Snm1B Interacts with PSF2

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Abstract
The protein Snm1B plays a key role in interstrand crosslink (ICL) repair. In a yeast two-hybrid screen we identified the protein PSF2 to bind Snm1B. PSF2 is a member of the GINS complex involved in replication initiation and elongation, and is known to play a role in ICL repair. Snm1B was shown to bind PSF2 in human cells through two regions, strongly to a 144 amino acid N-terminal region and weakly to a second smaller 37 amino acid C-terminal region. Ectopic expression of PSF2 increased the amount of Mus81, a protein component of the endonucleolytic complex involved in ICL repair, co-immunoprecipitating with Snm1B. Moreover, deleting the N-terminal, but not C-terminal region of Snm1B reduced the amount of co-immunoprecipitating Mus81. Conversely, the telomere-binding protein TRF2 competed with PSF2 for binding to the C-terminus of Snm1B, and deletion of this region, but not the N-terminal region, reduced Snm1B chromatin association. We speculate that the N-terminal region of Snm1B forms a complex containing PSF2 and Mus81, while the C-terminal region is important for PSF2-mediated chromatin association.

Introduction
Interstrand crosslinks (ICLs) are toxic lesions that covalently attach opposite strands of DNA [1]. One protein involved in the repair of ICLs is Snm1B (Apollo/Dclre1B) [2,3,4,5,6,7]. Snm1B is a 60 kDa protein belonging to the β-CASP family of proteins, which also contains Ssm1A (Dclre1A) and Ssm1C (Artemis/Dclre1C) [8]. These proteins are characterized by β-CASP and Metalloβ-Lactamase domains responsible for nucleic acid hydrolysis [8], and all three proteins have inherent 5'-3' DNA exonuclease activity [6,9,10,11]. In mammalian and chicken cells, Ssm1A plays a role in ICL repair, likely in a different repair pathway than Snm1B [12,13,14]. Ssm1C is involved in homologous end joining and has a structure-specific endonuclease activity dependent upon binding DNA-PKcs [9,15]. Snm1B is required for proper ICL repair, as knockdown of this protein leads to sensitivity of cells to ICLs [2,3,4,13] and blocks the formation of double-strand breaks (DSBs) that occur as an intermediate in ICL repair [3,16]. The enzymatic activity of Snm1B appears to be dispensable for ICL repair, although the conserved β-CASP and Metalloβ-Lactamase domains are required [3]. Interestingly, Ssm1B associates with Mus81 through the Metalloβ-Lactamase domain [3]. Mus81 and Emel form the structure-specific endonuclease complex Mus81/Emel [17,18] that is important for cleavage of replication fork substrates in vivo [17,18], and for the formation of DSBs after ICL formation during replication in vivo [19]. These results suggest that association of Ssm1B with Mus81 is important for ICL repair during DNA replication [3].

In addition to the role of Ssm1B in ICL repair, Ssm1B protects replicating telomeres from being detected as DNA damage through a C-terminal interaction with the telomere-binding protein TRF2 [5,6,7,20]. Moreover, knockdown of Ssm1B leads to an accumulation of DNA repair proteins on telomeres [7], indicative of telomere dysfunction [21]. The telomere protection and ICL repair functions of Ssm1B appear to be separate from one another. Specifically, a patient with a rare form of dyskeratosis congenital termed Hoyeraal–Hreidarsson syndrome, was reported to encode a truncated form of Ssm1B that failed to bind TRF2 and exhibited telomere DNA damage, but was not sensitive to ICL-inducing agents [22].

