the plant journal

SEB Society for Experimental Biology

*The Plant Journal* (2016) doi: 10.1111/tpj.13283

# AtRAD5A is a DNA translocase harboring a HIRAN domain which confers binding to branched DNA structures and is required for DNA repair *in vivo*

**Daniela Kobbe, Andy Kahles, Maria Walter, Tobias Klemm, Anja Mannuss, Alexander Knoll, Manfred Focke and Holger Puchta***

*Botanical Institute II, Karlsruhe Institute of Technology (KIT), Karlsruhe 76131, Germany*

Received 10 June 2016; revised 20 July 2016; accepted 22 July 2016.
*For correspondence (e-mail holger.puchta@kit.edu).

## SUMMARY

**DNA lesions such as crosslinks represent obstacles for the replication machinery. Nonetheless, replication can proceed via the DNA damage tolerance pathway also known as postreplicative repair pathway. SNF2 ATPase Rad5 homologs, such as RAD5A of the model plant *Arabidopsis thaliana*, are important for the error-free mode of this pathway. We able to demonstrate before, that RAD5A is a key factor in the repair of DNA crosslinks in Arabidopsis. Here, we show by *in vitro* analysis that AtRAD5A protein is a DNA translocase able to catalyse fork regression. Interestingly, replication forks with a gap in the leading strand are processed best, in line with its suggested function. Furthermore AtRAD5A catalyses branch migration of a Holliday junction and is furthermore not impaired by the DNA binding of a model protein, which is indicative of its ability to displace other proteins. Rad5 homologs possess HIRAN (Hip116, Rad5; N-terminal) domains. By biochemical analysis we were able to demonstrate that the HIRAN domain variant from Arabidopsis RAD5A mediates structure selective DNA binding without the necessity for a free 3′OH group as has been shown to be required for binding of HIRAN domains in a mammalian RAD5 homolog. The biological importance of the HIRAN domain in AtRAD5A is demonstrated by our result that it is required for its function in DNA crosslink repair *in vivo*.**

**Keywords: DNA damage tolerance, postreplicative repair, DNA crosslink repair, HIRAN domain, DNA translocase, *Arabidopsis thaliana*, Rad5, DNA-binding domain, replication fork, At5g22750.**

## INTRODUCTION

DNA lesions during the S-phase of the cell cycle endanger genomic integrity and proper replication. The mechanisms of the DNA damage tolerance (DDT) pathways allow lesion bypass without the requirement of direct lesion repair (Svoboda and Vos, 1995; Pagès and Fuchs, 2003). In *Saccharomyces cerevisiae*, DDT is regulated by the ubiquitination of proliferating cell nuclear antigen (PCNA) (Ulrich and Jentsch, 2000; Xiao *et al.*, 2000; Broomfield *et al.*, 2001; Hoege *et al.*, 2002; Ulrich, 2005; Andersen *et al.*, 2008). The monoubiquitination of PCNA by Rad6/Rad18 leads to translesion synthesis (TLS), which is error prone. The error-free DDT pathway involves template switching and is initialized by the polyubiquitination of PCNA mediated by the E3 ubiquitin ligase Rad5, which recruits Mms2/Ubc13 to Rad6/Rad18. There are two not mutually exclusive modes of error-free DNA damage bypass. One mode involves replication fork regression, whereby the new leading strand anneals to the new lagging strand. The resulting four-way junction is called a chicken foot structure; due to the previous uncoupled and ongoing synthesis of the nascent lagging strand, this strand serves as the template for leading strand synthesis. The chicken foot structure is subsequently reversed, restoring the replication fork. The second error-free DDT mode involves template switching via strand invasion and D-loop (displacement loop) formation in a homologous recombination-like mechanism. *In vitro*, Rad5 physically interacts with branched DNA substrates and catalyses the regression and reversal of model replication forks via its ATP-dependent translocase activity (Blastyák *et al.*, 2007) and also catalyses strand invasion and D-loop formation (Burkovics *et al.*, 2014). *In vivo* investigations showed that blocked replication forks cannot be restarted in cells lacking Rad5 and that abnormal DNA structures arise. Both the

ubiquitin ligase and the ATPase activity are involved in restarting blocked replication forks (Minca and Kowalski, 2010). Recently it was shown that the Walker B motif of the helicase domain not only plays a role for the ATPase activity of Rad5, but is also required for PCNA polyubiquitination (Choi *et al.*, 2015). In summary, Rad5 is not only involved in the error-free DDT pathway via its ubiquitin ligase activity but might also participate in both proposed error-free DDT modes through physical interactions with DNA.

DNA damage tolerance is conserved in eukaryotes. In humans, there are two functional Rad5 orthologs that are also involved in DDT: SNF2 histone linker PHD RING helicase, first described as a potential tumor suppressor protein, and helicase-like transcription factor (HLTF) (Sood *et al.*, 2003; Motegi *et al.*, 2006, 2008; Unk *et al.*, 2006). Both human homologs interact with human RAD18, UBC13, and PCNA and with each other (Motegi *et al.*, 2006, 2008). *In vitro*, HLTF is able to process replication forks, including the displacement of associated proteins, and catalyses D-loop formation; *in vivo*, HLTF restarts stalled replication forks (Blastyák *et al.*, 2010; Achar *et al.*, 2011; Burkovics *et al.*, 2014). Several *Rad5* homologs are found in the genome of the model plant *Arabidopsis thaliana;* however, only *RAD5A* is involved in DNA repair. AtRAD5A shares the domain organization of yeast Rad5 and human HLTF (Chen *et al.*, 2008) (Figure 1a). Investigations of RAD5A-deficient plants revealed reduced plant growth on media containing DNA crosslinking agents and methylating genotoxins, which can block replication. Additionally, RAD5A is involved in double-stranded break repair via homologous recombination (Chen *et al.*, 2008; Mannuss *et al.*, 2010). REV3 is one of several known proteins involved in TLS in Arabidopsis (Sakamoto *et al.*, 2003; Kobbe *et al.*, 2015). The analysis of *rad5a* and *rev3* single and double mutant lines have demonstrated that both the error-free and the error-prone pathways of DDT are conserved in Arabidopsis (Wang *et al.*, 2011). RAD5A seems also to be involved in the regulation of DDT in plants, as Arabidopsis PCNA1 and PCNA2 are ubiquitinated due to the RING domain and ubiquitin ligase function of RAD5A (Strzalka *et al.*, 2013).

The RING domain is flanked by the two helicase subdomains with the seven helicase motifs typical of superfamily 2 helicases (SF2) (Figure 1a). The helicase domain is involved in ATP binding and hydrolysis and therefore in DNA translocation, causing regression and reversion of a stalled replication fork (Blastyák *et al.*, 2007; Unk *et al.*, 2010). In the N-terminus an HIRAN (Hip116, Rad5p, N-terminal) domain is identified by bioinformatic algorithms. The HIRAN domain was first described as a domain that exists as a stand-alone unit in several bacteria and prophages or is a module of proteins with other domains known to be involved in DNA processing in eukaryotes. On

