The Human Suv3 Helicase Interacts with Replication Protein A and Flap Endonuclease 1 in the Nucleus.

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Abbreviations: dsDNA, double stranded DNA; FEN1, flap endonuclease 1; GEN, gap endonuclease activity; mtSSB, mitochondrial single strand binding protein; PNPase, polynucleotide phosphorylase; RPA, replication protein A; SCE, sister chromatid exchange; ssDNA, single stranded DNA

SYNOPSIS

The human Suv3 helicase (hSuv3) has been shown to be a major player in mitochondrial RNA surveillance and decay, but its physiological role might go beyond this functional niche. hSuv3 has been found to interact with BLM and WRN, members of the RecQ helicase family involved in multiple DNA metabolic processes, and in protection and stabilization of the genome. Here, we have addressed the possible role of hSuv3 in genome maintenance by examining its potential association with key interaction partners of the RecQ helicases. By analysis of hSuv3 co-immunoprecipitation complexes, we identify two new interaction partners of hSuv3: the Replication Protein A (RPA) and Flap Endonuclease 1 (FEN1). Utilizing an *in vitro* biochemical assay we find that low amounts of RPA inhibits helicase activity of hSuv3 on a forked substrate. Another single strand binding protein, mtSSB, fails to affect hSuv3 activity, indicating that the functional interaction is specific for hSuv3 and RPA. Further *in vitro* studies demonstrate that the flap endonuclease activity of FEN1 is stimulated by hSuv3 independently of flap length. hSuv3 is generally thought to be a mitochondrial helicase, but the physical and functional interactions between hSuv3 and known RecQ helicase associated proteins strengthen the hypothesis that hSuv3 may play a significant role in nuclear DNA metabolism as well.

Key words: hSuv3 helicase, Flap Endonuclease 1, Replication Protein A, RecQ helicase family, genome stability, DNA repair

INTRODUCTION

The Suv3 helicase (SUV3, hSUV3p, SUPV3L1) was originally identified in a study on mitochondrial RNA metabolism in *Saccharomyces cerevisciae* [1]. Together with the nuclease Dss1p, *S. cerevisiae* Suv3 (ScSuv3p) forms the mitochondrial degradosome complex, which plays a pivotal role in mitochondrial RNA metabolism, and consequently in mitochondrial and cellular homeostasis [2, 3]. Suv3 is ubiquitously present in all eukaryotes [4]. The human *SUV3* gene was identified based on a high level of sequence conservation [4], and characterized as a DExH-box RNA helicase of the Ski2 superfamily capable of unwinding RNA/RNA, RNA/DNA and DNA/DNA duplexes *in vitro* [4, 5]. Originally, hSuv3 was found to primarily unwind substrates in the 5'-3' direction [5], however, recently the directionality was reported to be 3'-5' [6].

Expression analysis in human tissues found the gene to be expressed in all tissues, with the highest levels in the liver [4]. In mice, Suv3 expression begins at the blastocyst stage and becomes extensive in all cell types throughout life, with expression in the mature animal being highest in the brain, sensory organs and testis [7]. Complete loss of the *SUV3* gene in mice results in embryonic lethality [8], while conditional post-natal loss of Suv3 function in mice leads to an accelerated aging-like phenotype [9].

Human Suv3 localizes mainly to the mitochondrial matrix [10, 11]. It appears to have a central role in mitochondrial RNA metabolism, and its knockdown is associated with accumulation of truncated mitochondrial RNA species, decrease in mitochondrial DNA copy number and mitochondrial protein expression, eventually resulting in cell death [12-14]. To date, no ortholog of the Dss1p nuclease has been found in humans, and results proposing the polynucleotide phosphorylase (PNPase) as a possible alternative [6, 15] remain a matter of controversy due to the lack of PNPase in the mitochondrial matrix [16].

In addition to the mitochondrial localization, a small fraction of hSuv3 has been detected in the nucleus [13]. Knockdown of hSuv3 in HeLa cells led to an increase in homologous recombination during mitosis as measured by sister chromatid exchange (SCE) [8], indicating a potential nuclear role of hSuv3. However, no nuclear function of hSuv3 has yet been reported. Nonetheless, a high throughput interaction screen in *S. cerevisiae* found that ScSuv3p interacts with Sgs1, the sole yeast RecQ helicase [17]. Importantly, the interaction seems to be conserved as hSuv3 was found to interact with two of the human RecQ helicases, WRN and BLM [8]. The RecQ helicase family is well known for their crucial role in genome maintenance, with deficiencies leading to severe DNA instability associated with premature aging syndromes. These helicases participate in an intricate interplay with a variety of DNA metabolic proteins to achieve an optimal activity for each of their numerous roles [18]. One such partner is RPA, a heterotrimeric single-stranded DNA-binding protein complex, consisting of 70, 32 and 14 kDa subunits, which was identified for its role in SV40 DNA replication [19]. RPA has been shown to stimulate helicase activities of RecQ helicases RECQ1 [20], BLM [21], WRN [22], RECQL4 [23] and RECQ5β [24]. Through its binding to DNA, RPA appears to actively coordinate assembly and disassembly of DNA processing proteins on ssDNA, thereby serving multiple roles in DNA metabolism spanning from DNA replication and telomere maintenance to homologous recombination and repair [25].

FEN1 is another well documented partner of the RecQ helicases [18]. It is a multifunctional structure-specific nuclease, displaying an endonuclease activity on 5'-flap single-stranded DNA or RNA substrates, a low efficiency 5'-3' exonuclease activity on DNA structures [26] and a gap endonuclease activity [27]. FEN1 plays a critical role in DNA repair, homologous recombination, telomere maintenance, RNA primer removal and resolution of stalled replication forks [27-30]. While at present a potential relationship between FEN1 and RECQ1 has not been characterized [31], stimulation of FEN1 in the presence of BLM [32], WRN [33], RECQL4 [34] and RECQ5β [35] has been reported.

We speculate that hSuv3 might participate in genome maintenance *via* a RecQ helicase like complex through interactions with members of the RecQ family and other proteins known to be key partners of the RecQ helicases. In this study we present evidence that hSuv3 interacts with FEN1 and RPA, and examine the functional consequences of these interactions.

