function in DNA damage response.

DNA replication

DNA replication is one of the biggest challenges for genomic integrity as the events of replication intrinsically render DNA vulnerable to damage. Therefore, cells employ extreme measures to ensure exact duplication of the genome. Among these measures is tight control of replication initiation.

An important component of replication control is the spatial-temporal regulation of replisome assembly. Replisomes are assembled at specific sites termed origins of replication, where eukaryotic replication initiates. Central to spatial regulation of replication initiation, Origin Recognition Complex (ORC) binds specifically to replication origins and serves as a platform to recruit pre-replication complex (pre-RC) components (Figure 1) [1-3]. ORC-dependent recruitment of pre-RC components Cdc6 and Cdt1 is required for the subsequent loading of Mcm2-7, the replicative helicase [4-6]. The replication origins that are bound by the pre-RC are competent for replication initiation and therefore are referred as 'licensed origins'. However, MCM2-7 is not competent for DNA unwinding in the context of the pre-RC. Origin DNA unwinding

requires recruitment of another set of proteins as part of the conversion of pre-RC to pre-initiation complex (pre-IC), providing an additional regulatory mechanism at the level of enzymatic activity besides sequential protein recruitment [7].

Some of the steps in pre-IC formation are unclear and may differ in different organisms but some underlying principles are conserved [1, 8-10]. The requirement for Cdc7 and Cdk2 kinase activities and subsequent recruitment of Cdc45 and GINS for the formation of the active helicase complex appears to be universal. Additional factors that function at this step include Mcm10, Ctf4, Dpb11, Sld2 and Sld3 in yeast [11-16] and Mcm10, And1, TopBP1, RecQ4, Treslin/ticcr and GEMC1 in higher eukaryotes [17-25].

Research in *Schizosaccharomyces pombe* demonstrated that Cdk phosphorylation of Sld2 and Sld3 is required for their interaction with Dpb11 [15, 16]. Disruption of the interactions between Dpb11 and both Sld2 and Sld3 inhibits replication initiation. The function of CDK-dependent phosphorylation is conserved in humans in the context of TopBP1 (human Dpb11 homolog) and Treslin (human Sld3 homolog) [26, 27], but appears to be dispensable for RecQ4 (human Sld2 homolog) association with TopBP1 [17, 18].

Sld3 association with Dpb11, similar to Treslin association with TopBP1, brings Cdc45 to replication origins [13, 27]. Sld2, on the other hand, recruits GINS and polymerase ϵ to origins via its interaction with Dpb11 [28]. Interestingly, GINS loading onto replication origins does not require RecQ4 [17]. Stable association of MCM2-7 with Cdc45 and GINS, facilitated by MCM10 and RecQ4 [20], leads to formation of the active helicase complex termed **Cdc45/MCM2-7/GINS** (CMG) [4, 29].

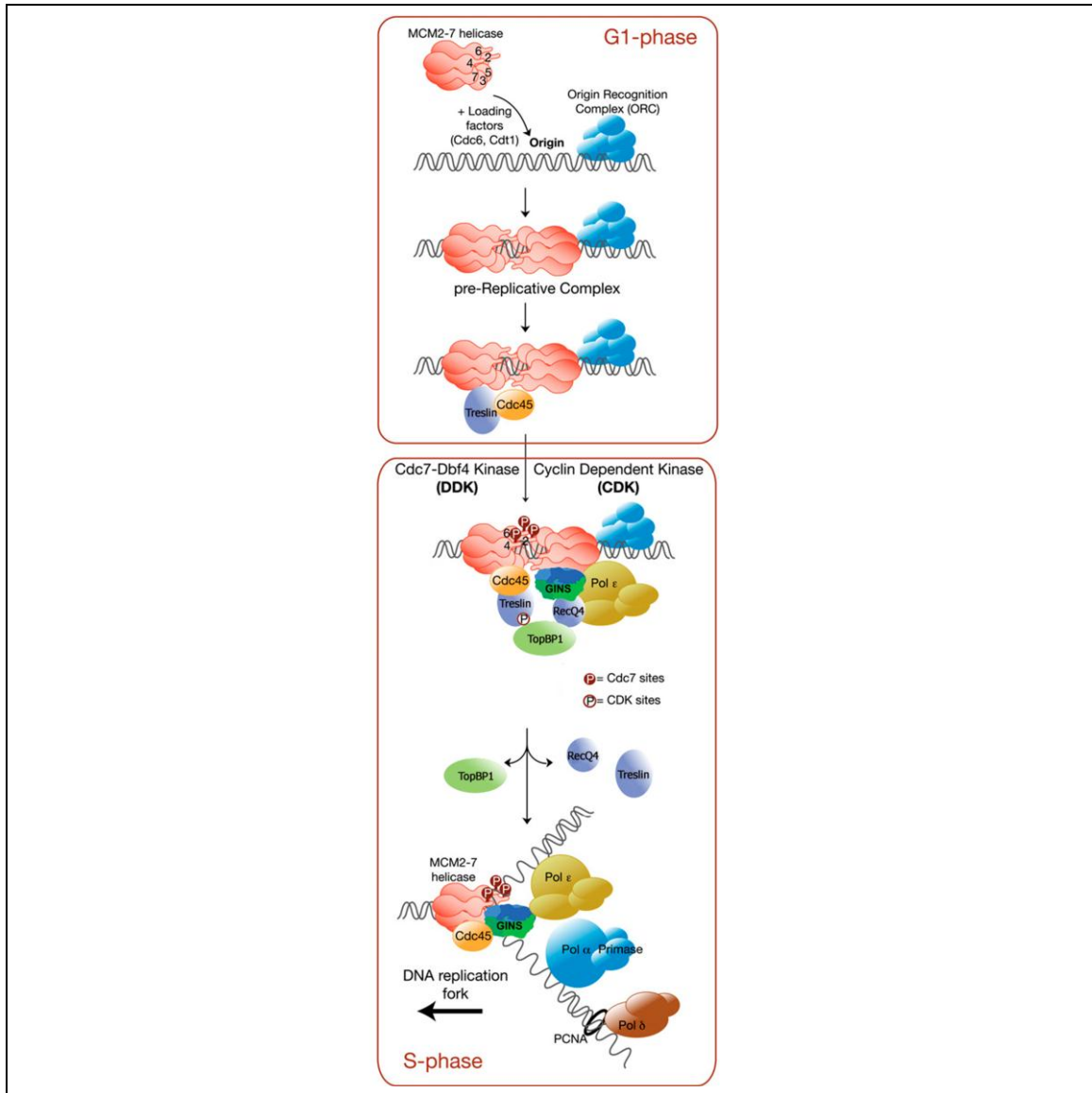


Figure 1. Replisome assembly at origins of replication.

ORC1-6 complex binding to replication origins in G1 phase initiates the replisome assembly process. Subsequently, Cdc6- and Cdt1-dependent activities load the inactive MCM2-7 helicase as a double hexamer onto origins, concluding the pre-RC assembly. Activation of the MCM2-7 helicase requires the transformation of the pre-RC to pre-IC through recruitment of additional proteins. CDK-phosphorylated Treslin (human Sld3 homolog) associates with TopBP1 (human Dpb11 homolog), bringing Cdc45 to replication origins. Although CDK-dependent phosphorylation is also required for Sld2 association with Dpb11 in yeast, RecQ4 (human Sld2 homolog) binding to TopBP1 (human Dpb11 homolog) does not seem to require CDK phosphorylation. Subsequent loading of GINS and pol ε is Sld2-dependent in yeast but RecQ4-independent in human. Pol α loading requires both TopBP1 and RecQ4 in *Xenopus*. Cdc45, MCM2-7 and GINS (CMG) complex formation leads to an active replicative helicase. CMG, along with the other components, such as the replicative polymerases pol ε, pol δ and pol α, constitutes the replication progression complex that is required for the initiation of replication. Figure adapted from [1].

Activation of the replicative helicase and subsequent unwinding of the origin DNA is followed by loading of polymerase α -primase (pol-prim); the only DNA polymerase capable of synthesizing a template de novo on ssDNA. Prior to pol-prim loading, origin DNA needs to be unwound [30]. As the origin DNA is melted, RPA binds to the single stranded DNA (ssDNA), keeping the strands from re-annealing. This event also precedes binding of pol-prim. However, *in vitro* studies using purified proteins have shown that pol-prim cannot synthesize primers on RPA-coated ssDNA [31-33]. SV40 T antigen can release the RPA-dependent inhibition on primer synthesis by pol-prim [31, 32]. The cellular factor(s) that facilitate this reaction (termed primosome activity) is not determined yet. Although the exact mechanism is elusive at this point, pol-prim loading to origins requires Cdc45 [34], TopBP1 [35], Mcm10 [36, 37], RecQ4 [18] and And1 [37]. It remains to be determined whether the protein machinery involved in pol-prim origin loading is the same or separate from that required for primosome activity.

DNA replication stress

A variety of circumstances can present major challenges for DNA replication with potentially detrimental consequences. A comprehensive DNA damage repair toolbox helps cells deal with different kinds of damage. Below is a brief review of different types of DNA damage that induce replication stress and the mechanisms cells employ to cope with replication stress.

Types of replication stress

Different types of DNA damage challenge faithful and complete duplication of the genome. These challenges may arise from intrinsic properties of the genome as in secondary structures. They may also be caused by DNA lesions induced by accumulation of genotoxic cellular metabolites (e.g. reactive oxygen species), or exogenous DNA damage (e.g. ultraviolet radiation). Furthermore, perturbation of the activities required for DNA replication or DNA damage, through genetic mutations or chemical inhibition of the enzymes necessary for replication, can also hinder DNA replication.

Evolutionarily conserved repeat sequences dispersed throughout the genome are hotspots for genomic instability. Secondary structures adopted by unwound DNA templates at these repeat sequences can stall replication forks, leading to repeat expansions or deletions if not dealt with properly [38]. The location of the repeat region with respect to the replication origin is thought to affect the stability of the repeat [39]. Hereditary diseases associated with repeat expansion include Huntington's syndrome and Fragile X syndrome [40]. Mechanistic understanding of replication associated repeat instability can be beneficial for therapeutic purposes.

Highly transcribed regions of the genome present another type of impediment at replication forks. Despite the temporal separation of most transcription from DNA replication, some genes, such as tRNA and rRNA genes, are continuously transcribed during S phase, which permits collision between the transcription complex and the replication fork. Collision with the transcription complex can lead to replication fork pausing. Cells have evolved mechanisms to evade genomic instability due to such

collisions, which involves DNA helicases capable of removing proteins bound to template DNA [41, 42].

DNA lesions in the template can also induce replication stress. Oxidative stress induced by accumulated metabolic byproducts called reactive oxygen species (ROS) generates a variety of DNA lesions, including base damage and sugar damage, some of which can interfere with replication fork progression [43]. In addition, exposure to exogenous factors, such as UV radiation or various chemicals, can also produce DNA damage. Exogenous genotoxins may damage DNA indirectly through inducing oxidative stress, as in the case of UVA radiation, or directly, by altering the DNA molecule, as in the case of UVB and UVC radiation [44]. UV irradiation at doses above 10 J/m^2 cannot be dealt with efficiently via the nucleotide excision repair pathway. Replication fork encounters with DNA photoadducts, mainly cyclobutane-type pyrimidine dimers (CPDs) and [6-4]pyrimidine-pyrimidone (6-4PP), disrupts ongoing replication by stalling replicative polymerases. Cisplatin exposure similarly stalls replication forks primarily through induction of DNA crosslinks. Replicative polymerase stalling by such DNA lesions on the leading strand can cause the uncoupling of the replicative helicase from the replicative polymerase, resulting in the generation of extended ssDNA regions [45]. These regions containing DNA lesions can later be bypassed by translesion polymerases η and ι [46] or recombination-like mechanisms.

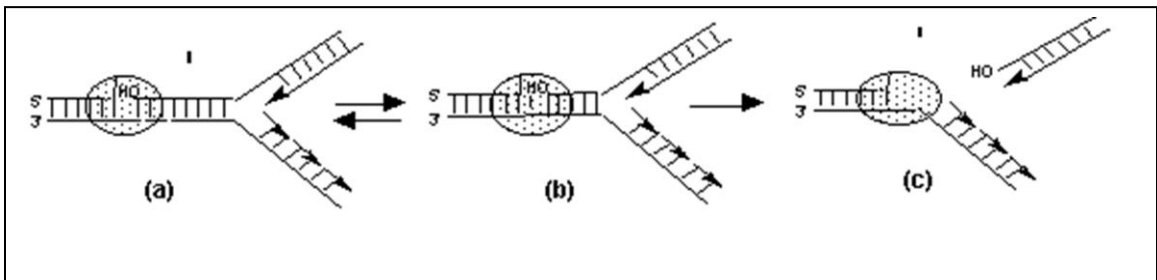


Figure 2. Replication associated double strand break formation after CPT exposure. (A) Topoisomerase I catalyzes ssDNA breaks needed to relax overwound DNA. (B) CPT binding to topoisomerase I inhibits the re-ligation of the ssDNA breaks that are formed by topoisomerase I. (C) Collision of the replication fork with the CPT-Topoisomerase I-DNA ternary complex leads to DSB formation. Accumulation of replication-associated DSBs results in CPT cytotoxicity. From [54].

Chemical or mutational perturbations of the enzymatic activities required for DNA replication are another source for replication stress. Examples of such chemical genotoxins include hydroxyurea (HU), aphidicolin (APH) and camptothecin (CPT). HU inhibits replication by interfering with the activity of ribonucleotide reductase whose activity is required to sustain the dNTP pool that are used to synthesize nascent DNA strands during replication [47]. Aphidicolin, on the other hand, directly interferes with nascent DNA synthesis through inhibition of B family polymerases; pol α , pol δ and pol ϵ [48-50].

CPT is a topoisomerase I poison whose derivatives are widely used in cancer chemotherapy. Topoisomerase activity is required to relieve the torsional stress that occurs as a result of dsDNA unwinding during replication or transcription [51]. Topoisomerase accomplishes this by creating a transient break in the sugar-phosphate backbone of the DNA, and then annealing the break. Topoisomerase poisons freeze the DNA-topoisomerase complex after catalysis of the strand break but before ligation, thereby resulting in a permanent DNA break in the template and topoisomerase-DNA complex. Collisions between replication forks and topoisomerase- DNA complexes results in double strand breaks (DSBs) [52] (Figure 2), accumulation of which eventually leads to cell death [53].

Checkpoint signaling

Central to the eukaryotic cellular response to replication stress is Ataxia Telangeicia-related (ATR) signaling. ATR is a phosphoinositide 3-kinase related kinase family member. ATR is activated upon accumulation of certain DNA protein structures

that recruit DNA damage response proteins to stalled replication forks (Figure 3) [55]. RPA-bound ssDNA is a central component of this DNA damage sensing mechanism [56]. Various types of replication stress stereotypically lead to RPA-bound ssDNA accumulation, which serves as a universal signal for checkpoint activation. RPA-bound ssDNA accomplishes checkpoint activation through recruiting many DNA damage response proteins including ATRIP, Rad9, Mre11 to stalled forks [56]. Accumulated RPA recruits the ATR binding partner, ATRIP, through a direct interaction between the acidic ATRIP checkpoint recruitment domain and the basic cleft of RPA70N [57]. ATR is recruited to stalled forks through this interaction. Rad9-1-1 complex is also recruited through a similar mechanism that employs a direct interaction between the acidic Rad9 checkpoint recruitment domain and the RPA70N basic cleft [58].

Apart from RPA-ssDNA, Rad9-1-1 loading additionally requires free 5' ends at the primer-template junction [60, 61]. Consistent with the primer-template junction-dependent Rad9-1-1 recruitment to stalled forks, primer synthesis by polymerase α -primase is required for checkpoint activation upon replication stress [62, 63]. It should be noted that primer synthesis at stalled forks can serve other functions in addition to Rad9-1-1 loading.

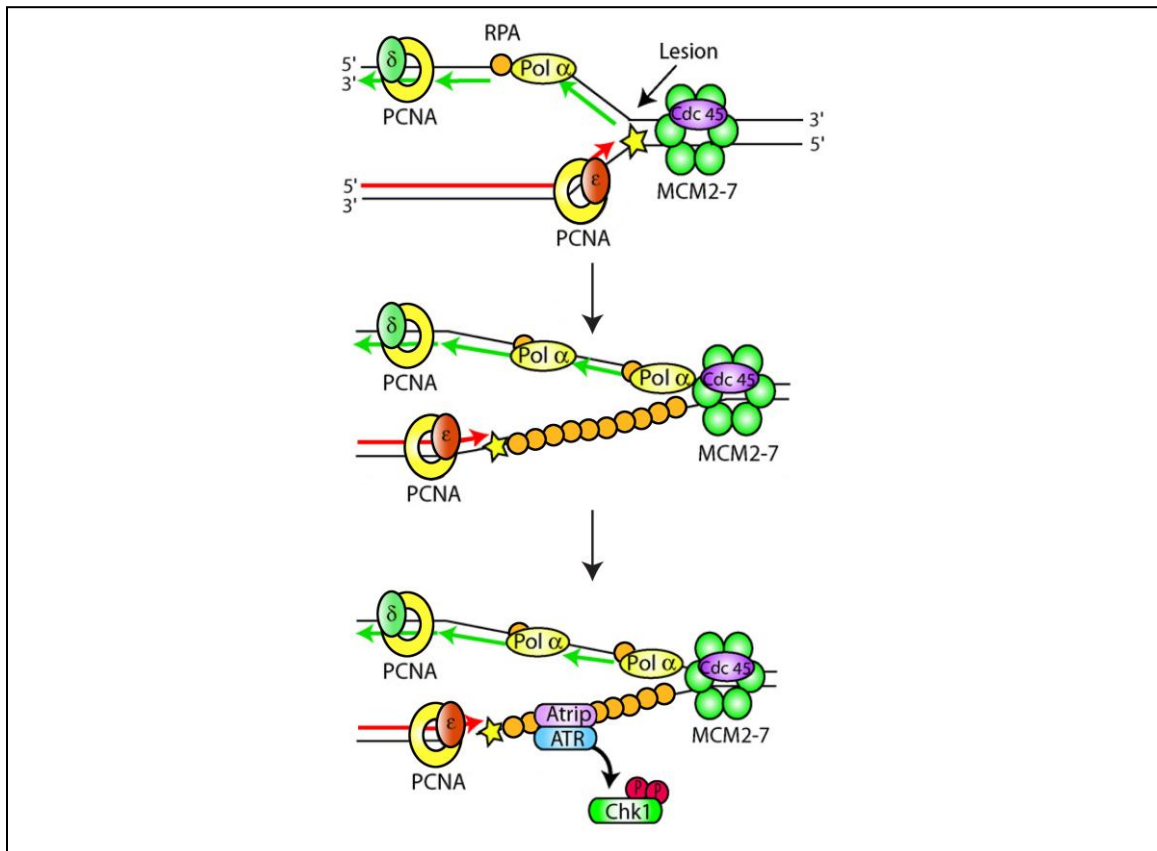


Figure 3. Checkpoint signaling activation after replication fork stalling.

At a normal replication fork, unwinding of the parental DNA by MCM2-7 generates single stranded DNA that is replicated by leading and lagging strand polymerases. During this process, a limited amount of ssDNA is exposed, which is readily bound by the ubiquitous single stranded DNA binding protein RPA. If the replication fork encounters DNA lesions that stall the replicative polymerase, replicative helicase uncouples from the replicative polymerase leading to the accumulation of ssDNA bound RPA at stalled replication forks. Accumulation of ssDNA-bound RPA results in the recruitment of DNA damage response proteins including Rad9/1/1 and the ATRIP/ATR complex followed by subsequent activation of ATR kinase. ATR then phosphorylates the checkpoint protein Chk1 to initiate checkpoint signaling. Adapted from [59].

TopBP1 is another protein interaction scaffold required for ATR activation. The exact mechanism for TopBP1 recruitment to stalled replication forks is not known. One of the functions of TopBP1 in checkpoint activation is recruitment of polymerase α -primase [64, 65]. It is not yet clear whether TopBP1 accomplishes this function through direct physical interactions or through other mediator proteins. Nevertheless, TopBP1-mediated pol-prim recruitment to stalled replication forks is important for Rad9-1-1 loading [65]. TopBP1 also functions as a direct protein activator of ATR kinase through its interaction with ATRIP [66, 67] and Rad9-TopBP1 interaction is important for TopBP1-mediated ATR activation [68, 69].

It is important to note that of the checkpoint activating DNA-protein structures identified so far, RPA-bound ssDNA and the 5' ends of template-nascent primer junctions, occur naturally at the replication forks, albeit in amounts insufficient to trigger a global checkpoint response. It is possible that the factors that are important for checkpoint activation associate with the fork even in the absence of damage, albeit more transiently. Transient association of checkpoint activating proteins would limit their action at the fork and prevent activation of checkpoint signaling (Figure 4). Indeed, ATR and Chk1 were found to be important for genomic stability in the absence of damage as well [70-72], suggesting that ATR and Chk1 are activated and operational at basal levels even in the absence of exogenous damage. This may enable a 'fork surveillance mechanism' where the cellular factors that are important for responding to damage are kept in close proximity to the active replication fork for prompt response when damage is encountered.

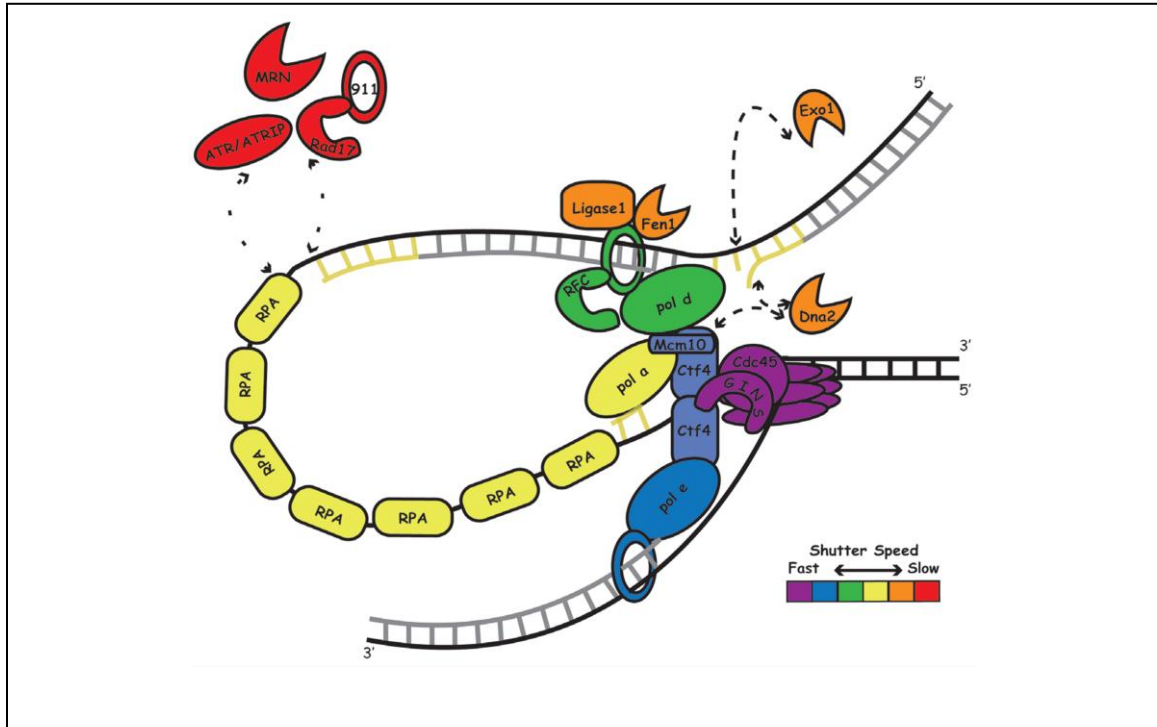


Figure 4. Snapshot of the emerging replisome¹.

During unperturbed replication, CMG helicase unwinds parental DNA as pol epsilon synthesizes the leading strand. The discontinuous nature of lagging strand synthesis requires the replisome to be remodeled as proteins synthesize or process the Okazaki fragments. Multiple weak interactions among the proteins in the replisome, of which only a few are depicted, enable replisome remodeling through exchange of interaction partners. This provides the plasticity crucial to adapt to different circumstances, e.g., replication through “slow zones” or damaged templates, which lead to transient accumulation of specialized proteins, e.g., ATRIP/ATR and Rad911. From [73].

¹ This figure was published in Guler and Fanning (2010) The replisome: a nanomachine or a dynamic dance of protein partners? *Cell Cycle* 9(9):1680-1.

Activated ATR kinase regulates a wide range of cellular responses to replication stress through phosphorylating a plethora of substrates [74]. One of the best characterized ATR substrates is Chk1 (Figure 5). ATR-dependent phosphorylation activates Chk1, which then modulates global responses to replication stress, including inhibition of new origin firing and cell cycle progression by Cdc25 inactivation [75, 76]. ATR signaling also targets the components of the replication fork, either directly or indirectly through Chk1 [59]. Although mechanistic details remain to be investigated, ATR signaling increases fork stability, preventing the dissociation of the replisome components from the stalled forks, which aids in subsequent recovery from replication stress. ATR is further implicated in modulating replicative helicase activity following replication stress-induced helicase-polymerase uncoupling. In addition, ATR regulates activities of proteins involved in repair and recovery from the replication block such as WRN [77] and BLM [78]. Through such phosphorylation events, ATR signaling seems to affect repair pathway choice.

Replication restart

The ultimate goal of replication stress response is to resume replication once the damage is dealt with in order to accomplish complete and faithful replication of the genome. Eukaryotic restart mechanisms are not fully understood, but appear to be composed of alternative and complementary pathways.

