The nuclease hSNM1B/Apollo is linked to the Fanconi anemia pathway via its interaction with FANCP/SLX4

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The recessive genetic disorder Fanconi anemia (FA) is clinically characterized by congenital defects, bone marrow failure and an increased incidence of cancer. Cells derived from FA patients exhibit hypersensitivity to DNA interstrand crosslink (ICL)-inducing agents. We have earlier reported a similar cellular phenotype for human cells depleted of hSNM1B/Apollo (siRNA). In fact, hSNM1B/Apollo has a dual role in the DNA damage response and in generation and maintenance of telomeres, the latter function involving interaction with the shelterin protein TRF2. Here we find that ectopically expressed hSNM1B/Apollo co-immunoprecipitates with SLX4, a protein recently identified as a new FA protein, FANCP, and known to interact with several structure-specific nucleases. As shown by immunofluorescence analysis, FANCP/SLX4 depletion (siRNA) resulted in a significant reduction of hSNM1B/Apollo nuclear foci, supporting the functional relevance of this new protein interaction. Interestingly, as an additional consequence of FANCP/SLX4 depletion, we found a reduction of cellular TRF2, in line with its telomere-related function. Finally, analysis of human cells following double knockdown of hSNM1B/Apollo and FANCP/SLX4 indicated that they function epistatically. These findings further substantiate the role of hSNM1B/Apollo in a downstream step of the FA pathway during the repair of DNA ICLs.

INTRODUCTION

Fanconi anemia (FA) is a rare recessive genetic disease characterized by congenital defects, bone marrow failure and an increased incidence of cancer. Cells derived from FA patients show chromosomal and genomic instability, and hypersensitivity to DNA interstrand crosslink (ICL)-inducing agents (1,2). The disease has a high degree of genetic heterogeneity, with 15 genes known so far. The proteins encoded by these FANC genes and by a growing number of associated genes with no mutations in FA patients found thus far constitute the FA/BRCA pathway embedded within the DNA damage response. The FA/BRCA pathway is activated by recognition of ICL, involving a complex of the proteins FANC, FAAP24 and MHF, followed by the recruitment of the FA core complex, possessing PHD E3 ubiquitin ligase activity mediated by FANCL. Subsequently, two proteins, FANCD2 and FANCI, are activated by monoubiquitination (and phosphorylation) and recruit downstream repair proteins such as BRCA1, FANCD1/BRCA2, FANCN/PALB2 and FANCJ/BRIP1/BACH1 (reviewed in 2).

A recent addition to this group of downstream repair proteins of the FA/BRCA pathway is SLX4 as shown by the identification of biallelic mutations in FA patients (3,4) and functional studies (5–8). This new FA protein, FANCP/SLX4, is a highly conserved regulator of structure-specific endonucleases (SSEs) (7,9). These enzymes catalyze DNA cleavage, which is required for the processing of DNA ICLs.

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repair intermediates. FANCP/SLX4 interacts directly with the SSeS SLX1, XPF-ERCC1 and MUS81-EME1 (5–7,10). Several observations suggest that another nuclease, hSNM1B/Apollo, is linked to the FA/BRCA pathway. We originally identified the human SNM1B/Apollo based on its homology to the S. cerevisiae PSO2(SNM1) (11), a protein involved in resistance towards ICL-inducing agents (reviewed in 12).

Similarly, siRNA-mediated depletion of hSNM1B/Apollo results in increased sensitivity to mitomycin C (MMC), as shown by survival and chromosome breakage analysis of HeLa cells (11,13). The analysis of genetic interactions between hSNM1B/Apollo and the FA pathway revealed that the FA gene FANCD2 and hSNM1B/Apollo function epistatically in response to MMC-induced ICL damage (13). Our earlier finding of hSNM1B/Apollo being dispensable for mono-ubiquitination of FANCD2, a key step of the FA pathway, suggests a function downstream of the FA core complex (11). Altogether, multiple lines of evidence point to a role for hSNM1B/Apollo within the FA/BRCA pathway; however, the exact nature of this alliance remains elusive.