EXPERIMENTAL

Cell culture

HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 50 µg/ml streptomycin in a humidified 5% CO_2 incubator at 37ºC. GM38 primary fibroblasts were grown in modified Eagle's medium supplemented with 15% fetal bovine serum, non-essential amino acids, essential amino acids, 50 U/ml penicillin and 50 µg/ml streptomycin in a humidified 5% CO_2 incubator at 37ºC.

hSuv3 Plasmids and Purification

Plasmid expressing 6xHis-MBP-Δ46NhSuv3, a fusion of His-tagged maltose binding protein and amino acids 47-786 of hSuv3 was generated as previously described [13]. The above plasmid was modified by PCR-mediated C-terminal deletion with primers: hSUV3-722Cdel-F2, 5'-TAGACCCAGCTTTCTTGTACAAAGTGGTG-3', and hSUV3-722Cdel-R, 5'-CTCAGTAGCTTTACTCCCTAGAGCTTTGGT-3', resulting in a construct pEXPHisMBP-hSuv3(47-722)-WT encoding residues 47-722 of the hSuv3 with N-terminal 6xHisMBP tag. Furthermore, point mutation of the conserved lysine 213 (Walker A motif) was introduced into construct pEXPHisMBP-hSuv3(47-722)-WT with primers: hSUV3-K213A-F, 5'-GCGACTTATCACGCAATCCAGAAATAC-3' and hSUV3-K213A-R, 5'-TCCACTGTTTGTGGGGCCTGAATG-3', generating pEXPHisMBP-hSuv3(47-722)-K213A. Both plasmids, pEXPHisMBP-hSuv3(47-722)-WT and pEXPHisMBP-hSuv3(47-722)-K213A, were verified by direct sequencing. Protein expression and purification was carried out as previously described [13], with the following modifications. Protein expression was induced with 0.2 mM IPTG for 18 hrs at 20°C. Metal affinity chromatography steps were performed on Ni-charged HisTrap FF columns (GE Healthcare) equilibrated with Buffer A (50 mM HEPES NaOH, pH 7.5, 300 mM KCl, 10 mM β-mercaptoethanol, 10% (v/v) glycerol, 25 mM imidazole) and developed with Buffer A supplemented with 250 mM imidazole. Size exclusion chromatography was carried on Superdex 200 16/60 column (GE Healthcare) equilibrated with Buffer A.

Recombinant proteins

The recombinant RPA was purified as a complex from *E. coli* by methods described previously [36]. Recombinant mtSSB was purchased from Abnova. Purified FEN1 was a generous gift from Dr. David M. Wilson, III (NIA, Baltimore).

Preparation of cell extracts

Whole cell extracts were prepared by incubating harvested HeLa cells with the CelLytic™ M reagent (Sigma) for 30 minutes at 4ºC, followed by centrifugation at 18,000*g* for 20 min. The supernatant was used directly for further analysis. Nuclear extracts were prepared as described previously [37].

Antibodies

Polyclonal anti-hSuv3 antibodies were raised against the recombinant purified hSuv3(47-722) in rabbits by Covance, Inc. The antibodies were purified from whole serum using AminoLink Plus Immobilization Kit (Pierce) according to manufacturer instructions. The following antibodies were purchased: mouse monoclonal anti-RPA (Santa Cruz Biotechnology, sc-81372), mouse

monoclonal anti-FEN1 (Abcam, Ab462), mouse monoclonal anti-Cdc47 (Thermo scientific, MS862-P1), rabbit polyclonal anti-calregulin (Santa Cruz Biotechnology, sc11398) and rabbit polyclonal anti-VDAC (Abcam, ab15895).

Co-immunoprecipitation
The cell extracts were pre-cleared with Recombinant Protein A agarose beads for 2 hours at 4˚C. The pre-cleared extracts were incubated with 4 µg of antibody overnight at 4ºC. Protein A agarose beads were pre-blocked with 0.1 µg/ml BSA, before incubation with the lysate-antibody complexes, for 2 hours at 4˚C. The beads were washed three times in the indicated wash buffer: hSuv3-RPA: 50 mM Tris-HCl pH 7.4, 150 mM NaCl and 0.01% Triton X-100; hSuv3-FEN1: 20 mM HEPES (pH 7.9), 20% glycerol, 300 mM KCl, and 0.1% Tween 20. The final complexes were eluted by boiling the beads in reducing SDS-PAGE loading buffer for 5 minutes at 95ºC. The immunoprecipitates were analyzed by Western blotting. In the case of treatment, the nuclear extracts were incubated with 0.2 U/(µg of protein) DNase I for 20 min at 37˚C or 50 µg/ml ethidium bromide (EtBr) prior to immunoprecipitation.

Western blotting
Extracts were separated on an 8-16% Tris-glycine gradient SDS gel followed by transfer to a PVDF membrane. The membrane was blocked for 1 hour at 37ºC with TBST (150 mM NaCl, 20 mM Tris-HCl pH 7.4 and 0.1% Tween 20) containing 3% non-fat dry milk, and incubated with the primary antibodies for 1 hour at room-temperature. The membrane was further incubated with horseradish peroxidase-linked secondary antibodies (Sigma) and visualized using ECL® plus chemiluminescent detection kit.

Oligonucleotide substrates
PAGE-purified oligonucleotides (Integrated DNA Technologies) were used for preparation of substrates: D50: 5'-GGGACGCGTCGGCCTGGCACGTCGGCCGCTGCGGCCAGGCACCCGATGGC-3', D49: 5'-TTTGTTTGTTTGTTTGTTTGTTTGCCGACGTGCCAGGCCGACGCGTCCC-3', D50-2: 5'-GGGACGCGTCGGCCTGGCACGTCGGCTTTGTTTGTTTGTTTGTTTGTTTT-3', D25F: 5'- CCGACGTGCCAGGCCGACGCGTCCC-3', D25-2: 5'-GGGACGCGTCGGCCTGGCACGTCGG-3', FLAP1: 5′-AGTAAAACGACGGCCAGTGC-3', FLAP15: 5'-TTTTTTTTTTTCCAAGTAAAACGACGGCCAGTGC-3', FLAP template (T44): 5'-GCA CTG GCC GTC GTT TTA CGG TCG TGA CTG GGA AAA CCC TGG CG-3'
FLAP upstream (F25): 5′-CGC CAG GGT TTT CCC AGT CAC GAC C-3′.
5'-end labeling was performed by incubating the oligonucleotide in the presence of γ^{32}P-ATP and T4 PNK at 37 ºC for one hour. For annealing, 5'-^{32}P-labeled oligonucleotides were mixed with 2-fold excess of the unlabeled complementary strand in 40 mM Tris-HCl pH 8.0, 50 mM NaCl and incubated at 70ºC for 10 min, followed by slow-cooling to 30ºC. Finally, the resulting substrates were purified using G-25 spin-columns.