The above observations suggest protein-protein interactions may mediate the different functions of Ssm1B. We screened a yeast two-hybrid library for novel protein interactions using the very C-terminus of Ssm1B as a bait. This region was already known to interact with at least one protein, TRF2 [5,6,7], and moreover, a C-terminal deletion mutant was both poorly expressed and reduced the viability of cells, at least in ectopic over-expression settings [23], suggesting a functional contribution. The two-hybrid screen revealed an interaction with the protein PSF2. PSF2 is a 21 kDa protein that is one of four proteins that form the GINS complex. The GINS complex is a highly conserved protein complex [24] required for DNA replication and initiation through interacting with Cdc45 [25,26,27]. Of interest with regards to Ssm1B, knockdown of PSF2 was recently shown to sensitize cells to ICLs [28]. We thus investigated whether PSF2 was a bona fide interacting protein of Ssm1B, and the impact of deleting the regions responsible for this binding in Ssm1B on TRF2 and Mus81 associations and chromatin loading.

Materials and Methods

Plasmids
All Ssm1B expression constructs were derived from PCR amplification of a Flag-Ssm1B cDNA [5] and sequenced to confirm correct. The yeast two-hybrid Ssm1B bait vector was created by cloning two tandem in-frame copies of the sequence...
Figure 1. Snm1B interacts with PSF2. A, Diagram of Snm1B mutants generated. The two putative PSF2-binding regions (PSF2 BR1 and BR2) are denoted by grey bars. WT, wild type. * denotes a mutation that reduces TRF2 binding. B, Growth of yeast expressing PSF2-Gal4AD (PSF2) and the Gal4BD (vector), PSF2-Gal4AD and Gal4BD fused to the C-terminus of Snm1B (Snm1B), or positive control TRF2-Gal4AD (TRF2) and Snm1B-GalBD on SD/-leu/-trp or SD/-ade/-his/-leu/-trp dropout media in the presence of x-a-gal. C, E, The indicated Flag epitope-tagged Snm1B (Flag-Snm1B) or the indicated GFP-tagged Flag-Snm1B (GFP-Flag-Snm1B) transiently expressed in 293T cells in the presence of ectopic Myc epitope-tagged PSF2 (Myc-PSF2) were immunoprecipitated (IP) with either an anti-Flag or anti-GFP antibody and immunoblotted (IB) with an anti-Flag antibody to visualize Flag-Snm1B or an anti-Myc antibody to visualize Myc-PSF2. Data are representative of at least two independent experiments. doi:10.1371/journal.pone.0049626.g001
encoding the last 37 amino acids of Snm1B into pGBK7 (Clontech). Previously described Flag-Snm1B-WT, Flag-Snm1B 1-494, and Flag-Snm1B 1N2 cDNAs [23] were PCR amplified and subcloned into pLPC (a gift from Susan Smith). Flag-Snm1B 1-494 was created by two separate PCR amplifications to generate regions spanning amino acids 1 to 221 and 364 to 532, which were then simultaneously ligated into pLPC, resulting in the described internal deletion having the addition of the sequence 5’-CCGGGCGCCG-3’ (a NotI restriction site and one base pair to keep in frame) between amino acids 221 and 364. Flag-Snm1B 1-529 and Flag-Snm1B 1-532 were cloned by PCR amplification to include a 5’ Flag epitope tag and sequence corresponding to the indicated amino acids, and then subcloned into pEGFP-C3 (Clontech). Flag-Snm1B 1-529 and Flag-Snm1B 1-532 were cloned by PCR amplification to include a 5’ Flag epitope tag and sequence corresponding to the indicated amino acids, and then subcloned into pEGFP-C3 (Clontech). Flag-Snm1B 1-529 and Flag-Snm1B 1-532 were cloned into pCMV-MYC (Clontech). Flag-Snm1B was PCR amplified from clone MGC-673 (ATCC) and cloned into pCMV-MYC (Clontech). The F120A mutation was introduced into pcDNA3-myc-TRF2 [5] by site-directed mutagenesis. HA-MUS81 was created by PCR amplification of clone MGC:14953 (Imagenes) with primers that included a 5’ amino acids of the construct and a 3’ primer designed to create a Flag epitope-tag at the indicated amino acids of the construct and a 3’ primer corresponding to the original 3’ of Flag-Snm1B, and then subcloned into pEGFP-C3. PSF2 was PCR amplified from clone MGC-673 (ATCC) and cloned into pCMV-MYC (Clontech). The F120A mutation was introduced into pcDNA3-myc-TRF2 [5] by site-directed mutagenesis. HA-MUS81 was created by PCR amplification of clone MGC:14953 (Imagenes) with primers that included a 5’ HA epitope tag and cloned into pcDNA3 (Invitrogen).