Here we report on the physical interaction between hSNM1B/Apollo and FANCP/SLX4. The close relationship between the two molecules was also evident in another set of our experiments showing epistasis between hSNM1B/Apollo and FANCP/SLX4 in response to MMC and ionizing radiation (IR)-induced DNA damage. Our findings link hSNM1B/Apollo to the group of downstream repair proteins acting at later steps within the FA/BRCA pathway. In addition to its function in the response to DNA damage, hSNM1B/Apollo is involved in telomere generation and maintenance (14–17). We and others have previously shown that hSNM1B/Apollo cooperates directly with the shelterin component TRF2 (14,15,18). Co-immunoprecipitation (co-IP) studies suggested that FANCP/SLX4 is also a physical interactor of TRF2 (7). Here we show that cellular TRF2 protein levels depend on FANCP/SLX4. This finding further substantiates the dual function of FANCP/SLX4 in the cellular response to DNA damage and in telomeric maintenance, a characteristic which also applies to hSNM1B/Apollo and TRF2.

RESULTS

hSNM1B/Apollo co-immunoprecipitates with FANCP/SLX4

The cellular characteristics of FA patient cells consist of hypersensitivity to ICLs and in some cases to IR, of increased chromosome breakage following treatment with ICL-inducing agents such as MMC or cisplatin and of cell cycle abnormalities. We found similar features in human cells depleted for hSNM1B/Apollo (siRNA) and hypothesized a role for hSNM1B/Apollo in the FA/BRCA pathway (11,18). Several others also found evidence for hSNM1B/Apollo functioning in this pathway (13,19); however, a convincing physical interaction with molecules of the FA/BRCA pathway has not been shown yet. Similar to hSNM1B/Apollo, the SLX4 gene, which was recently identified as the 15th FA gene, FANCp, has a role in both, the cellular response to DNA damage and telomere maintenance (6–8,20). This putative functional overlap prompted us to explore the possibility of a physical interaction between FANCP/SLX4 and hSNM1B/Apollo in more detail by employing co-IP analysis. We and others have so far been unable to detect endogenous hSNM1B in western blots even after enrichment by co-IP (11,13,15), presumably because of its extremely low expression level. Therefore, HEK293T cells were transiently transfected with hSNM1B-Flag, or an empty vector control, followed by IP with antibodies against FANCP/SLX4 or hSNM1B/Apollo. Total rabbit IgG antibodies from a control non-immunized rabbit were used as a negative control in the co-IP studies. The western blot was probed with antibodies directed against SLX4/FANCP and subsequently against the Flag-tag. Transiently expressed hSNM1B/Apollo-Flag was specifically co-immunoprecipitated along with the endogenous FANCP/SLX4 from lysates of cells transfected with the plasmid encoding hSNM1B/Apollo-Flag, whereas no signals were detected from control cells transfected with the empty vector as well as from the negative controls with total IgG antibodies added (Fig. 1A). The reverse IP using the Flag or hSNM1B/Apollo antibodies did not, however, co-immunoprecipitate the endogenous FANCP/SLX4 (data not shown), similar to the observation we have previously made with the well-established hSNM1B/Apollo interactor, TRF2 (18). In order to detect DNA damage and TRF2-dependent changes in the FANCP/SLX4–hSNM1B/Apollo interaction, we transfected HEK293T cells with the hSNM1B/Apollo expression construct (pCMV–Tag2B/hSNM1B/Apollo) and treated them with the radiomimetic drug bleomycin or depleted them for TRF2 (siRNA), followed by co-IP analysis. As shown in Supplementary Material, Figure S1, induction of DNA damage or siRNA-mediated depletion of TRF2 did not reduce the amount of transiently expressed hSNM1B/Apollo co-immunoprecipitated along with FANCP/SLX4.

FANCP binds to the N-terminal part of hSNM1B/Apollo

To further explore the association of FANCP/SLX4 and hSNM1B/Apollo, we mapped the interaction domain of hSNM1B/Apollo. We transiently expressed Flag or EGFP-tagged fragments of hSNM1B/Apollo in HEK293T cells and immunoprecipitated from corresponding lysates with antibodies against FANCP/SLX4. The western blots were probed with antibodies directed against Flag or GFP to detect hSNM1B/Apollo and its derivatives. Three deletion mutants were generated lacking portions of the C-terminus (Flag-Apollo 1–401 and Flag-Apollo 1–299) or the N-terminal 267 amino acids (Apollo 268–532-GFP). While lack of the C-terminal parts of hSNM1B/Apollo did not interfere with FANCP/SLX4 binding, loss of the N-terminal portion of the protein did (Fig. 1B and C). The lower band of the hSNM1B-EGFP doublet (Fig. 1C), which we have noticed before (21), may represent a degradation product of hSNM1B or might be a consequence of GFP fusion, for which there is some evidence in the literature (22). In either event, the doublet is irrelevant for the interpretation of the experiment. A schematic overview of the hSNM1B/Apollo proteins and protein fragments used to map the FANCP/SLX4 binding region of hSNM1B/Apollo and the results of the co-IP experiments is shown in Figure 1D.
hnSNM1B/Apollo foci formation is affected by FANCP/SLX4 depletion