Helicase activity assay
The reactions contained 0.5 nM substrate and the indicated concentration of protein in 20 mM HEPES NaOH, pH 7.5, 1 mM ATP, 3 mM $MgCl_2$, 1 mM DTT and 5% glycerol. Reactions for the interaction studies with RPA and mtSSB were performed in 20 mM Tris-HCl pH 7.4, 1mM ATP, 3 mM MgCl2, 1 mM DTT, 5% glycerol and 0.1 mg/ml BSA. The reactions were started by

addition of the 5'-end labeled substrate, incubated at 37°C for 30 min, terminated by addition of helicase stop buffer to a final concentration of 10 mM Tris HCl, pH 8.0, 10 mM EDTA, 10% glycerol, 0.3% SDS, 0.01% bromophenol blue, 15 nM unlabeled oligonucleotide and 10 ng/µl Proteinase K (New England Biolabs), and incubated 10 min. at 37°C. The products were resolved on a non-denaturing 8% polyacrylamide gel and detected using PhosphorImager followed by analysis with ImageQuant software.

Incision activity assay
Reactions were prepared as described for the helicase activity assay in 20 mM HEPES NaOH, pH 7.5, 1 mM ATP, 3 mM $MgCl_2$, 1 mM DTT, 5% glycerol. After incubation with the substrate for 15 min at 37°C, the reactions were terminated by addition of Formamide Loading Dye to a final concentration of 30% formamide (v/v), 3.3 mM EDTA, 0.003% bromophenol blue. Samples were heated at 95ºC for 5 minutes, and loaded onto 7 M urea, 15% polyacrylamide gel. Results were analyzed as for the helicase activity assay.

RESULTS

Substrate specificity and directionality of hSuv3
Initial biochemical characterization of hSuv3 helicase activity conducted by Shu *et al.* [5] revealed that the enzyme possesses 5'-3' directionality. Although slight increase of the product indicating the reverse 3'-5' direction was shown, it was concluded that the hSuv3 preferentially unwinds tailed substrates in the 5'-3' direction. More recently, the same group reported an opposite finding demonstrating 3'-5' directionality of hSuv3 [6] . In the second study the tailed substrates used were exclusively unwound in the 3'-5' fashion with no evidence for activity in the reverse direction. In light of the reported discrepancy in directionality of hSuv3, we decided to analyze the helicase activity of hSuv3 on multiple DNA duplex substrates. To do so, we expressed and purified to apparent homogeneity hSuv3(47-722)-WT and its corresponding hSuv3(47-722)-K213A point mutant lacking ATPase activity (Figure 1A). To verify that the helicase activity was solely from hSuv3 and not from a co-purifying *E. coli* helicase, both the WT and the mutant protein were tested on a forked DNA substrate D49/D50-2*. The WT protein unwound DNA only in the presence of ATP and the activity was abolished when the energy co-factor was substituted by a non-hydrolyzable analog ATPγS. No helicase activity was observed when the hSuv3(47-722)-K213A point mutant was used (Figure 1B). To test the directionality of hSuv3 helicase we used D49/D50-2* forked substrate with 26 bp duplex region and 24-23 nt single-stranded arms, and corresponding tailed substrates in which one of the single-stranded regions was removed. We found that hSuv3 translocates in the 3'-5' direction during unwinding of DNA duplexes, as demonstrated by strand separation of the 3'-tailed D25F/D50-2* substrate (Figure 1C). No helicase activity was observed with the 5'-tailed D49*/D25-2 substrate. Additionally, our results demonstrate that the enzyme preferentially unwound the forked DNA/DNA duplex (D49/D50-2*) over the corresponding tailed substrate (D25F/D50-2) (Figure 1C).

Physical interaction betweenhSuv3 and RPA *in vivo*
Studies have shown that Suv3 interacts with several RecQ helicases, WRN and BLM in human cells [8], and Sgs1 in yeast [17]. In order to explore the possibility of the involvement of hSuv3 in DNA metabolism through a RecQ-helicase like complex, we decided to search for interactions between hSuv3 and well-documented protein binding partners of RecQ family members. To investigate a potential association of endogenous hSuv3 and RecQ helicase partners, we performed co-immunoprecipitation using HeLa cell lysates. RPA was one of the proteins we were able to detect by Western blotting in the anti-hSuv3 immunoprecipitates (Figure 2A). The result was further supported by the reciprocal experiment, in which hSuv3 was immunoprecipitated by the anti-RPA antibody. As controls, normal IgGs (from mouse or rabbit) were used under the same assay conditions, and neither hSuv3 nor RPA were detected.

To date, RPA has been described as an exclusively nuclear protein [38], while hSuv3 localizes to both the mitochondrial matrix [10] and the nucleus [13]. Based on this, we hypothesized that it is the nuclear fraction of hSuv3 that is binding RPA. To test this, we repeated the co-immunoprecipitation experiment using HeLa nuclear extract (Figure 2B). Indeed, the result was confirmed and even stronger interaction was seen in the nuclear fraction. The quality of the nuclear extract was analyzed using subcellular markers (Figure 2C). The nuclear extract was negative for calregulin and VDAC, used as cytoplasmic and mitochondrial markers, respectively, while enriched for the nuclear protein Cdc47.