Yeast Two-Hybrid Assay

Yeast strain AH109 expressing pGBK7 encoding the bait protein comprised of the aforementioned Snm1B C-terminal region fused in frame to the Gal4 DNA-binding domain (Gal4BD) was used to screen the Matchmaker™ Pretransformed Human Hela Library, according to the manufacturer’s protocol (Clontech). Y187 yeast were then transformed with the prey vector pGADT7 encoding either PSF2 or TRF2 (identified in this screen) fused in frame to the Gal4 DNA-binding domain (Gal4BD) and mated with AH109 yeast containing either empty pGBK7 or pGBK7 expressing the bait and analyzed for growth on SD/- leu/-trp, as a control, and SD/-ade/-his/-leu/-trp drop-out plates supplemented with X-α-gal (Sigma) to show the interaction of the indicated proteins.

Transient Transfection

293T and Hela cells were transiently transfected with Fugene 6 (Roche) at approximately 40%-60% confluency according to the manufacturer’s protocol. The cells were collected 36 to 48 hours later for analysis.

Co-Immunoprecipitation Analysis

The indicated transiently transfected 293T cells were lysed with RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40 supplemented with 1mM PMSF, 2 μg/mL aprotinin, and 1 μg/mL leupeptin) by homogenization through a 25G needle. Lysates were rotated at 4°C for 5–10 minutes and spun at 16,000g to remove debris and the protein concentration was determined by DC™ Protein Assay (Bio-Rad). To immunoprecipitate Flag-tagged Snm1B protein, an equal amount of lysate (1–2 mg depending on experiment) was incubated with 15 μL of M2 affinity gel (Sigma) and rotated at 4°C overnight. To immunoprecipitate GFP-tagged Flag-Snm1B protein, an equal amount of lysate (1–2 mg depending on experiment) was incubated with 4 μg of anti-GFP antibody (Roche), rotated at 4°C overnight, after which 10 μL of GammaBind G Sepharose (GE/Amersham) was added and samples were rotated an additional hour. To immunoprecipitate Myc-tagged PSF2 protein, an equal amount of lysate (1–2 mg depending on experiment) was incubated with 4 μg of anti-Myc antibody (Invitrogen), rotated at 4°C overnight, after which 10 μL of GammaBind G Sepharose (GE/Amersham) was added and samples were rotated an additional hour. Immunoprecipitates were then washed twice with RIPA buffer, resolved by polyacrylamide gel electrophoresis, and immunoblotted with mouse anti-Flag M2 (1:1000 Sigma), mouse anti-Myc (1:5000 Invitrogen) or mouse anti-Mus81 (1:1000, Abcam) antibodies to detect Flag-tagged Snm1B, GFP-tagged Flag-Snm1B, Myc-tagged PSF2 and TRF2, or Mus81 proteins, respectively. Exposures were optimized to detect the different proteins, and hence vary between different antibodies.