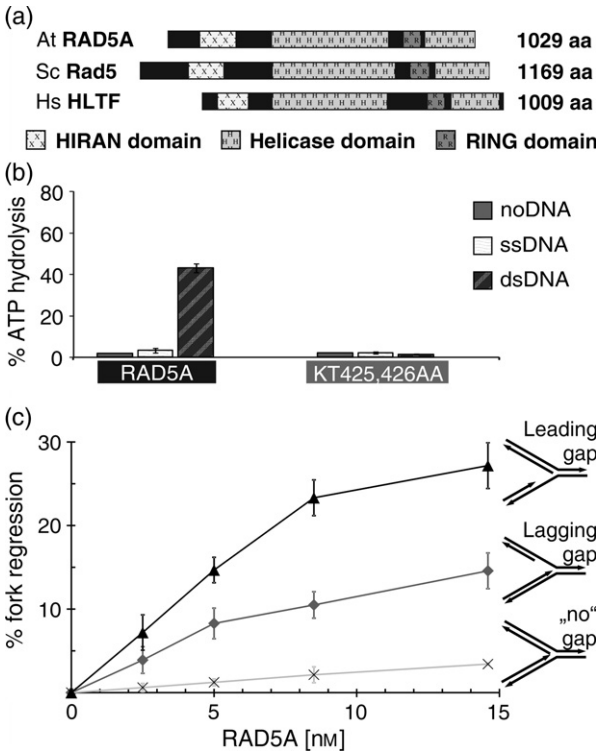


**Figure 1.** Different Rad5 homologs and the ATPase and fork regression properties of Arabidopsis RAD5A.
(a) Domain structure of selected Rad5 homologs. At, *Arabidopsis thaliana*; Sc, *Saccharomyces cerevisiae*; Hs, *Homo sapiens.*
(b) Quantification of ATP hydrolysis in the presence of different DNA substrates by 5.75 nM enzyme.
(c) Quantification of fork regression of different replication forks.
Each data point represents the mean of at least three independent replicates. Error bars indicate standard deviations.

this basis, it was postulated that the HIRAN domain is involved in specific interactions with DNA (Iyer *et al.*, 2006). This assumption was recently confirmed for the HIRAN domain of human HLTF by structural and biochemical data (Hishiki *et al.*, 2015; Kile *et al.*, 2015). Both groups were able to resolve the structure of this HIRAN domain in complex with DNA. Additionally it was shown, that HLTF is necessary for restraining the further progression of replication forks under dNTP shortage *in vivo* probably by catalysing fork regression and that the HIRAN domain is crucial for this function (Kile *et al.*, 2015). Also data from *Schizosaccharomyces pombe* Rad8 (the Rad5 homolog in S. p.) implicate the HIRAN domain in DNA damage response *in vivo* (Ding and Forsburg, 2014).

Interestingly, the residues defined for HLTF for interactions with DNA are not conserved in the HIRAN domain of AtRAD5A and AtRAD5A was not yet biochemically characterized. Therefore, in this study, we analyzed the biochemical properties of *Arabidopsis thaliana* RAD5A and investigated the function of its HIRAN domain variant *in vitro* and *in vivo.*

## RESULTS

### RAD5A of *Arabidopsis thaliana* is a double-stranded DNA-dependent ATPase able to regress replication forks with preference for forks with a leading strand gap

RAD5A of *Arabidopsis thaliana* was expressed in *Escherichia coli* and purified via a C-terminal Strep-Tag II and gel filtration. RAD5A-KT425,426AA, with two amino acid substitutions of lysine and threonine in the Walker A motif that as shown for HsHLTF (MacKay *et al.*, 2009) or ScRad5 (Chen *et al.*, 2005; Minca and Kowalski, 2010), abolishes the ATPase activity, and was prepared in the same manner.

We could show that RAD5A hydrolyzed ATP in the presence of double-stranded DNA, but not in the absence of DNA or when using single-stranded DNA, which would be in line with it being a double-stranded DNA translocase (Figure 1b). The ATPase-deficient variant RAD5A-KT425, 426AA showed negligible ATP hydrolysis, indicating the absence of contaminating bacterial proteins such as helicases in the protein preparation.

As Rad5 homologs have been shown to be able to regress replication forks we asked if also AtRAD5A is able to perform this reaction. So far, most fork regression assays for Rad5 homologs were performed with replication forks without significant gaps at the fork. However in nature single-stranded regions occur. While undisturbed replication is correlated with a lagging strand gap, at stalled forks uncoupling of replication leads to leading strand gaps. Therefore we compared the ability of RAD5A to process replication forks with a leading strand gap, a lagging strand gap and without a significant gap. The gap length was chosen to be 30 nt. AtRAD5A is able to regress replication forks (Figures S2 and 1c) and – in line with one of its proposed functions in DDT namely the regression of stalled replication forks – replication forks with a leading strand gap are processed most efficiently at 8.5 nM of enzyme 23% (±2%) for the fork regression product compared with 11% (±2%) for the fork with lagging strand gap and 2% (±1%) for the fork without significant gap. With the negative control (KT-AA) 0% (±0%), 2% (±3%) and 0% (±1%) were detected respectively. Values in brackets represent standard deviation after correction for background without enzyme.

### RAD5A is able to catalyse branch migration of Holliday junctions unaffected by protein blockades

A double-stranded DNA translocase is assumed to translocate dsDNA using the hydrolysis of a nucleoside triphosphate as a source of energy. This reaction can lead to the remodeling of DNA structures as shown, or to the removal of proteins bound to the DNA or both. We decided to further analyze the possible translocase activity of RAD5A within the biologically relevant context of a Holliday junction. Holliday junctions are central intermediates in homologous recombination and additionally mimic the chicken foot structure that arises in error-free DDT. *In vivo* data obtained with *rad5a* mutant plants suggest functions of RAD5A in those two pathways (Chen *et al.*, 2008; Mannuss *et al.*, 2010). The Holliday junction substrate (Gari *et al.*, 2008) used is partially mobile (pmHJ) because of two homologous and two heterologous arms, and processing by a dsDNA translocase via branch migration yields two double-stranded DNA products (Figure S1c). Indeed, RAD5A was able to process this pmHJ substrate via branch migration (Figure 2a,b). Because of the homology, pmHJ is also forming the two duplex structures slowly by DNA breathing in the absence of enzyme. Binding of RAD5A-KT425,426AA stabilizes the structure and leads to the apparent 'formation' of pmHJ in the quantification (Figure 2b). In contrast, a model Holliday junction without sequence homology (X0-HJ) (Figure S1c) could not be processed by RAD5A, indicating its inability for strand separation and branch migration over long heterologous regions (Figure 2a). Branch migration products were observed when using an intermediate Holliday junction with a homologous core but heterologous ends (X26-HJ), albeit in reduced amounts compared with a Holliday junction with homologous arms (Figure 2a,b).

Using pmHJ as the preferred Holliday junction substrate, we assessed whether the branch migration activity of RAD5A is dependent on the presence of (deoxy)ribonucleoside triphosphates (Figure S3). Although ATP or dATP were the preferred cosubstrates, other (d)NTPs could also be used but led to reduced branch migration activity. Interestingly, common ATP analogs, which are often used as negative controls, were also used for branch migration if the anhydride linkage between the β-phosphate position and γ-phosphate position was intact (ATP-α-S, ATP-γ-S, ApCpp). Further ATP analogs with modified linkage of the γ-phosphate to the β-phosphate (AppCp, AMPPNP) could not be used for branch migration (Figure S3).

As dsDNA translocases may also be involved in the removal of bound proteins, we investigated the branch migration activity of RAD5A on a protein-bound Holliday junction by creating a partially mobile Holliday junction with one *Bam*HI binding sequence (pmHJ_*Bam*HI, Figure S1c). Modified *Bam*HI–E111A, in which glutamate-111 is replaced by alanine, can bind to the *Bam*HI recognition site but is not able to cut the DNA (Dorner *et al.*, 1999; Engler *et al.*, 2001). First, we determined the optimal *Bam*HI–E111A concentration, such that the binding sequence of pmHJ–*Bam*HI was nearly saturated after preincubation with *Bam*HI–E111A (Figure S4). Interestingly, branch migration of RAD5A was not affected by the obstacle induced by *Bam*HI–E111A: pmHJ_*Bam*HI was processed

 