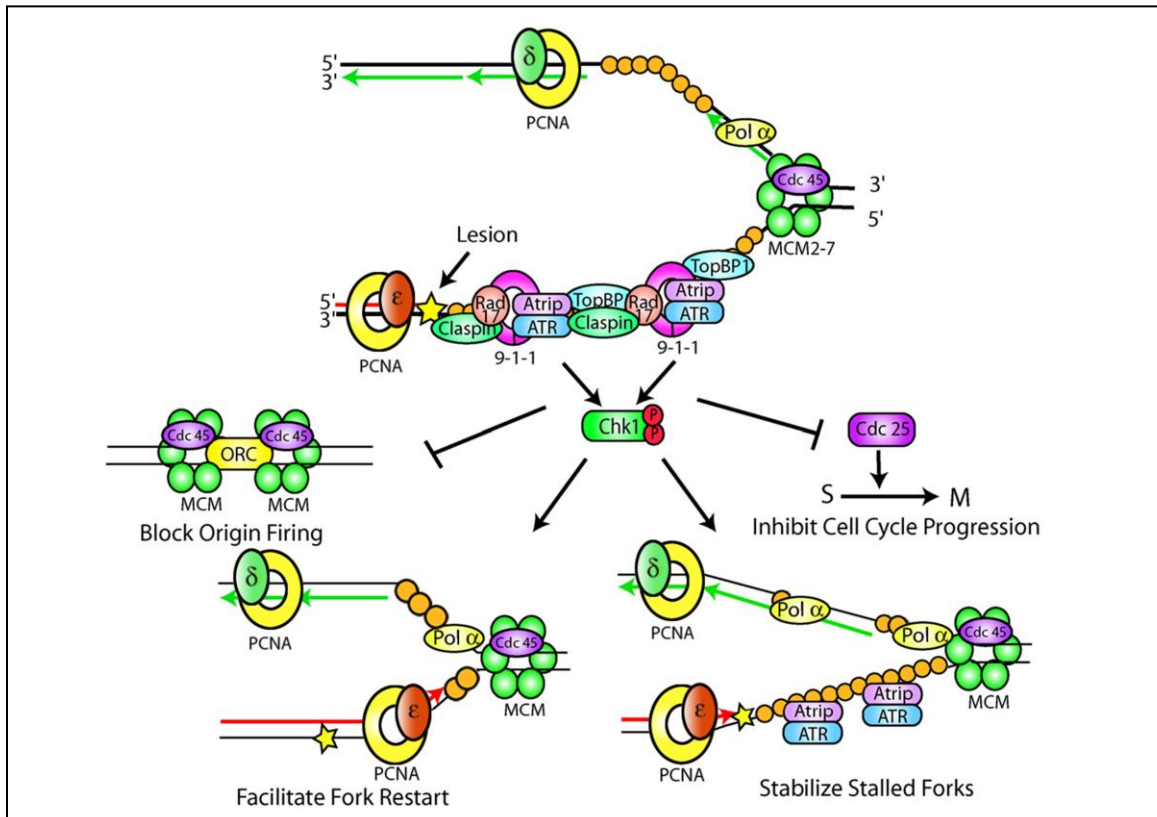


Figure 5. Checkpoint signaling upon replication fork stalling.

Uncoupling of the replicative helicase from the replicative polymerases due to polymerase stalling at sites of DNA lesions leads to accumulation of RPA-bound ssDNA. Accumulated RPA-ssDNA facilitate recruitment of DNA damage response proteins and subsequently activate ATR signaling. Among many ATR substrates is checkpoint protein 1 (Chk1). Activated Chk1 coordinates the checkpoint response to replication stalling which includes suppression of new origin firing, blocking of cell cycle progression through Cdc25 inhibition, stabilization of the fork and facilitating replication fork restart. Modified from [59].

One of the factors that affect the recovery pathway decision is the stability of the fork after the replication stress. Certain types of replication arrest, such as those induced by short exposure to HU, inhibit replication fork progression but does not interfere with the association of the fork components with replicating chromatin, therefore leaving the replisome intact. These stalled replication forks are stable in checkpoint-competent cells [79, 80] and can resume replication once the arresting factor is removed [81]. Such direct restart from stalled forks enables rapid reactivation of replication. On the other hand, replication forks may collapse when the replisome components dissociate from the replicating chromatin under replication stress conditions such as prolonged exposure to HU or replication fork collisions with CPT-trapped topoisomerase DNA complexes. When replication forks are inactivated due to collapse, replication has to resume via alternative pathways which include replication by new origin firing and/or recombination-like replication restart [82].

Several damage response proteins are recruited to damaged forks for damage repair or bypass to allow replication resumption after replication arrest. Among these are specialized DNA helicases that remodel the replication fork for reactivation. The annealing helicase Smarcal1 is proposed to promote replication restart by annealing extended stretches of ssDNA accumulated at forks due to uncoupling of the replicative helicase and the replicative polymerases [83-88] (Figure 6A). A second helicase with ssDNA annealing activity was recently discovered [89], however its cellular function is not yet known.

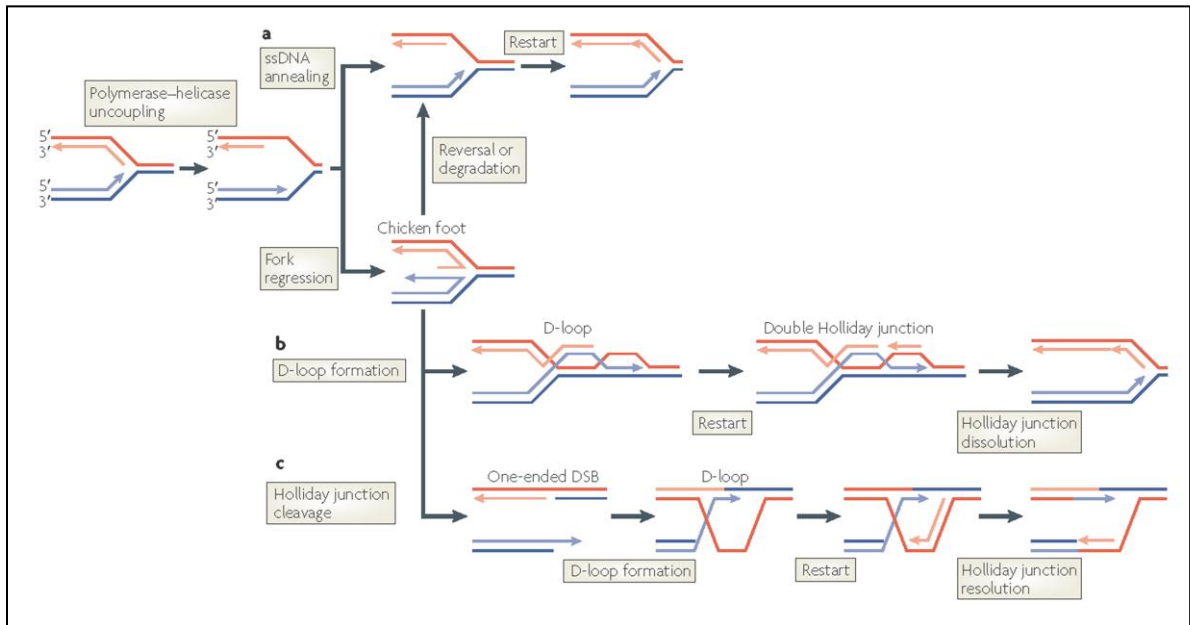


Figure 6. Models for replication fork restart.

Uncoupling of the replicative helicase from the replicative polymerase lead to accumulation of ssDNA at the stalled fork. **(A)** Replication restart by fork remodeling can be accomplished through re-annealing of the excess ssDNA generated upon uncoupling, possibly through Smarcal1 activity. **(B)** Replication fork regression, possibly catalyzed by several DNA helicases including BLM, WRN, FancM or HLTF, involves the annealing of the nascent strands to each other to generate a Holliday junction that resembles a ‘chicken foot.’ Double strand ends that are generated by this mechanism can then initiate recombination through a pathway that involves Parp1 and Mre11. Displacement loops (D-loops) formed enables replication restart. **(C)** If Holliday junctions are resolved by a nuclease that results in a one-ended DSB, then replication can be reactivated through a mechanism similar to break-induced replication where the DSB end invades the sister chromatid to result in a D loop. Holliday junction can be resolved by Mus81 activity after DNA replication restart. From [82].

Stalled forks may also be remodeled into a structure called a ‘chicken foot’ by fork regression, where the nascent strands are separated from the template strands to allow re-annealing of the template strands while the nascent strands anneal to each other (Figure 6B). A variety of helicases exhibit fork regression activity *in vitro*. Helicases implicated in replication restart through fork regression include Fanconi anemia complementation group member M (FANCM) [90, 91], RecQ helicase family members BLM and WRN [92, 93], and Rad5 ortholog HLTF [94, 95]. Additional specialized activities associated with these proteins, either directly, or through their interaction partners, can determine the subsequent steps of the replication stress recovery process, leading into divergent restart pathways.

Annealing of the nascent strands to each other by fork regression allows use of one nascent strand to serve as a template for the other nascent strand. This template switching process enables bypass of the DNA lesion or replication fork block (Figure 7). Alternatively, template switching can occur at long distance, where the nascent strand anneals with a genomic region that contains some degree of homology. Repetitive sequences are possible candidates for this type of long-distance template switching events [96].

Annealing of these homologous or highly similar sequences is very similar to events required for homologous recombination (HR). Thus, it is not surprising that several HR proteins are implicated in replication fork regression and template switching. In addition to WRN and BLM helicases mentioned above, HR proteins Mre11, Rad51, PARP, and Xrcc3 contribute to replication stress recovery [81, 98].

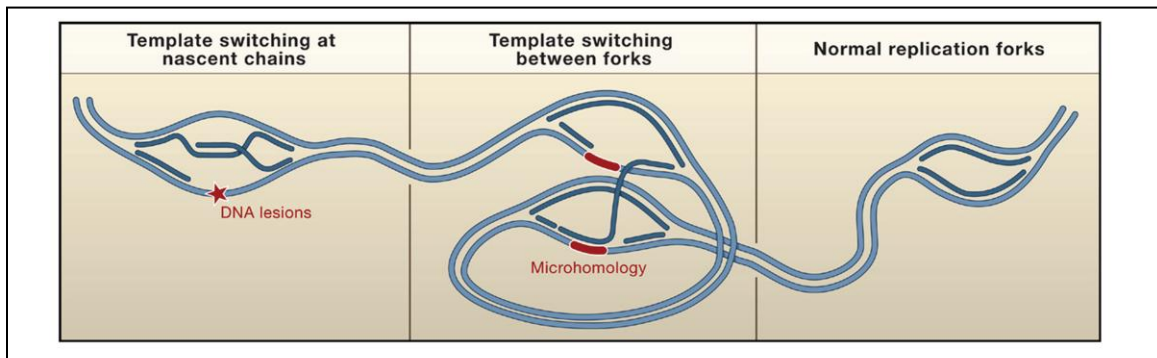


Figure 7. Template Switching.

Replication forks can bypass DNA lesions (red star) by template switching where one of the nascent strands utilizes the other as the template to replicate the DNA. Forks may also utilize repeat elements with microhomology (red bars) from a different nearby fork to bypass replication stall sites, however, this mechanism may lead to genomic rearrangement. From [97].

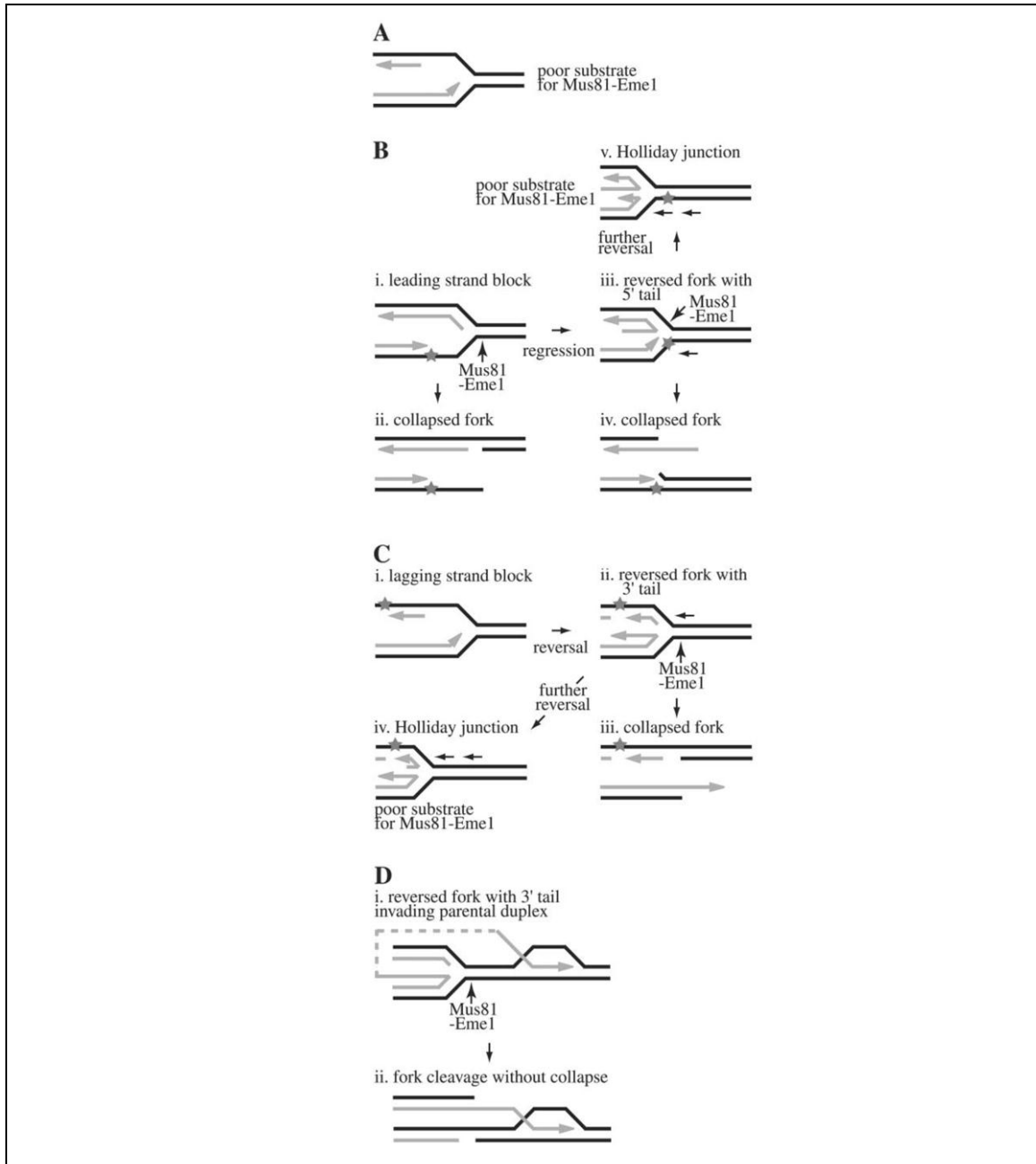


Figure 8. Mus81/Eme1-dependent processing at the stalled replication forks.

Models generated based on substrate specificity of Mus81/Eme1 *in vitro*. A. Normal replication forks are poor substrates for Mus81/Eme1. B-D. DNA lesions in the leading (B) or lagging (C) strand template can result in replication fork stalling. In the absence of fork remodeling, these stalled forks may be good substrates for Mus81/Eme1 nuclease activity. Fork reversal and subsequent processing at the fork may result in three or four way junctions with strand discontinuity or Holliday junctions. Mus81/Eme1 cleaves substrates with junctions containing strand discontinuity more efficiently than Holliday junctions [101].

HR-like pathways for replication restart may be activated by DSBs at the replication fork. In the case of CPT-induced replication stress, fork collision with the CPT-trapped topoisomerase-DNA complex and associated ssDNA breaks directly results in the generation of a DSB [52] (Figure 2), which activates an HR-mediated repair pathway [99]. On the other hand, DSBs can be actively generated by enzymatic processing at the stalled fork as part of the repair process after replication stalling. Nascent strand annealing through fork regression mediated by DNA helicases such as BLM results in DSBs that can potentiate a HR-mediated pathway. Additionally, the eukaryotic endonuclease Mus81 specifically recognizes branched substrates and generates DSBs [100, 101] important for Rad51 foci formation and recombination-mediated replication repair in the absence of WRN helicase [102] (Figure 8).

HR requires a 3' overhang on which the Rad51 assembles as a filament to initiate homologous sequence search. Recent work suggests that PARP1 functions at this step by recruiting Mre11 [98] (Figure 9). Mre11-dependent degradation of nascent strands is tightly regulated and unwanted Mre11 nuclease activity is restricted by both BRCA2 and Rad51 [103, 104]. The 3' overhangs generated by Mre11 nuclease activity are first bound by RPA, which is then replaced by Rad51. This process is highly regulated as several proteins, such as Mms22L/TONSL complex and Rnf8 [105-107], were shown to promote Rad51 loading, whereas others were shown to disrupt Rad51 filament formation [108]. Strand invasion by Rad51 filament results in the formation of D-loops which enable loading of the replication machinery to continue replication by bypassing the stall site. Mus81/Eme1 endonuclease is implicated in resolving the resulting double Holliday junction and promoting sister chromatid exchange (Figure 8). On the other hand, Mus81

independent mechanisms are proposed to exist to dissolve the double Holliday junction in a way that avoids formation of recombinogenic products.

Dormant origins provide another mechanism by which complete genome duplication can be achieved when replication is challenged. Under normal replication conditions, only a fraction of the origins that are licensed during G1 are actually utilized for DNA replication in S phase. MCM2-7 loaded on the licensed origins is inactive as a helicase and requires Cdc45 and GINS for its activation [29]. Cdc45 protein levels seem to be the limiting factor for replication initiation at a limited number of origins [110]. Replication stress seems to activate dormant origins by a mechanism that remains largely undetermined [111]. One possible mechanism suggests an indirect role for checkpoint signaling, where checkpoint-dependent inhibition of new replication factories possibly directs replication initiation activity towards replication factories that are already activated to allow activation of dormant origins specifically located at replication stall sites [112]. However the distinct mechanisms involved in dormant origin activation remain to be investigated. Nevertheless, use of dormant origins seems to be an integral component of the cellular replication restart apparatus to preserve genomic integrity after fork stalling even in the absence of exogenous DNA damage [113].

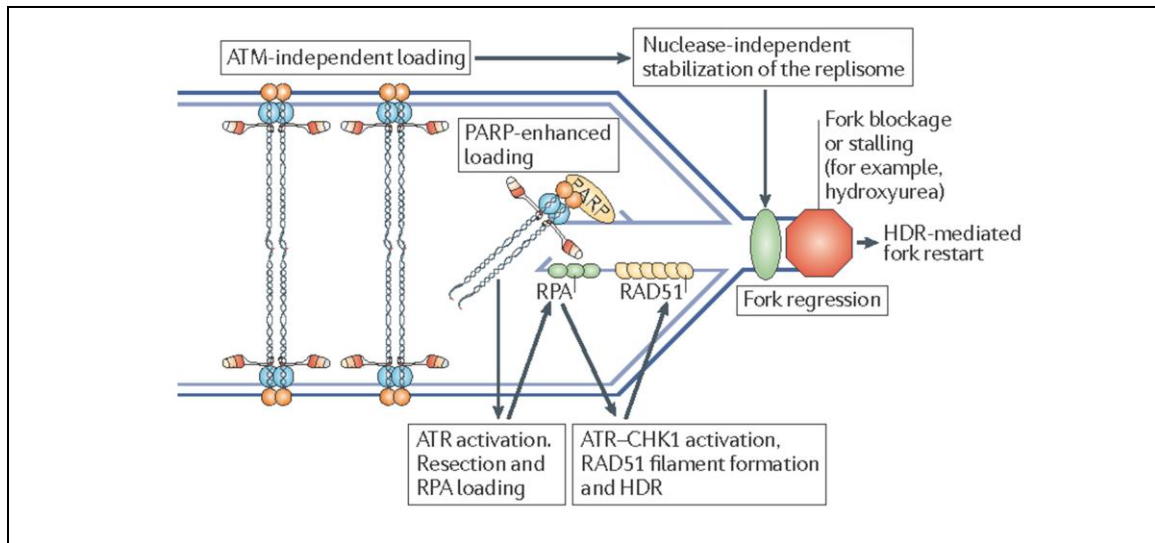


Figure 9. The MRE11 complex in response to stalled replication forks.

Nascent strands annealing to one another due to fork regression (as shown in figure) or ssDNA breaks at the template result in double stranded ends at replication forks. PARP enhances MRE11 complex recruitment to stalled replication forks. Mre11-dependent end resection at double stranded ends generates the 3' overhang which is first coated by RPA. RPA is then replaced by Rad51, which then initiates the search for a homologous sequence for recombination-dependent replication restart [109].

DNA replication, DNA damage response and cancer

Cancer is a leading cause of death in the world, accounting for the 13% of total number of deaths². Uncontrolled cellular proliferation is the hallmark of cancer. This characteristic is commonly accompanied by genomic instability. Increased knowledge of the molecular mechanisms that enable uncontrollable cell proliferation under conditions that challenge genome integrity is important to understand cancer, and can potentially benefit cancer therapy through identification of novel therapeutic targets or biomarkers for molecular diagnostics.

Given that the hallmark of this malady is uncontrolled cellular proliferation, it is no surprise that DNA metabolism is among the prime targets for cancer therapy. Among the therapeutic agents that target DNA metabolism are inhibitors of enzymatic activities required for cellular proliferation such as the CPT-derived topoisomerase I inhibitors topotecan and irinotecan [114]. Other agents may target DNA directly. DNA crosslinking by cisplatin or mitomycin C [115], DNA strand break formation by bleomycin [116] or DNA intercalation by doxorubicin [117] are exploited for therapy in a wide range of cancer types including lung, ovarian, cervical, breast and colon cancer. In addition to these chemotherapy agents, radiation therapy, which is used as standard of care for many types of cancer, also works by damaging the DNA.

Despite the therapeutic advantages of using genotoxins for killing cancer cells, use of genotoxins presents a major challenge in terms of specificity. Significant side effects may prevail with these agents, since they affect all proliferating cells in the body. One recent advance in cancer therapy that utilizes the concept of targeting DNA

² <http://www.who.int/mediacentre/factsheets/fs297/en/>

metabolism for sensitivity while increasing specificity is a synthetic lethality. This approach exploits the increased dependence of cancer cells for specific DNA damage pathways. As discussed in previous sections, normal cells have multiple overlapping pathways that function in cellular DNA damage response. These back up mechanisms serve as the cell's insurance for controlled DNA replication with the assurance of correct and complete genome duplication even when one pathway becomes dysfunctional. However, since the biogenesis of cancer is linked closely with de-regulation of these pathways, cancer cells end up relying on one of these pathways when the complementary pathway is disrupted. The 'synthetic lethality' approach exploits this property of cancer cells with respect to normal cells to achieve effective and selective cancer therapy.

Successful clinical application of the synthetic lethality approach was documented recently with poly (ADP-ribose) polymerase-1 (PARP1) inhibitor olaparib in cancer patients with BRCA1/BRCA2 mutations [118, 119]. Replication-associated DSBs that accumulate upon PARP1 inhibition, which can be efficiently repaired by BRCA1/2-dependent HR, become lethal in BRCA1/2 deficient cancer cells while sparing normal cells [120]. These PARP1 studies illustrated synthetic lethality as a viable approach for efficient and selective cancer therapy.

Despite significant advances achieved by this approach, the response rates in clinical studies for the PARP1 inhibitor olaparib did not exceed 41% in BRCA-deficient cancer patients [121], suggesting that additional backup pathways exist. Our knowledge of these backup pathways is far from complete. Comprehensive understanding of the plasticity of the cellular replication stress response that enables cells to tolerate and adapt to a plethora of replicative challenges is likely to lead to novel therapeutic opportunities.

DNA helicases

Helicases perform important functions for DNA and RNA metabolism. The human genome encodes a large number of helicases, equipping the cell with a helicase toolbox. Each helicase accomplishes specialized functions required for cellular activities ranging from DNA replication and DNA repair to transcription, RNA splicing and RNA silencing. The specialized nature of these enzymes suggests that their function is not only confined to simple unwinding or translocation, but also that they help drive the processing and progression of specific biological pathways. These specialized functions are most often performed through specific interactions with other proteins or specific DNA/protein complexes, and/or additional enzymatic activities.

Biochemical and structural properties of DNA helicases

Helicases are enzymes that couple the energy released from ATP hydrolysis for translocation on DNA and/or RNA. Most helicases are specific for their substrates, so they translocate on either DNA, or RNA, or DNA/RNA hybrids. DNA helicases translocate on dsDNA or ssDNA to unwind dsDNA into ssDNA or remove proteins bound to DNA. Translocation occurs with a directionality based on the intrinsic polarity of the DNA molecule.

Helicases are classified into six superfamilies based on their ‘helicase’ motifs [122, 123]. Of note, not all proteins containing these signature motifs were found to possess unwinding activity [122]. Therefore, these superfamilies represent ‘motor’ proteins that hydrolyze ATP for translocation on DNA/RNA. DNA helicases can be

found in different oligomeric states, where some function as monomers and others as hexamers.

Despite significant variation among the helicases that belong to different superfamilies, they share a common structural framework that enables harnessing the energy from ATP hydrolysis to translocation on DNA/RNA. Central to this framework are Walker A and B motifs responsible for ATP binding and ATP hydrolysis, respectively, and the arginine finger which is also important for ATP hydrolysis (Figure 10).

Helicases most often possess additional domains that can regulate helicase function by several mechanisms. One mechanism is through affecting helicase activity, in terms of processivity or rate, by post translational modifications or protein/DNA interactions. Examples include chi-site recognition-induced pausing of RecBCD [124], Cdc7/Dbf4-dependent phosphorylation and Cdc45/GINS binding-dependent activation of Mcm2-7 [4, 125-127], and fine-tuning of *Sulfolobus solfataricus* Hel308 processivity by an autoinhibitory domain that binds to the unwound ssDNA [128]. Helicase function can also be modulated through regulating subcellular localization, or even sub-compartmental localization within, as observed in the recruitment of DNA helicases to stalled replication forks by RPA [83, 86, 87]. Additional domains may also possess enzymatic functions other than helicase activity, therefore coupling other enzymatic activities such as the nuclease activity in RecB, Dna2 or WRN [129-131].