Since both hSuv3 and RPA are able to bind DNA, we wanted to rule out the possibility that the observed interaction was mediated through DNA. Pretreatment of the nuclear extract with either DNase I or ethidium bromide (EtBr) did not decrease the signal compared to the non-treated control, supporting the notion that the interaction is protein-protein mediated (Figure 2D).

To investigate if the finding could be extended to non-transformed cells, additional nuclear extracts were prepared from GM38 primary fibroblasts and used for co-immunoprecipitation. Again, precipitation of Suv3 with RPA was achieved (Figure 2E). The reciprocal co-immunoprecipitation was unsuccessful. Still, these results support the notion that the interaction may have a biological relevance in normal cell function

RPA inhibits hSuv3 helicase activity.
Previous studies have shown that RPA can stimulate DNA unwinding activity of several RecQ helicases, including RECQ1 [20], BLM [21], WRN [22], RECQL4 [23] and RECQ5β [24]. In light of the observed interaction, we considered that the same could be the case for hSuv3. To test potential functional consequence of the physical interaction between hSuv3 and RPA, we performed the helicase activity assay on a forked duplex substrate in the presence of hSuv3 and increasing concentrations of RPA. The presence of RPA led to inhibition of the unwinding of a 50-bp forked substrate by hSuv3 (Figure 3A-B). The highest amounts of RPA alone resulted in a small amount of product as well, which can be explained by the double strand destabilization by RPA due to its inherent polarity binding [19].
To determine whether this functional effect was specific to RPA, the human mitochondrial single-stranded DNA binding protein (mtSSB) was used in a parallel hSuv3 unwinding assay. The mtSSB had no significant effect on the helicase activity of hSuv3 on the forked substrate at all concentrations tested (Figure 3C-D). The capability of both RPA and mtSSB to bind the

substrate was verified by electromobility shift assay (EMSA) (data not shown). Consequently, the lack of effect by mtSSB supports the idea that a specific protein-protein interaction between hSuv3 and RPA is responsible for the decreased unwinding of the DNA substrate.

Physical interaction between hSuv3 and FEN1 *in vivo*.

To learn more about a potential hSuv3 multiprotein complex, we examined the co-immunoprecipitate from HeLa nuclear extracts for other robust RecQ helicase partners. The FEN1 protein was detected in the anti-hSuv3 immunoprecipitates from nuclear extracts (Figure 4A). Again, neither normal mouse, nor rabbit IgG were able to co-immunoprecipitate either one of the above proteins.

Also, we could demonstrate the presence of hSuv3 protein in the anti-FEN1 immunoprecipitate. Together these results support the notion that nuclear hSuv3 interacts with FEN1. The interaction was not affected by the presence of either EtBr or DNase I, demonstrating that it is not DNA-mediated, but rather a direct protein-protein interaction (Figure 4B). These results were also reproduced using GM38 primary fibroblasts (Figure 4C), again supporting a biological role of the interaction.

Stimulation of flap incision in the presence of hSuv3

Several of the RecQ helicases, including BLM [32], WRN [33], RECQL4 [34] and RECQ5β [35], have been found to stimulate the 5'-flap incision of FEN1. Hence, based on the physical interaction observed between hSuv3 and FEN1, we tested whether the interaction could modulate the catalytic activity of one or both of the proteins. In order to characterize the effect of hSuv3 on the rate of FEN1 incision, we performed an incision assay utilizing a 44 bp DNA substrate with a 15 nucleotide flap, adjacent to an upstream 19 bp duplex. In the presence of FEN1 alone, 17% of the substrate was incised. When adding increasing amounts of hSuv3-WT to the reaction, an approximately 3-fold stimulation was observed using 10 fold excess of hSuv3-WT compared to the effect of BSA (Figure 5A). hSuv3-K213A did also cause significant stimulation at similar conditions though the effect was less pronounced compared to hSuv3-WT. Considering that the flap structure could be important for the stimulatory effect, we performed the assay on a similar substrate with a shorter 1 nucleotide flap (Figure 5C-D). Again, 2-3 fold stimulation was achieved by adding the hSuv3 proteins, indicating that hSuv3 stimulates FEN1 incision independently of flap length. Importantly, hSuv3 alone did not catalyze any significant cleavage of the 5'-flap DNA substrates.

DISCUSSION

The importance of the mitochondrial function of conserved Suv3 helicase has been demonstrated in several organisms, ranging from the protozoan *Trypanosoma brucei* to *S. cerevisiae* and mammals [2, 12, 39]. However, evidence of the nuclear role is limited. Interactions with the RecQ helicases demonstrated in *S. cerevisiae* (Sgs1) [17], and in humans (WRN and BLM) [8] may provide clues to the elucidation of Suv3's nuclear function. Deficiencies in either BLM or WRN are associated with premature aging, sharing striking similarity to the phenotype caused by disruption of Suv3 in mice, which includes sarcopenia, kyphosis, skin defects and premature death [9, 18, 40, 41]. However, only BLM deficiency is associated with elevated sister chromatid exchange, also seen in the hSuv3 knockdown [8, 42]. It was previously suggested that hSuv3 could work together with WRN and BLM in resolving replication fork crises. This could explain the elevated sister chromatid exchange (SCE) levels in cells with knocked-down hSuv3, possibly as a result of alternative repair by illegitimate recombination [8]. Identification of interacting protein partners is crucial to a further characterization of the potential function of hSuv3 in DNA metabolism. This manuscript describes, for the first time, non-mitochondrial interactions of hSuv3 in an *in vivo* experimental setup. Previous data have been based either on yeast two-hybrid systems [43] or *in vitro* interactions [8].

In this study, we find that hSuv3 directly interacts with the nuclear protein RPA. RPA is considered to be the primary eukaryotic single-stranded DNA binding protein, serving functional roles in replication, recombination and repair [38]. Hence, the results suggest that hSuv3 cooperates with RPA in one of these essential processes. Interestingly, RPA inhibits hSuv3 unwinding of a forked substrate. This differs from the results previously obtained with RecQ helicases [20-22, 24], where RPA was shown to stimulate DNA unwinding activity in a dose-dependent manner. Another single stranded binding protein, mtSSB, does not cause the same effect. Therefore, the effect of hSuv3's helicase activity by RPA cannot be ascribed to the mere presence of ssDNA binding protein on the exposed single strands blocking hSuv3 entry.
One possible explanation for the decrease in hSuv3 helicase activity caused by RPA could be that post-translational modifications of one or both proteins, dictated by a specific cellular event, might be needed to stabilize and cause stimulation. Alternatively, the role of the hSuv3-RPA interaction might be independent of helicase activity, and rather be limited to coordinate loading of other proteins facilitating a specific functional outcome. As such, the BLM helicase promotes homologous recombination between diverged sequences through a novel ATPase-independent mechanism [44].