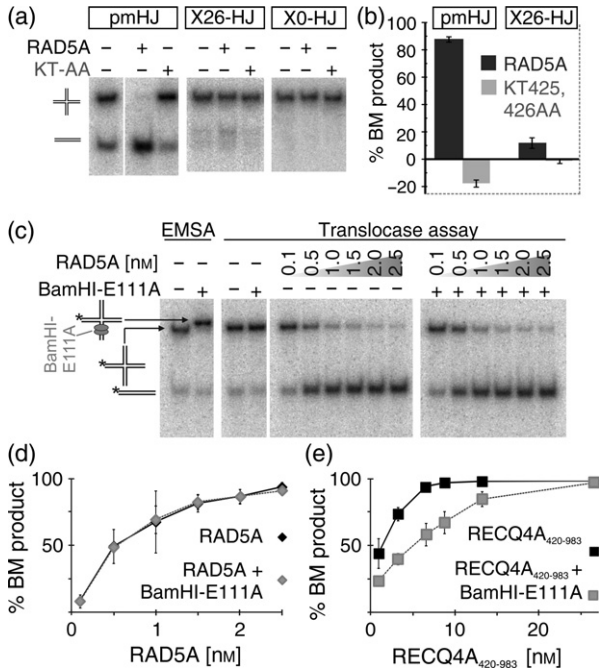


**Figure 2.** Branch migration activity of RAD5A.
(a, b) Branch migration (BM) activity of 5.75 nM RAD5A with different HJ substrates, with decreasing extent of homology from left to right (Figure S1c).
(a) Phosphorimager data.
(b) Quantification: the stabilization of pmHJ by the binding of RAD5A-KT425,426AA compared to the no-enzyme control, in which HJ slowly decomposes by breadthening, leads to the apparent 'formation' of pmHJ (negative value of branch migration).
(c–e) Branch migration in the presence of another DNA-binding protein. Branch migration by RAD5A (0–2.5 nM) is analyzed in the presence and absence of *Bam*HI–E111A pre-bound to the partially mobile Holliday junction pmHJ_*Bam*HI–E111A (Figure S1c). For comparison, the catalytic core of the RECQ4A helicase of Arabidopsis (RECQ4A-420-983) was used. (c) Phosphorimager data. (d) Quantification for RAD5A. (e) Quantification for RECQ4A-420-983. Figure S4 justifies the use of 5 nM *Bam*HI–E111A. Each data point represents the mean of at least three independent replicates. Error bars indicate standard deviations; γ-setting for phosphorimager data was 2.6.

identically, irrespective of whether *Bam*HI–E111A was pre-bound or not (Figure 2c,d). To compare these findings with another branch migration-catalysing enzyme, we performed the same experiments with the helicase RECQ4A-420-983; this catalytic active core fragment of *Arabidopsis thaliana* RECQ4A is an ATP-dependent helicase that can process synthetic replication forks by fork regression and branch migration (Schröpfer *et al.*, 2014). In contrast to RAD5A, the activity of RECQ4A-420-983 following preincubation with *Bam*HI–E111A was reduced (Figure 2c,e). Thus, the unaffected branch migration activity of RAD5A by a protein obstacle is not a common feature of proteins processing Holliday junctions, and the results suggest that RAD5A is directly involved in the disassociation of proteins bound to DNA.

### The HIRAN domain of Arabidopsis RAD5A is different from the characterized HIRAN domain of human HLTF

The structure of the HIRAN domain of human HLTF in complex with single-stranded DNA was recently solved by three groups (Achar *et al.*, 2015; Hishiki *et al.*, 2015; Kile *et al.*, 2015) and the importance of specific amino acids was analyzed. Kile *et al.* showed that most of the amino acids involved in DNA interaction (indicated by numbers in Figure 3) were conserved in HIRAN domains of proteins from different domains of life so that they concluded that the HIRAN domain evolved from a general nucleic acid binding architecture to a domain that specifically binds to 3′ ends. Indeed they also identified the HIRAN domain of SM3L1 (At5g05130) of *Arabidopsis thaliana.* However this protein was not yet shown to be involved in DNA metabolism, in line with our own preliminary data that the growth of a T-DNA insertion mutant in this gene is not sensitive to the genotoxins bleomycin and mitomycin C. However, as already stated in the introduction, we identified a functional homolog of ScRAD5 and HsHLTF, namely AtRAD5A (At5g22750) (Chen *et al.*, 2008). A multiple sequence alignment suggests that in plants at least two different HIRAN domain variants exist (Figure 3). The HIRAN domain of SM3L1 homologs is similar to the characterized HIRAN domain of human HLTF, but for AtRAD5A homologs the amino acids identified for interaction with the 3′ end of DNA are not conserved. Without further information it is unclear if the HIRAN domain variant of Arabidopsis RAD5A will have the same properties as the characterized one of HsHLTF and this situation could lead to different biochemical properties of the whole proteins. Therefore we wanted to assess the biochemical properties of the RAD5A HIRAN domain.

### The HIRAN domain-containing N-terminus of RAD5A preferentially binds to branched DNA structures without the need of a free 3′ hydroxyl group

To investigate the potential DNA-binding function of the HIRAN domain variant of Arabidopsis RAD5A, we purified an N-terminal fragment of RAD5A that contains the HIRAN domain (RAD5A-1-242) and performed electrophoretic mobility shift assays (EMSA) with different DNA substrates (Figure 4a). To guarantee comparability and to exclude potential sequence dependency, the DNA structures all shared the same radioactively labeled oligonucleotide as a common origin. Interestingly branched DNA structures (splayed arm, 3′ and 5′ flaps, replication fork and Holliday junction) were the preferred substrates for binding by RAD5A-1-242 compared to single stranded, double-stranded or 3′ or 5′ overhang substrates. As the replication fork is the central element of DDT we continued with this substrate and asked, whether a free 3′ OH group at the junction point is important for efficient DNA binding. For

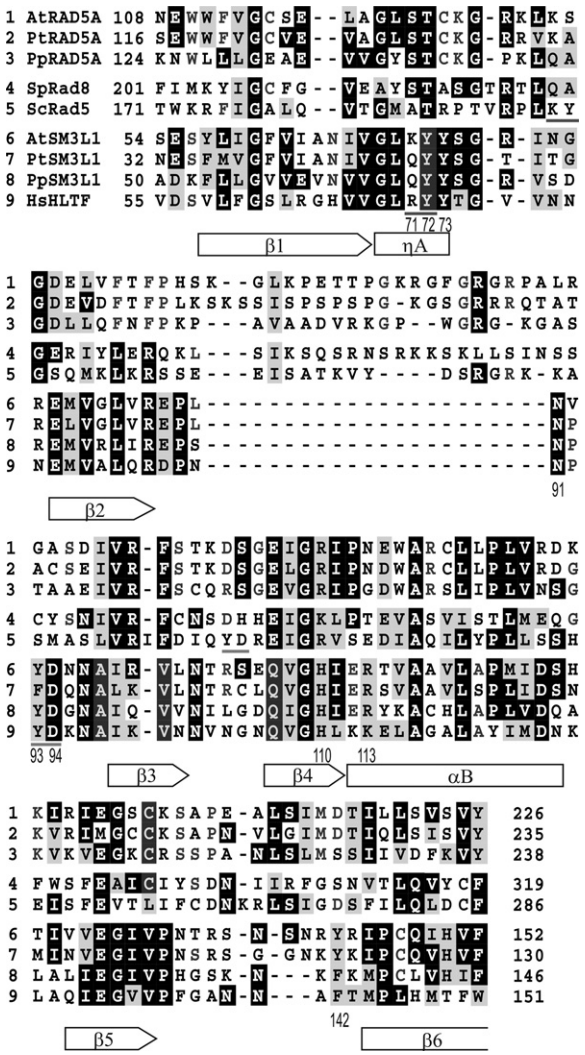


**Figure 3.** The HIRAN domain of Rad5 homologs are only partially conserved on the amino acid level.

Multiple sequence alignment of different selected HIRAN domains. 1, AtRAD5A; 2, PtRAD5A; 3, PpRAD5A; 4, SpRad8; 5, ScRad5; 6, AtSM3L1; 7, PtSM3L1; 8, PpSM3L1; 9, HsHLTF. At, *Arabidopsis thaliana*; Pt, *Populus trichocarpa*; Pp, *Physcomitrella patens*; Sp, *Schizosaccharomyces pombe*; Sc, *Saccharomyces cerevisiae*; Hs, *Homo sapiens*. The sequences were retrieved after NCBI BLAST search. For AtSM3L1 the protein start was assumed as deposited (Uniprot Q9FF61). Missing N-terminal sequences for PtSM3L1 were taken from the phytosome Potri006 g035700 entry and refined for PpSM3L1 (XP_001765135.1) using the genomic data deposited at jgi (gw1.73.11.1). The alignment was calculated by Expresso using default settings (Di Tommaso *et al.*, 2011) (the structure automatically used was 4s0n.pdb) and refined manually. The secondary structure of the HIRAN domain of HsHLTF is given below the sequence (Kile *et al.*, 2015) and amino acids important for DNA interaction (Hishiki *et al.*, 2015; Kile *et al.*, 2015) for HsHLTF HIRAN are indicated by the respective amino acid number. The two main differences of this alignment of the HIRAN domains of ScRAD5 and HsHLTF to the recently published alignment (Kile *et al.*, 2015) are indicated by gray bars under the two sequences.