Figure 2. Myc-TRF2 disrupts the interaction between Flag-Snm1B and Myc-PSF2. A, B, The indicated wild type (WT) or N2 mutant Flag epitope-tagged Snm1B (Flag-Snm1B) and Myc epitope-tagged PSF2 (Myc-PSF2) proteins transiently expressed in 293T cells in the absence or presence of wild type (WT) or the F120A mutant of Myc epitope-tagged TRF2 (Myc-TRF2) were immunoprecipitated (IP) with anti-Flag antibody and immunoblotted (IB) with either an anti-Flag antibody to visualize Flag-Snm1B or an anti-Myc antibody to visualize Myc-PSF2 or Myc-TRF2. Bottom: normalized Myc-PSF2:Flag-Snm1B ratio. Data are representative of at least two independent experiments.
Chromatin Fractionation and Immunoblot Analysis
The chromatin and soluble fractions of the indicated transiently transfected 293T cells were isolated with 0.1% Triton X-100 CSK buffer (10 mM PIPES pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 1 mM EGTA, 1 mM EDTA, 50 mM NaF, 5 mM Na3VO4 supplemented with the addition 1 mM PMSF, 2 μg/mL aprotinin, and 1 μg/mL leupeptin) as previously described [28]. The soluble fraction was centrifuged at 16,000 g to remove remaining debris. The insoluble pellet was resuspended in 1x laemmli buffer (60mM Tris-Cl pH 6.8, 2% SDS, 5% glycerol, 100 mM dithiothreitol, and 0.01% bromophenol blue) and sonicated for 30 seconds at 25% amplitude on a Fisher Scientific sonic dismembrator model 150. Whole cell lysates were isolated with RIPA buffer as described above. 50 μg of protein from the soluble, chromatin, and whole cell lysates were resolved by SDS polyacrylamide gel electrophoresis and immunoblotted as above with anti-Flag M2, mouse anti-Myc, mouse anti-Mus81, anti-lamin B1 (1:1000, Invitrogen), anti-histone H3 (1:1000, Cell Signaling) and anti-β-tubulin (1:1000, Sigma) antibodies to detect Flag-Snm1B, Myc-PSF2, Mus81, the nuclear protein laminin B1, the chromatin protein histone H3, and the cytosolic protein β-tubulin, respectively. Exposures were optimized to detect the different proteins, and hence vary between different antibodies.

ImageJ Quantification
Quantification of immunoblots was performed with ImageJ [29]. The ratio of co-immunoprecipitated Myc-PSF2 to Flag-Snm1B was determined by quantifying the densitometry signals of Myc-PSF2 and Flag-Snm1B immunoblots with the Myc-PSF2/Flag-Snm1B (WT) ratio normalized to one. The fold increase in Mus81 co-immunoprecipitated was calculated for each Flag-Snm1B protein (WT, 1–495, or Δ222–363) by dividing the value of the co-immunoprecipitated Mus81 when Myc-PSF2 is expressed by the value of the co-immunoprecipitated Mus81 when Myc-PSF2 is not expressed.

Results
A Yeast Two-Hybrid Screen Identified an Interaction Between Snm1B and PSF2
The very C-terminus of Snm1B may serve a regulatory role [23] and was already known to bind to at least one protein, the telomere-binding protein TRF2 [5,6,7,20]. Thus, we used a tandem repeat of the last 37 amino acids of human Snm1B as bait (Figure. 1a) to screen a human cDNA prey library by the yeast two-hybrid methodology for potential protein interactions. A total of 134 clones were sequenced corresponding to 8 unknown sequences and 35 annotated genes, including the aforementioned TRF2 (Table. S1). One of these potential interacting proteins was the PSF2 subunit of the GINS complex, which was of particular interest given that PSF2, like Snm1B, is required for ICL repair [28,30]. We confirmed that the PSF2 prey vector required the Snm1B bait vector to activate the Ade, His, and Mel1 reporter genes, as indicated by blue colony growth of the reporter strain of yeast on the appropriate dropout media supplemented with X-a-gal (Figure. 1B). This growth was less than that observed with the positive control TRF2 vector, suggesting a weak interaction. We conclude that the C-terminus of Snm1B binds, likely directly, to the GINS subunit PSF2.