In *S. cerevisiae* ScSuv3p was found to interact with Ddc1 and MEC3 [17]. Ddc1 and MEC3 are DNA damage checkpoint proteins recruited to unrepaired recombination intermediates, and are homologues of human Rad9 and Hus1 of the 9-1-1 checkpoint complex (SGD yeast database: http:/db.yeastgenome.org/cgi-bin/locus.pl?locus=dde). The 9-1-1 complex is a multifunctional protein complex, loaded onto DNA in response to various types of genotoxic stresses, which participates in checkpoint signaling at stalled replication forks [45]. RPA is known to bind to the Rad9 subunit, hence the three proteins in complex could be involved in the resolving of blocked replication forks.

Our investigation also uncovered an interaction between hSuv3 and FEN1 in the nuclear fraction of human cells. hSuv3 could potentially modulate the activity of FEN1 *in vivo* in one of its many functions in DNA repair, telomere maintenance, Okazaki fragment maturation or resolving stalled replication forks [46, 47]. In this case, hSuv3 was shown to stimulate the incision activity of FEN1, independently of flap length and only in part on helicase activity. The K213A amino acid substitution is located in a Walker A consensus motif in which the lysine residue is believed to be important for ATP binding [48]. Therefore hSuv3-K213A would be expected to lack any conformational change associated with ATP binding, which could explain the difference between the effects of hSuv3-WT and hSuv3-K213A on FEN1 incision. On the other hand, the stimulatory effect by hSuv3-K213A might still be sufficient to fulfill the nuclear role of hSuv3. A potential ATP-independent role of hSuv3 could explain why expression of a nuclear dominant negative version of hSuv3 did not affect morphology or growth rate of the cell [15].

Helicase-independent stimulation of FEN1 activity has also been shown for WRN, BLM, RecQ4 and RecQ5 [32-35]. The interaction between WRN and FEN1 has previously been proposed to be involved in initiation of recombination pathways at stalled replication forks by employing FEN1's gap endonuclease activity (GEN) [27]. The FEN1 GEN activity was also stimulated in the presence of phosphorylated RPA (pRPA). Replication fork arrest is one event that has been reported to lead to phosphorylation of RPA [49], and consequently it has been speculated that FEN1 may coordinate with RPA in a protein complex located at the replication fork [47]. Our findings could suggest involvement of hSuv3 in this putative complex. Meanwhile, high amounts of RPA were found to inhibit FEN1 flap cleavage, an effect believed to be important in regulation of endonuclease switching in Okazaki fragment maturation [50]. This could implicate hSuv3 in proper lagging strand synthesis during Okazaki fragment processing.

To summarize, we present evidence of physical and functional interactions of hSuv3 with the RPA and FEN1 proteins. These findings support our hypothesis that hSuv3 participates in a multi-protein complex involved in nuclear DNA metabolism, making hSuv3 a potential new player in the field of genome maintenance.

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FIGURES

Figure 1: Substrate Specificity and Directionality of hSuv3. (A) SDS-PAGE of purified hSuv3(47-722)-WT and -K213A mutant, 500 ng per lane, 4-15% gel, silver stained. (B) Helicase assay performed as described in Materials and Methods with D49/D50-2* forked substrate. WT and K213A hSuv3(47-722) were used at 20 nM concentration. Energy cofactors used are indicated. Triangle denotes heat-denatured substrate (C). Helicase assay with indicated DNA substrates. hSuv3(47-722)-WT used at indicated concentrations.

Figure 2: Interaction of RPA with hSuv3. (A) Interaction of RPA with hSuv3 in HeLa whole cell extract. Total cell lysates were used for co-immunoprecipitation (co-IP) assays with anti-RPA, anti-hSuv3 or normal IgG. (B) RPA and hSuv3 interact in the nuclear fraction. HeLa nuclear extracts (1 mg) were used for co-IP as in A. (C) Western blot analysis of nuclear extract. The nuclear extract used in B and whole cell extract were analyzed for the presence of subcellular markers calregulin (cytoplasma), VDAC (mitochondria) and Cdc47 (nucleus). (D) The hSuv3-RPA interaction is independent of DNA. HeLa nuclear extracts (1 mg) were either untreated or treated with 0.2 U/(µg of protein) DNase I (Ambion) for 20 min at 37˚C or 50 µg/ml ethidium bromide (EtBr) for 30 min on ice, before co-IP as in A. (E) RPA and hSuv3 interact in the nuclear fraction of primary human fibroblast GM38. Nuclear extracts were used for co-IP as in B.

Figure 3: Inhibition of hSuv3 helicase activity by RPA. (A) Unwinding activity of hSuv3 on a forked substrate in the presence of RPA. Purified recombinant proteins were mixed (50 nM hSuv3 and RPA as indicated) and the reaction was started by addition of the radioactively labeled DNA substrate (0.5 nM), followed by incubation at 37˚C for 30 min. (B) Quantification from A. hSuv3helicase activity in the presence of RPA relative to helicase activity by hSuv3 alone. Product from RPA alone were subtracted from helicase activity values. Error bars represent standard deviation (mean value of three experiments). * $p < 0.001$ in Student t-test for different from hSuv3 alone, ** $p < 0.0001$. (C) Unwinding activity of hSuv3 in the presence of mtSSB as in A. (D) Quantification of fold stimulation from C. Error bars represent standard deviation (mean value of three experiments).