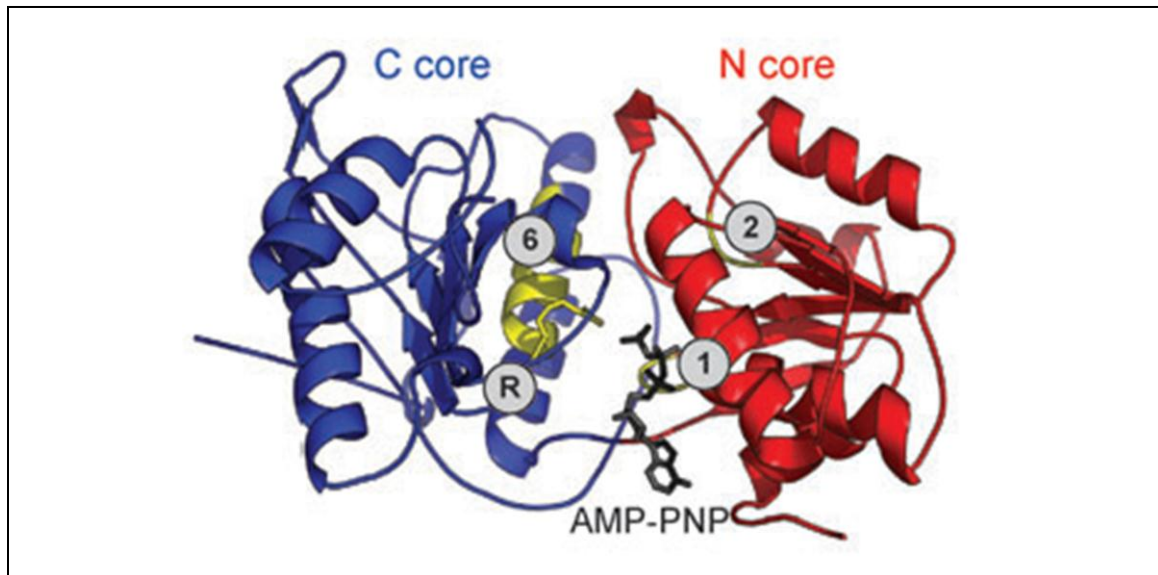


Figure 10. Common structural themes among helicases from different superfamilies. Superfamily 1 member PcrA helicase core structure is shown as representative to illustrate common structural elements observed among helicases from different superfamilies (shown in yellow). Two Rec-A like core domains, located within the same monomer in superfamily 1 and 2 helicases, form a crevice. One side of this crevice harbors NTP binding and hydrolysis motifs called Walker A (1) and Walker B (2) located on the N-terminal Rec-A like core (N core), while the arginine finger (R) within Motif 6 (6) that modulates NTP binding or hydrolysis is located on the other side located at the C-terminal Rec-A like core (C core). The two Rec-A like core domains that form the crevice are contributed by two adjacent subunits in the case of hexameric superfamily 3 helicases. From [122].

Recently, a novel ATP-dependent activity was characterized for helicases. Yusufzai and Kadonaga showed that the HepA-related protein HARP (also known as Smarcal1) has ATP-dependent ssDNA annealing activity [85]. A second example of this type of helicase is annealing helicase 2 AH2 [89]. Although ssDNA annealing activity was shown for BLM, WRN and RecQ5 [132-135], these helicases do not use ATP for this function, unlike the case of Smarcal1 and AH2 where the reaction is ATP-dependent [85, 89]. Indeed, ATP inhibits ssDNA annealing by BLM, WRN and RecQ5. Nevertheless, strand annealing activity by these proteins is proposed to be important for Holliday junction migration and remodeling of the stalled replication forks into chicken foot structures for replication restart.

DNA helicases in DNA replication and repair

Separation of double stranded parental DNA is necessary for the replication machinery to synthesize nascent strands. This unwinding activity is accomplished through the replicative helicase that travels with the replication fork. The Mcm complex, a member of the superfamily 6, is thought to be the replicative helicase in archaea and eukaryotes [136]. The heterohexameric Mcm2-7 helicase is well conserved among eukaryotes whereas a homohexameric Mcm complex serves as the archeal replicative helicase [136, 137]. Our current understanding is these proteins function as a double hexamer at the replication fork.

The helicase activity of Mcm2-7 is tightly regulated. Mcm2-7 loaded at the origins in G1 phase does not unwind origin DNA until the start of S phase. Mcm2-7 origin unwinding is activated after Cdc7 and CDK dependent phosphorylation and

subsequent binding of Cdc45 and GINS [1]. Indeed, purified Mcm2-7 has little if any helicase activity *in vitro*, yet is active when in complex with Cdc45 and GINS [4, 29].

In addition to Mcm2-7, other helicases have been identified that associate with the fork. One example is a superfamily 1 member RecD family helicase called Rrm3 in *S. cerevisiae* [138]. Rrm3 moves with the fork and provides additional power to help the fork move through genomic regions that are hard to replicate such as DNA templates with repeats or tightly bound proteins. Indeed, Rrm3 deletion leads to replication fork pausing at not only centromeres and rDNA regions but also telomeres and tRNA genes [41, 139, 140]. In higher eukaryotes, RecQ4 was identified as a pre-RC component [141] and part of a stable complex with replication fork components through its association with Mcm10 [20]. RecQ4 downregulation decreases cellular replication and proliferation [141], however, its specific function remains to be identified.

The idea of ‘accessory helicases’ is rather new in the eukaryotic replication field. Bacterial helicases Rep, UvrD and DinG have been shown to be important for replication fork progression through genomic regions with tightly bound proteins such as highly transcribed segments [142-146]. The additional helicase power harnessed through direct interactions between Rep and the replicative helicase DnaB was shown to be important in sustaining rapid genome duplication rates in *E. coli* [146]. Similar ‘accessory helicase’ impact on genome duplication would be consistent with the recent data, which shows that replication forks move at a fairly uniform speed throughout the genome in yeast [147].

Having an additional helicase traveling with the fork can provide additional advantages apart from removal of high affinity nucleoprotein complexes ahead of the fork. Homologous recombination machinery is a well characterized complex with two

helicase motors, RecB and RecD, which translocate on the opposite strands of the DNA. Having two motors with opposite polarities within the same complex appears to promote increased processivity by enabling the complex to go through templates with nicks or lesions in one of the strands [148, 149].

In addition to helicases that travel with the fork, other helicases with functions in DNA damage response are recruited to replication forks when forks encounter an impediment. These helicases perform specialized functions facilitated by substrate specificity and protein interactions and play important roles in determining how the damage is processed.

One such helicase is Smarcal1 annealing helicase, which is recruited to stalled replication forks through a direct interaction between Smarcal1 N terminus and RPA32 C terminal domain [83, 87]. It is suggested that Smarcal1 promotes replication restart by remodeling the fork into a chicken foot structure with its ssDNA annealing activity. Helicase superfamily 2 member Rad5-related human helicase HLTF was also shown to be capable of promoting fork regression *in vitro* in addition to being required for efficient replication restart in cells exposed to replication stress [94, 95].

RecQ helicase family members WRN and BLM are also implicated in replication stress response. In addition to *in vitro* fork regression activity [92, 93], WRN and BLM can catalyze branch migration at Holliday junctions [150, 151]. Through activities including, but not limited to, fork regression and Holliday junction migration, WRN and BLM can regulate multiple steps in recombination-mediated repair to promote replication restart.

Other less characterized DNA helicases with functions in replication stress response include FANCM, FANCI, HLTF, Fbh1 and Hel308 [95, 108, 152-155]. Synergistic and/or complementary functions performed by these helicases involved in replication restart remain largely to be revealed. This will be of further interest particularly considering the potential of using helicases as targets for cancer therapy.

Human DNA Helicase B

HDHB: Biochemical properties and role in DNA replication

DNA helicase B (DHB) was identified by biochemical fractionation of mouse FM3A cells [156]. The purified helicase has ssDNA-stimulated ATPase activity [157] and ATP-dependent helicase activity with 5' to 3' polarity and specificity for DNA as substrate [158-160]. The biochemical characteristics of mouse DHB are preserved in human homolog [161].

DHB is well conserved among vertebrates without an obvious homolog in lower eukaryotes (Figure 11). The DHB helicase domain contains seven conserved superfamily 1 helicase motifs with sequence similarity to HR proteins RecD and bacteriophage T4 dda (Figure 12). The human DHB (HDHB) C terminus contains a phosphorylation-dependent subcellular localization domain (PSLD) [164]. The PSLD is responsible for nuclear localization in G1 phase and phosphorylation-dependent nuclear export of most of the cellular HDHB pool at G1/S transition [164]. The HDHB C-terminus contains other potential phosphorylation sites [165, 166] but the functional importance of these potential modifications remains to be determined. The N-terminal domain, also well conserved, lacks a functional domain identifiable from primary structure and its function

remains to be identified. Of note, the HELB gene produces an alternatively spliced transcript, corresponding to mainly the N terminal domain of HDHB; residues 1-575. However, the biological function of this isoform is not yet characterized.

Interestingly, DNA helicase B activity co-purifies with polymerase- α primase activity from mouse FM3A cells and purified mDHB stimulates DNA synthesis and primosome activity by polymerase- α primase [167-169]. In accordance with these results, HDHB interacts with pol-prim and stimulates DNA synthesis by polymerase- α primase and primosome activity [161]. DHB primosome activity strongly suggests that HDHB might be involved in cellular functions that require primosome activity, namely DNA replication initiation, lagging strand synthesis, and checkpoint activation after replication stress.

Consistent with a role for DHB in DNA replication, a temperature sensitive mouse allele in mouse that inactivates mDHB helicase activity are unable to replicate and proliferate when cultured at the non-permissive temperature [169]. Furthermore, an HDHB Walker B mutant inhibits DNA replication when microinjected into HeLa cell nuclei in early G1 phase, whereas injection of WT HDHB has no effect [161]. Additional support for a potential DHB function in DNA replication comes from ChIP experiments that showed HDHB localization to chromosomal origins during G1 phase (Gerhardt and Fanning, unpublished data). Altogether, these data suggest that DHB helicase activity may be important for chromosomal DNA replication. However, the mechanistic details of HDHB involvement in DNA replication remain to be investigated.

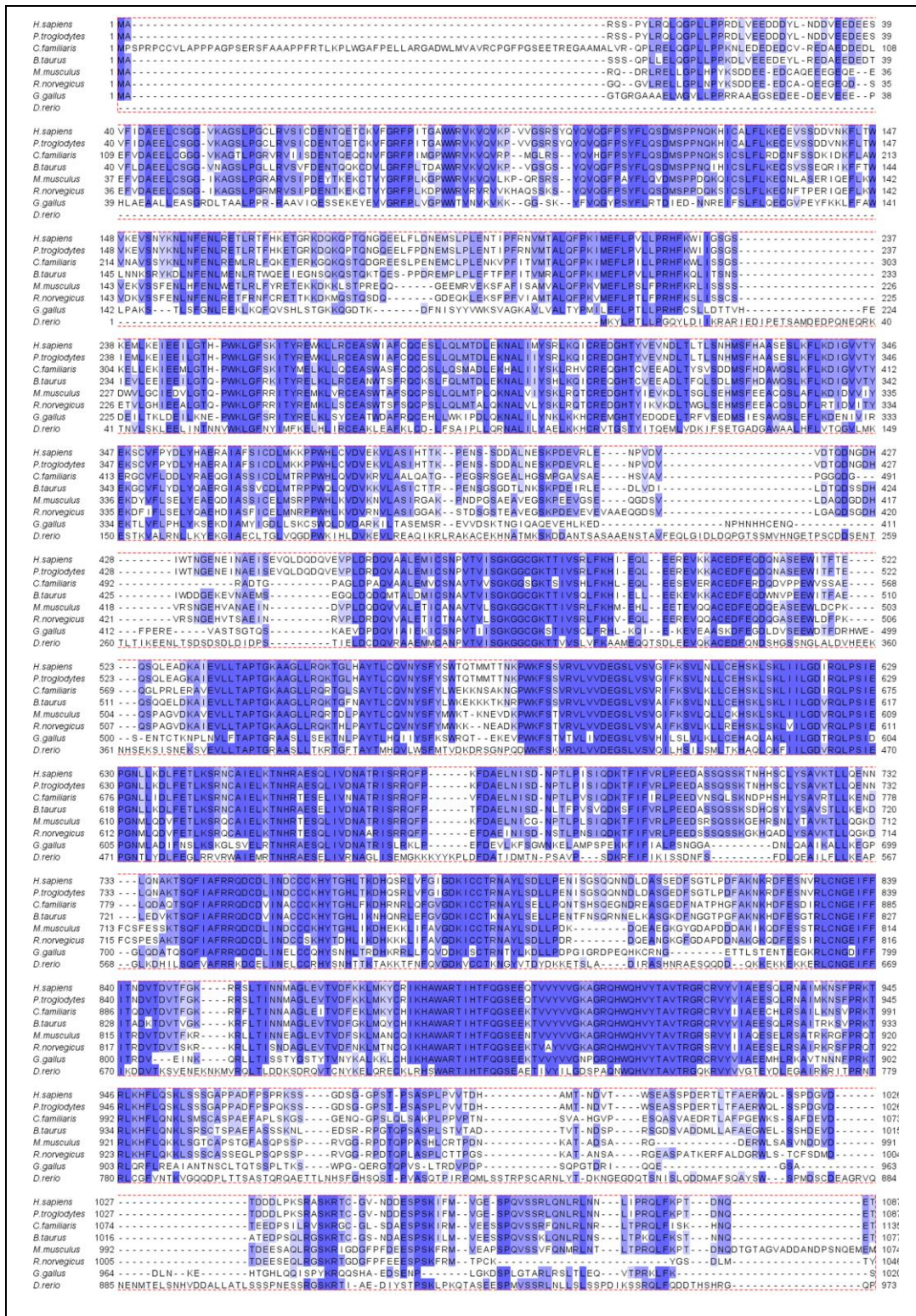


Figure 11. Protein sequence conservation in DHB among vertebrates. Multiple sequence alignment for vertebrate DHB protein sequences was performed using TCooffee [162] and visualized with Jalview [163]. Dark blue highlights residues that are identical whereas light blue denotes similar residues.

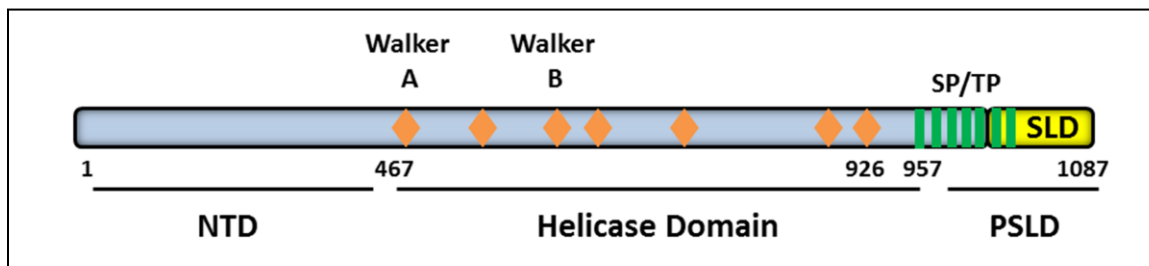


Figure 12. Schematic representation of HDHB.

HDHB can be divided into three functional domains as determined by sequence analysis. The N-terminal domain (NTD) does not contain any known sequence motifs despite being well-conserved among vertebrate DNA helicase B orthologs. The helicase domain contains seven conserved helicase motifs found in superfamily 1 (indicated by orange rhombi). A phosphorylation-dependent subcellular localization domain (PSLD), located at the C-terminus, has seven SP/TP motifs (indicated by green rectangles) that are putative cyclin dependent kinase (CDK) sites. Phosphorylation of Ser 967 located at the PSLD results in the nuclear export of the protein during G1/S transition.

Implications for HDHB function in DNA damage response

Analysis of the HDHB primary structure reveals potential PIKK phosphorylation sites within the helicase domain. Indeed, HDHB was identified as a potential ATM/ATR substrate for phosphorylation after DNA damage by mass spectrometry analysis [74]. This phosphorylation modifies S709, which is located in the helicase domain, between motifs IV and V. However, it is yet to be determined how this modification affects HDHB function and/or helicase activity in response to DNA damage.

Ectopically overexpressed GFP-tagged HDHB forms nuclear foci, particularly in G1 phase cells [164]. Consistent with a role for HDHB in DNA damage response, treatment of cells with DNA damaging agents, particularly the topoisomerase poisons CPT and etoposide, elevates the number of GFP- HDHB foci per cell [164]. Nuclear foci formed by overexpressed GFP-HDHB contain DNA damage response proteins Mre11 (Gu and Fanning, unpublished data), Rad51 and Rad52 (Yan and Fanning, unpublished data). Moreover, HDHB interacts with Mre11 (Gu and Fanning, unpublished data) and Rad51 (Liu and Fanning, unpublished data). In accordance with its interactions with these HR proteins, HDHB silencing leads to decreased DSB-induced HR (Liu and Fanning, unpublished data) and increased sensitivity to mitomycin C induced DNA damage (Liu and Fanning unpublished data), recovery from which depends on functional recombination machinery [170]. Endogenous HDHB was also found in protein complexes with the mismatch repair protein PMS1 by mass spectrometry analysis [171]. Interestingly, PMS1, one of the human homologs of mismatch repair protein MutL, is implicated in regulating HR [172]. Together, these findings strongly implicate HDHB function in DNA damage response.

Amplification and overexpression of genes with functions in the control of cell proliferation or DNA damage response are associated with tumorigenesis and drug resistance in cancer. Interestingly, HELB is among the highly amplified genes in osteosarcoma [173]. Furthermore, HDHB was found to be overexpressed in pancreatic cancer [174]. Although a direct causal relationship between tumorigenesis and HELB amplification or HDHB overexpression events is not established yet, accumulating evidence implicates HDHB at the intersection of replication and DNA damage response making HDHB functional studies intriguing in the context of cancer biology.

CHAPTER II

HUMAN DNA HELICASE B (HDHB) BINDS TO REPLICATION PROTEIN A AND FACILITATES CELLULAR RECOVERY FROM REPLICATION STRESS³

Introduction

DNA helicase activity is a vital component of all DNA transactions that require separation of the two strands of DNA, including DNA replication, DNA repair, and recombination. The abundant variety of DNA helicases, which greatly exceeds that of DNA polymerases, has hindered efforts to elucidate their functional role in DNA processing pathways, particularly in vertebrates. The conserved vertebrate DNA helicase B (HELB) was initially discovered in extracts of a temperature-sensitive mouse cell line as a thermolabile ATPase whose activity depended on single-stranded DNA (ssDNA) [157, 158, 175]. Subsequent biochemical studies revealed that the ATPase displayed ssDNA-dependent helicase activity [160, 168, 169]. More recently, analysis of mouse and human HELB cDNAs revealed their sequence homology and biochemical similarity to several prokaryotic superfamily 1B helicases that unwind DNA with 5'-3' polarity [122, 161, 176].

A potential role for HELB in chromosomal replication was initially suggested by studies of the mutant mouse cell line expressing temperature-sensitive HELB helicase

³ Bulk of this chapter was published in Guler GD*, Liu H*, Vaithiyalingam S, Arnett DR, Kremmer E, Chazin WJ and Fanning E. Human DNA Helicase B (HDHB) binds to replication protein A and facilitates cellular recovery from replication stress. *J. Biol. Chem.* In press. * Equal contribution.

activity: when shifted to the non-permissive temperature, the cells accumulated in early S-phase [169]. Consistent with this finding, microinjection of purified recombinant human HELB (HDHB) protein with a substitution in the Walker B motif, i.e. helicase-dead, into human cells in G1 inhibited DNA synthesis in up to 70% of the injected cells, whereas injection of the wild type protein did not [161]. Also of note, purified mouse and human HELB were found to interact functionally with purified DNA polymerase alpha-primase, displaying primosome activity on replication protein A (RPA)-coated ssDNA in vitro [160, 161]. These activities would be consistent with a role for HELB in initiation of chromosomal replication, in lagging strand synthesis, or possibly in recovery from DNA damage by re-priming the leading strand template downstream of forks stalled at a lesion [177, 178]. HDHB has also been identified in proteomic screens as a potential target of the ataxia telangiectasia-mutated (ATM) checkpoint kinase [74] and as part of a mismatch repair complex [171]. Taking these findings together, we reasoned that HDHB might function in chromosomal replication, perhaps at the interface of replication with repair, and set out to explore this possibility.

Here we demonstrate that in S phase cells exposed to replication stress, HDHB accumulates on chromatin in a checkpoint signaling independent, RPA-dependent manner. We identify in detail direct physical interactions of HDHB with RPA, which closely resemble those that recruit S phase checkpoint signaling proteins ATRIP and Rad9 to stalled forks. HDHB depletion does not disrupt activation of S phase checkpoint signaling, but instead slightly stimulates it. Moreover, HDHB depletion reduces viability of cells exposed to camptothecin, and increases chromosomal breaks and gaps in cells

exposed to aphidicolin. Based on these results, we propose that HDHB functions to counteract replication stress.

Experimental Procedures

Cell culture, synchronization, and genotoxin treatment

U2OS, HCT116, HeLa, and 293 Phoenix retroviral packaging cells were grown as monolayers in Dulbecco-modified Eagle medium supplemented with 10% fetal bovine serum at 37°C and 5% CO₂. U2OS cells were synchronized at G1/S by incubation for 17 h with 2.5 mM thymidine (Sigma-Aldrich), followed by a 12 h release, and another 17 h incubation with thymidine. Cells were released from the second thymidine incubation for 3 h into S phase and for 9 h into G2/M phase. To enrich U2OS cells in G1 phase, cells were cultured with 30 ng/ml nocodazole (Sigma-Aldrich) for 16 h and then released for 4 h. Cells were irradiated with UV-C at 254-nm in a Stratalinker (Stratagene).

Antibodies against HDHB.

Polyclonal rabbit antibodies were described previously [164]. To generate monoclonal antibodies, purified recombinant T7-tagged HDHB protein [161] (50 µg) was injected intraperitoneal (*i.p.*) and subcutaneously (*s.c.*) into LOU/C rats using CpG2006 (TIB MOLBIOL) as adjuvant. After 8 weeks, a boost of antigen was given *i.p.* and *s.c.* Three days later, fusion of P3X63-Ag8.653 myeloma cells with the rat spleen cells was performed according to standard procedures [179]. Hybridoma supernatants were tested in a solid phase immunoassay using T7-tagged HDHB protein adsorbed to polystyrene

microtiter plates. Crude *E. coli* extract served as a negative control. Hybridoma cells expressing mAb 4C11 and mAb 5C9 were stably subcloned and used to produce antibodies for further analysis (Figure 13A; Figure 19E, F). Rat IgG was purified using Melon gel IgG purification kit (Pierce) according to the manufacturer's instructions, and dialyzed into 25 mM HEPES-KOH pH 7.5 and 50 mM NaCl. Nonimmune rat IgG was purchased from Jackson ImmunoResearch.

Cell fractionation and western blotting.

To obtain whole cell extract, cells were lysed in RIPA buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 10 mM NaF, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, 1 mM leupeptin) on ice for 30 min, and centrifuged at 12,500 rpm for 15 min. Chromatin fractionation was performed as described [180] except that the final Triton X-100 concentration used for separation of cytoplasmic proteins from nuclei was 0.05% for U2OS or HCT116 and 0.1% for HeLa cells. Antibodies used for western blotting were: rabbit anti-HDHB [164], mouse monoclonal anti-RPA 70C or 34A [181], anti-Chk1 phospho-S317 (Cell Signaling), anti-RPA32 phospho-S4/S8 (Bethyl), total RPA32 (RPA2, Calbiochem), glyceraldehyde 3-phosphate dehydrogenase (Santa Cruz), mouse anti-PCNA (PC-10, Santa Cruz), mouse anti-tubulin (NeoMarkers or Santa Cruz), mouse anti-histone H1 (Santa Cruz), mouse anti-Chk2 (Upstate), rabbit anti-Chk2 phospho-T68 (Cell Signaling), mouse anti-Chk1 (Santa Cruz), rabbit anti-Chk1 phospho-S345 (Cell Signaling), mouse anti-FLAG antibody (Sigma-Aldrich), mouse anti-His antibody (Genscript). Rabbit polyclonal anti-GST and anti-Orc2 came from Fanning Lab stocks.

Fluorescence microscopy

To visualize FLAG-HDHB localization by microscopy, U2OS cells grown on coverslips were transfected with pFLAG HDHB plasmids diluted 1:5 with empty pFLAG-CMV2 vector for HDHB expression closer to physiological level. For staining, cells were pre-extracted with cytoskeleton buffer and Triton X-100 prior to fixation to visualize chromatin-bound proteins [182]. EdU was stained using Click-iT® EdU Alexa Fluor® 647 Imaging Kit (Invitrogen). Cells were then blocked with 5% FBS and 0.3% Triton X-100 in PBS for 1 h at room temperature. Antibodies were diluted in 1% BSA and 0.3% Triton X-100 in PBS. Primary incubations with rabbit anti-DYKDDDDK Tag Antibody (Cell Signaling), mouse anti-RPA2 (Calbiochem), mouse anti- γ H2AX phospho-S139 (Upstate) were done at 4°C overnight. Secondary antibody incubations with AlexaFluor 488 conjugated donkey anti-mouse (Invitrogen) and AlexaFluor 555 conjugated donkey anti-rabbit (Invitrogen) were done at room temperature for 1 h. Coverslips were mounted in ProLong Antifade (Molecular Probes). Images were taken on a Zeiss Z1 Axio-Observer Apotome microscope.

Recombinant proteins.