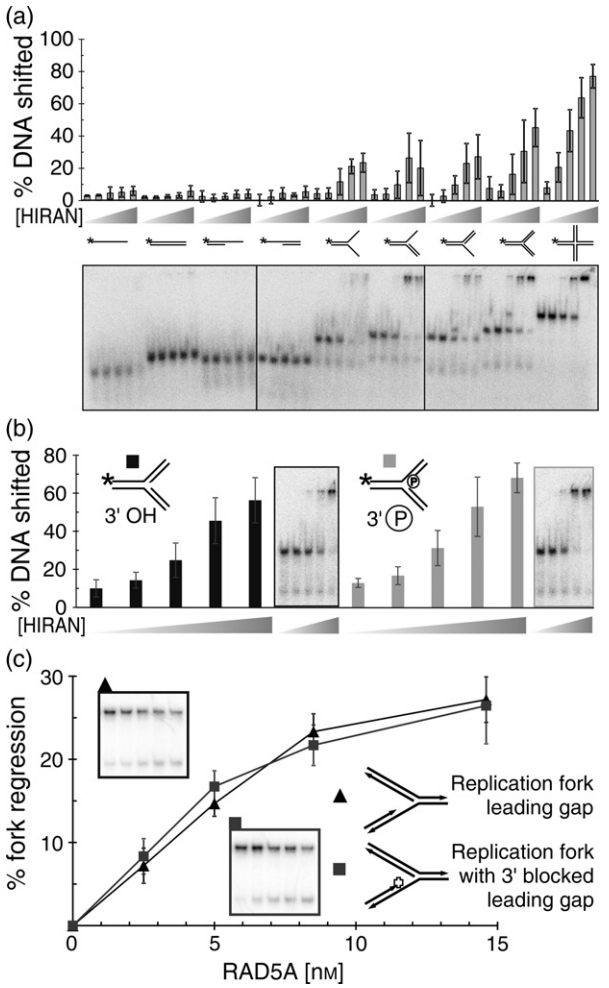


**Figure 4.** DNA-binding properties of the HIRAN domain of RAD5A and the influence of a blocked 3′ end on replication forks.

(a) Increasing concentrations of RAD5A-1-242 containing the HIRAN domain (0, 86, 172, 259 and 345 nM) were incubated with different (non-migratable) DNA structures as symbolized (single-stranded DNA, double-stranded DNA, 3′ overhang, 5′overhang, splayed arm, 3′ flap, 5′ flap, replication fork and Holliday junction) and the binding was analyzed on 6% TBE-PAGE gels and quantified.

(b) Increasing concentrations of RAD5A-1-242 containing the HIRAN domain (0, 86, 172, 259 and 345 nM) were incubated with non-migratable replication fork structures without single-stranded regions at the junction point and either a 3′ OH or a 3′ phosphate group at the leading strand. Binding was analyzed by 6% TBE-PAGE and quantified.

(c) Fork regression was quantified for the indicated concentrations of full-length RAD5A in the presence of a migratable replication fork with leading strand gap or the same fork with a TAMRA modification at the 3′ end of the leading strand. Each data point represents the mean of at least three independent replicates. Error bars indicate standard deviations.

the HIRAN domain of human HLTF it was shown that the 3′ hydroxyl group of single-stranded DNA was necessary for the binding of the HIRAN domain to this single-stranded DNA (Hishiki *et al.*, 2015; Kile *et al.*, 2015). However a 3′ phosphate group at the junction point on the leading strand did not impair DNA binding of the Arabidopsis RAD5A HIRAN variant (Figure 4b).

As binding by the HIRAN domain variant was not impaired by a blocked 3′ end we next asked if the fork

regression activity of the full-length protein is influenced. Using the replication fork with leading strand gap as analyzed before (Figure 1c) either with or without the fluorophore TAMRA as a potential blockage of the 3′ end of the leading strand we did not detect significant differences in fork regression (Figure 4c). This result supports the hypothesis that the HIRAN domain variant of Arabidopsis RAD5A is recruiting RAD5A to branched DNA structures irrespectively of the presence of a 3′ OH group.

### The HIRAN domain is essential for DNA repair *in vivo*

To define the role of the HIRAN domain *in vivo*, we transformed *rad5a*-mutant plants with constructs encoding RAD5A or RAD5A-ΔHIRAN. The coding sequence was placed under control of the natural *RAD5A* promoter and terminator. The plants were challenged with the genotoxic agent cisplatin for sensitivity assays. The structures preferentially formed by cisplatin are intrastrand crosslinks (Eastman, 1985). After treatment with 5 μM cisplatin, the *rad5a-2* mutant exhibited a strong impairment in growth that was one-fifth that of the wild type (wt) (Figure 5a). Four independent lines carrying the full-length RAD5A ORF had a relative dry weight comparable to wt (*rad5a-2* RAD5A #1-#4). However, *rad5a-2* mutant lines transformed with the construct encoding RAD5A-ΔHIRAN (*rad5a-2 RAD5A-ΔHIRAN* #1-#4) showed strong hypersensitivity comparable with the *rad5a-2* mutant. Taken together, these results demonstrate that the function of RAD5A in DNA repair *in vivo* is dependent on the presence of its HIRAN domain.

To analyze whether the respective proteins were indeed expressed *in planta* at comparable levels as wild type RAD5A, we performed western blotting. RAD5A proteins encoded by the complementation constructs were detected in plantlets at quantities similar to that in wild type (Figure 5b).

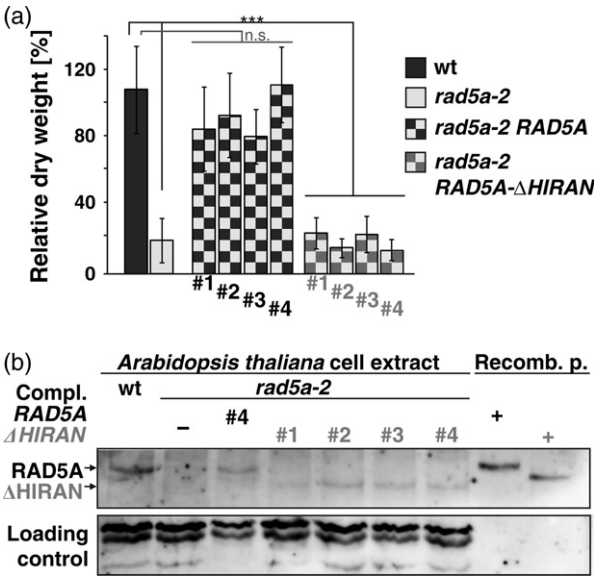


**Figure 5.** The HIRAN domain is essential for DNA repair *in vivo*.
(a) Analysis of the DNA damage response of *rad5a*-2 mutant plants complemented with cDNA constructs of either *RAD5A* or *RAD5A-ΔHIRAN*. The relative dry weight per plant of at least 24 seedlings after 22 days of growth on medium containing 5 μM cisplatin was determined. The relative dry weight per plant is given as a percentage and is normalized to the relative dry weight per plant of each line grown on genotoxin-free medium. Each assay was performed at least three times, and the mean values including the standard deviations (error bars) are depicted. For each construct, four independent integration lines were tested (#1–#4). ***Signifies a *P*-value of less than 0.001 if performing Student's *t*-test.
(b) Western blot analysis of complemented *rad5a*-2 mutant plants with cDNA constructs of either *RAD5A* or *RAD5A-ΔHIRAN* with an anti-RAD5A antibody; 500 pg of recombinant protein purified from *Escherichia coli* is loaded for comparison. Unspecific signals at approximately 55 kDa are used to judge the quantity of the total protein loaded.