Figure 4: Interaction of FEN1 with hSuv3. (A) Interaction of FEN1 with hSuv3 in the nuclear fraction of HeLa cells. HeLa nuclear extracts were used for co-immunoprecipitation assays with anti-FEN1, anti-hSuv3 or normal IgG. (B) The hSuv3-FEN1 interaction is independent of DNA. Nuclear extracts were either untreated or treated with DNase I or EtBr, before co-IP as in A. (C) FEN1 and hSuv3 interact in the nuclear fraction of primary human fibroblast GM38. Nuclear extracts were used for co-IP as in A.

Figure 5: Stimulation of FEN1 incision activity by hSuv3. (A) FEN1 incision on a long (15 nt) flap in the presence of hSuv3-WT, hSuv3-K213A or BSA. Purified recombinant proteins were mixed (1 nM FEN1) and the reaction started by addition of the radioactively labeled DNA substrate (0.5 nM), followed by incubation at 37˚C for 15 min. (B) Quantification of fold stimulation from A. Error bars represent standard deviation (mean value of five experiments). (C) FEN1 incision on a short (1 nt) flap in the presence of hSuv3-WT, hSuv3-K213A or BSA. (D) Quantification of fold stimulation from C. Error bars represent standard deviation (mean value of four experiments). P values were determined using Student's t-test.

Figure 1

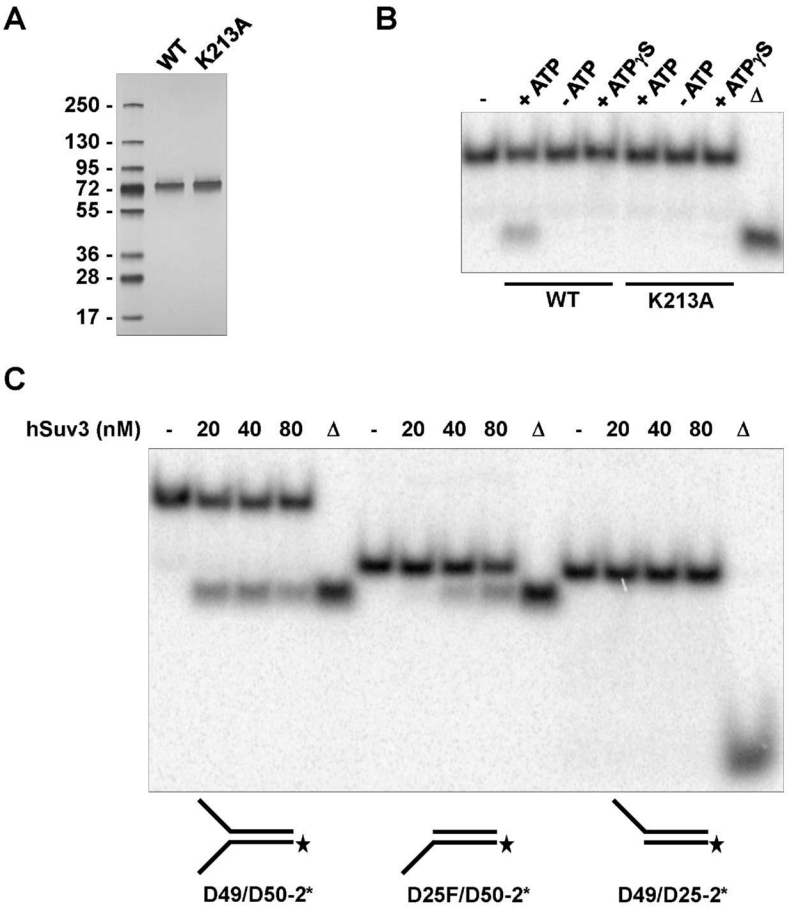

A
WT
K213A
250 -
130 -
95 -
72 -
55 -
36 -
28 -
17 -
B
-
+ ATP
- ATP
+ ATPγS
+ ATP
- ATP
+ ATPγS
Δ
WT
K213A
C
hSuv3 (nM) - 20 40 80 Δ - 20 40 80 Δ - 20 40 80 Δ
D49/D50-2*
D25F/D50-2*
D49/D25-2*