WT and mutant HDHB were purified from Hi5 insect cells as previously described [161]. Mutant 3xA HDHB was generated by site-directed mutagenesis in pFLAGWT HDHB plasmid [164] using mutagenic primers E499A (AGTTGGAAGAAAGAGCAGTAAAAAAGC CTG), D506A (AAGCCTGTGAAGCTTTTGAACAAGA), D510A

(GAAGATTTTGAACAAGCCCAGAATGCTTCAGAAG). Correct mutagenesis was confirmed by DNA sequencing. The 3xA HDHB coding sequence excised from the pFLAG vector using *NotI/SalI* was cloned into pFast-Bac HT (Sigma-Aldrich) using *NotI/XhoI*. Baculovirus for 3xA HDHB expression was generated in Sf9 cells using Bac-to-Bac Baculovirus expression system (Invitrogen).

HDHB truncation mutants were designed based on predicted secondary structure (psipred, predictprotein) [183, 184], disorder (DisEMBL) [185], and sequence conservation among vertebrate DHBs (T-Coffee) [162], and cloned in pET28 for bacterial expression of His-tagged proteins. Plasmids used for bacterial expression of His-tagged RPA truncation mutants were: pET15b-RPA70(1-120) for RPA70N [57] from Dr. C. Arrowsmith, pET15b-RPA32(172-270) for RPA32C [186], pET11d-RPA70(1-168) for RPA70N+L [187], from Dr. M. Wold, pET15b-RPA70(181-422) for RPA70AB [188] and pET15b-RPA70(436-616/32(43-171)/14 for RPA70C/32D/14 [189], both from Dr. A. Bochkarev. His-tagged RPA and HDHB truncation mutants were expressed in *E. coli* and purified over Ni-NTA column (Qiagen). GSTtagged WT and R41/43E mutant RPA70(1-120) constructs, kindly provided by Drs. D. Cortez and X. Xu [58], were expressed in *E. coli*, then purified over glutathione Sepharose beads (Sigma-Aldrich). Wild type human RPA was expressed from pET11d-WT RPA (from Dr. M. Wold) in *E. coli*, and purified as described [190].

Co-immunoprecipitation and GST pulldown assays.

For co-immunoprecipitations using FLAG M2 beads, extracts from cells transfected with FLAG-HDHB or control plasmid were lysed in 50 mM Tris HCl pH 7.4, 150 mM NaCl, 1 mM EDTA and 1% Triton X-100, and then incubated with FLAG M2 antibody agarose (Sigma-Aldrich) for 2 h. FLAG-HDHB that co-immunoprecipitated endogenous RPA was washed three times with FLAG IP wash buffer (50 mM Tris HCl pH 7.4 and 150 mM NaCl), 10 min each, and analyzed by western blotting. For FLAG coimmunoprecipitations with RPA truncation mutants, FLAG-HDHB-containing cell extracts were bound to FLAG M2 resin as above. The beads were washed with FLAG IP high salt wash buffer (50 mM Tris HCl pH 7.4 with 800 mM NaCl, and then with 1 M NaCl), and incubated with purified His-tagged RPA truncation mutants for 30 min in Tris HCl pH 7.4, 150 mM NaCl. Proteins bound to beads were analyzed by western blotting after three washes with FLAG IP wash buffer.

For other co-immunoprecipitations, protein A beads pre-bound to rabbit anti-HDHB antibody were incubated with purified HDHB for 1 h at 4°C in binding buffer (30 mM Hepes-KOH pH 7.8, 10 mM KCl, 7 mM MgCl₂) containing 2% milk. Beads were then washed with binding buffer and incubated with purified WT RPA or RPA truncation mutants for 30 min at 4°C. Beads were washed once with binding buffer, three times with wash buffer (30 mM Hepes-KOH pH 7.8, 75 mM KCl, 7 mM MgCl₂, 0.25% inositol, 0.01% NP-40, 10 μM ZnCl₂), once more with binding buffer, 10 min each, and bound proteins were analyzed by SDS-PAGE and western blotting.

For GST pull-downs, purified GST alone or GST-tagged WT or R41/43E RPA70N were allowed to bind to glutathione beads overnight in binding buffer with 2% milk. The beads were then incubated with purified HDHB truncation mutants or with whole cell extracts from cells expressing FLAG-tagged WT- or 3xA-HDHB for 30 min, washed, and analyzed as described above.

Helicase assay

M13mp18 circular ssDNA (USB) annealed to a 33-nucleotide DNA (5'-TCGACTCTAGAGGATCCCCGGGTACCGAGCTCG-3'), ³²P-radiolabeled at the 5' end, served as a partial duplex DNA substrate. Helicase reactions (10 µl) contained 20 mM Tris-HCl (pH 7.5), 8 mM DTT, 1 mM MgCl₂, 1 mM ATP, 20 mM KCl, 4% (w/v) sucrose, 80 µg/ml BSA, 8 ng of ³²P-labeled helicase substrate, and 6 to 24 ng of HDHB. In negative control reactions, ATP was omitted. Reactions were incubated at 37°C for 30 min. At the end of incubations, reactions were stopped by addition of 10 µl stop buffer (for a final concentration of 0.3% SDS, 10 mM EDTA, 5% glycerol, and 0.03% bromophenol blue). Samples were electrophoresed on 12% native polyacrylamide gels in 89 mM Tris borate, 2 mM EDTA. The wet gel was wrapped in plastic and exposed to a PhosphorImager screen for visualization and quantification. The average background density determined from no-enzyme and no ATP samples was subtracted from the unwound product values. Percentage of unwound DNA was calculated with the formula: % unwound = 100 x [product / (remaining DNA substrate + product)].

Isothermal titration calorimetry.

HDHB peptide (EQLEEREVKKACEDFEQDQNASEEW) was purchased (Genscript) and further purified by high-performance liquid chromatography to >90% purity. RPA70N and HDHB peptide were exchanged into 20 mM Tris (pH 7.2) 75 mM NaCl, and 2 mM β -mercaptoethanol. Binding affinity of RPA70N with HDHB peptide was measured using a MicroCal VP-isothermal titration calorimeter. Titration experiments were performed by first injecting 2 μ l of 1 mM HDHB peptide into 75 μ M of RPA70N in the sample cell, followed by additional 10 μ l injections. Data were analyzed using Origin software. Thermodynamic parameters and binding constant (K_d) were calculated by fitting the data to the best binding model using a nonlinear least-squares fitting algorithm.

NMR spectroscopy.

NMR experiments were performed using Bruker DRX 500-MHz or 600-MHz spectrometers equipped with cryoprobes. ^{15}N - ^1H heteronuclear single-quantum coherence (HSQC) spectra were acquired using 1024 complex points in the ^1H dimension and 128 complex points in the ^{15}N dimension. ^{15}N -enriched RPA70N sample was prepared at 100 μ M in a buffer containing 20 mM Tris (pH 7.5), 75 mM NaCl and 2 mM DTT. A series of ^{15}N - ^1H HSQC spectra were collected at RPA70N/HDHB peptide molar ratios of 1:0, 1:0.5, 1:1, 1:2, and 1:4. All spectra were processed by Topspin v2.0 (Bruker, Billerica, MA) and analyzed with Sparky (University of California, San Francisco, CA).

Gene silencing

The pRetro-Super (pRS) was kindly provided by R. Agami [191]. HDHB shRNA (CAGGTGCTTGGTGGAGAGT) and control shRNA (GACCCGCGCCGAGGTGAAG) were cloned into pRS. The pRS plasmids were then transfected into the retrovirus packaging cell line Phoenix 293 as described on the Nolan lab website (<http://www.stanford.edu/group/nolan>) with minor modifications. Briefly, cells were transfected with each pRS-derived plasmid and selected with 5 µg/ml puromycin (Sigma-Aldrich). To harvest virus, cells at 75% confluence were incubated for 16 h at 37°C. Collected media were passed through a 0.45-µm syringe filter (Pall Corporation). To obtain stable HDHB knock-down in HCT116, cells were infected with virus stock pre-incubated with 4 µg/ml polybrene (Sigma-Aldrich). After overnight incubation, cells were replated in growth medium, and selected in 5 µg/ml puromycin for 7–10 days.

For transient HDHB knock-down, HeLa cells were transfected with pGIPZ HDHB 33141 (shRNA1:GCAAGACTGTGATCTAATT) or 33143 (shRNA2:CCAGTTCTCAGTCATCTAA) (Open Biosystems) and selected with 3 µg/ml puromycin. Non-silencing pGIPZ was used as control. RPA70 siRNA (AACACUCUAUCCUCUUUCAUG) and control siRNA (AUGAACGUGAAUUGCUCAA) (Dharmacon) were transfected into HeLa cells exactly as described [58]. Transfections were done using Lipofectamine 2000 (Invitrogen) for HeLa cells and Fugene HD (Roche) for U2OS cells according to the manufacturer's protocol. Cells transiently silenced for HDHB or RPA70 were analyzed 72 h post-transfection.

Clonogenic survival assay.

Stably HDHB or control-silenced HCT116 cells were seeded in 60-mm dishes (~800 per dish). Cells were allowed to attach to the dish for 12 h, then treated in triplicate with different concentrations of CPT for 12 h, washed twice with PBS, and incubated in fresh growth medium for 10 days. Cell colonies were fixed and stained with 0.5% crystal violet in 70% ethanol. Visible colonies were counted. Experiments were repeated at least 3 times.

Chromosome analysis in metaphase spreads.

HCT116 cells stably silenced for HDHB or control-silenced were cultured in 100 mm dish to 30% confluence, followed by addition of aphidicolin (0.2 or 0.4 μ M) for 24 h, and then 100 ng/ml colcemid (Roche) for 2 h. Cells were trypsinized, washed once with PBS, resuspended in 10 ml pre-warmed 75 mM KCl, and incubated for 10 min at 37°C. Cells were collected by centrifugation (5 min, 800 rpm) and resuspended in 0.2 ml 75 mM KCl. To fix the cells, 5 ml prechilled acetic acid/methanol (1:3) was dropped into the cell suspension while vortexing, mixed immediately, and incubated for at least 30 min on ice. For staining, cells were collected by centrifugation, washed once with cold fresh fixative, then resuspended in fresh fixative (0.5 ml for ~10⁷ cells), and dropped onto wet cold slides (slides were kept in 70% ethanol at -20°C) on ice from ~10 cm height. Slides were air-dried, baked at 65°C for 2 h, and stained with 4% Giemsa in 10 mM phosphate buffer for 15 min. Slides were rinsed with water, dried, and mounted on cover slips with

Cytoseal 60 (Richard-Allan Scientific). Slides were observed under bright field microscope, and 100 cells, each with 45-46 chromosomes, per sample, were counted.

Results

DNA damage induces accumulation of HDHB on chromatin

To elucidate potential roles for HDHB in chromosomal replication, we reasoned that variation in its subcellular localization as a function of the cell cycle, in particular its association with chromatin, might correlate with its function. Since HDHB was easily detectable in whole cell extracts of U2OS, HeLa, and HCT116 tumor cells, but much less abundant in primary cells (Figure 13B), U2OS cells were biochemically fractionated using an established method (Figure 13C). U2OS cells were released from a nocodazole block, fractionated at 3-hour intervals, and proteins in each sample were analyzed in western blots (Figure 14A). Tubulin was detected in the soluble fraction and Orc2 in the chromatin fraction, as expected. Chromatin-bound PCNA was absent in G1, and began to accumulate in early S (6 h after release), as expected. HDHB was found mainly in the soluble fraction throughout the cell cycle, and the level of chromatin-bound HDHB remained very low, with a barely detectable increase in S phase (Figure 14A, lanes 5-8). Cells released from a double thymidine block displayed a similar subcellular distribution of HDHB (Figure 14B). The results indicate that unlike the authentic replication fork protein PCNA, the subcellular distribution of HDHB fluctuated little during the cell cycle.

To examine the possibility that the low level of chromatin-bound HDHB might function in DNA repair rather than in bulk DNA replication, asynchronously growing U2OS cells were treated with DNA damaging agents, biochemically fractionated and analyzed by western blot. Exposure to ultraviolet irradiation (UV), the topoisomerase I inhibitor camptothecin (CPT), or the ribonucleotide reductase inhibitor hydroxyurea (HU) induced accumulation of HDHB on chromatin (Figure 14C). The level of chromatin-bound HDHB correlated with the amount or duration of exposure to each genotoxin (Figure 15A-F). A similar increase in chromatin-bound HDHB was observed in HCT116 cells treated with UV, CPT or HU (Figure 14D). Chromatin-bound PCNA was clearly decreased in the CPT-treated cells (Figure 14D, lane 4), an observation consistent with the ability of CPT-topoisomerase I cleavage complexes to cause replication fork collapse [51]. All three agents induced a modest, but clearly detectable, increase in chromatin-bound RPA, suggestive of replication stress [55, 56, 58] (Figure 14D, compare lanes 2, 4, 6 with lanes 1, 3, 5).

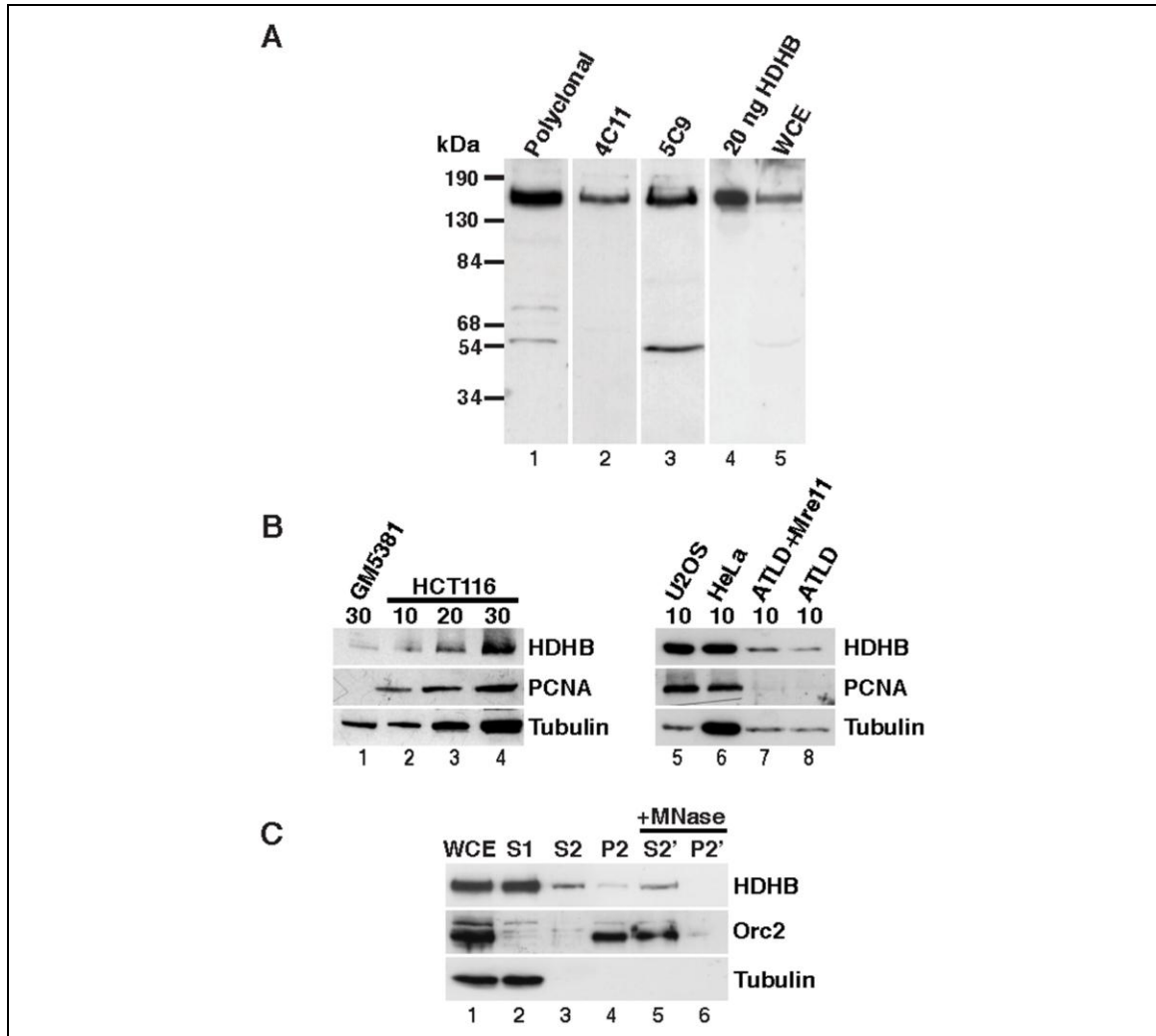


Figure 13. HDHB antibody specificity, HDHB expression levels and subcellular distribution.

(A) U2OS whole cell extract (WCE) (lanes 1-3, 5) and purified HDHB (lane 4) were analyzed by SDS-PAGE and western blot using polyclonal rabbit (lanes 1, 4, 5) and monoclonal rat antibodies (lanes 2, 3). (B) Whole cell extracts (10-30 μ g as indicated) from GM5381 (primary skin fibroblast) (lane 1), HCT116 (colorectal carcinoma cell line) (lanes 2-4), U2OS (osteosarcoma cell line) (lane 5), HeLa (cervical carcinoma cell line) (lane 6), ATLD+Mre11 (Mre11-complemented ATLD cells) (lane 7), ATLD (Mre11-deficient ataxia-telangiectasia-like disorder cells immortalized by hTERT expression) (lane 8) were separated by SDS-PAGE and analyzed by western blotting with antibodies against HDHB, tubulin, or PCNA, as indicated. (C) Whole cell extract (WCE), cytoplasmic (S1), nuclear soluble (S2), and chromatin (P2) fractions from asynchronously growing U2OS cells were prepared as described in Materials and Methods. In lanes 5, 6, nuclei were treated with micrococcal nuclease (MNase) before separating the nuclear soluble fraction from chromatin fraction. Proteins were visualized by western blotting with the antibodies indicated. Experiments in panels A, C and right panel of B were performed by Dr. Hanjian Liu.

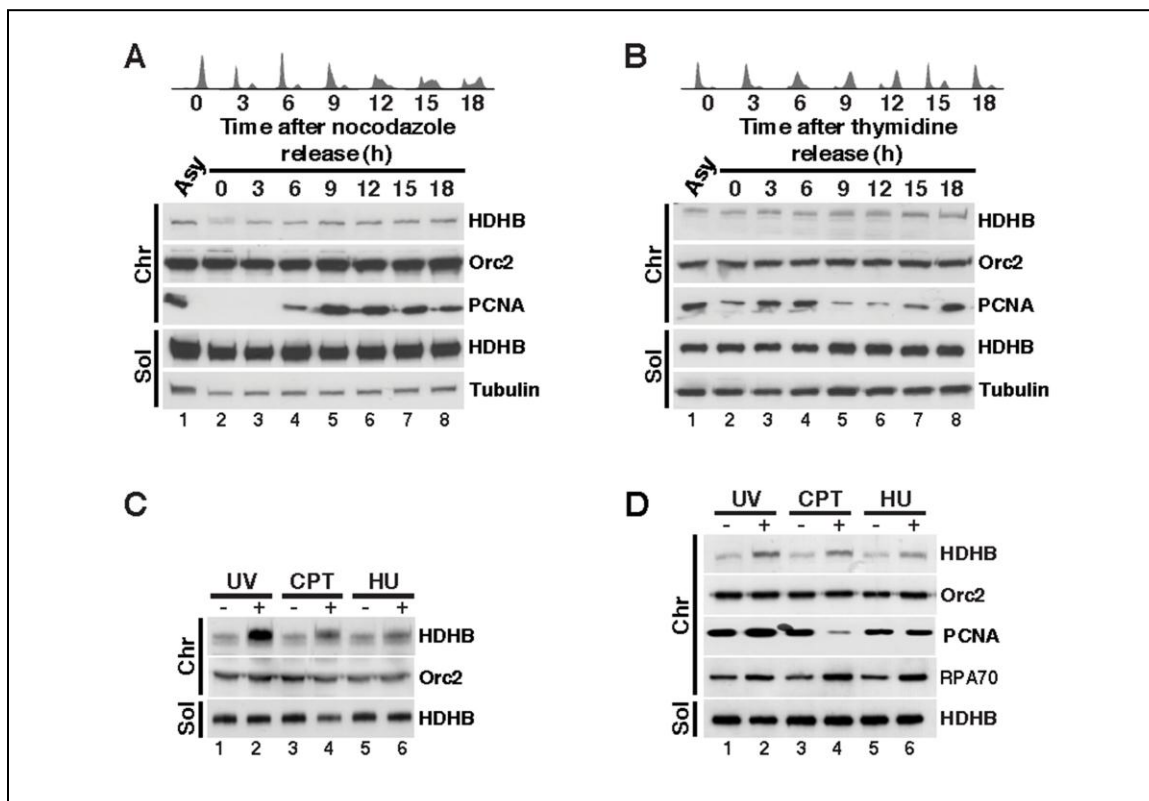


Figure 14. DNA damage induces HDHB accumulation on chromatin.

(A) U2OS cells were released from a nocodazole block for the indicated times and cell cycle distribution was characterized by flow cytometry (upper panels). Cells from each time point were separated into soluble (Sol) and chromatin fractions (Chr) (16) and analyzed by western blot with the indicated antibodies (lower panels). Fractions from asynchronous cells were analyzed in parallel (Asy). (B) U2OS cells were released from a double thymidine block for the indicated times and cell cycle distribution was characterized by flow cytometry (upper panels). Cells from each time point were separated into soluble (Sol) and chromatin fractions (Chr) (16) and analyzed by western blot with the indicated antibodies (lower panels). Fractions from asynchronous cells were analyzed in parallel (Asy). (C, D) Asynchronous U2OS (C) or HCT116 (D) cultures treated (+) with 100 J/m² UV, 10 μM camptothecin (CPT) or 5 mM hydroxyurea (HU) for 2 h, or left untreated (-), were fractionated as in (A, B) and analyzed by western blotting with the indicated antibodies. Experiments were performed by Dr. Hanjian Liu.

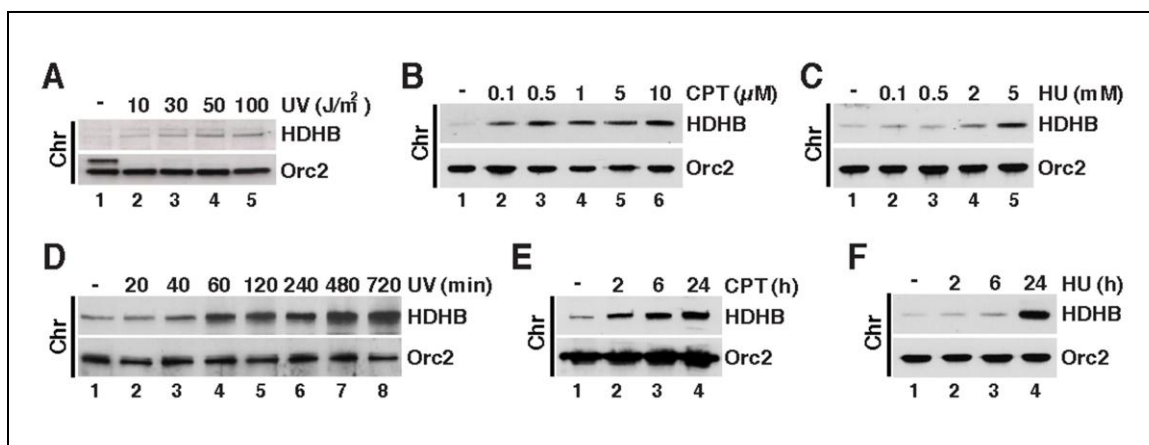


Figure 15. Damage-dependent accumulation of HDHB on chromatin.

(A-C) Asynchronously growing U2OS cells were treated with indicated doses of UV (A), CPT (2 h) (B), or HU (2 h) (C) as indicated, then fractionated and analyzed as described in Figure 14C. (D-F) Asynchronous cultures of U2OS cells were treated with 100 J/m² UV (D), 0.1 μM CPT (E), or 2 mM HU (F) for the indicated time periods, then fractionated and analyzed as described in Figure 14C. Panels A and D were performed by Dr. Hanjian Liu.

If the damage-induced accumulation of HDHB on chromatin were indeed associated with replication stress, one would expect to observe it preferentially in S phase cells. This prediction was tested by enriching U2OS cells in G1, early-mid S phase, or G2/M, then exposing them to HU, CPT, UV, or ionizing radiation (IR), followed by biochemical fractionation and western blot analysis. HU, CPT, and UV induced the accumulation of chromatin-bound HDHB almost exclusively in S phase cells, with little or no increase observed in G1 or G2/M cells (Figure 16AC). These findings are consistent with replication stress-induced accumulation of HDHB on chromatin. In addition, cells exposed to IR also displayed a modest induction of chromatin-bound HDHB in G1, S, and G2/M (Figure 16D, compare lanes 1, 3, 5 with lanes 2, 4, 6). IR-induced damage includes DNA double strand breaks, which undergo limited processing by nucleases to generate short stretches of ssDNA that facilitate repair [192-195]. Thus, chromatin structures generated in response to IR may display features in common with the extended stretches of RPA-coated ssDNA in replication-stressed chromatin. These findings indicate that HDHB accumulates on chromatin in response to replication stress, and to a lesser extent, in response to IR-induced damage.