## DISCUSSION

In this study, we biochemically characterized RAD5A of *Arabidopsis thaliana* and demonstrated that the conserved HIRAN domain variant is indeed a DNA-binding domain with a preference for the binding of branched DNA without the need of a 3′ hydroxyl group. Deletion of the HIRAN domain of Arabidopsis RAD5A leads to an impaired DNA damage response. It was already known by *in vivo* investigations of plants lacking RAD5A that this enzyme is required for repairing DNA crosslinks and methylation damage. These kinds of DNA damage can hinder replication and cause stalling of the replication fork leading to the assumption that the role of Arabidopsis RAD5A is similar to that of Rad5 in DDT in *Saccharomyces cerevisiae* (Chen *et al.*, 2008). Uncoupling of replication synthesis leads to a leading strand gap and, interestingly, RAD5A catalyses fork regression of such a replication fork with a leading strand gap better than one with lagging strand gap or without gap at the junction point. Also HLTF was shown to regress replication forks with a leading strand gap (Achar *et al.*, 2011) but mostly replication forks without gaps at the junction were used and the processing of the different replication fork substrates by Rad5 homologs were not compared (Blastyák *et al.*, 2007, 2010; Achar *et al.*, 2011, 2015). However the preference demonstrated for RAD5A of Arabidopsis is in line with a direct function of Rad5 homologs in the error-free mode of DDT.

Continuing studies demonstrated a further involvement of Arabidopsis *RAD5A* in the repair of double-stranded breaks via homologous recombination (Chen *et al.*, 2008; Mannuss *et al.*, 2010). Both the model of error-free DDT and the model of homologous recombination involve cruciform DNA intermediates (Holliday junctions), which need to be processed to canonical double-stranded DNA. As shown in this study with recombinant RAD5A and a partially mobile Holliday junction, RAD5A can process such cruciform DNA structures via branch migration.

Thus, similar to yeast Rad5 and human HLTF, Arabidopsis RAD5A is most likely not only involved in the control of

plant DDT, as recently demonstrated by the ability of RAD5A to function as an E3-ubiquitin ligase that polyubiquitinates Arabidopsis PCNA1 and PCNA2 (Strzalka *et al.*, 2013), but also in the error-free pathway of DDT by processing and resolving the chicken foot intermediate to restart replication after stalling and bypassing the lesion through fork regression (Ulrich and Jentsch, 2000; Hoege *et al.*, 2002; Branzei *et al.*, 2004; Blastyák *et al.*, 2007, 2010; Motegi *et al.*, 2008; Unk *et al.*, 2008, 2010; Masuda *et al.*, 2012).

In contrast to a Holliday junction with sequence homology (representing the situation in nature), a static Holliday junction without sequence homology (X0-HJ; Ip *et al.*, 2008) cannot be processed by RAD5A but is used as a co-substrate for ATP hydrolysis, indicating that RAD5A has no canonical strand-separating helicase activity.

We showed that ATP hydrolysis is dependent on the presence of double-stranded DNA. In contrast, Arabidopsis RAD5A does not hydrolyze ATP in the presence of single-stranded DNA. In agreement, human HLTF is also not able to hydrolyze ATP in the presence of oligonucleotide-based single-stranded DNA (Blastyák *et al.*, 2010). Further biochemical analysis showed that in contrast to *Saccharomyces cerevisiae* Rad5 and human HLTF, a broad spectrum of (d)NTPs can be used by RAD5A (Blastyák *et al.*, 2007, 2010). Nonetheless, ATP and (d)ATP are the preferred nucleoside triphosphates.

During branch migration, the activity of RAD5A is not impaired by prebound *Bam*HI–E111A as a model of a blockade by DNA-bound proteins. We also wanted to compare this behavior with other helicases or translocases involved in the DNA damage response and recombination in Arabidopsis. We have previously characterized AtRECQ2 (Kobbe *et al.*, 2008), AtRECQ3 (Kobbe *et al.*, 2009), AtRECQ4A-420-983 (Schröpfer *et al.*, 2014) and AtSRS2 (Blanck *et al.*, 2009) HsBLM has already been analyzed with respect to *Bam*HI–E111A displacement (Sommers *et al.*, 2014), and *Bam*HI was found to impair the translocation of BLM. AtRECQ4A is the functional homolog of human BLM (Hartung *et al.*, 2007), the RecQ helicase that is defective in the human disease Bloom syndrome. Therefore, we chose to use AtRECQ4A-420-983 in the present study. In contrast to RECQ4A-420-983, RAD5A's branch migration activity is not affected by the presence of *Bam*HI–E111A, which is in line with it belonging to the family of Snf2-like proteins. Snf2-like proteins are described as chromatin remodelers that must address proteins bound to DNA during translocation (Flaus *et al.*, 2006).

As shown in Figure 1(a) Rad5 homologs have an N-terminal HIRAN domain. Although this domain was already described in 2006 on a bioinformatic level with functions proposed on the basis of the occurrence of HIRAN domains (Iyer *et al.*, 2006), different groups solved the structure of the HIRAN domain of human HLTF in complex with DNA only in 2015 and characterized the human HLTF HIRAN domain variant biochemically (Achar *et al.*, 2015; Hishiki *et al.*, 2015; Kile *et al.*, 2015). While the focus of two studies was on the HIRAN domain as a single-stranded DNA-binding domain with interactions with the 3′ OH end of single-stranded DNA (Hishiki *et al.*, 2015; Kile *et al.*, 2015) the third study showed that while single-stranded DNA is bound, still a replication fork is bound preferentially (Achar *et al.*, 2015).

We show, that in Arabidopsis at least two HIRAN domain variants exist (Figure 3). Interestingly the HIRAN domain variant of the functional Arabidopsis Rad5 homolog, RAD5A, is different with respect to some amino acids shown to be important for DNA interaction by the human HLTF HIRAN variant. Therefore we analyzed the DNA-binding properties of the HIRAN domain variant of Arabidopsis RAD5A.

Also the HIRAN domain of RAD5A binds better to a replication fork than to single-stranded DNA as does the HIRAN domain of HLTF (Achar *et al.*, 2015). Binding of the HLTF HIRAN domain, to the replication fork does not need a single-stranded DNA region (Achar *et al.*, 2015). As in our study, the replication fork used in the study of Achar *et al.* (2015) did not expose single-stranded DNA. However before, the HTLF HIRAN domain was shown to require single-stranded DNA with a 3′ OH end for binding (Hishiki *et al.*, 2015; Kile *et al.*, 2015). Therefore the study by Achar *et al.* extended and specified the binding spectrum of the HIRAN domain of HTLF. However the effect of the presence of the 3′ OH group at the junction point of the replication fork was not further analyzed. However a 3′ OH at the junction point is important for DNA binding (Hishiki *et al.*, 2015; Kile *et al.*, 2015). We showed for the HIRAN domain variant of Arabidopsis RAD5A that a substitution of the 3′ OH by a 3′ phosphate group did not interfere with the binding of the HIRAN domain to the replication fork. Also fork regression by the full-length protein was not impaired by a substitution of the 3′ OH by the fluorophore TAMRA. Furthermore we were able to show that the HIRAN domain variant of Arabidopsis RAD5A does not need an accessible end at all for binding as also a static Holliday junction was bound with high efficiency.

The HIRAN domain of *Schizosaccharomyces pombe* Rad8 (the Rad5 homolog in S. p.) (Ding and Forsburg, 2014) and HLTF (Achar *et al.*, 2015) are required for DNA repair.