Figure 2

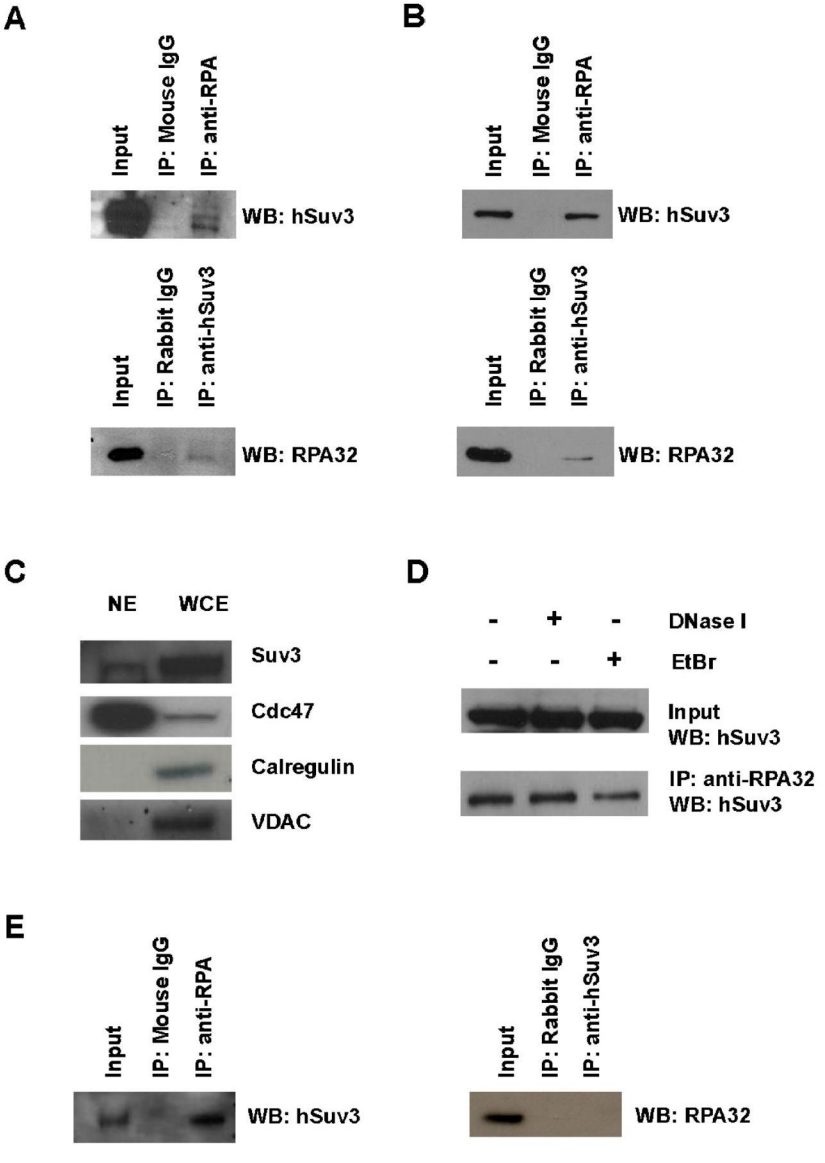

A
Input
IP: Mouse IgG
IP: anti-RPA
WB: hSuv3
Input
IP: Rabbit IgG
IP: anti-hSuv3
WB: RPA32
B
Input
IP: Mouse IgG
IP: anti-RPA
WB: hSuv3
Input
IP: Rabbit IgG
IP: anti-hSuv3
WB: RPA32
C
NE
WCE
Suv3
Cdc47
Calregulin
VDAC
D
- + - DNase I
- - + EtBr
Input
WB: hSuv3
IP: anti-RPA32
WB: hSuv3
E
Input
IP: Mouse IgG
IP: anti-RPA
WB: hSuv3
Input
IP: Rabbit IgG
IP: anti-hSuv3
WB: RPA32

Figure 3

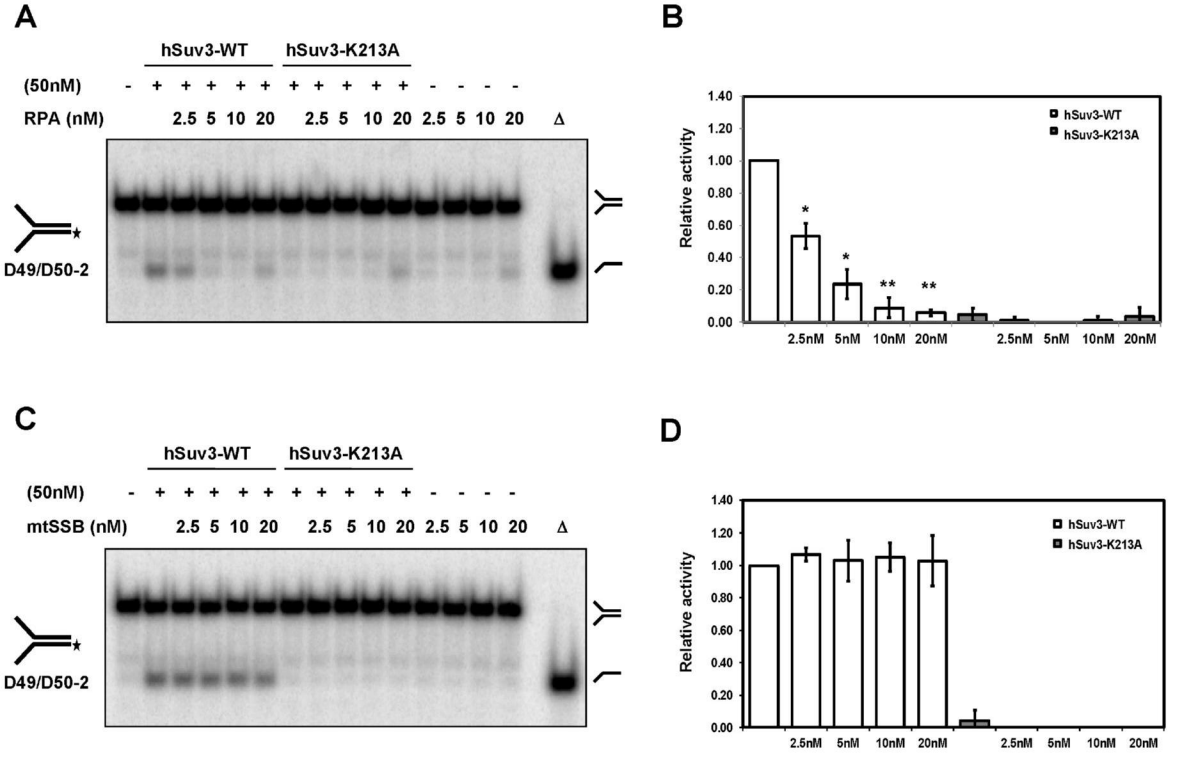

A
hSuv3-WT
hSuv3-K213A
(50nM)
RPA (nM)
2.5 5 10 20 2.5 5 10 20 2.5 5 10 20
Δ
D49/D50-2
B
Relative activity
hSuv3-WT
hSuv3-K213A
2.5nM 5nM 10nM 20nM 2.5nM 5nM 10nM 20nM
C
hSuv3-WT
hSuv3-K213A
(50nM)
mtSSB (nM)
2.5 5 10 20 2.5 5 10 20 2.5 5 10 20
Δ
D49/D50-2
D
Relative activity
hSuv3-WT
hSuv3-K213A
2.5nM 5nM 10nM 20nM 2.5nM 5nM 10nM 20nM