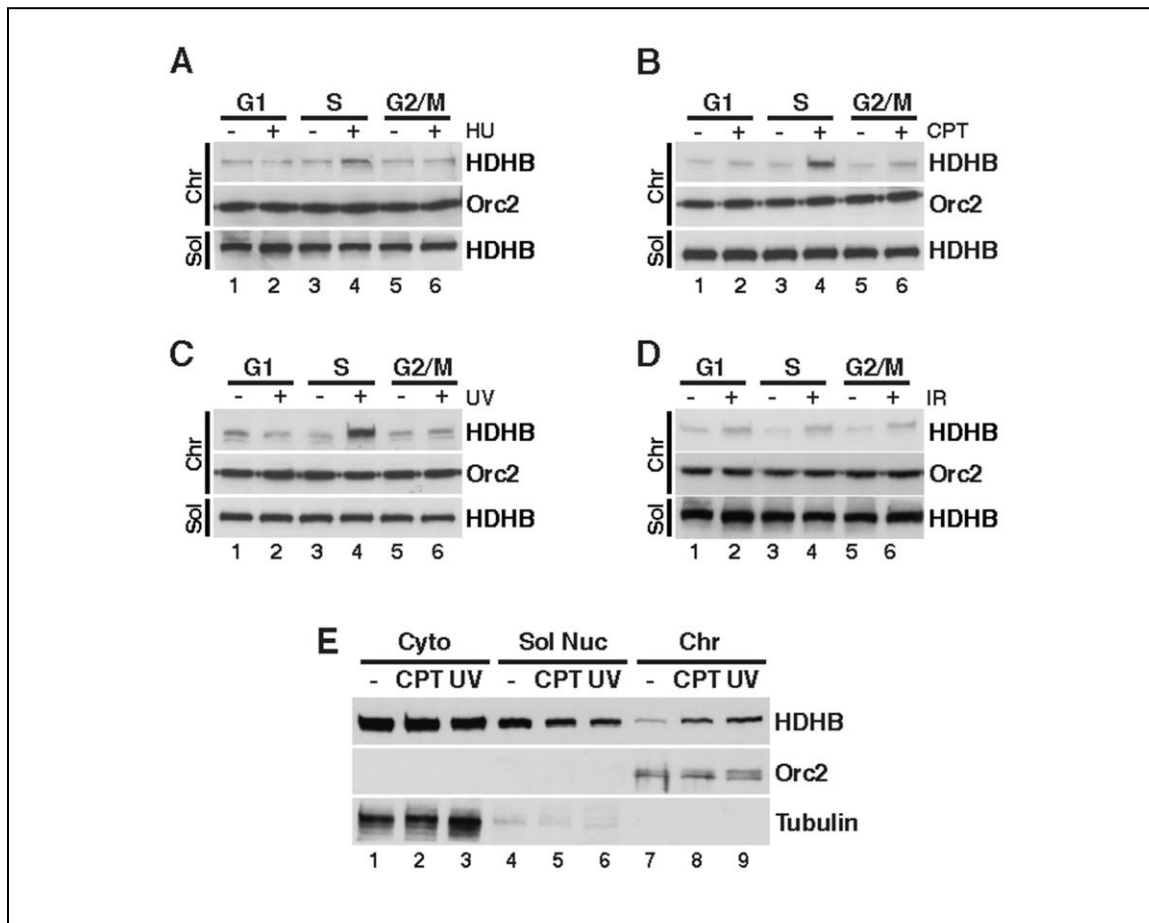


Figure 16. HDHB accumulates on chromatin in response to replication stress.

(A-D) U2OS cells enriched in G1, S, or G2/M phase as described in Materials and Methods were treated with (A) 5 mM HU, (B) 10 μ M CPT, or (C) 100 J/m² UV, or (D) 20 Gy ionizing radiation (IR), or left untreated (-) and analyzed as described in (Figure 14C). (E) U2OS cells synchronized in S phase and treated with UV or CPT as in Figure 14C, or left untreated, were extracted using digitonin in isotonic buffer to release cytosolic proteins as described previously (14). Nuclei were extracted to separate soluble nuclear from chromatin-bound proteins. Fractions were analyzed by western blotting with the indicated antibodies. Experiments were performed by Dr. Hanjian Liu.

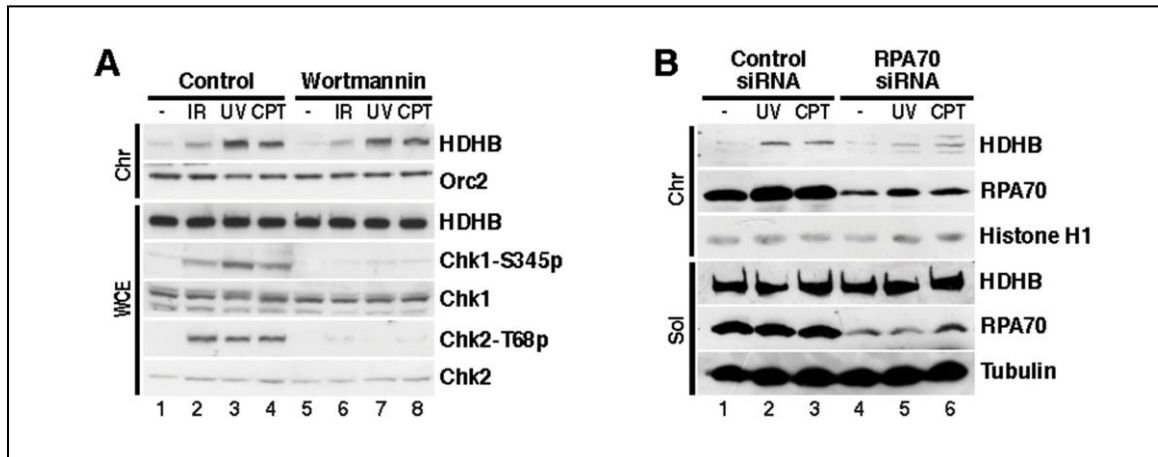


Figure 17. Replication stress-induced redistribution of HDHB does not require checkpoint signaling, but correlates with the level of RPA on chromatin.

(A) U2OS cells were pre-treated with DMSO (control) or 200 μ M wortmannin for 30 min, and then exposed to 20 Gy IR, 100 J/m² UV, or 10 μ M CPT for 2 h. Whole cell extracts (WCE) or chromatin fractions (Chr) were analyzed by western blotting with the indicated antibodies. Experiment was performed by Dr. Hanjian Liu. (B) HeLa cells transiently transfected with RPA70 siRNA or control siRNA were treated with UV or CPT as in Figure 14C, or left untreated (-) as a control. Soluble (Sol) and chromatin (Chr) fractions (16) were analyzed by western blotting with the indicated antibodies.

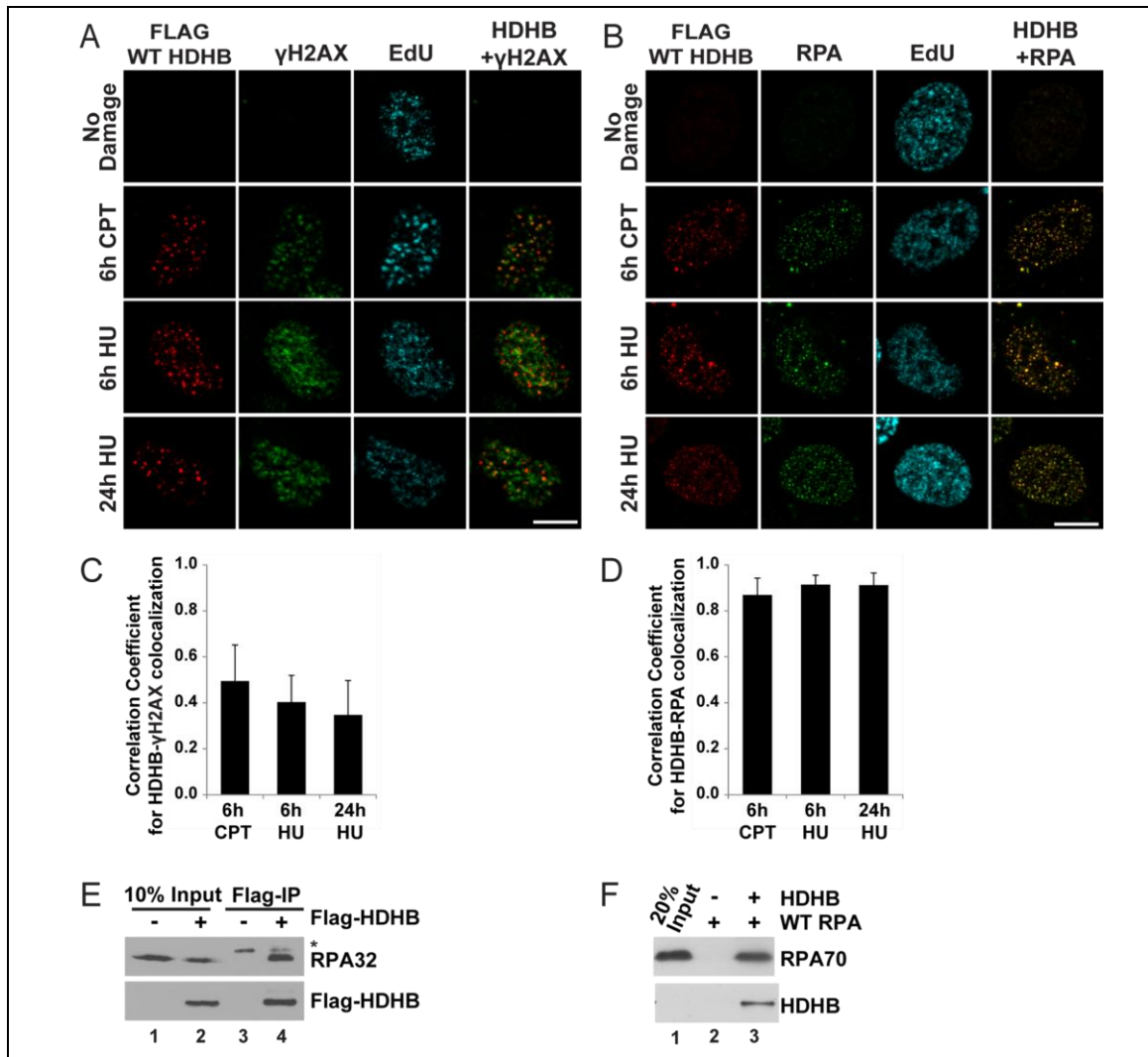


Figure 18. HDHB co-localizes and associates with RPA.

(A, B) U2OS cells transiently expressing FLAG-tagged WT HDHB were pre-labeled with EdU for 20 min, and treated with 1 μ M CPT for 6 h or 2 mM HU for 6 h or 24 h, or left untreated as control (No Damage). Cells were then pre-extracted to remove soluble proteins, fixed, and stained to visualize EdU and chromatin-bound FLAG, γ H2AX (A) or RPA32 (B). Scale bar, 10 μ M. (C, D) Pearson correlation coefficients for HDHB co-localization with γ H2AX (C) or RPA (D) were calculated in 19-22 cells for each sample using ImageJ software. (E) Whole cell extracts (WCE) prepared from HeLa cells transiently transfected with pFLAG-HDHB (+) or empty pFLAG (-) were incubated with anti-FLAG-M2 agarose beads. Proteins bound to the beads were analyzed by SDS-PAGE and western blotting with anti-FLAG or monoclonal anti-RPA antibody 34A as indicated. * Non-specific band. (F) Purified WT heterotrimeric RPA was incubated in the absence (-) or presence (+) of purified HDHB as indicated with Protein A-beads pre-bound to anti-HDHB antibody. Proteins bound to the beads were analyzed by SDS-PAGE and western blotting with monoclonal RPA70C and rabbit anti-HDHB.

This DNA damage-induced accumulation of chromatin-bound HDHB could reflect a redistribution of soluble HDHB to chromatin or an increased level of total HDHB after damage. To distinguish between these possibilities, S phase cells that had been treated with CPT, UV, or left untreated were biochemically fractionated with a different protocol to generate separate cytosolic, soluble nuclear, and chromatin-associated protein fractions. We found that the soluble nuclear fractions from CPT- and UV-treated cells contained less HDHB than did that from control cells (Figure 16E, top row, compare lane 4 with lanes 5, 6). Conversely, the chromatin fractions from the CPT- and UV- treated cells contained more HDHB than that from untreated cells (Figure 16E, lanes 7-9). The level of HDHB in the cytosolic fraction of CPT- or UV-treated cells was not detectably different than that of untreated cells (compare lane 1 with lanes 2, 3). The results are most consistent with a replication stress-induced recruitment of soluble nuclear HDHB to chromatin.

Requirements for HDHB recruitment to chromatin

To determine the requirements for HDHB recruitment to chromatin in response to replication stress, we first considered that phosphoinositide-3 kinase-related protein kinases (PIKK) ATM, ATR, and DNA-PK activated by DNA damage might recruit HDHB to chromatin. Consistent with this possibility, HDHB contains several predicted PIKK phosphorylation sites and was identified by mass spectrometry as a target for ATM/ATR after DNA damage [74]. To test for a possible role for PIKK activity in recruitment of HDHB to chromatin, we briefly treated cells with wortmannin, a broad-spectrum inhibitor of PIKK family kinases [196] before exposing them to DNA

damaging agents. As expected, exposure to IR, UV, and CPT resulted in robust phosphorylation of Chk1 serine 345 and Chk2 threonine 68 in control cells, with little effect on total Chk1 or Chk2 (Figure 17A, compare lane 1 with lanes 2-4) and checkpoint signaling was strongly inhibited in the presence of wortmannin (compare lane 5 with lanes 6-8). Importantly, HDHB recruitment to chromatin after genotoxin treatment was virtually identical in the presence and absence of checkpoint signaling (Figure 17A, top row, compare lanes 1-4 with lanes 5-8). Thus inhibition of PIKK activity does not reduce DNA damage-induced recruitment of HDHB to chromatin.

Replication stress-induced recruitment of S phase checkpoint proteins, e.g. ATRIP, to chromatin depends on their ability to bind to the RPA-coated ssDNA that accumulates at sites of DNA damage [55, 56, 58]. To test the possibility that damage-induced recruitment of HDHB may be mediated by RPA, HeLa cells transiently depleted of RPA70 were exposed to UV or CPT and then biochemically fractionated. The level of RPA70 in the soluble and chromatin fractions was substantially lower in RPA-silenced cells than in control-silenced cells, validating the knock-down (Figure 17B, rows 2 and 5, compare lanes 1-3 with lanes 4-6). Consistent with published evidence [58], RPA70 depletion slightly increased the fraction of cells in S phase, and reduced S phase checkpoint signaling. Also as expected, the level of RPA70 on chromatin increased in response to UV and CPT, both in control-silenced and in RPA-silenced cells (Figure 17B, row 2, compare lanes 2, 3 with lane 1, and lanes 5, 6 with lane 4). RPA70 silencing did not affect the level of HDHB in the soluble fraction (row 4, compare lanes 1-3 with 4-6). Importantly, however, the level of HDHB recruited to chromatin after UV- and CPT treatment in RPA70-silenced cells was lower than in control-silenced cells (Figure 17B,

top row, compare lanes 2, 3 with lanes 5, 6). Thus HDHB recruitment to chromatin in response to replication stress correlates with the level of chromatin-bound RPA.

The recruitment of soluble nuclear HDHB to chromatin in response to replication stress, the dispensability of PIKK activity for this recruitment, and the correlation of HDHB recruitment with the RPA level on chromatin led us to question whether HDHB recruitment to chromatin might be mediated by RPA. To assess this possibility, we first asked whether it co-localizes with the DNA damage marker γ H2AX or the RPA that accumulates at stalled forks [55]. Since none of our anti-HDHB antibodies was capable of staining the endogenous protein in immunofluorescence microscopy, FLAG-tagged HDHB was expressed in human cells. S-phase cells were marked by pulse-labeling with the thymidine analog EdU, then exposed to CPT or HU, or left untreated as a control. After pre-extraction of soluble proteins, chromatin-bound HDHB and γ H2AX (Figure 18A) or RPA (Figure 18B) were visualized by immunofluorescence microscopy. Exposure to HU or CPT resulted in nuclear foci of FLAG-HDHB, γ H2AX and RPA. FLAG-HDHB co-localized significantly with RPA foci (Figure 18B and D) and partially with γ H2AX (Figure 18A and C), similar to a pattern previously observed for ssDNA and RPA co-localization with γ H2AX after replication stress [152, 197]. These results suggest that HDHB localizes to ssDNA accumulated following replication fork stalling.

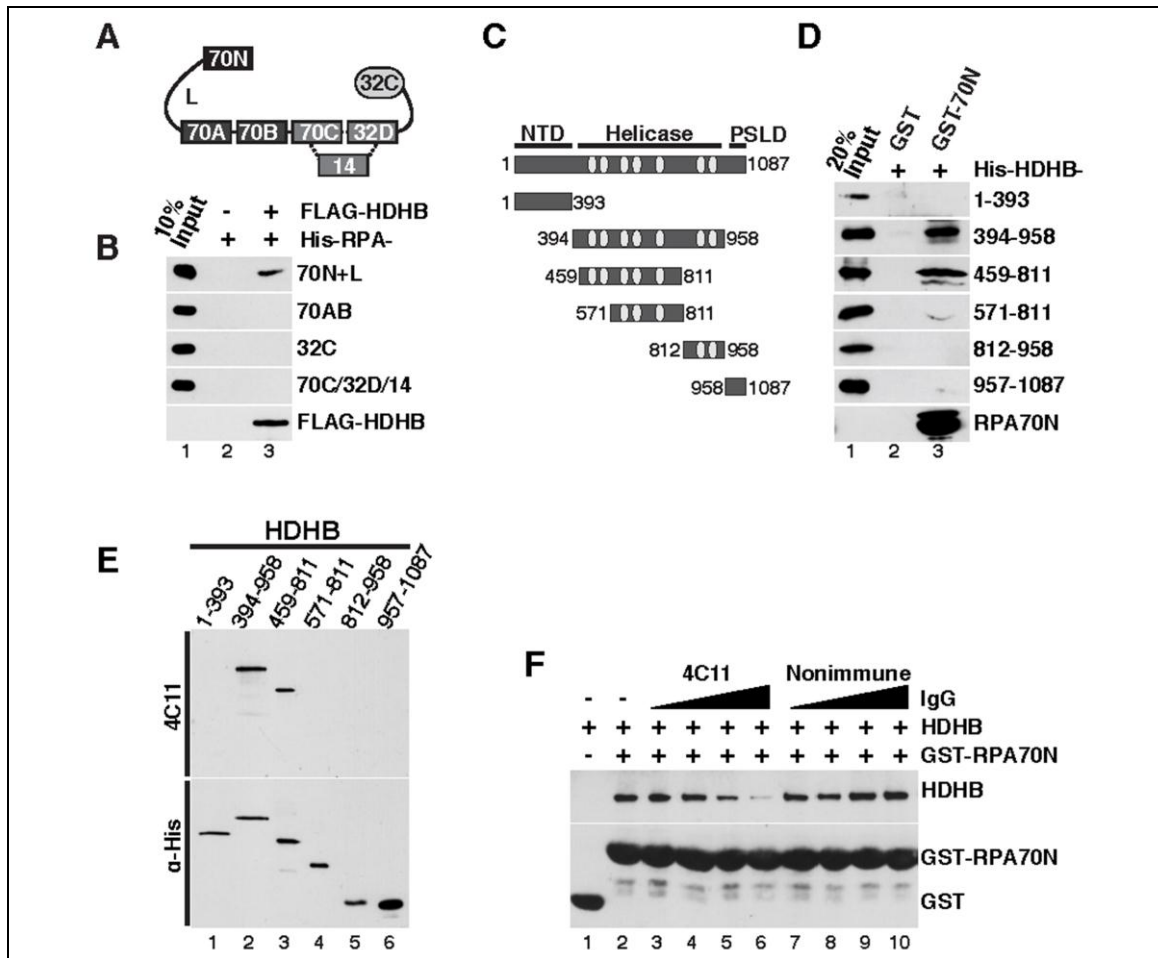


Figure 19. Direct physical interaction of HDHB with RPA.

(A) Modular domain architecture of RPA. The three subunits RPA70, 32, and 14 interact through a threehelix bundle (dotted lines). Compact domains (OB-folds, filled rectangles; winged helix, filled oval) joined by flexible linkers (L, lines) [198]. (B) Purified His-tagged RPA constructs captured on anti-FLAG antibody beads in the presence (+) or absence (-) of whole cell extract as in (A) were analyzed by western blotting with anti-His (top four panels) or anti-FLAG. (C) Diagram of HDHB domains and truncation constructs. NTD, N-terminal domain; PSLD, phosphorylation-regulated subcellular localization domain [164]. The seven conserved helicase motifs I, Ia, and II–VI of superfamily I are indicated by light gray ovals. The first and last residue numbers of each construct are indicated. (D) Glutathione beads pre-bound to purified GST or His-GST-RPA70N were incubated with purified His-tagged HDHB truncation mutants. Proteins captured on the beads were analyzed by western blotting with anti-His antibody. (E) His-tagged HDHB truncation mutants (E) were separated by SDS-PAGE and visualized by western blotting performed with HDHB monoclonal antibody 4C11 (top panel) or anti-His antibody (lower panel). (F) Glutathione beads pre-bound with GST (lane 1) or GST-RPA70N were incubated with purified HDHB (0.5 μ g) in the absence (lanes 1 and 2) or presence of increasing amounts (0.3, 0.6, 1.2, 2.4 μ g) of monoclonal IgG 4C11 (lanes 3-6) or of non-immune rat IgG (lanes 7-10).

If chromatin-bound RPA were directly responsible for recruiting soluble HDHB to chromatin, one would expect HDHB to interact physically with RPA. Consistent with this prediction, endogenous RPA was co-precipitated with FLAG-HDHB from extracts of cells transiently expressing FLAG-HDHB, but not FLAG-vector (Figure 18E, compare lanes 3 and 4). Importantly, purified RPA bound to anti-HDHB antibody beads in the presence of purified recombinant HDHB (Figure 18F, lane 3), but not in its absence (lane 2), demonstrating a direct physical interaction between the two proteins.

HDHB interacts specifically with the N terminal region of RPA70

Detailed biochemical mapping and structural analysis of the RPAHDHB interaction was then pursued in order to more fully define the molecular basis for HDHB localization to replication-stressed chromatin. RPA interacts with partner proteins utilizing four of its seven structural domains: the N-terminal domain of RPA70 (RPA70N), the tandem high affinity ssDNA binding domains A and B of RPA70 (RPA70AB), and the C-terminal domain of RPA32 (RPA32C) [198-202] (Figure 19A). To map the HDHB binding site(s) in RPA, purified His-tagged RPA domains were added to FLAG antibody beads pre-incubated with control or FLAG-HDHB extracts and RPA domains captured on the beads were detected by immunoblotting with anti-His antibody. Under these conditions, HDHB interacted specifically with RPA70N, but not with RPA70AB, RPA32C or the trimerization core RPA70C/32D/14 (Figure 19B).

RPA70N serves as a chromatin recruitment domain for DNA damage response proteins [56, 58], raising the possibility that HDHB might be recruited to RPA-ssDNA by

docking with RPA70N. To search for a specific RPA70Ninteracting region in HDHB, we first designed HDHB fragments using tools for secondary structure, disorder, and fold prediction, as described in Methods (Figure 19C), and expressed them as His-tagged polypeptides in *E. coli*. The purified His-tagged HDHB fragments (Figure 19D, lane 1) were incubated with glutathione beads bound to GST or GST-RPA70N. HDHB residues 394-958 and 459-811 bound specifically to GSTRPA70N beads, but not to GST (lanes 2, 3). Other HDHB polypeptides did not bind to either GST or GST-RPA70N, demonstrating a specific interaction between HDHB 459-811 and RPA70N. Since this same fragment of HDHB was also recognized by the monoclonal antibody 4C11 (Figure 19E; Figure 13A), we asked whether the antibody might compete with RPA70N to bind HDHB. Interestingly, pre-incubation of HDHB with increasing concentrations of 4C11 IgG inhibited binding of HDHB to GST-RPA70N, whereas non-immune control IgG had no effect (Figure 19F). We conclude that the HDHB residues 459-811 are sufficient to interact directly and specifically with RPA70N.

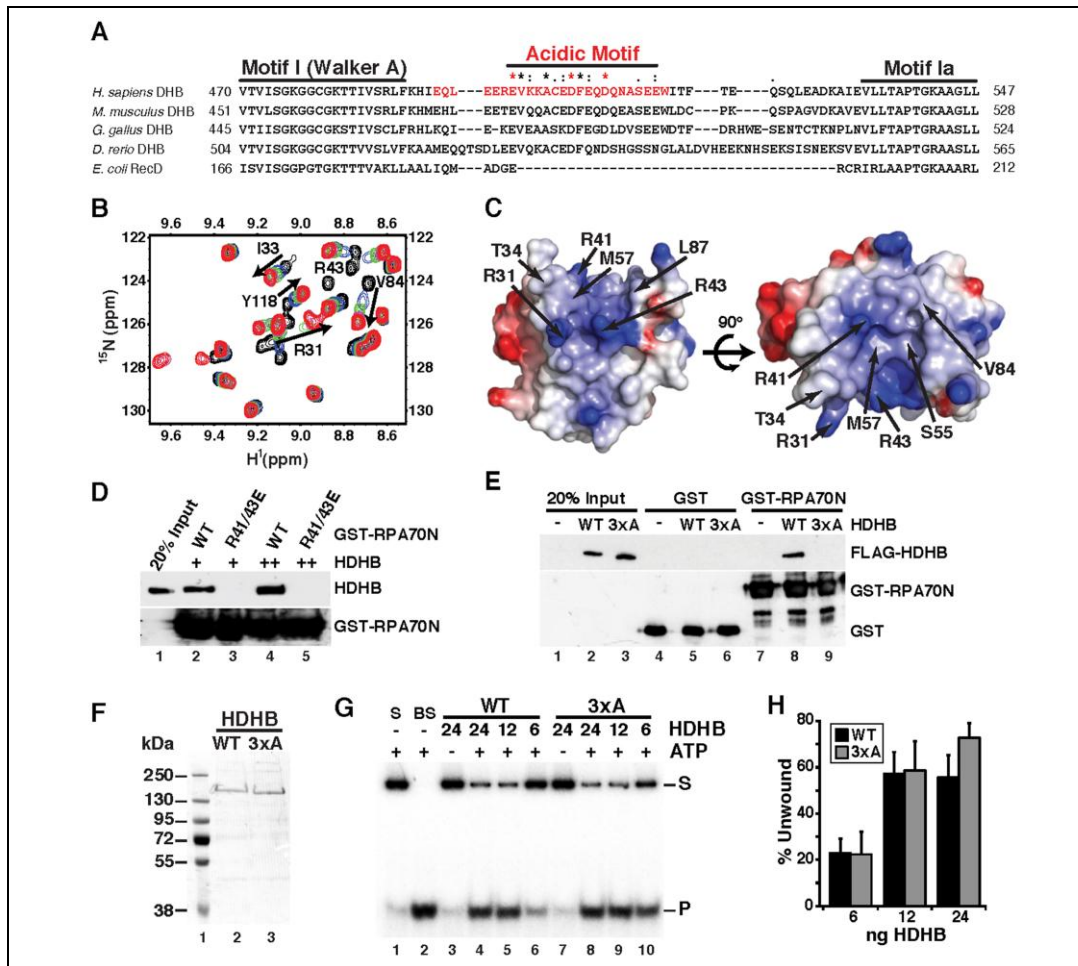


Figure 20. The basic cleft of RPA70N physically interacts with a conserved acidic motif in HDHB.