To demonstrate the importance of the RAD5A HIRAN domain variant, we complemented *rad5a*-mutant plants with *RAD5A* and *RAD5A-ΔHIRAN.* The *rad5a-RAD5A-ΔHIRAN* plants show no complementation and a *rad5a*-like DNA repair phenotype, illustrating the importance of the HIRAN domain for repairing DNA crosslinks, which can cause replication fork stalling. Strictly speaking, deletion of the HIRAN domain might not only abolish the ability of

AtRAD5A to bind to branched DNA structures but might also perturb interactions with other cellular proteins, which might also contribute to the detected defect in DNA repair. In any case, our western blot data exclude the possibility that the phenotype is merely due to the misfolding of the mutant protein because this would most probably result in imminent degradation. Thus, although the HIRAN domain of Arabidopsis RAD5A is not conserved with respect to certain amino acids shown to be involved in protein-DNA interactions by human HLTF, it is important for DNA repair *in vivo*. This action could be mediated by binding of the HIRAN domain to defined branched DNA structures, thus guiding the protein to provide a correct and precise protein localization.

Thus, although with the present sets of data one cannot finally conclude whether or not the human HLTF HIRAN domain variant might bind some branched DNA structures also without the necessity of a 3′ OH group as it is the case for the RAD5A HIRAN variant, our findings generalize the potential role of structure specific DNA binding even by different HIRAN domain variants and support the proposed function of Arabidopsis RAD5A in DDT.

## EXPERIMENTAL PROCEURES

### Cloning of expression vectors

The coding sequence for RAD5A (accession no. AK228695, AED93070.1) was cloned into a modified pET-Duet-1 vector (Novagen; Merck KGaA, Darmstadt, Germany), giving rise to an expression construct with the following additional N- and C-terminal sequences: MGSSHHHHHHSQDPN SSSARLQVD[1029]GTLEVLFQGPTGSTSA**WSHPQFEK**. The StrepII-Tag is shown in bold. For RAD5A-KT425,426AA, point mutations were introduced in the Walker A box, leading to –LG**AA**VM– instead of –LG**KT**VM–. For RAD5A-ΔHIRAN, 119 amino acids were deleted, starting at aa position 107, leading to –VSG[Δ]INS– instead of –VSG[119] INS–. This corresponds to the HIRAN domain as described (Iyer *et al.*, 2006). The N-terminal RAD5A fragment was expressed with the following sequence: MGSSHHHHHH SQDP**M[240]T**GTLEVLFQGPTGSTSAWSHPQFEK (bold: first 242 aa of RAD5A).

### Recombinant protein preparation

RAD5A and its variants were expressed in ER2566 (NEB) and purified via the StrepII-Tag followed by gel filtration. RAD5A-1-242 was purified via the StrepII-Tag. Details can be found as in Methods S1. Fractions were mixed with an equal volume of 100% glycerol, and fractions of interest were evaluated by SDS-PAGE and branch migration activity and pooled. Aliquots of the purified protein were stored at −80°C for further characterization. All recombinant RAD5A variants were purified in the same manner. The protein concentration was determined by colloidal Coomassie-stained SDS-PAGE using BSA (BioRad, Hercules, CA, USA) as the standard.

### DNA substrates

Appropriate oligonucleotides (Constantinou *et al.*, 2001; Gari *et al.*, 2008; Ip *et al.*, 2008; Bétous *et al.*, 2013) (Figure S1) were labeled with [γ-32P]-ATP (6000 Ci/mmol, Hartmann Analytic) and T4 polynucleotide kinase (NEB, Ipswich, MA, USA) and subsequently purified using Illustra MicroSpin G-25 columns (GE Healthcare, Pittsburgh, PA, USA). For the building up of substrates having either a 3′ TAMRA or a 3′ phosphate modification T4 PNK 3′ phosphatase minus (NEB) was used.

The replication fork substrates were prepared similarly to the HJ substrates following the protocol described (Bétous *et al.*, 2013) with the exception of elution of the substrates after gel extraction by diffusion at 4°C overnight in annealing buffer.

For the HJ substrates used for the data in Figure 2 oligonucleotides types A (labeled) and B as well as C and D were annealed by slowly decreasing the temperature after heating for 5 min at 95°C; oligonucleotides used for the generation of double-stranded DNA were prepared in the same manner. AB and CD were then annealed at 37°C for 30 min followed by cooling to room temperature.

The substrates used for the EMSAs shown in Figure 4(a, b) were prepared by slowly decreasing the temperature after heating for 5 min at 95°C of equimolar mixtures of the constituting oligonucleotides (Figure S1d) in annealing buffer as used for the preparation of the replication fork substrates.

### Translocase assay

Reactions were performed in translocase reaction buffer (40 mM Tris–HCl pH 8, 20 mM NaCl, 1.35 mM $MgCl_2$, 50 μg $ml^{-1}$ BSA, 6 mM DTT, 1.8 mM ATP) with 150 pM radioactively labeled HJ or RF. RAD5A enzyme was used in the indicated concentrations in a 20 μl reaction. To prebind *Bam*HI–E111A (NEB) to pmHJ_*Bam*HI–E111A, 5 nM *Bam*HI–E111A was added for 15 min at 30°C. The translocase reaction was initiated with the addition of RAD5A enzyme and was incubated at 37°C for 30 min.

The reactions were terminated with a one-third volume of stop solution (50 mM EDTA, 0.6% SDS, 0.1% xylene cyanol, 0.1% bromophenol blue, and 20% glycerol). To determine background values, parallel reactions were performed without enzyme. After native TBE-PAGE at 4°C, the radioactive DNA was visualized by autoradiography using the CR-35-Bio Scanner (Raytest, Straubenhardt, Germany) and quantified (Kobbe *et al.*, 2008, 2009; Blanck *et al.*, 2009) with an AIDA Image Analyzer 4.5 (Raytest).

### ATPase assay

An ATPase assay was performed with 5.75 nM of enzyme at 37°C for 30 min in ATPase-reaction buffer (40 mM

Tris–HCl pH 8, 20 mM NaCl, 25 μM $MgCl_2$, 50 μg $ml^{-1}$ BSA, 6 mM DTT) with 50 nM DNA substrate, 5 nM ATP and 0.4 nM [γ-32P]-ATP as a tracer. The reactions and analysis were performed as described for the translocase assay using 20% TBE-PAGE.

### Electrophoretic mobility shift assay

EMSA reactions were performed at 37°C for 10 min in EMSA-reaction buffer (40 mM Tris–HCl pH 8, 20 mM NaCl, 50 μg $ml^{-1}$ BSA, 6 mM DTT) with 75 pM radioactively labeled DNA substrate (supplied in one-tenth volume of annealing buffer); 0, 86, 172, 259 and 345 nM of RAD5A-1-242 were used in a 20 μl reaction. After adding one-third of the volume of loading solution (0.1% bromophenol blue, and 40% glycerol), the analysis was performed as described above.

### *In vivo* analysis

Genomic sequences of the promoter (763 bp upstream of the start codon) and terminator (518 bp downstream of the stop codon) regions of *RAD5A* and the coding sequences of RAD5A and RAD5A-ΔHIRAN were cloned into pPZP221 (Hajdukiewicz *et al.*, 1994). Using the GV3101::pMP90 *Agrobacterium* strain, the constructs were transformed into Arabidopsis plants via the floral dip method (Clough and Bent, 1998). T1-Transformants were selected on solid GM selection medium (60 mg $L^{-1}$ gentamycin), and a segregation analysis was used to identify single-locus lines in generation T2. Homozygous T-DNA containing plants were selected on selection medium in generation T3 and subsequently used in sensitivity assays, as described (Mannuss *et al.*, 2010).