We next explored the ability of FANCP/SLX4-depleted (siRNA) cells to form hnSNM1B/Apollo nuclear foci. The FANCP/SLX4 siRNA (si#3) used here has been validated before (5) and FANCP/SLX4 knockdown in U2OS cells was tracked for each experiment by western blot analysis (Fig. 2A). The fraction of hnSNM1B/Apollo foci-positive cells was determined by counting nuclei containing at least one focus in indirect immunofluorescence (IF) experiments. This revealed a modest but highly significant (P < 0.0001) reduction in foci formation as a consequence of FANCP/SLX4 knockdown, reducing the fraction of positive nuclei from 67.6% in the control-siRNA-treated cells (mean of two independent experiments with at least 500 nuclei/experiment analyzed) to 53.5% in the cells transfected with the FANCP/SLX4-siRNA (Fig. 2B).

Reduced cellular TRF2 as a consequence of FANCP/SLX4 deficiency

In order to analyze the impact of FANCP/SLX4 knockdown on TRF2 foci formation, we counted the number of TRF2 foci per cell. No significant difference in TRF2 foci formation
was observed between FANCP/SLX4 siRNA-treated cells and controls when nuclei with >20 TRF2 foci were counted (data not shown). However, earlier experiments have indicated that efficient knockdown of TRF2, as detected by western blot analysis, is not necessarily accompanied by a reduction of nuclear TRF2 foci as detected by IF, suggesting that the residual TRF2 is sufficient to form foci detectable by IF. We therefore assessed the amount of TRF2 in western blot analysis following siRNA-mediated knockdown of FANCP/SLX4. Interestingly, this revealed a striking reduction of detectable TRF2 in the FANCP/SLX4 knockdown cells (Fig. 2A) with only 30.7% of the TRF2 (mean of three independent experiments), when compared with the cells treated with the control siRNA. We next analyzed FA patient cells, 457–2, for the TRF2 expression level by western blot analysis. This lymphoblast line is carrying a 1 bp deletion (c.1093delC, p.Gln365Ser) and a splice site mutation (c.1163+5C→T, p.Arg317_Phe387 del) in the FANCP/SLX4 gene, leading to one truncated allele and the other with exon 5 skipping and an in-frame deletion of 71 amino acids in the corresponding protein (4). Quantification of western blot signals showed that TRF2 was reduced by ∼33% in the FA patient cells of complementation group P (FA-P) when compared with FANCP/SLX4-proficient control cells (Fig. 2C).

FANCP/SLX4 and hSNM1B/Apollo function epistatically in the cellular response to MMC and IR-induced DNA damage

A key characteristic of FA patient cells is their hypersensitivity to ICLs. To gain further insight into the interaction of hSNM1B/Apollo and the FA/BRCA pathway during the response to DNA damage, we examined the survival of human U2OS cells depleted for hSNM1B/Apollo and FANCP/SLX4 (siRNA), either alone or in combination, following exposure to increasing concentrations of MMC. We have previously evaluated the functionality of the hSNM1B/Apollo-siRNAs on both, the protein level (11,18) and the mRNA level (data not shown). The extent of hSNM1B/Apollo knockdown was tracked for each experiment by indirect IF using anti-hSNM1B/Apollo antibodies. In a typical experiment, the proportion of hSNM1B/Apollo foci-positive cells was reduced to 5–15% compared with ∼40% in cells transfected with control siRNAs (Fig. 3A and Supplementary Material, Fig. S2). The FANCP/SLX4 knockdown was tracked by western blot analysis (Fig. 3B). Cells transfected with siRNAs were treated with MMC for 1 h and were then analyzed for their ability to form colonies in comparison to cells transfected with a control siRNA. Depletion of both FANCP/SLX4 and hSNM1B/Apollo did not further increase the MMC sensitivity above the level observed in cells depleted for either of the two genes alone (Fig. 3C).