(A) Sequence alignment of HELB from four vertebrate species and *E. coli* RecD. Consensus sequence in the acidic motif is based on all available vertebrate HELB sequences: Star (*), identical; colon (:), conserved; dot (.), semi-conserved residues. Red asterisk, residues substituted by alanine to generate the HDHB 3xA mutant (see E below). Red font, a synthetic HDHB peptide E493-W517 used in NMR (B, C) and ITC (Figure 21A) experiments. (B) Overlaid ^{15}N - ^1H -HSQC spectra of RPA70N in the absence (black) and presence of HDHB peptide at 1:0.5 (blue), 1:1 (green), 1:2 (pink), and 1:4 (red) molar ratios. The black arrows show chemical shift perturbations that result from binding of RPA70N with HDHB peptide. (C) Molecular surface diagram of RPA70N with the significantly perturbed residues labeled. Red, acidic residues; blue, basic residues. (D) GST-pulldown assays of purified full length HDHB with wild type (WT) or mutant (R41/43E) GSTRPA70N. Bound proteins were detected by immunoblotting with anti-HDHB (upper panel) or anti-GST (lower panel). (E) Extracts from HeLa cells transiently expressing FLAG-WT or -3xA HDHB were mixed with glutathione beads pre-bound to GST or GST-RPA70N. Proteins bound to the beads were analyzed by SDS-PAGE and immunoblotting with anti-FLAG (upper panel) or anti-GST (lower panel) antibodies. (F) Purified His-tagged WT and 3xA HDHB were analyzed by SDS-PAGE and stained with Coomassie. (G, H) Helicase activity of purified His-tagged WT and 3xA HDHB proteins was assayed as described [161] using a radiolabeled partial duplex DNA substrate (S) and the indicated amounts of purified HDHB. Helicase activity was visualized by phosphorimaging and quantified (lanes 4-9) after subtraction of radiolabeled background (product band P) detected in the DNA substrate (lane 1) and in the absence of ATP (lanes 3 and 7). BS, boiled substrate. Brackets show standard error of the mean ($n \geq 3$). Panels B and C were performed by Dr. Sivaraja Vaithiyalingam.

A conserved acidic motif in HDHB interacts physically with the basic cleft of RPA70N

The RPA70N-interacting surfaces of p53, ATRIP, Rad9 and Mre11 have been mapped to an acidic stretch of residues in each protein [58]. We used this information, together with the mapping data in Figure 19 and amino acid sequence alignment of HDHB-related helicases, to search for potential RPA70N-binding motifs. The search revealed a phylogenetically conserved acidic peptide, residues 493-517, inserted between the superfamily 1B helicase motifs I and Ia (Figure 20A). This sequence motif is absent in the corresponding region of *E. coli* RecD, the prototype member of helicase superfamily 1B, implying that it may not be necessary for helicase activity and might have another role. To investigate the interaction of this region of HDHB with RPA70N, we designed a synthetic peptide that contains the conserved acidic residues of HDHB. Isothermal titration calorimetry (ITC) experiments were performed to measure the affinity of interaction between RPA70N and the peptide. The binding isotherm was fit with a single site binding model and resulted in a K_d of $15 \pm 0.05 \mu\text{M}$ (Figure 21A).

In order to map the specific binding surface of the HDHB peptide on RPA70N, NMR chemical shift perturbation experiments were performed on ^{15}N -enriched RPA70N. The series of ^{15}N - ^1H HSQC spectra acquired with an increasing concentration of unlabeled peptide added into the solution revealed perturbations to a select number of peaks in the spectrum. This observation indicates that the binding interface between RPA70N and the peptide is specific (Figure 20B). The disappearance of signals at a sub-stoichiometric molar ratio of peptide to RPA70N is consistent with the $15 \mu\text{M}$ K_d estimated by ITC. Analysis of the data showed that, in addition to the RPA70N basic residues (R31, R41, and R43), hydrophobic residues (I33, Y42, L44, F56, M57, L58,

A59, V84, L87, I95, L96, and L99) are involved in binding with HDHB (Figure 20B, C; Figure 21B). Mapping of these residues onto the structure of RPA70N reveals that HDHB binds to the basic cleft of the OB-fold domain (Figure 20C). This binding surface in RPA70N resembles that recognized by p53, Rad9, ATRIP, and Mre11 [58, 203, 204].

The importance of the RPA70N basic cleft and the HDHB acidic motif in the binding interaction was then tested in pull-down experiments with wild type and mutant proteins. GST-RPA70N interacted with HDHB as expected, and charge reverse substitutions in the basic cleft of GST-RPA70N (R41/43E) abolished HDHB binding (Figure 20D). A 3xA mutant form of FLAGHDHB with alanine substitutions in HDHB acidic residues E499, D506 and D510 was generated to test the role of this motif in binding to RPA70N. Full length FLAG-HDHB WT was pulled down by GST-RPA70N, but FLAG-HDHB 3xA as not (Figure 20E). The results confirm the roles of the acidic motif in HDHB and the basic cleft of RPA70N in the interaction.

We purified recombinant WT and 3xA HDHB proteins to assess the specificity of the 3xA substitution on HDHB loss of function (Figure 20F). The 3xA and WT HDHB displayed comparable helicase activity (Figure 20G, H), demonstrating that the 3xA substitution did not perturb the helicase domain stability, overall fold, or activity, but specifically impaired the ability of HDHB to bind to RPA70N. Altogether, these results establish a direct physical interaction between the HDHB acidic motif and the RPA70N basic cleft.

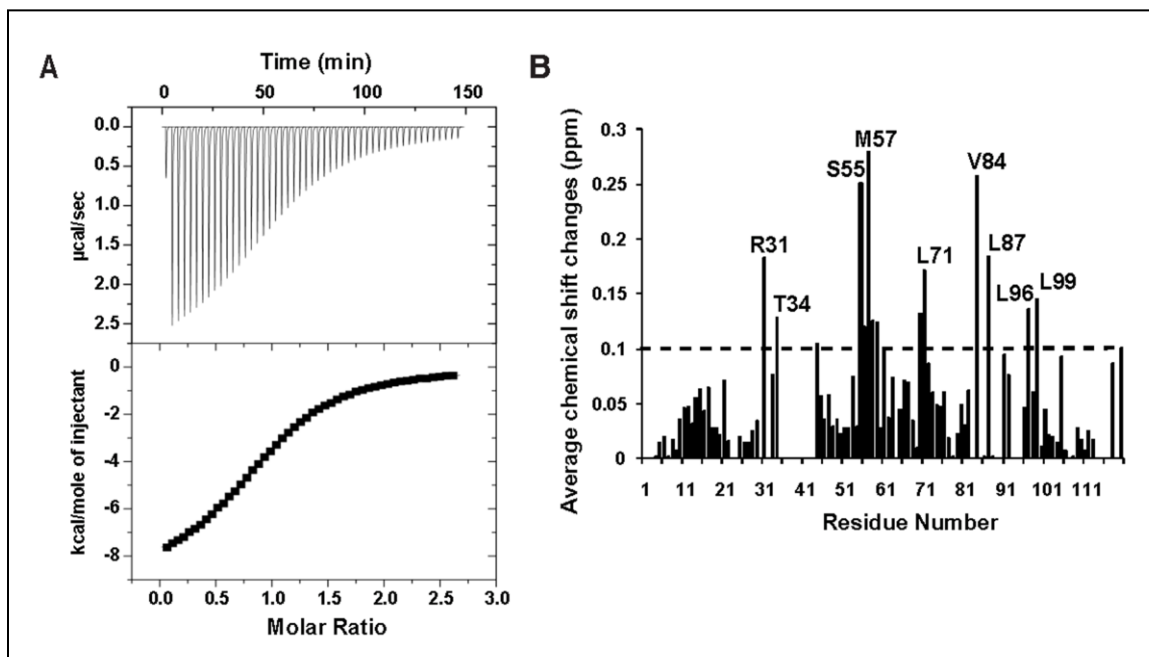


Figure 21. Analysis of the binding of HDHB peptide to RPA70N.

(A) The binding isotherm of interaction between RPA70N and HDHB peptide showing the heat changes (upper) and the integrated heat changes that fit with a single site binding model (lower). K_d $15 \pm 0.05 \mu\text{M}$. (B) The plot of the NMR chemical shift perturbations in RPA70N induced by the binding of HDHB (residues 493-517). The dashed line indicates one standard deviation above the mean. The absence of a bar for a given residue indicates that the peak for this residue was not assigned or that the peak disappeared upon the binding of the peptide. Experiments were performed by Dr. Sivaraja Vaithiyalingam.

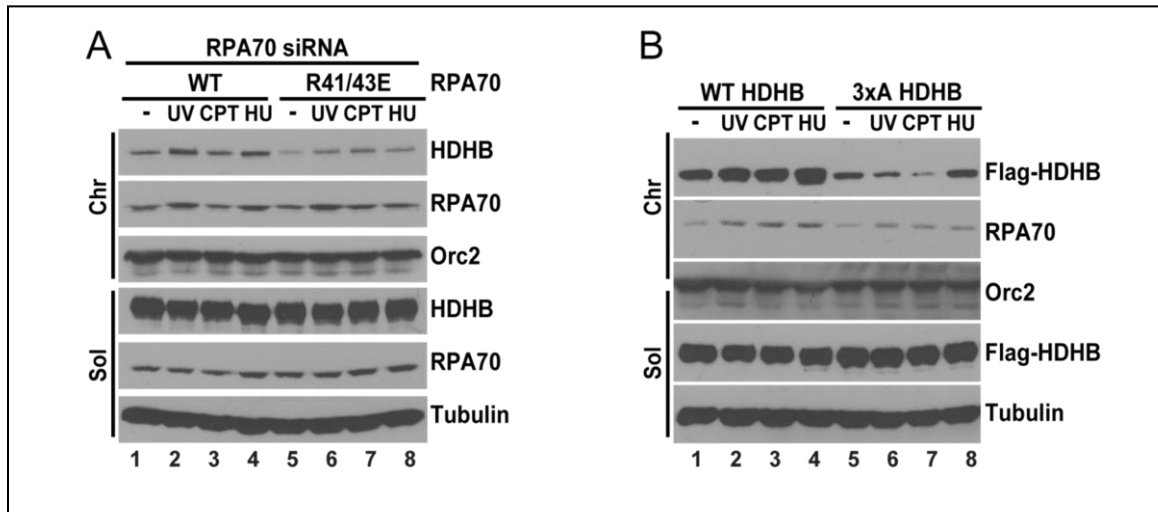


Figure 22: RPA70N-HDHB interaction interface is important for efficient chromatin recruitment of HDHB in response to DNA damage.

(A) HeLa cells transfected with RPA70 siRNA with silencing-resistant WT or R41/43E RPA70 expression plasmids were treated with the indicated DNA damaging agents as in Figure 17, or left untreated (-) as a control. Soluble (Sol) and chromatin (Chr) fractions [180] were analyzed by western blotting with the indicated antibodies. (B) HeLa cells transiently expressing FLAG-tagged WT or 3xA HDHB were treated with UV, CPT, or HU, fractionated, and analyzed by western blotting with anti-FLAG, anti-RPA70, anti-Orc2, or anti-tubulin as indicated.

RPA70N -HDHB interaction surface promotes damage-dependent chromatin recruitment of HDHB

RPA70N basic cleft is known to function as a recruitment scaffold for several DNA damage response proteins including ATRIP and Rad9 [57, 58]. The striking similarity in HDHB-RPA70N binding to RPA70N interactions with ATRIP and Rad9 prompted us to ask whether RPA70N-HDHB interaction can play a role in damage-induced HDHB recruitment to chromatin. To test the contribution of RPA70N basic cleft to HDHB recruitment, cells co-transfected with RPA70 siRNA and silencing-resistant variants of both R41/43E and WT RPA70 were treated with genotoxins and then fractionated as described in Figure 17. WT and R41/43E RPA70 were recruited to chromatin at comparable levels after UV, CPT and HU treatment (Figure 22A). However, cells expressing R41/43E RPA70 recruited less HDHB to chromatin than did the WT RPA70 expressing cells, implicating RPA70N basic cleft in damage-induced recruitment of HDHB to chromatin.

If damage-induced HDHB recruitment defect observed in RPA silenced (Figure 17B) or R41/43E RPA70 expressing cells (Figure 22A) is due to a requirement for a direct interaction between HDHB and RPA at DNA damage sites, HDHB acidic motif mutations 3xA, which perturbed RPA70N interaction (Figure 20E), should also disrupt HDHB recruitment to chromatin after DNA damage. To determine the potential contribution of the HDHB acidic motif in damage-induced HDHB recruitment to chromatin, asynchronously growing cells transiently expressing FLAG-WT or -3xA HDHB were exposed to UV, CPT or HU, biochemically fractionated as in Figure 17, and proteins in each fraction were analyzed by immunoblotting. As expected, both FLAG-

WT HDHB and RPA accumulated on chromatin after exposure to UV, CPT, and HU (Figure 22B, lanes 1-4). RPA accumulated on chromatin also in damaged cells expressing FLAG-3xA HDHB (lanes 5-8). Despite similar protein levels of WT- and 3xA-HDHB in soluble fractions, chromatin fractions from undamaged control cells contained less 3xA-HDHB than WT-HDHB. Furthermore, UV-, CPT- or HU-induced accumulation of FLAG-3xA HDHB on chromatin was reduced (lanes 5-8). Interestingly, we noted that after UV and CPT exposure, 3xA HDHB bound to chromatin actually decreased compared to undamaged control sample. This surprising result can be explained if HDHB normally travels with the fork and dissociates from chromatin upon CPT- or UV-induced fork collapse, as observed for other replisome components [102, 208], while the disruption of RPA70N interaction in 3xA hinders damage induced-recruitment. Altogether, our results suggest that RPA70N-HDHB interaction surface is important for efficient HDHB recruitment to chromatin after DNA damage.

HDHB depletion reduces recovery from replication stress

The evidence presented above demonstrates a replication stress-induced, RPA-dependent, PIKK activity-independent redistribution of soluble nuclear HDHB to chromatin in tumor cell lines, together with the specific, direct physical interaction between the RPA70 basic cleft and a conserved peptide in HDHB. These findings would be consistent with the hypothesis that the unidentified role of HDHB in replication may lie in mitigating replication stress. To establish a basis to address this hypothesis, shRNAs H1 and H2, targeting two different HDHB sequences, were expressed in HeLa cells and selected for co-expression of puromycin resistance (Figure 23A). Comparison

of HDHB- and control-silenced cells by two-dimensional flow cytometry revealed that the substantial reduction in HDHB levels had little effect on cell cycle distribution in the absence of overt damage (Figure 23B).

Based on the RPA-dependence of HDHB recruitment in response to replication stress (Figure 17B, Figure 22A) and the specific interaction of HDHB with the basic cleft of RPA70N (Figure 19, Figure 20), we first considered the possibility that HDHB recruitment to chromatin might be important for activation of checkpoint signaling. To assess this possibility, HDHB- or control-silenced HeLa cells were exposed to HU to induce replication stress, or left untreated. Analysis of whole cell extracts by western blotting confirmed HDHB silencing (Figure 23C, lanes 2, 3, 5, 6). We observed robust induction of phospho-Chk1 and N-terminally phosphorylated RPA32 in HU-treated extract, and not in untreated extracts, with stronger checkpoint signaling in HDHB-silenced extracts than in control-silenced extract (compare lanes 5, 6 with lane 4). We conclude that HDHB is not important for HU-induced activation of S phase checkpoint signaling. On the contrary, HDHB depletion modestly enhanced replication stress signaling, consistent with the possibility that HDHB might somehow counteract replication stress.

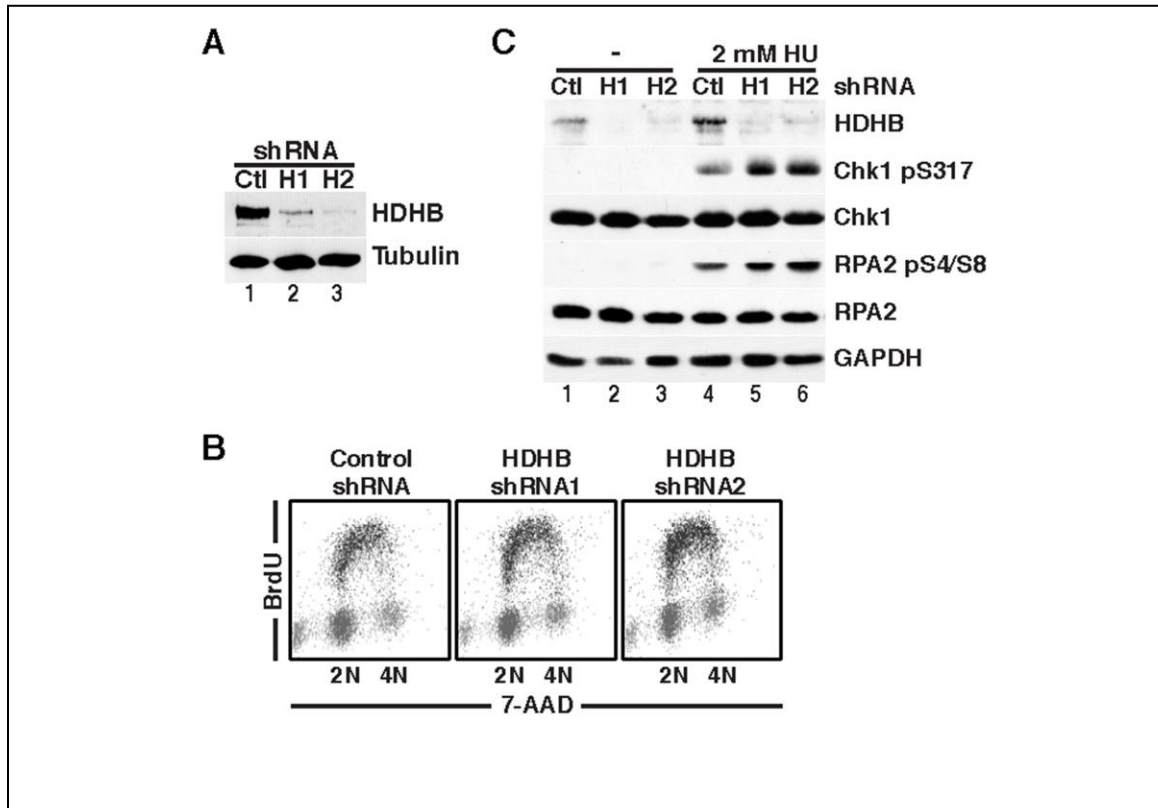


Figure 23. Transient HDHB depletion does not disrupt replication stress-induced checkpoint signaling, but impairs recovery of HeLa cells from replication stress.

(A) Whole cell extracts of HeLa cells transiently expressing non-silencing (Ctl) or HDHB-silencing shRNAs (H1 and H2) were analyzed by western blotting with the indicated antibodies. (B) HeLa cells transiently expressing non-silencing (Ctl) or HDHB-silencing shRNAs (H1 and H2) were incubated with 10 μ M BrdU for 30 min, stained for BrdU and total DNA, and analyzed by flow cytometry. (C) HeLa cells transiently expressing non-silencing (Ctl) or HDHB-silencing shRNAs (H1 and H2) as in (A) were exposed to 2 mM HU for 2 h or left untreated (-) as indicated. Whole cell extracts were then analyzed by SDS-PAGE and western blotting with antibodies against HDHB, Chk1 phospho-S317, RPA32 phospho-S4, S8, total RPA32 (RPA2), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a loading control.

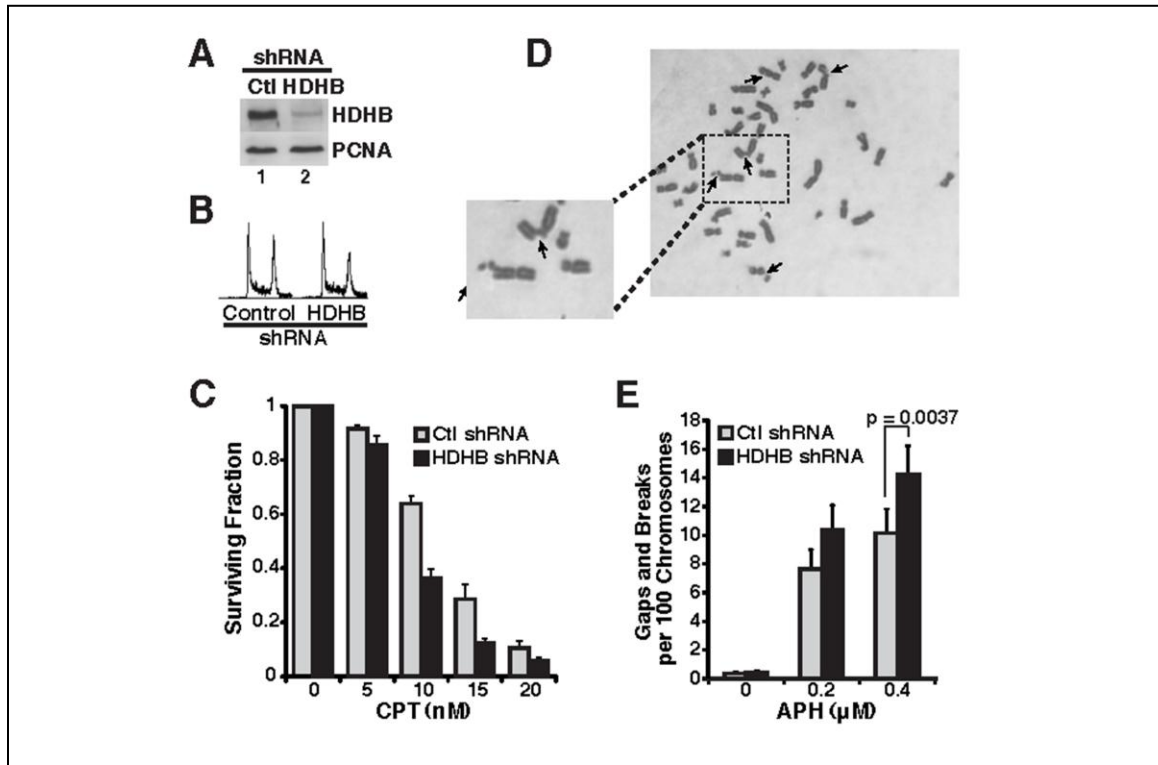


Figure 24. Delayed recovery from replication stress in HCT116 cells stably depleted of HDHB.

(A) Extracts (WCE) of HCT116 cells stably expressing control or HDHB shRNAs were analyzed by immunoblotting with indicated antibodies. (B) Flow cytometry of HDHB- and control-depleted HCT116 cells. (C) Colonies formed by HDHB-depleted (black) or control-depleted (gray) HCT116 cells after exposure to the indicated doses of CPT for 12 h were quantified. The surviving fraction of colonies formed by untreated cells was set to 1. Values for CPT-treated cells represent the average from three independent experiments; brackets indicate standard deviation. (D) Images of metaphase chromosomes from HDHB-silenced HCT116 cells exposed to 0.4 μM aphidicolin (APH) for 24 h. Arrows indicate chromosome gaps and breaks. (E) Quantification of chromosome gaps and breaks in metaphase spreads from control- (light gray) and HDHB-silenced (black) HCT116 cells after exposure to the indicated concentrations of APH for 24 h. Brackets indicate standard deviations $n \geq 3$. P-value was calculated using two-tailed Student's t-test. Experiments were performed by Dr. Hanjian Liu.

We next examined the possible role of HDHB in cellular recovery from replication stress induced by CPT. HDHB was stably knocked down in HCT116 cells using a different shRNA expression vector that targeted a third HDHB sequence. The cell cycle distribution of the HDHB-silenced cells was comparable to that of the control-silenced cells (Figure 24A, B). The role of HDHB in recovery from replication stress induced by CPT was then monitored in clonogenic survival assays. Equal numbers of HDHB- and control- depleted HCT116 cells were cultured in the absence or presence of CPT for 12 h and colonies formed by surviving cells were counted after 10 days. Exposure to 10, 15 or 20 nM CPT reduced colony formation of HDHB-depleted cells to about half that of control-depleted cells (Figure 24C), suggesting that HDHB-depletion sensitizes cells to CPT-induced damage.

We also monitored the ability of stably HDHB-silenced HCT116 cells to recover from replication stress induced by exposure to partially inhibitory concentrations of aphidicolin, which uncouples DNA synthesis from DNA unwinding at the fork, resulting in extended stretches of RPA-ssDNA and expression of common fragile sites [62, 205-207]. Metaphase chromosomes prepared from HDHB-silenced cells exposed to aphidicolin displayed breaks and gaps (Figure 24D, arrows). As expected, few chromosomal aberrations were observed in HDHB- or control-silenced cells cultured without aphidicolin (Figure 24E). Evaluation of chromosomal breaks and gaps in aphidicolin treated cells revealed a dose-dependent increase in chromosomal instability, with significantly more aberrations in HDHB-silenced than in control-silenced cells (Figure 24E). These results further confirm a role of HDHB in recovery from replication stress.