### Immunologic detection of protein expression of complementation constructs

Three-day old Arabidopsis seedlings were ground in liquid nitrogen. The proteins in the cell powder were extracted by adding per 100 mg of powder 1 ml low salt buffer (20 mM Tris–HCl pH 8, 5 mM $MgCl_2$, 1 mM DTT, 1× protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) and 1 ml urea buffer (8 M urea, 20 mM Tris–HCl pH 8, 5 mM $MgCl_2$, 20 mM DTT, 1× protease inhibitor cocktail (Sigma-Aldrich). Both after the addition of low salt buffer and urea buffer the sample was treated for 5 min in a cold ultra sound bath. After centrifugation for 30 min at 16 000 ***g***, the proteins in the supernatant were precipitated overnight at −20°C by adding four times the volume of a solution containing 10% trichloroacetic acid dissolved in acetone and 20 mM DTT. The pellet after centrifugation was washed twice with acetone containing 20 mM DTT and dissolved in 30 mM Tris pH 8.5, 7 M urea, 2 M thiourea, and 65 mM CHAPS. Sample buffer was added and after denaturation for 30 min at 80°C, and the proteins were separated by 8% SDS-PAGE. Western blotting was then performed. An anti-RAD5A antibody was generated in rabbits against denatured recombinant full-length RAD5A by BioGenes. Serum antibodies were purified using Protein A HP SpinTrap columns (GE Healthcare). Detection was carried out using an anti-rabbit IgG HRP-linked secondary antibody (CST, Danvers, MA, USA) and the SignalFire Elite ECL reagent (CST).

## ACKNOWLEDGEMENTS

The authors would like to thank Rebecca Kucera and Jurate Bitinaite from New England Biolabs for the kind gift of *Bam*HI–E111A as well as Jennifer Reuster, Maria Männle and Marie Weihnacht for technical assistance. Jasmin Jansen and Sandra Thies were also involved in cloning and preliminary biochemical characterization. This work was supported the European Research Council ERC [ERC-2010-AdG_20100317 COMREC to H.P.] and by the German Research Foundation DFG [grant No. KO 4049/1-1 to D.K.].

## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

**Figure S1.** Overview of the substrates used.

**Figure S2.** Phosphorimager data demonstrating fork regression.

**Figure S3.** Translocase activity dependence on different (deoxy) nucleoside triphosphates and ATP analogs.

**Figure S4.** Titration of *Bam*HI–E111A.

**Methods S1.** Detailed purification protocol.

## REFERENCES

**Achar, Y.J., Balogh, D. and Haracska, L.** (2011) Coordinated protein and DNA remodeling by human HLTF on stalled replication fork. *Proc. Natl Acad. Sci. USA*, **108**, 14073–14078.

**Achar, Y.J., Balogh, D., Neculai, D., Juhasz, S., Morocz, M., Gali, H., Dhe-Paganon, S., Venclovas, Č. and Haracska, L.** (2015) Human HLTF mediates postreplication repair by its HIRAN domain-dependent replication fork remodeling. *Nucleic Acids Res.* **43**, 10277–10291.

**Andersen, P.L., Xu, F. and Xiao, W.** (2008) Eukaryotic DNA damage tolerance and translesion synthesis through covalent modifications of PCNA. *Cell Res.* **18**, 162–173.

**Bétous, R., Couch, F.B., Mason, A.C., Eichman, B.F., Manosas, M. and Cortez, D.** (2013) Substrate-selective repair and restart of replication forks by DNA translocases. *Cell Rep.* **3**, 1958–1969.

**Blanck, S., Kobbe, D., Hartung, F., Fengler, K., Focke, M. and Puchta, H.** (2009) A SRS2 homolog from *Arabidopsis thaliana* disrupts recombinogenic DNA intermediates and facilitates single strand annealing. *Nucleic Acids Res.* **37**, 7163–7176.

**Blastyák, A., Pintér, L., Unk, I., Prakash, L., Prakash, S. and Haracska, L.** (2007) Yeast Rad5 protein required for postreplication repair has a DNA helicase activity specific for replication fork regression. *Mol. Cell*, **28**, 167–175.

**Blastyák, A., Hajdú, I., Unk, I. and Haracska, L.** (2010) Role of double-stranded DNA translocase activity of human HLTF in replication of damaged DNA. *Mol. Cell. Biol.* **30**, 684–693.

**Branzei, D., Seki, M. and Enomoto, T.** (2004) Rad18/Rad5/Mms2-mediated polyubiquitination of PCNA is implicated in replication completion during replication stress. *Genes Cells*, **9**, 1031–1042.

**Broomfield, S., Hryciw, T. and Xiao, W.** (2001) DNA postreplication repair and mutagenesis in *Saccharomyces cerevisiae. Mutat. Res.* **486**, 167–184.

**Burkovics, P., Sebesta, M., Balogh, D., Haracska, L. and Krejci, L.** (2014) Strand invasion by HLTF as a mechanism for template switch in fork rescue. *Nucleic Acids Res.* **42**, 1711–1720.

**Chen, S., Davies, A.A., Sagan, D. and Ulrich, H.D.** (2005) The RING finger ATPase Rad5p of *Saccharomyces cerevisiae* contributes to DNA double-strand break repair in a ubiquitin-independent manner. *Nucleic Acids Res.* **33**, 5878–5886.

**Chen, I.-P., Mannuss, A., Orel, N., Heitzeberg, F. and Puchta, H.** (2008) A homolog of ScRAD5 is involved in DNA repair and homologous recombination in Arabidopsis. *Plant Physiol.* **146**, 1786–1796.

**Choi, K., Batke, S., Szakal, B., Lowther, J., Hao, F., Sarangi, P., Branzei, D., Ulrich, H.D. and Zhao, X.** (2015) Concerted and differential actions of two enzymatic domains underlie Rad5 contributions to DNA damage tolerance. *Nucleic Acids Res.* **43**, 2666–2677.

**Clough, S.J. and Bent, A.F.** (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735–743.

**Constantinou, A., Davies, A.A. and West, S.C.** (2001) Branch migration and Holliday junction resolution catalyzed by activities from mammalian cells. *Cell*, **104**, 259–268.

**Di Tommaso, P., Moretti, S., Xenarios, I., Orobitg, M., Montanyola, A., Chang, J.-M., Taly, J.-F. and Notredame, C.** (2011) T-Coffee: a web server for the multiple sequence alignment of protein and RNA sequences using structural information and homology extension. *Nucleic Acids Res.* **39**, W13–W17.

**Ding, L. and Forsburg, S.L.** (2014) Essential domains of *Schizosaccharomyces pombe* Rad8 required for DNA damage response. *G3 (Bethesda)*, **4**, 1373–1384.

**Dorner, L.F., Bitinaite, J., Whitaker, R.D. and Schildkraut, I.** (1999) Genetic analysis of the base-specific contacts of *Bam*HI restriction endonuclease. *J. Mol. Biol.* **285**, 1515–1523.

**Eastman, A.** (1985) Interstrand cross-links and sequence specificity in the reaction of cis-dichloro(ethylenediamine)platinum(II) with DNA. *Biochemistry*, **24**, 5027–5032.

**Engler, L.E., Sapienza, P., Dorner, L.F., Kucera, R., Schildkraut, I. and Jen-Jacobson, L.** (2001) The energetics of the interaction of *Bam*HI endonuclease with its recognition site GGATCC. *J. Mol. Biol.* **307**, 619–636.

**Flaus, A., Martin, D.M., Barton, G.J. and Owen-Hughes, T.** (2006) Identification of multiple distinct Snf2 subfamilies with conserved structural motifs. *Nucleic Acids Res.* **34**, 2887–2905.

**Gari, K., Décaillet, C., Stasiak, A.Z., Stasiak, A. and Constantinou, A.** (2008) The Fanconi anemia protein FANCM can promote branch migration of Holliday junctions and replication forks. *Mol. Cell*, **29**, 141–148.

**Hajdukiewicz, P., Svab, Z. and Maliga, P.** (1994) The small, versatile pPZP family of Agrobacterium binary vectors for plant transformation. *Plant Mol. Biol.* **25**, 989–994.

**Hartung, F., Suer, S. and Puchta, H.** (2007) Two closely related RecQ helicases have antagonistic roles in homologous recombination and DNA repair in *Arabidopsis thaliana*. *Proc. Natl Acad. Sci. USA*, **104**, 18836–18841.