Two Regions of Snm1B Mediate Co-Immunoprecipitation with PSF2 in Human Cells
To evaluate whether full-length Snm1B binds to full-length PSF2 in human cells, human 293T cells were co-transfected with expression plasmids encoding N-terminal Flag epitope-tagged human Snm1B (Flag-Snm1B) and N-terminal Myc epitope-tagged human PSF2 (Myc-PSF2). The expressed Flag-Snm1B protein was

Figure 3. Flag-Snm1B co-immunoprecipitates with Mus81 and Myc-PSF2. The indicated wild type (WT) or mutant (Δ222–363 or 1–494) Flag epitope-tagged Snm1B (Flag-Snm1B) and HA epitope-tagged Mus81 (HA-Mus81) proteins transiently expressed in 293T cells in the absence or presence of Myc epitope-tagged PSF2 (Myc-PSF2) were immunoprecipitated (IP) with anti-Flag antibody and immunoblotted (IB) with an anti-Flag antibody to visualize Flag-Snm1B (WT, Δ222–363, or 1–494), anti-Myc antibody to visualize Myc-PSF2, or an anti-Mus81 antibody to visualize Mus81. Bottom: fold increase in Mus81 co-immunoprecipitating with Flag-Snm1B. Data are representative of at least two independent experiments. doi:10.1371/journal.pone.0049626.g003
Figure 4. Flag-Snm1B is enriched in the chromatin fraction in the presence of Myc-PSF2. A. B. The indicated wild type (WT) or mutant (Δ222–363 or 1–494) Flag epitope-tagged Snm1B (Flag-Snm1B) proteins were transiently expressed in 293T cells alone or in the presence of either Myc epitope-tagged PSF2 (Myc-PSF2), HA epitope-tagged Mus81 (HA-Mus81), or both. Soluble, chromatin (insoluble), and whole cell lysates were immunoblotted with anti-Flag, anti-Myc, anti-Mus81, anti-β-tubulin, anti-lamin B1 or anti-histone H3 antibodies to visualize Flag-Snm1B (WT, Δ222–363, or 1–494), Myc-PSF2, Mus81, β-tubulin, lamin B1 or histone H3 proteins, respectively. Immunoblot exposures were optimized for each antibody, although the soluble and chromatin fractions immunoblotted for tubulin, histone H3, or lamin B1 were exposed for the same time. Exposure times for lanes 10–13 in B were different from lanes 1–9, as these samples were resolved on a different gel. Data are representative of at least two independent experiments.

doi:10.1371/journal.pone.0049626.g004
then immunoprecipitated by virtue of the Flag tag from these cells and immunoblotted with an anti-Myc antibody to detect Myc-PSF2, revealing that the two proteins co-immunoprecipitated (Figure. 1C). The reciprocal was also true, namely that Flag-Snm1B co-immunoprecipitated with Myc-PSF2, although this only occurred in the presence of HA-Mus81 (see below, Figure. S1A, B). Interestingly, when the experiment was performed with Flag-Snm1B<sup>1–494</sup>, a mutant of Flag-Snm1B lacking the portion of Smn1B used in the two-hybrid screen (Figure. 1A), Smn1B still co-immunoprecipitated with Myc-PSF2, although at a reduced efficiency (Figure. 1C). These results suggest the presence of another PSF2-binding region within Smn1B.