Discussion

Previous work on HELB implicated its helicase activity in chromosomal replication, but its functional role remained elusive. Here we show that although the level of HDHB on chromatin is quite constant throughout the cell cycle, additional HDHB is recruited to chromatin in cells that are exposed to a variety of DNA damaging agents. UV, CPT, and HU-induced HDHB recruitment to chromatin is dose-dependent and occurs preferentially in S phase cells. This recruitment does not depend on checkpoint kinase activity, but does correlate with the level of RPA on chromatin, a pattern consistent with replication stress-dependent recruitment analogous to that of S phase checkpoint proteins. Consistent with this interpretation, HDHB interacts directly with the N-terminal domain of the RPA70 subunit, a primary recruitment scaffold for multiple S phase checkpoint proteins [58, 203, 204]. Importantly, HDHB silencing did not impair S phase checkpoint signaling in response to replication stress, but did delay cellular recovery from replication stress. Based on these results, we suggest that the primary role of HDHB in chromosomal replication is to mitigate replication stress.

Replication stress-dependent recruitment of HDHB to chromatin

The checkpoint-independent, RPA-dependent accumulation of HDHB on chromatin in response to replication stress (Figure 15, Figure 16, Figure 17), and the direct physical interaction of a conserved acidic peptide in HDHB with the basic cleft of RPA70N (Figure 19, Figure 20) are consistent with a role for RPA70N-HDHB interaction in recruiting HDHB to chromatin. Decreased chromatin recruitment of HDHB

upon mutagenesis of the RPA70N-HDHB interaction surface provides further evidence in support of this mode of damage-induced chromatin recruitment. Interestingly, RPA70N-HDHB interaction closely resembles that of RPA70N with ATRIP, Rad9 and Mre11 [57, 58, 204]. Qualitatively, acidic peptides from each of these proteins have been shown to bind to the same surface of RPA70N that binds the acidic peptide from HDHB [58]. Quantitatively, a sub-stoichiometric molar ratio of the HDHB peptide to RPA70N (0.5: 1) was sufficient to induce strong chemical shift perturbations in the RPA70N spectrum (Figure 20B; Figure 21B). In contrast, a ten-fold greater molar ratio of the ATRIP, Rad9 or Mre11 peptide over RPA70N (4-6: 1) was used under very similar experimental conditions to observe a comparable chemical shift perturbation in the RPA70N spectra [58]. The comparison indicates that the interaction of HDHB with RPA70N is considerably stronger than that of ATRIP, Rad9, and Mre11.

It is interesting to consider the potential functional implications of the quantitatively stronger binding of HDHB to RPA70N. The observation that S phase checkpoint kinase activity is not needed to recruit HDHB to chromatin in response to replication stress (Figure 17A) implies that HDHB is recruited in parallel with the S phase checkpoint signaling proteins. Based on the stronger HDHB-RPA70N interaction, it is possible that HDHB can be more readily recruited to RPA-coated ssDNA at sites of replication stress, that a shorter stretch of RPA-ssDNA, i.e. fewer RPA molecules, would be sufficient to attract HDHB. In this case, HDHB might act “on the fly” to bypass or otherwise counteract the cause of the replication stress, thereby obviating the need to assemble a checkpoint signaling complex. Should HDHB recruitment fail to promptly relieve the replication stress, longer stretches of RPA-ssDNA would accumulate and

serve as the scaffold for recruiting S phase checkpoint proteins. This speculation could provide a plausible explanation for the somewhat more intense checkpoint signaling observed in HDHB-silenced than in control-silenced cells (Figure 23C). The ability of HDHB to mitigate DNA damage and modulate the intensity of checkpoint signaling may be important in certain tissues, such as thymus and testis, or for tumor cell viability.

How does HDHB stimulate recovery from replication stress?

Depletion of HDHB led to increased checkpoint signaling in HU-treated cells, decreased viability of cells exposed to CPT, and increased chromosomal breaks and gaps in cells recovering from aphidicolin (Figure 23, Figure 24). The fact that the data were generated in two different cell lines, depleted transiently or stably with three different shRNAs expressed from two different vectors, and evaluated in three different assays suggests that the observed results are not likely to be the consequence of off-target silencing or another experimental peculiarity. We conclude that HDHB has one or more biochemical activities that stimulate cellular recovery from replication stress.

Consistent with the replication-defective mutant phenotypes that led to HELB discovery, the helicase activity of HDHB is likely to play a fundamental role in relieving replication stress. Superfamily 1 helicases working as a ‘cooperative inchworm’ can generate sufficient force to displace streptavidin from biotin-labeled DNA [209]. The superfamily 1B helicase Rrm3, which migrates with progressing replication forks in budding yeast, is thought to use this mechanism to displace stably bound transcription complexes that block replication fork progression [138, 210, 211]. In a possibly related

mechanism observed in prokaryotes, the 3'-5' superfamily 1 helicase Rep co-migrates with the 5'-3' hexameric replicative helicase at the fork and serves as an auxiliary helicase to overcome fork stalling [143, 146, 212]. The HDHB that accumulates upon replication stress may also make use of such mechanisms to clear obstacles that impede an advancing fork.

The primosome activity of HELB might also play a role in counteracting replication stress [160, 161]. RPA-coated ssDNA is refractory to primer synthesis by DNA polymerase alpha-primase [31, 213]. However, a mediator protein, e.g. the SV40 helicase large T antigen, that interacts physically with both proteins can displace RPA from the template, and in concert, load DNA polymerase alpha-primase on the exposed template [33, 214, 215]. HDHB interacts with both RPA (Figure 17, Figure 19, Figure 20) and DNA polymerase alpha-primase [161], suggesting that HDHB recruitment to chromatin might enable it to re-prime the leading strand downstream of stalled forks. Such damage bypass/fork re-priming mechanisms are observed among distantly related bacteria from *B. subtilis* to *E. coli* and were recently detected in eukaryotes [177, 178, 212, 216-218].

The sequence similarity of HELB with prokaryotic proteins involved in homology-dependent DNA repair suggests another potential mechanism for HDHB to mitigate replication stress. Of particular interest is the ability of the superfamily 1B helicase Dda from phage T4, in conjunction with the T4 recombinase UvsX, to rescue stalled forks through two sequential template-switching reactions [219, 220]. The possibility that HDHB might mitigate replication stress in part through homology-dependent fork recovery mechanisms merits further investigation [82, 221-223]. It will be

interesting to learn which of these several HDHB activities contribute to its ability to relieve different kinds of replication stress.

HDHB: a damage tolerance protein?

The results of this study demonstrate replication stress-induced recruitment of HDHB to chromatin in a checkpoint-independent and RPA-dependent manner, and provide evidence that HDHB functions to relieve replication stress. The molecular features of HDHB interaction with RPA closely resemble those of proteins that initiate the assembly of S phase checkpoint complexes at sites of replication stress, yet we have not detected any HDHB contribution to checkpoint signaling. Instead, HDHB joins a diverse group of damage tolerance proteins that are recruited to sites of replication stress through interactions with different surfaces of RPA. Two prominent examples are the PCNA-modifying ubiquitin ligase Rad18, which binds to RPA70AB [224, 225] and the DNA translocase Smarcal 1/HARP ([226] and references therein), which binds to RPA32. Thus it will be important to elucidate the roles of HDHB in this network of damage tolerance proteins.

Chapter III

DISCUSSION AND FUTURE DIRECTIONS

Summary of this work

HDHB was previously implicated in DNA damage response. However, the molecular signals that HDHB responds to and its function in DNA damage response remained largely unknown. Studies presented in this thesis identified the molecular mechanism that recruits HDHB to chromatin after replication stress induction through exposure to various DNA damaging agents and provided insight into HDHB function in the absence and presence of DNA damage.

Mechanism for HDHB recruitment to chromatin after replication stress

Overexpressed GFP-tagged HDHB was previously reported to form nuclear foci that increase in number following cellular exposure to the topoisomerase inhibitor CPT [164]. Furthermore, previous work from the Fanning lab demonstrated HDHB accumulation on chromatin in cells exposed to various DNA damaging agents, including IR, UV, CPT and HU (Liu and Fanning, unpublished data). The diverse array of DNA damage that induced HDHB association with chromatin raised the question of how HDHB recognizes DNA damage induced by these genotoxins. Our results indicate that RPA-bound ssDNA is central to HDHB recruitment to chromatin following DNA

damage exposure (Chapter II). This finding provides an explanation as to how HDHB can ‘sense’ an assortment of DNA damage, since RPA-ssDNA is known to arise as a common intermediate following exposure to various genotoxins, particularly the types that induce replication stress [55, 59]. Consistent with an RPA-dependent recruitment model, we found that replication stress leads to HDHB localization to DNA-damage induced RPA foci. Direct physical interaction of RPA with HDHB mapped to the N-terminal domain of RPA 70-kDa subunit (RPA70N) and a conserved acidic motif in HDHB. NMR and isothermal titration calorimetry revealed an RPA70N-HDHB docking interface strikingly similar to that of RPA70N with p53, ATRIP, Rad9, and Mre11 [58]. Furthermore, our studies establish that damage-induced HDHB recruitment depends on RPA, but not on checkpoint signaling. Site-directed mutagenesis of the HDHB-RPA70N interaction interface established its contribution to damage-induced recruitment of HDHB to chromatin. These results suggest that the chromatin recruitment mechanism for HDHB upon replication fork stalling closely resembles that of ATRIP, Rad9 and Mre11.

HDHB function in replication stress response

Helicase-defective DHB mutants in both mouse and human cells were reported to inhibit cellular DNA replication [161, 169, 176]. These studies implicated DHB helicase activity in DNA replication but did not reveal its specific function.

In order to determine whether HDHB is essential for replication, we tested effects of transiently depleting HDHB on cell proliferation in the absence and presence of DNA damage. Interestingly, cell cycle profile of BrdU pulsed HDHB silenced HeLa cells did

not reveal any significant difference compared to control cells (Figure 23). We corroborated these results by examining thymidine analog EdU incorporation in control- and HDHB-depleted cells. In addition to no significant difference in cell cycle profiles, HDHB-depletion did not affect the percentage of EdU-positive cells, as expected from cell cycle analysis. (Figure 25D). Furthermore, EdU intensity was comparable in control- and HDHB-depleted cells (Figure 25E). One possible explanation for these surprising results is silencing inefficiency, where the residual levels of HDHB protein left in HDHB shRNA transfected cells are sufficient to enable the HDHB-dependent activities required for DNA replication. However, another possibility is that HelB is a non-essential gene for DNA replication under unperturbed conditions and an alternative pathway compensates for the absence of HDHB.

On the other hand, previous work from Fanning Lab implicated HDHB in DNA damage response [164] (Gu, Yan, Liu and Fanning, unpublished data). Work presented in this thesis further establishes HDHB function in DNA damage response, particularly in response to replication stress. A potential role for HDHB in preserving genomic stability after replication stress is underscored by the elevated number of aphidicolin-induced chromosome gaps and breaks (Figure 24E) and decreased cellular recovery from CPT as measured by clonogenic cell survival assay (Figure 24C) and γ H2AX immunostaining (Figure 27).

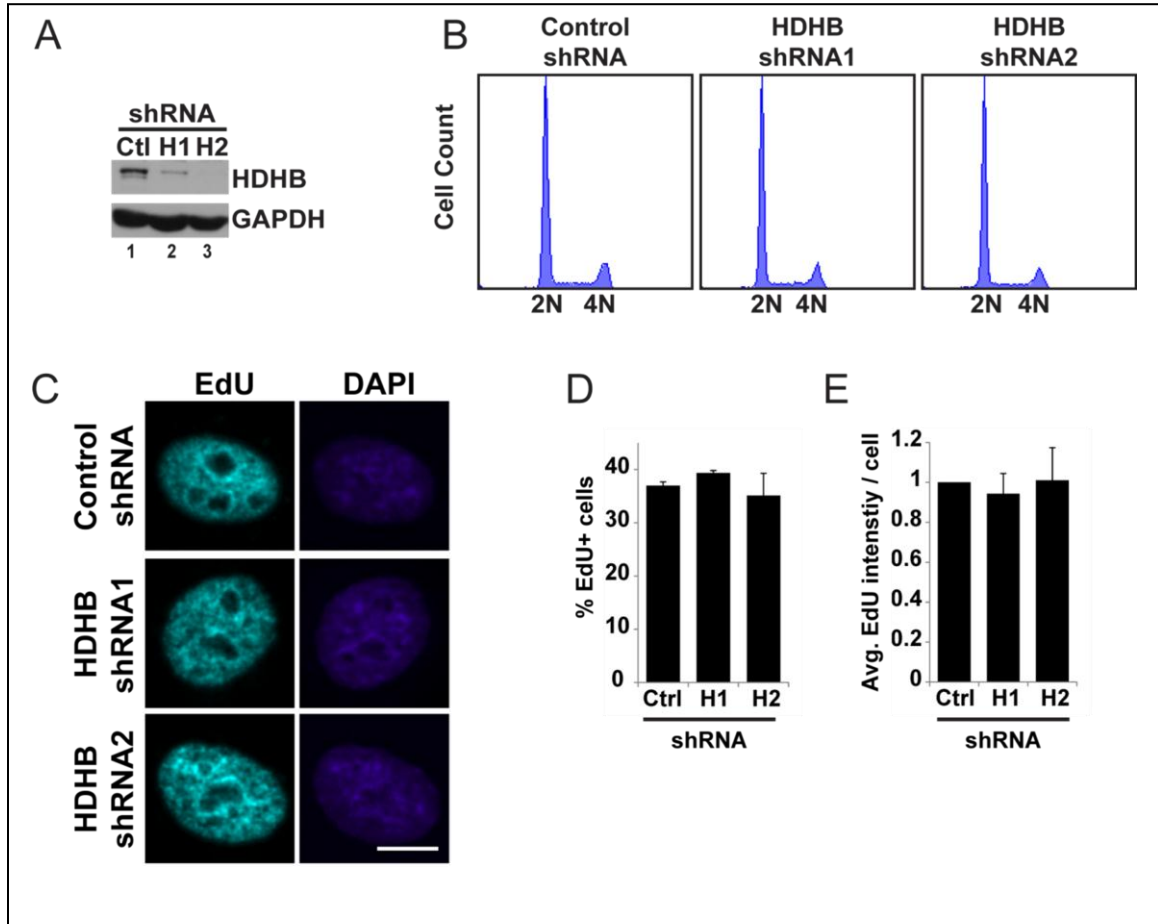


Figure 25. Effects of HDHB silencing on cell cycle distribution and cellular replication under unstressed conditions.

(A) Whole cell extracts of HeLa cells transiently expressing non-silencing (control = Ctl) or HDHB-silencing shRNAs (shRNA1 = H1 and shRNA2 = H2) were analyzed by western blotting with the indicated antibodies. (B) Cell cycle analysis of HeLa cells transiently expressing non-silencing (Control) or HDHB-silencing shRNAs (shRNA1 and shRNA2). (C) EdU incorporation in HeLa cells transiently expressing non-silencing (Control) or HDHB-silencing shRNAs (shRNA1 and shRNA2). Cells were incubated with 10 μ M EdU for 1 hour, then stained for EdU (cyan) and DAPI (blue). (D) Quantification of the percentage of EdU+ cells from samples described in (C). Minimum of 100 cells were evaluated for each set. Error bars show standard deviation from two independent experiments. (E) Average EdU intensity measured for each nuclei using Metamorph from samples described in (C). Minimum of 100 cells were evaluated for each set. Error bars show standard deviation from two independent experiments.

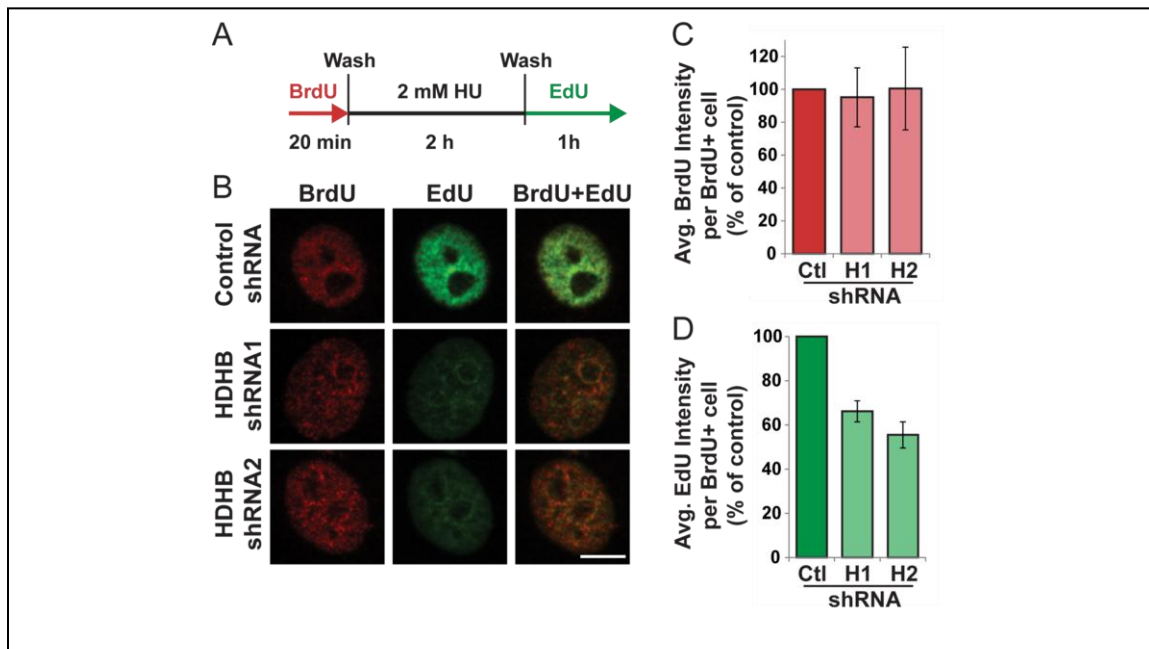


Figure 26. Replication restart after HU exposure in HDHB-depleted cells.

(A) Asynchronously growing HDHB- or control-silenced HeLa cells were labeled with BrdU before exposure to HU, and with EdU after removal of HU, as diagrammed, then fixed and stained for BrdU (red) and EdU (green). (B) Representative images of cells sequentially labeled as in (A). Scale bar, 10 μ M. (C, D) Quantification of average BrdU (C) or EdU (D) signal intensity per BrdU-positive nucleus measured using Metamorph from images generated as in (A, B). In each experiment, the average intensity from HDHB-silenced cells (H1 and H2) was expressed relative to that of the control-silenced cells (Ctl), which was set to 100%. The bar graphs display average intensities and standard deviation calculated from three independent experiments.

Replication stress response ultimately aims to restore replication to accomplish complete and faithful replication of the genome. Therefore, one possibility is that HDHB functions in replication stress response by promoting replication resumption after DNA damage. To test this possibility, we asked whether cells transiently depleted of HDHB would restart replication after exposure to hydroxyurea (HU). Asynchronously-growing silenced samples were pulse-labeled with BrdU to mark S-phase cells, then exposed to HU for 2 hours, and allowed to recover without HU in the presence of EdU (Figure 26A). Immunofluorescence microscopy was used to visualize and quantify the relative intensity of BrdU and EdU signals in individual cells (Figure 26B). As expected, HDHB- and control-silenced cells incorporated BrdU equally well in the absence of HU (Figure 26C), confirming that HDHB depletion did not significantly inhibit ongoing DNA synthesis (Figure 25). However, EdU incorporation after HU treatment was reduced in BrdU-positive HDHB-silenced cells relative to that in BrdU-positive control-silenced cells (Figure 26D), implicating HDHB in resuming replication efficiently after replication stress.

Resuming replication after stress requires a multi-layered process which includes checkpoint activation, stabilization of forks, and replication restart after repair or bypass of DNA damage. We did not observe any defect in Chk1 phosphorylation after HU exposure, suggesting that HDHB is dispensable for checkpoint activation (Chapter III). On the other hand, HDHB is implicated in homologous recombination (Liu and Fanning, unpublished data). Several proteins involved in homologous recombination were found to promote recovery from replication stress. It is possible that HDHB functions in these recombination-mediated replication stress recovery pathways. These results implicate

HDHB as part of a replication surveillance complex that helps recovery from replication stress, possibly through a homologous recombination-mediated repair pathway.

Implications of the results & future directions

Replication stress response is a multi-layered process with overlapping pathways that ultimately aims to restart replication for complete and accurate duplication of the genome. Replisome components and additional DNA repair proteins that are recruited after replication stress play important roles in reactivating damaged replication forks [55, 59, 82, 227], whereas new initiation events from dormant replication origins also contribute to this recovery process [111]. Evidence we have accumulated suggest that HDHB contributes to recovery from replication stress.

HDHB: A non-essential pre-RC component involved in replication stress response?

DHB helicase activity was previously reported to be important for chromosomal replication under normal conditions [161, 169, 176]. Consistent with these reports, HDHB is located at replication origins during G1 and G1/S phase, coinciding with the temporal localization of pre-RC proteins to replication origins (Gerhardt and Fanning, unpublished data). We also found that HDHB interacts with TopBP1 and Cdc45 (Figure 28, Gerhardt, Guler and Fanning, unpublished data). These results altogether suggest that HDHB may be a pre-RC component whose helicase activity is important for chromosomal DNA replication.

Surprisingly, we did not observe any significant defect in BrdU incorporation or cell cycle progression in HDHB-silenced cells (Figure 23, Figure 25), suggesting that HDHB function is not essential for replication under normal conditions. A possible explanation for these paradoxical results from helicase inactivation vs helicase silencing experiments is that helicase-dead HDHB blocks compensatory pathways for DNA replication when in complex with its interaction partners. On the other hand, when HDHB is absent, these compensatory pathways can take over the HDHB dependent step in DNA replication. Interestingly, a similar observation was made for the RecQ family member WRN helicase, where small molecule-mediated inhibition of the WRN helicase activity prevented chromosomal replication despite the lack of a similar replication defect when the WRN gene was silenced [228]. These results suggest that activities by additional helicases other than MCM2-7, such as HDHB, may be required for normal DNA replication, but that these functions can be compensated by activities from other helicases with overlapping functions. It will be interesting to determine the functional significance of HDHB localization to replication origins in the context of DNA replication initiation and the compensatory mechanisms that are in effect in the absence of HDHB.

If HDHB is part of the pre-RC complex that localizes to replication origins and moves away when DNA replication initiates, another potential question to be investigated is whether HDHB moves with the ongoing replication fork. Auxiliary helicases that travel with the fork were demonstrated to perform important functions for replication fork movement particularly through hard to replicate regions such as genomic loci undergoing high transcription [41, 139, 140]. Perhaps, HDHB travelling with the fork may facilitate

a similar function for efficient and proper replication under normal conditions to deal with spontaneous replication stress. Investigation of the effects of HDHB-depletion on DNA replication, expression of DNA damage markers, cell cycle profiles, and genomic integrity over longer periods of time may provide insight into the relevant efficacies of the HDHB-dependent and independent modes of DNA replication for faithful genome duplication.

If HDHB is part of the replisome, it may also have an effect on replication fork stability after replication stress. It is possible that the stability of the HDHB-containing replisome is different than the alternative replisome that forms in the absence of HDHB. This would have potential implications for cellular replication restart capability at stalled forks. Indeed, it may provide an explanation for the defects observed in replication restart after HU treatment in HDHB-silenced cells (Chapter III Figure 26). Effects of HDHB on replication fork stability can be determined by quantitative comparison of the chromatin association of replisome components, such as replicative polymerases and PCNA, before and after exposure to replication stress in control and HDHB-depleted cells.

HDHB is a potential component of the pre-RC complex that functions in replication initiation ([161, 169, 176] and Gerhard and Fanning, unpublished data), which brings forth the possibility that HDHB loaded at the origins may be important for replication restart following replication stress through activation of dormant origins. This possibility can be investigated by chromatin fiber preparation from HDHB silenced cells [229]. Identification of the HDHB-dependent step in replication initiation will also allow tests to determine if these functions are also important in the context of replication reactivation from dormant origins.

HDHB as part of replication surveillance machinery

Certain proteins or protein/DNA complexes that are integral components of the replisome play central roles in initiating the cellular response to replication stress. RPA-bound ssDNA has emerged as one of these components [55, 59]. This complex occurs naturally at ongoing replication forks and accumulates further when replication forks stall or collapse, possibly due to uncoupling of the replicative helicase from the replicative polymerase [59], or due to enzymatic processing at the damaged forks [109]. RPA-ssDNA is important for recruiting DNA damage response proteins ATRIP, Rad17, Rad9 and Mre11 to damaged chromatin [56-58, 61, 204]. Work presented in this thesis identified that RPA-ssDNA is also important for HDHB recruitment to chromatin upon UV-, CPT- or HU-induced replication stress. Direct interactions between HDHB and RPA observed in this study seem to be independent of any post translational modifications (Chapter II). This result suggests that HDHB may indeed be associating with the fork in the absence of DNA damage as well, since RPA-bound ssDNA is found at ongoing replication forks albeit at relatively lower amounts. This may actually explain the basal level of HDHB found in the chromatin fractions from S phase cells in the absence of any exogenous DNA damage. Our observation that HDHB is enriched at chromosomal fragile sites during S phase in the absence of DNA damage (Figure 29) would be consistent with this model as well. In such a model, constant HDHB association with the ongoing replication fork may ensure surveillance of the fork, where keeping HDHB in close proximity to the replication fork may facilitate rapid and efficient response to replication challenges as a first line of defense. A similar model was suggested in bacteria where SSB mediated association of PriA with the ongoing fork

helps efficient replication restart following replication stress [212]. In addition to HDHB 3xA mutant, HDHB monoclonal antibody 4C11 which disrupt HDHB-RPA70N interaction (Figure 19 and Figure 20) but not HDHB helicase activity (Figure 30 and Figure 20) can be utilized for investigating the functional importance of RPA-HDHB interaction in the absence and presence of DNA damage.