**Hishiki, A., Hara, K., Ikegaya, Y., Yokoyama, H., Shimizu, T., Sato, M. and Hashimoto, H.** (2015) Structure of a novel DNA-binding domain of helicase-like transcription Factor (HLTF) and its functional implication in DNA damage tolerance. *J. Biol. Chem.* **290**, 13215–13223.

**Hoege, C., Pfander, B., Moldovan, G.-L., Pyrowolakis, G. and Jentsch, S.** (2002) RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. *Nature*, **419**, 135–141.

**Ip, S.C., Rass, U., Blanco, M.G., Flynn, H.R., Skehel, J.M. and West, S.C.** (2008) Identification of Holliday junction resolvases from humans and yeast. *Nature*, **456**, 357–361.

**Iyer, L.M., Babu, M.M. and Aravind, L.** (2006) The HIRAN domain and recruitment of chromatin remodeling and repair activities to damaged DNA. *Cell Cycle*, **5**, 775–782.

**Kile, A.C., Chavez, D.A., Bacal, J., Eldirany, S., Korzhnev, D.M., Bezsonova, I., Eichman, B.F. and Cimprich, K.A.** (2015) HLTF's ancient HIRAN domain binds 3′ DNA ends to drive replication fork reversal. *Mol. Cell*, **58**, 1090–1100.

**Kobbe, D., Blanck, S., Demand, K., Focke, M. and Puchta, H.** (2008) AtRECQ2, a RecQ helicase homologue from *Arabidopsis thaliana*, is able to disrupt various recombinogenic DNA structures in vitro. *Plant J.* **55**, 397–405.

**Kobbe, D., Blanck, S., Focke, M. and Puchta, H.** (2009) Biochemical characterization of AtRECQ3 reveals significant differences relative to other RecQ helicases. *Plant Physiol.* **151**, 1658–1666.

**Kobbe, S., Trapp, O., Knoll, A., Manuss, A. and Puchta, H.** (2015) The translesion polymerase ζ has roles dependent on and independent of the nuclease MUS81 and the helicase RECQ4A in DNA damage repair in arabidopsis. *Plant Physiol.* **169**, 2718–2729.

**MacKay, C., Toth, R. and Rouse, J.** (2009) Biochemical characterisation of the SWI/SNF family member HLTF. *Biochem. Biophys. Res. Commun.* **390**, 187–191.

**Mannuss, A., Dukowic-Schulze, S., Suer, S., Hartung, F., Pacher, M. and Puchta, H.** (2010) RAD5A, RECQ4A, and MUS81 have specific functions in homologous recombination and define different pathways of DNA repair in *Arabidopsis thaliana*. *Plant Cell*, **22**, 3318–3330.

**Masuda, Y., Suzuki, M., Kawai, H., Hishiki, A., Hashimoto, H., Masutani, C., Hishida, T., Suzuki, F. and Kamiya, K.** (2012) En bloc transfer of polyubiquitin chains to PCNA in vitro is mediated by two different human E2-E3 pairs. *Nucleic Acids Res.* **40**, 10394–10407.

**Minca, E.C. and Kowalski, D.** (2010) Multiple Rad5 activities mediate sister chromatid recombination to bypass DNA damage at stalled replication forks. *Mol. Cell*, **38**, 649–661.

**Motegi, A., Sood, R., Moinova, H., Markowitz, S.D., Liu, P.P. and Myung, K.** (2006) Human SHPRH suppresses genomic instability through proliferating cell nuclear antigen polyubiquitination. *J. Cell Biol.* **175**, 703–708.

**Motegi, A., Liaw, H.-J., Lee, K.-Y. *et al.*** (2008) Polyubiquitination of proliferating cell nuclear antigen by HLTF and SHPRH prevents genomic instability from stalled replication forks. *Proc. Natl Acad. Sci. USA*, **105**, 12411–12416.

**Pagès, V. and Fuchs, R.P.** (2003) Uncoupling of leading- and lagging-strand DNA replication during lesion bypass in vivo. *Science*, **300**, 1300–1303.

**Sakamoto, A., Lan, V.T., Hase, Y., Shikazono, N., Matsunaga, T. and Tanaka, A.** (2003) Disruption of the AtREV3 gene causes hypersensitivity to ultraviolet B light and gamma-rays in Arabidopsis: implication of the presence of a translesion synthesis mechanism in plants. *Plant Cell*, **15**, 2042–2057.

**Schröpfer, S., Kobbe, D., Hartung, F., Knoll, A. and Puchta, H.** (2014) Defining the roles of the N-terminal region and the helicase activity of RECQ4A in DNA repair and homologous recombination in Arabidopsis. *Nucleic Acids Res.* **42**, 1684–1697.

**Sommers, J.A., Banerjee, T., Hinds, T., Wan, B., Wold, M.S., Lei, M. and Brosh, R.M.** (2014) Novel function of the Fanconi anemia group J or RECQ1 helicase to disrupt protein-DNA complexes in a replication protein A-stimulated manner. *J. Biol. Chem.* **289**, 19928–19941.

**Sood, R., Makalowska, I., Galdzicki, M., Hu, P., Eddings, E., Robbins, C.M., Moses, T., Namkoong, J., Chen, S. and Trent, J.M.** (2003) Cloning and characterization of a novel gene, SHPRH, encoding a conserved putative protein with SNF2/helicase and PHD-finger domains from the 6q24 region. *Genomics*, **82**, 153–161.

**Strzalka, W., Bartnicki, F., Pels, K., Jakubowska, A., Tsurimoto, T. and Tanaka, K.** (2013) RAD5a ubiquitin ligase is involved in ubiquitination of *Arabidopsis thaliana* proliferating cell nuclear antigen. *J. Exp. Bot.* **64**, 859–869.

**Svoboda, D.L. and Vos, J.M.** (1995) Differential replication of a single, UV-induced lesion in the leading or lagging strand by a human cell extract: fork uncoupling or gap formation. *Proc. Natl Acad. Sci. USA*, **92**, 11975–11979.

**Ulrich, H.D.** (2005) The RAD6 pathway: control of DNA damage bypass and mutagenesis by ubiquitin and SUMO. *Chembiochem*, **6**, 1735–1743.

**Ulrich, H.D. and Jentsch, S.** (2000) Two RING finger proteins mediate cooperation between ubiquitin-conjugating enzymes in DNA repair. *EMBO J.* **19**, 3388–3397.

**Unk, I., Hajdú, I., Fátyol, K., Szakál, B., Blastyák, A., Bermudez, V., Hurwitz, J., Prakash, L., Prakash, S. and Haracska, L.** (2006) Human SHPRH is a ubiquitin ligase for Mms2-Ubc13-dependent polyubiquitylation of proliferating cell nuclear antigen. *Proc. Natl Acad. Sci. USA*, **103**, 18107–18112.

**Unk, I., Hajdú, I., Fátyol, K., Hurwitz, J., Yoon, J.-H., Prakash, L., Prakash, S. and Haracska, L.** (2008) Human HLTF functions as a ubiquitin ligase for proliferating cell nuclear antigen polyubiquitination. *Proc. Natl Acad. Sci. USA*, **105**, 3768–3773.

**Unk, I., Hajdú, I., Blastyák, A. and Haracska, L.** (2010) Role of yeast Rad5 and its human orthologs, HLTF and SHPRH in DNA damage tolerance. *DNA Repair (Amst.)*, **9**, 257–267.

**Wang, S., Wen, R., Shi, X., Lambrecht, A., Wang, H. and Xiao, W.** (2011) RAD5a and REV3 function in two alternative pathways of DNA-damage tolerance in Arabidopsis. *DNA Repair (Amst.)*, **10**, 620–628.

**Xiao, W., Chow, B.L., Broomfield, S. and Hanna, M.** (2000) The *Saccharomyces cerevisiae* RAD6 group is composed of an error-prone and two error-free postreplication repair pathways. *Genetics*, **155**, 1633–1641.