To map the putative second PSF2-binding region, a series of N- and C-terminal truncation mutants of Flag-Snm1B were generated and fused in frame to GFP to increase the size of small fragments to facilitate expression and detection. These proteins were co-expressed in 293T cells with Myc-PSF2, after which protein association was determined by immunoprecipitating the GFP-Flag-Snm1B mutants followed by immunoblot to detect Myc-PSF2. This analysis revealed that any fragment of GFP-Flag-Snm1B containing the region spanning amino acids 221 to 363 co-immunoprecipitated with Myc-PSF2 (Figure. 1D). Indeed, the minimal GFP-Flag-Snm1B<sup>222–363</sup> fragment encoding only amino acids 221 to 363 co-immunoprecipitated with roughly three-fold more Myc-PSF2 than GFP-Flag-Snm1B<sup>WT</sup> (Figure. 1E). We suggest that two terminal regions accounting for most of the affinity of Smn1B for PSF2. This analysis revealed that any fragment of GFP-Flag-Snm1B<sup>WT</sup> containing the region spanning amino acids 221 to 363 co-immunoprecipitated with Myc-PSF2, although this was reduced by more than half (Figure. 2). This result was validated by mutational analysis. Specifically, we demonstrate that the C-terminal PSF2-binding region was responsible for this increase, the experiment was repeated with Flag-Snm1B<sup>Δ222–361</sup>, which lacks the first PSF2-binding region accounting for most of the association with PSF2, and Flag-Snm1B<sup>1–494</sup> which lacks the second weaker PSF2-binding region (Figure. 1A, 1C, 1E). The ability to co-immunoprecipitate more Mus81 in the presence of Myc-PSF2 was significantly reduced in Flag-Snm1B<sup>Δ222–361</sup>, but not Flag-Snm1B<sup>1–494</sup> (Figure. 3), although Myc-PSF2 could also co-immunoprecipitate with HA-Mus81 in the absence of Flag-Snm1B (Figure. S1B). Admittedly, the reduction in HA-Mus81 association with Flag-Snm1B<sup>Δ222–361</sup> in the presence of Myc-PSF2 could be due to reduced Mus81 binding to Smn1B, although the Mus81-binding site maps to amino acids 68–139 in Smn1B [3]. Additionally, while Myc-PSF2 co-immunoprecipitated with Flag-Snm1B, the reverse co-immunoprecipitation required ectopic HA-Mus81 (Figure. S1A, B). Taken together, we suggest that interactions between Smn1B, PSF2, and Mus81 promote the formation of a larger complex, with Smn1B binding to PSF2 through the N-terminal region.

**TRF2 Reduces the Amount of PSF2 Co-Immunoprecipitating with Smn1B**

Given that TRF2 binds to one of the regions of Smn1B associating with PSF2 [5], we evaluated the impact of TRF2 expression on the newly identified interaction of Smn1B with PSF2. Specifically, 293T cells, either untransfected or co-transfected with expression plasmids encoding Flag-Snm1B, Myc-PSF2, and/or Myc-TRF2, were lysed after which Flag-Snm1B was immunoprecipitated and immunoblotted with anti-Myc or anti-Flag antibodies to detect Flag-Snm1B, Myc-PSF2, and/or Myc-TRF2. As expected, Flag-Snm1B co-immunoprecipitated with Myc-TRF2 [3,6,7] and Myc-PSF2. However, when both these proteins were co-expressed with Flag-Snm1B, the amount of Myc-PSF2, but not Myc-TRF2, that co-immunoprecipitated with Flag-Snm1B was reduced (Figure. 2A). When the level of immunoprecipitated Myc-PSF2 was normalized to the level of Flag-Snm1B, to account for the increase in Flag-Snm1B when co-expressed with Myc-TRF2 [23], ectopic Myc-TRF2 reduced the amount of Myc-PSF2 co-immunoprecipitating with Flag-Snm1B by more than half (Figure. 2). This result was validated by mutational analysis. Specifically, we demonstrate that Myc-TRF2<sup>Δ122</sup>, a mutant of TRF2 that cannot bind Smn1B [31], failed to reduce the amount of Myc-PSF2 that co-immunoprecipitated with Flag-Snm1B. Similarly, the N2 mutant of Flag-Snm1B that has reduced binding to TRF2 [23,31] and data not shown) readily co-immunoprecipitated with Myc-PSF2 in the presence of wild type Myc-TRF2 (Figure. 2B) [23,31]. We therefore suggest that TRF2 can inhibit the association of Smn1B with PSF2.