HDHB function in replication stress response

It is interesting that same molecular surface in RPA is employed for recruitment of several DNA damage response proteins to stalled replication forks, implicating the RPA70N basic cleft as a molecular hub for trafficking multiple damage response components. The finding that RPA70N utilizes the same molecular interface for interacting with HDHB and its other previously characterized interaction partners ATRIP, Rad9 and Mre11 [58] suggests a potential competition. Indeed, competition for RPA70N binding between ATRIP and Rad9 was previously documented [58]. This competition can be affected by the presence of additional contacts -with each other, with other proteins or with the DNA- and relative local concentrations of these proteins in the vicinity of the replication stall site. For example, HDHB interactions with RPA70AB (Figure 31) or TopBP1 (Figure 28) are likely to affect the local concentrations of HDHB at the stalled replication forks. Nevertheless, through this competition, RPA can potentially modulate DNA damage response pathway decisions, promoting either pathway progression, pathway choice, or both.

Sequestering the factors that function together at the same damage site by recruitment through a similar surface and then maintaining them at this site through additional interactions that are compatible with each other may enable a molecular hand-off mechanism that facilitates DNA repair complex formation [186]. Indeed, ATRIP and Rad9 function together to activate ATR kinase and initiate checkpoint response [55]. We did not observe any checkpoint activation defect in HDHB silenced cells (Figure 32), suggesting that HDHB does not function in checkpoint activation. However, HDHB may instead be involved in the damage response process downstream or independent of checkpoint activation. HDHB contains several putative ATM/ATR phosphorylation sites and was indeed identified as a potential ATM/ATR substrate in a mass spectrometry analysis [74]. Functional implications of this phosphorylation on HDHB function remains to be investigated.

On the other hand, competition imposed through recruitment by the same scaffold may have implications for DNA damage response pathway decisions if the competing proteins are involved in different pathways. A related possibility would be HDHB-mediated suppression of checkpoint activation through competition with damage response proteins ATRIP and Rad9 for RPA70N-dependent recruitment. The high binding affinity of HDHB for RPA70N in comparison to ATRIP, Rad9 and Mre11 suggests that perhaps HDHB is the preferred binding partner of RPA70N relative to these other proteins. In such a scenario, HDHB recruitment could enable local repair at stalled forks without initiation of a global checkpoint response.

Table 1. HDHB interaction partners identified to date

Interaction Partner	Interaction Partner Residues	Method	Reference
Pol-prim	p180	Pull Down ^{a,b}	[161]
RPA	RPA70 (1-120) RPA70 (181-422)	Pull Down ^{b,c}	This study (Figure 19, Figure 31)
Cdc45	N-terminus (1-182)	Pull Down ^{a,b}	Gerhardt, Arnett and Fanning, unpublished data
TopBP1	ND	Pull Down ^{a,b,c}	This study (Figure 28), Gerhardt and Fanning, unpublished data
Mre11	ND	Pull Down ^c	Gu and Fanning, unpublished data
Rad51	ND	Pull Down ^b	Liu and Fanning, unpublished data
PMS1	ND	Pull Down ^d	[171]

*can contain additional HDHB interaction sites in the N terminus

^a Pull downs performed with lysate from insect cells co-expressing both proteins

^b Pull downs performed with purified proteins

^c Pull downs performed with lysate from human cells overexpressing FLAG-tagged HDHB

^d Pull downs performed with lysate from human cells overexpressing FLAG-tagged HDHB

This type of competition between checkpoint activation and local repair pathways would be consistent with the threshold theory for checkpoint activation upon replication fork stalling. It was shown previously that short stretches of ssDNA at the replication fork are not enough to trigger checkpoint response [205, 230, 231]. Different lengths of ssDNA that accumulate at or behind the fork may recruit different sets of dynamic complexes and therefore determine the repair pathway to be employed. Such a mechanism would allow the cell to locally deal with replication problems that cause only limited stretches of ssDNA, without initiating a global replication arrest through a first response team that does not require checkpoint activity for its activation. When the damage cannot be efficiently dealt with via local repair, the accumulating RPA-coated ssDNA can then recruit ATR signaling components and activate the checkpoint to stop DNA replication and allow cell time to deal with the damage. Deregulation of the balance between local repair and checkpoint activation, for instance through changes in protein abundance, would potentially influence the damage tolerance capacity of the cell. HDHB overabundance or HelB copy number variations observed in some cancer cell lines should be considered from this perspective as well (Figure 13B) [173, 174]. Future studies on the potential interplay between HDHB-dependent pathways and checkpoint signaling that take into consideration not only the absence and presence of the wild type or mutant protein, but also relative protein abundance, will be interesting.

Our results suggest that HDHB functions in recovery from HU- and CPT-induced replication stress. Homologous recombination (HR)-mediated pathways were implicated in recovery from both HU and CPT [81, 99]. We, and others, have determined that HDHB interacts with several proteins implicated in regulation of HR, such as Rad51 and

Mre11 (Table 1). Interestingly, the RPA70N basic cleft is also the interaction surface for p53 [203]. RPA70N-p53 interaction was found to be important for p53-mediated suppression of HR [232]. Consequently, previous results that illustrate an HR defect in HDHB silenced cells (Liu and Fanning, unpublished data) can also be attributed to a potential increase in p53 binding to RPA70N in the absence of HDHB. On the other hand, p53 can suppress HR by preventing HDHB accumulation on chromatin by competing for the RPA70N binding site. The RPA70N basic cleft possibly contributes to HR as the *rfa-t11* mutation (Rfa1-K45E) in yeast, which corresponds to the basic cleft mutations that disrupt HDHB interaction (Figure 20D), lead to HR defects [233]. It will be interesting to investigate the potential interactions of HDHB-dependent functions with RPA70N- and p53-mediated pathways, particularly in the context of HR.

HDHB may also contribute to restart through its primosome activity in a mechanism similar to PriA-mediated re-priming downstream of the replication stall site observed in prokaryotes [178, 234]. Re-priming of the leading strand has been well documented in both *E. coli* and *B. subtilis* [212, 235]. Currently, there is no direct evidence that demonstrates the existence of a similar mechanism in eukaryotes. However, re-priming could explain observations such as ssDNA accumulation behind the forks [104, 216-218, 236] and hyper-accumulation of chromatin-bound polymerase- α primase and primed DNA after fork stalling [62, 63]. Interestingly, both HDHB and its mouse homolog have primosome activity *in vitro* [160, 161]. A potential role for HDHB in primosome activity after replication stalling remains to be investigated.

Concluding Remarks

Several genotoxins that interfere with DNA replication, such as camptothecin-derivatives or cisplatin, are commonly used in cancer therapy. Effectiveness of at least some of these therapies in killing cancer cells seems to be closely related to their ability to interfere with cellular replication [237]. Understanding the cellular mechanisms involved in the response to these genotoxins can benefit cancer therapy by molecular profiling of responsive and non-responsive cancer types, in addition to providing insight into some of the mechanisms responsible for developing resistance to chemotherapeutic genotoxins.

Our results identified HDHB as a novel component of the elaborate network of cellular factors that are responsible for protecting genome integrity by ensuring complete and faithful duplication of the genome under replication stress. Mass spectrometry approaches to characterize the protein complexes HDHB associates with can provide clues to the specific replication recovery mechanisms HDHB is involved in. On the other hand, co-depletion studies with different components of replication restart pathways will shed light on recovery pathways that are important for survival and recovery in the absence of HDHB. Although a large scale shRNA screen would be most informative, proteins involved in regulation of recombination-dependent pathways for replication restart, such as Rad51, Mus81, WRN and BLM, are interesting candidates for co-depletion studies to investigate potential functional interactions with HDHB.

Identification of an RPA-dependent mechanism for HDHB recruitment to chromatin upon DNA damage suggests that HDHB can respond to a variety of replication challenges. Due to the central nature of this recruitment mode in coordinating different

DNA damage response pathways, it is very likely that HDHB-dependent mechanisms function as part of a complex network, competing with certain pathways while collaborating with others. Applications of approaches that can capture the plasticity of this interconnected network which is coordinated at least in part through molecular hubs such as RPA-ssDNA, will be highly valuable for future studies. The dynamic nature of the DNA damage response network is likely a significant contributor to cancer heterogeneity and relevant clinical outcomes such as drug resistance. Understanding these complex networks of interactions can potentially reveal novel approaches that would be both specific and therefore more effective than current cancer therapies.

APPENDIX

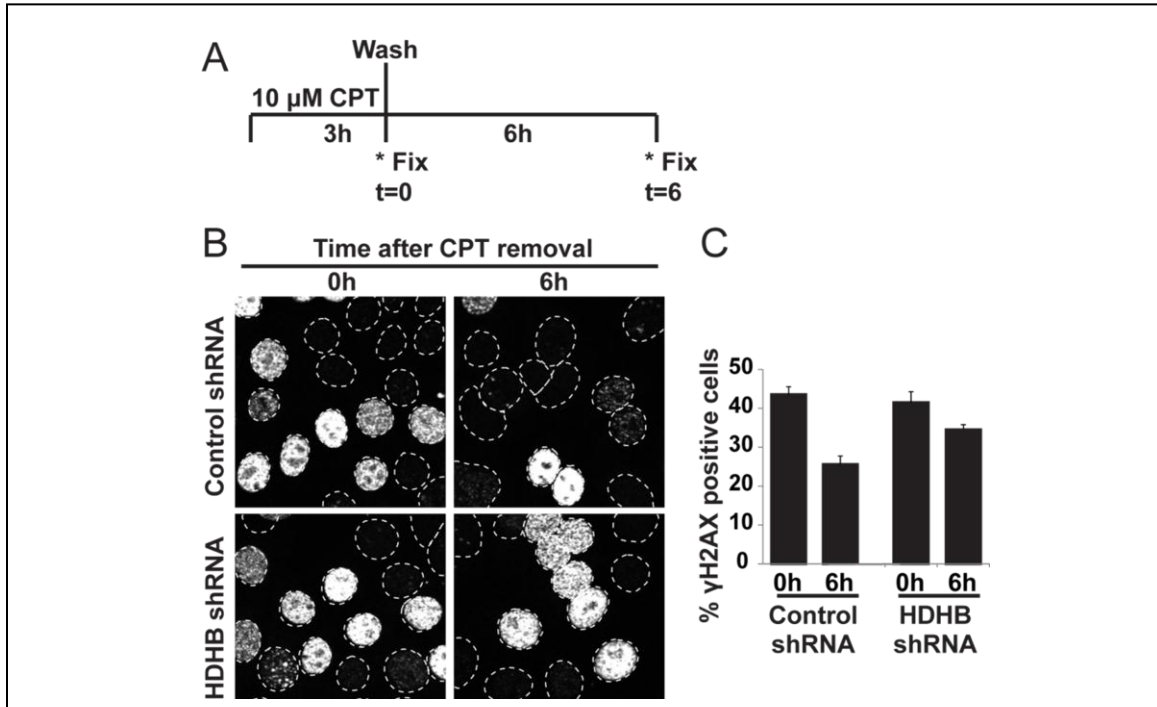


Figure 27. HDHB silencing perturbs cellular recovery from CPT induced damage.

(A) Experimental set-up to detect γ H2AX in cells recovering from CPT exposure. HeLa cells transiently expressing control- or HDHB-shRNA were treated with CPT, then washed and incubated in fresh media, and processed for γ H2AX immunofluorescence at the indicated times. (B) Representative images of control- and HDHB-silenced cells stained for γ H2AX (red) after CPT treatment at the indicated times are shown. Dashed white lines encircle nuclei. (C) Bar graph showing the fraction of the control- and HDHB-silenced cell populations displaying γ H2AX staining at 0h (gray bars) or 6h (black bars) the indicated time points. Brackets indicate standard deviation.

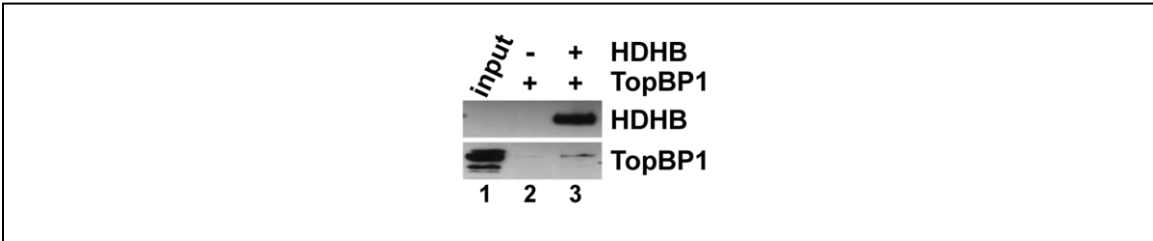


Figure 28. HDHB associates with Topoisomerase II binding partner 1 (TopBP1).

Purified TopBP1 (C) or Cdc45 (D) was incubated with Protein A-beads pre-bound with anti-HDHB antibody in the absence (-) or presence (+) of purified HDHB as indicated. Proteins bound to the beads were analyzed by western blotting. agarose beads pre-bound with HDHB antibody could pull down a small fraction of input TopBP1 in the presence, but not absence, of purified HDHB (Figure 23C), suggesting that HDHB can interact with TopBP1.

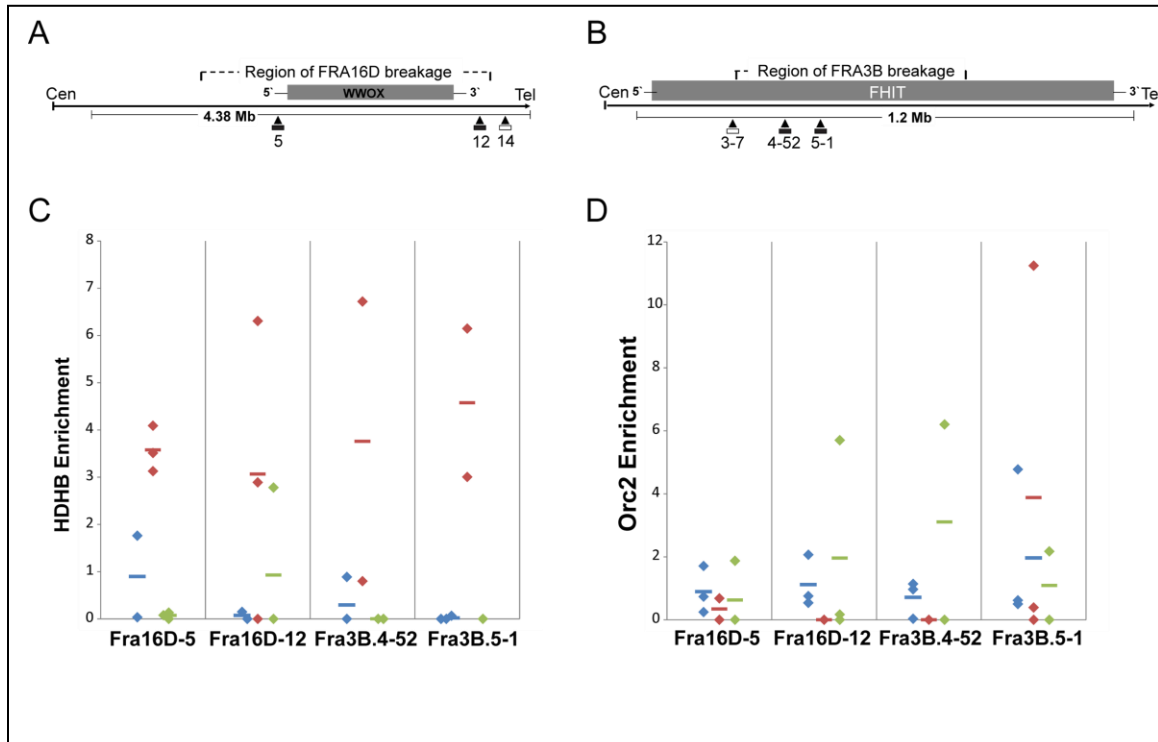


Figure 29. HDHB localization at common fragile sites Fra16D and Fra3B.

(A,B) Schematic representation of common fragile site Fra16D (A) and Fra3B (B) breakage region on chromosome 16 and chromosome 3, respectively. Gray bars mark the location of WWOX (A) and FHIT (B) genes. Primer sets used for quantitative PCR are denoted as filled bars (for primer sets localized to breakage region) or open bars (for primer sets outside of the breakage region). (C,D) Enrichment of HDHB (C) and Orc2 (D) at Fra16D and Fra3B breakage regions relative to . HDHB (C) and Orc2 binding to fragile sites were determined in Hct116 cells synchronized in G1 (blue diamonds), S (red diamonds) and G2/M (green diamonds) phases by ChIP followed by quantitative real time PCR with the primer sets indicated in panels A and B. Abundance of immunoprecipitated DNA was calculated by the target sequence detected in the HDHB- or Orc2-immunoprecipitate subtracted by the target sequence detected in non-immune rabbit IgG precipitate, divided by the target sequence detected in input (pre-IP) chromatin sample. Enrichments were calculated by normalization against the abundance determined using primer sets outside of the breakage region (open bars).

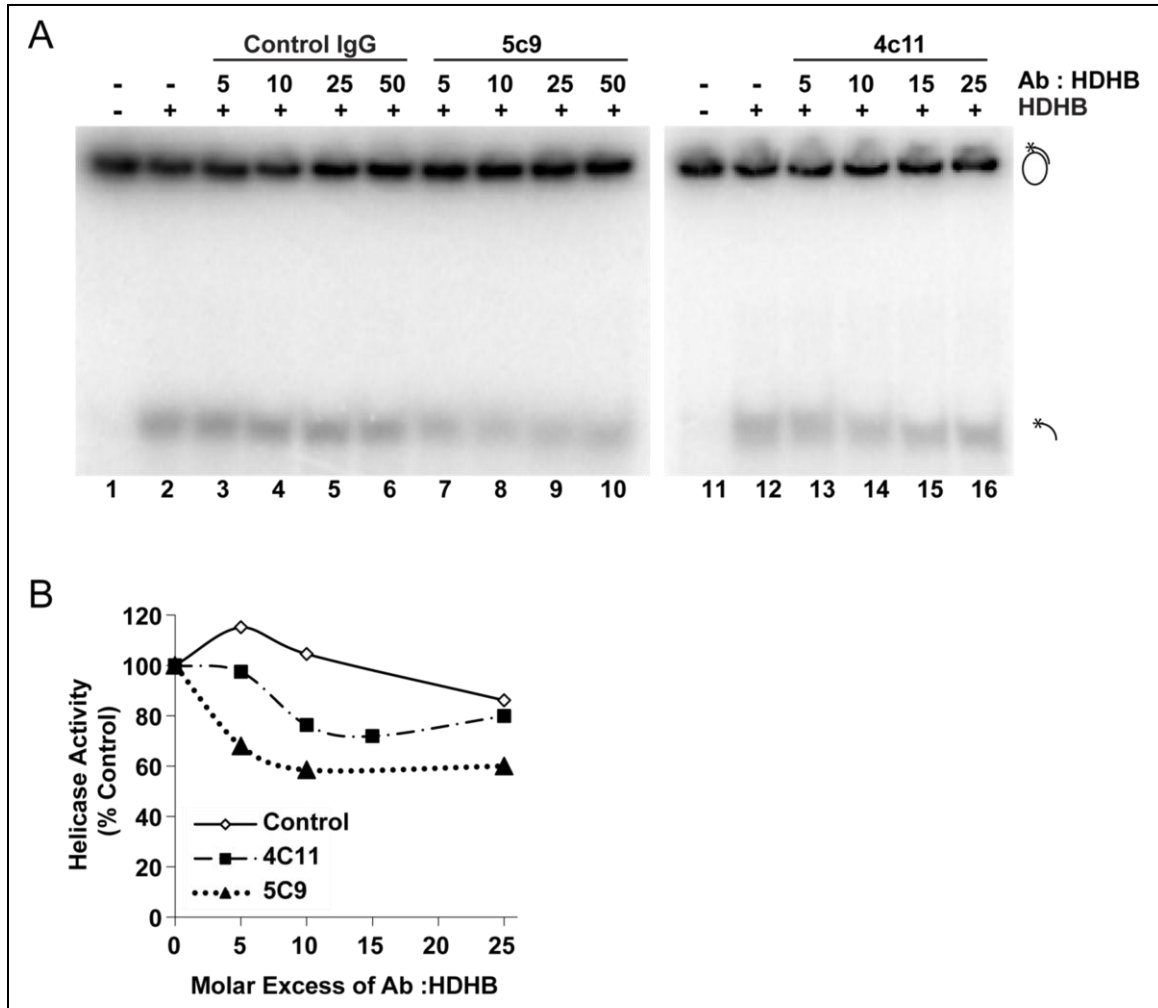


Figure 30. Effect of HDHB monoclonal antibodies on HDHB helicase activity. (A) Non-immune rat IgG (Control) or anti-HDHB monoclonal antibodies 5C9 or 4C11 were titrated in molar excess as indicated into HDHB helicase reactions containing 50 fmol of HDHB and 8 ng of M13 ssDNA annealed with radiolabeled primer as helicase substrate. (B) % Unwound substrates were quantified for each lane in panel A and helicases activities observed in the presence of antibodies were normalized to the helicase activity observed in the absence of any antibody.

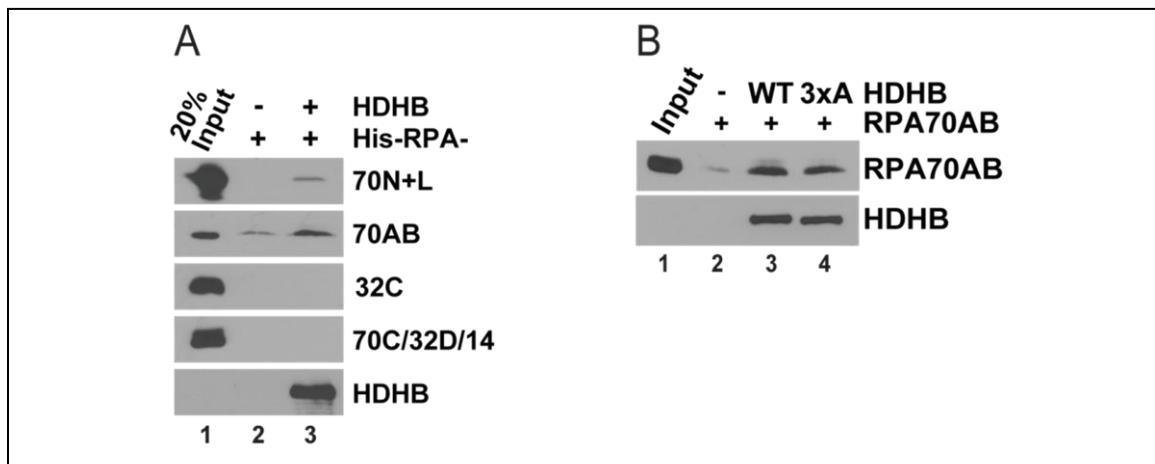


Figure 31. HDHB interactions with RPA.

(A) Purified His-tagged RPA constructs were captured on anti-HDHB antibody beads in the presence (+) or absence (-) of HDHB and analyzed by western blotting with anti-His (top four panels) or anti-HDHB. (B) HDHB antibody pre-bound beads were incubated with purified His-tagged RPA70AB in the absence (lane 2) or presence of purified His-tagged WT or 3xA HDHB (lanes, 3, 4). Proteins bound to the beads were analyzed by western blotting with anti-His antibody.

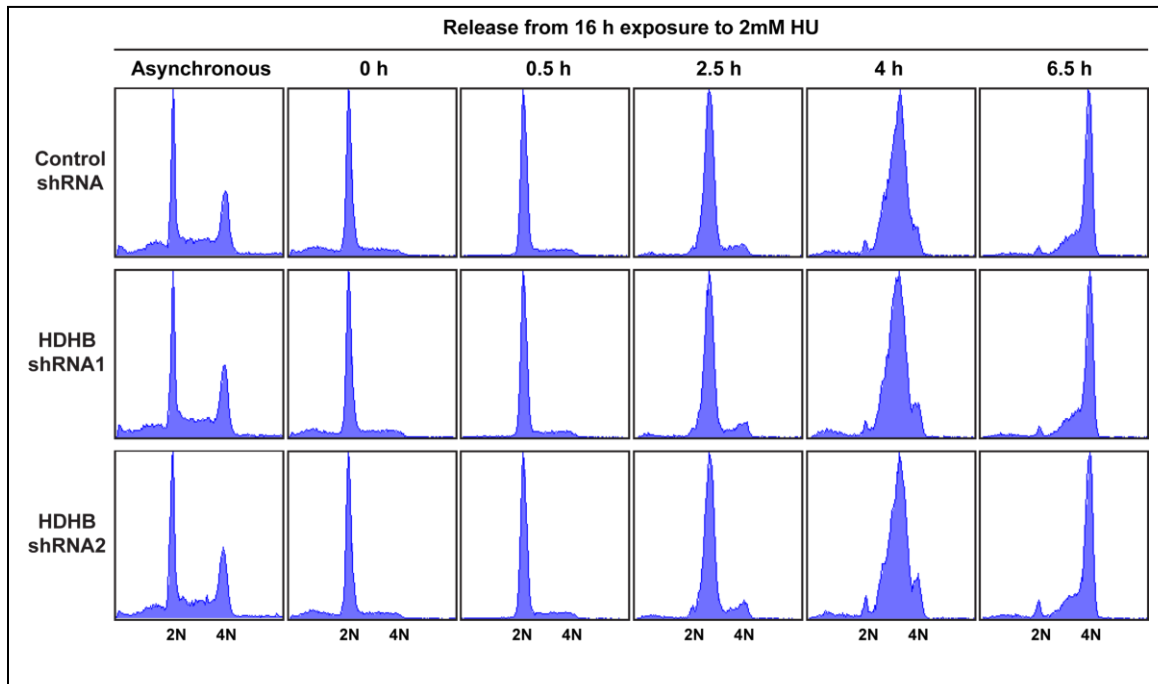


Figure 32. S phase progression after HU exposure.

Asynchronously growing HDHB- or control-silenced HeLa cells were exposed to 2 mM HU for 16 hours, and then released in nocodazole containing medium. S phase progression was monitored by collecting the cells at the indicated timepoints after release from HU treatment followed by propidium iodide staining for flow cytometry analysis of cellular DNA content.

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