Figure 4

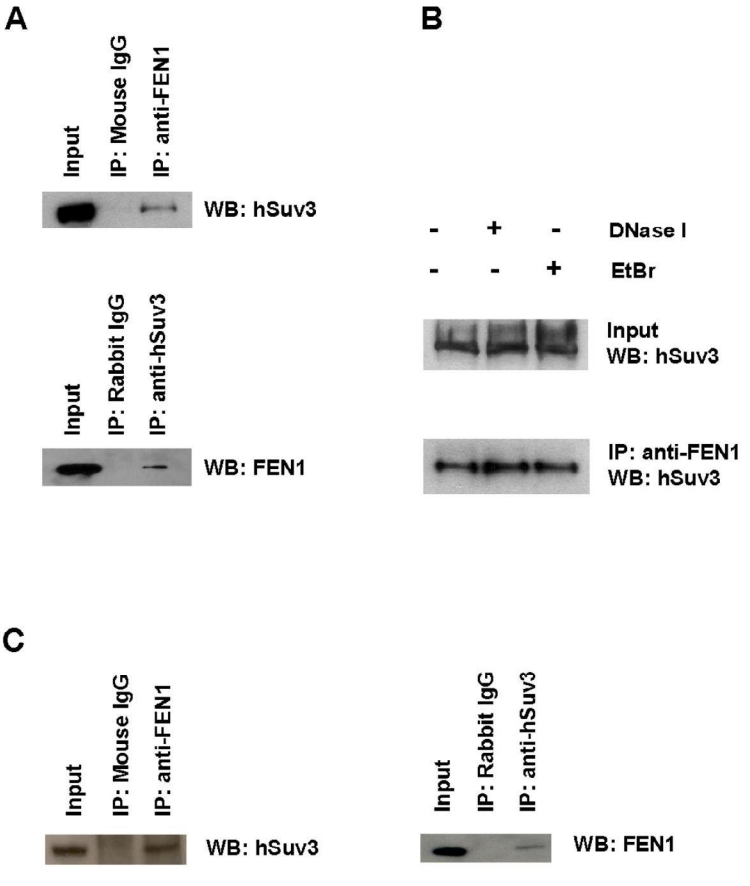
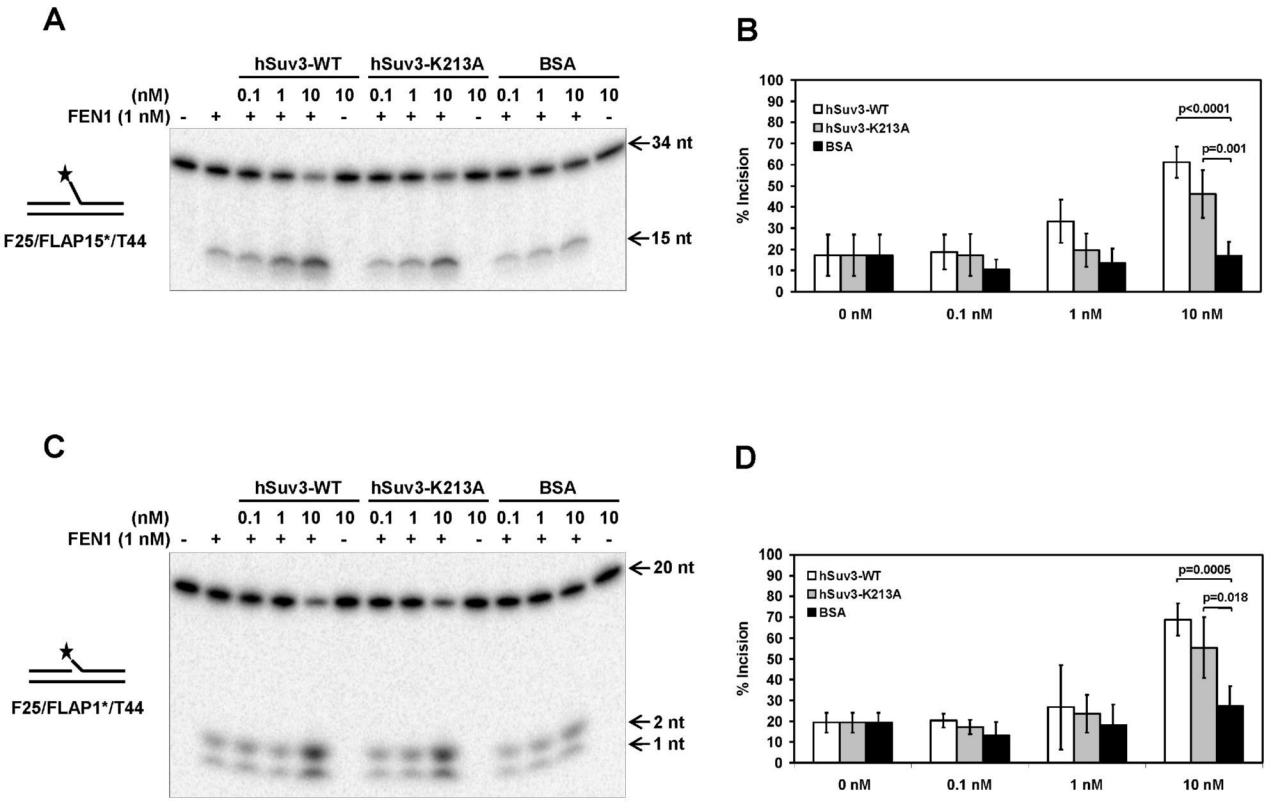

A
Input
IP: Mouse IgG
IP: anti-FEN1
WB: hSuv3
Input
IP: Rabbit IgG
IP: anti-hSuv3
WB: FEN1
B
- + - DNase I
- - + EtBr
Input
WB: hSuv3
IP: anti-FEN1
WB: hSuv3
C
Input
IP: Mouse IgG
IP: anti-FEN1
WB: hSuv3
Input
IP: Rabbit IgG
IP: anti-hSuv3
WB: FEN1

Figure 5


A
hSuv3-WT
hSuv3-K213A
BSA
(nM)
0.1 1 10 10 0.1 1 10 10 0.1 1 10 10
FEN1 (1 nM) - + + + + - + + + + - + + + -
34 nt
15 nt
F25/FLAP15*/T44
B
hSuv3-WT
hSuv3-K213A
BSA
% Incision
p<0.0001
p=0.001
0 nM
0.1 nM
1 nM
10 nM
C
hSuv3-WT
hSuv3-K213A
BSA
(nM)
0.1 1 10 10 0.1 1 10 10 0.1 1 10 10
FEN1 (1 nM) - + + + + - + + + + - + + + -
20 nt
2 nt
1 nt
F25/FLAP1*/T44
D
hSuv3-WT
hSuv3-K213A
BSA
% Incision
p=0.0005
p=0.018
0 nM
0.1 nM
1 nM
10 nM