**PSF2 Increases the Amount of Mus81 Co-Immunoprecipitating with Smn1B**

The Mus81 subunit of the structure specific endonuclease complex Mus81/Eme1 is required for the formation of DSBs after ICL formation during replication in vivo, and binds to Smn1B [3,19]. Given these observations and that PSF2, as part of the GINS complex, is a member of the replication fork [32], we measured the impact of PSF2 expression on the association of Smn1B with Mus81. Specifically, 293T cells, either untransfected or transiently co-transfected with expression plasmids encoding Flag-Snm1B, Myc-PSF2, and/or HA-Mus81, were lysed, after which Flag-Snm1B was immunoprecipitated and immunoblotted to detect Flag-Snm1B, Myc-PSF2, or Mus81. As previously reported, Flag-Snm1B co-immunoprecipitated with Mus81 [3]. However, the addition of Myc-PSF2 increased this association by more than two fold (Figure. 3). To determine which of the two PSF2-binding regions was responsible for this increase, the experiment was repeated with Flag-Snm1B<sup>Δ222–361</sup>, which lacks the first PSF2-binding region accounting for most of the association with PSF2, and Flag-Snm1B<sup>1–494</sup> which lacks the second weaker PSF2-binding region (Figure. 1A, 1C, 1E). The ability to co-immunoprecipitate more Mus81 in the presence of Myc-PSF2 was significantly reduced in Flag-Snm1B<sup>Δ222–361</sup>, but not Flag-Snm1B<sup>1–494</sup> (Figure. 3), although Myc-PSF2 could also co-immunoprecipitate with HA-Mus81 in the absence of Flag-Snm1B (Figure. S1B). Admittedly, the reduction in HA-Mus81 association with Flag-Snm1B in the presence of Myc-PSF2 could be due to reduced Mus81 binding to Smn1B, although the Mus81-binding site maps to amino acids 68–139 in Smn1B [3]. Additionally, while Myc-PSF2 co-immunoprecipitated with Flag-Snm1B, the reverse co-immunoprecipitation required ectopic HA-Mus81 (Figure. S1A, B). Taken together, we suggest that interactions between Smn1B, PSF2, and Mus81 promote the formation of a larger complex, with Smn1B binding to PSF2 through the N-terminal region.

**Snm1B is Enriched in the Chromatin Fraction through the C-Terminal Interaction with PSF2**

Since PSF2 accumulates in chromatin through forming a complex with Cdc45 and MCM2-7 [26,33] and associates with Smn1B (Figures. 1, 2, and 3), we tested whether PSF2 localized Smn1B onto chromatin. 293T cells were transiently transfected with an expression vector encoding Flag-Snm1B or co-transfected with expression vectors encoding Flag-Snm1B and Myc-PSF2. Chromatin and soluble fractions were isolated and their purity validated by the appropriate absence or presence of the nuclear protein lamin B1 or the chromatin protein histone H3 and the cytosolic protein tubulin. Both fractions were then immunoblotted to detect Flag-Snm1B and Myc-PSF2, revealing that the amount of Flag-Snm1B in the chromatin fraction was increased upon co-expressing Myc-PSF2 (Figure. 4A).

To determine which PSF2-binding region was responsible for this enrichment, the experiment was repeated with Flag-Snm1B<sup>Δ222–361</sup> and Flag-Snm1B<sup>1–494</sup>, which lack the N- and C-terminal PSF2-binding regions, respectively. This analysis revealed that the C-terminal PSF2-binding region was responsible for this enrichment. Specifically, while the levels of both wild type and Δ222–363 mutant Flag-Snm1B proteins in the chromatin fraction were increased in the presence of Myc-PSF2, very little Flag-Snm1B<sup>1–494</sup> was detected in the chromatin fraction in the presence of Myc-PSF2 (Figure. 4A). We suggest that PSF2 promotes recruitment of Smn1B to chromatin through the C-terminal PSF2-binding region.
We then tested if the inability of Flag-Snm1B1-494 to associate with chromatin could be overcome by the expression of HA-Mus81. 293T cells were transiently co-transfected with expression vectors encoding the wild type or the 1-494 mutant versions of Flag-Snm1B with no transgene, Myc-PSF2, and/or HA-HA-Mus81 (Figure 4B). As already noted, very little Flag-Snm1B1-494 was detected in the chromatin fraction, with most of the protein remaining in the soluble fraction, even in the presence of Myc-PSF2 or HA-Mus81. However, in cells expressing both Myc-PSF2 and HA-Mus81, Flag-Snm1B1-494 was enriched in the chromatin fraction (Figure 4B). This recruitment to chromatin is likely dependent upon the N-terminal PSF2-binding region of Snm1B. Specifically, when the experiment was repeated with Flag-Snm1B or Flag-Snm1B1-222-363, each protein was still enriched in the chromatin fraction upon expression of Myc-PSF2, but this was not enhanced in the presence of HA-Mus81 (Figure 4B). Thus, we suggest that Snm1B can be recruited to chromatin through the C-terminus directly by PSF2 and the N-terminal PSF2-binding region in a complex with PSF2 and Mus81.

**Discussion**

We now report that PSF2 binds to Snm1B in the yeast two-hybrid assay and co-immunoprecipitates with Snm1B in human cells, indicating that PSF2 associates with Snm1B, probably directly. Mapping revealed that PSF2 bound to two distinct regions in Snm1B: strongly to an N-terminal region encompassed by amino acids 221 to 363 and weakly to a C-terminal region encompassed by amino acids 496 to 332. Interestingly, these two interaction regions impacted Snm1B functions differently. The C-terminal region competed with TRF2 binding and was required for chromatin association in the presence of PSF2, whereas the N-terminal region promoted an association with Mus81. We thus suggest that the interaction with PSF2 is related to Snm1B function.

The N-terminal region of Snm1B promoted complex formation between PSF2 and Mus81, which led to the accumulation of Snm1B on chromatin in the presence of both Mus81 and PSF2. Given the role of Mus81 in the cleavage step of ICL repair, perhaps the N-terminus fosters this step. Admittedly, assigning different roles to the N- and C-terminal PSF2-binding regions is likely an oversimplification, as both the Flag-Snm1B1-222-363 and Flag-Snm1B1-495 mutants may alter more than PSF2 binding. Nevertheless, ectopic expression of PSF2 promoted the association of Snm1B with Mus81 and chromatin, independently supporting a role for these PSF2-binding regions in different functions of Snm1B. Another caveat to these interpretations is that Snm1B was over-expressed due to the inability to detect endogenous Snm1B [2,6]. Nevertheless, indirect evidence corroborates the suggestion that the interaction with PSF2 has an impact on Snm1B function.

**Supporting Information**

Figure S1 Myc-PSF2 co-immunoprecipitates Flag-Snm1B in the presence of HA-Mus81. A, B, The indicated Flag epitope-tagged Snm1B (Flag-Snm1B) proteins transiently expressed in 293T cells alone, with Myc epitope-tagged PSF2 (Myc-PSF2), or with Myc-PSF2 and HA epitope-tagged Mus81 (HA-Mus81) were immunoprecipitated (IP) with an anti-Myc antibody and immunoblotted (IB) with an anti-Flag antibody to visualize Flag-Snm1B, anti-Myc antibody to visualize Myc-PSF2, or an anti-Mus81 antibody to visualize Mus81. (TIF)

**Table S1 Yeast Two-Hybrid Analysis.** (PDF)

**Acknowledgments**

We thank Shuqun Yang, Tohru Yonekawa, and Brian Freiblein for thoughtful discussions.

**Author Contributions**

Conceived and designed the experiments: JRS CMC. Performed the experiments: JRS. Analyzed the data: JRS CMC. Contributed reagents/materials/analysis tools: JRS. Wrote the paper: JRS CMC.

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