Increased IR sensitivity has been described for HeLa and HEK293 cells deficient for hSNM1B/Apollo and FANCP/SLX4, respectively (6,11); however, cells from FANCP/SLX4 knockout mice and DT40 chicken cells deficient for FANCP/SLX4 or hSNM1B/Apollo do not show increased IR sensitivity (8,20,23). We therefore assessed the IR sensitivity of a different human cell line, U2OS, following siRNA-mediated depletion of either FANCP/SLX4 or hSNM1B/Apollo. Knockdown of hSNM1B/Apollo and FANCP/SLX4 resulted in increased IR sensitivity, confirming the earlier reports on human cells using siRNA-mediated depletion (Fig. 3D). Double knockdown of both genes in U2OS cells did not change IR sensitivity when compared with cells depleted for either of the two proteins (Fig. 3D). These results indicate that FANCP/SLX4 and hSNM1B/Apollo function epistatically in the cellular response to both ICLs and DNA double-strand breaks (DSBs) and are both members of the FA/BRCA pathway involved in the repair of this type of lesions.

DISCUSSION

In this study, we investigated the relationship between the nucleases hSNM1B/Apollo and the FA/BRCA pathway with respect to protein–protein and genetic interactions. FA is characterized by a remarkable genetic heterogeneity with so far 15 different genes found mutated in FA patients. This genetic diversity reflects the complexity of the repair of ICLs involving the FA/BRCA pathway. In addition to these FA genes, additional members of the FA/BRCA pathway have been identified with no mutations found in FA patients yet, for example, the recently identified nuclease FAN1 (24) and several FA-associated proteins (FAAPs), such as FAAP100, FAAP24 or FAAP20.
We have earlier identified another putative member of the latter group of FA/BRCA pathway proteins, hSNM1B/Apollo, as proposed on the basis of the FA-like phenotype of cultured human cells depleted for hSNM1B/Apollo (siRNA) (11). Further evidence for such a role came from studies showing that hSNM1B/Apollo is involved in the activation of S and G2/M phase checkpoints in response to DNA damage (18,19) and the report of an interaction between hSNM1B/Apollo and FANCD2 as revealed by co-IP studies, although this interaction was not confirmed by functional studies (19). As recently demonstrated by epistasis analysis, hSNM1B/Apollo indeed functions in the FA/BRCA pathway in response to ICLs (13).

By employing co-IP, we here identified FANCP/SLX4 as a new binding partner of hSNM1B/Apollo. As previously described, the analysis of hSNM1B/Apollo protein interactions is hindered by its low expression, and this was accompanied by another obstacle in the present study: While co-IP using anti-FANCP/SLX4 antibodies consistently brought down hSNM1B/Apollo, we were not able to co-immunoprecipitate endogenous FANCP/SLX4 by using different antibodies against hSNM1B/Apollo or the Flag-tag (to immunoprecipitate hSNM1B/Apollo N-terminally fused to the Flag-tag). We have made a similar observation previously in co-IP experiments performed to confirm the hSNM1B/Apollo interaction with the telomere factor TRF2, initially revealed by yeast two-hybrid experiments (18) and independently found by others (14,15,27).

The processing of intermediates arising during the repair of ICLs requires the activity of various structure-specific nucleases. Several recent studies suggest that FANCP/SLX4 executes its DNA repair function by providing a scaffold for various nucleases with different substrate specificities, such as SLX1, XPF-ERCC1 and MUS81-EME1. The latter two have been shown to be important for the repair of ICLs and the resolution of homologous recombination (HR) intermediates with FANCP/SLX4 enhancing their activity (5,6,28–35). hSNM1B/Apollo possesses 5′ to 3′ exonuclease activity (14) and adds, as shown in the present study, to the set of nucleases guided by FANCP/SLX4. This assigns hSNM1B/Apollo closer to the group of proteins with a direct function in the repair of ICLs and DSBs.

The finding of an FANCP/SLX4–hSNM1B/Apollo interaction was further supported by our experiments showing a significant reduction of hSNM1B/Apollo nuclear foci following FANCP/SLX4 knockdown, indicating that both proteins are functionally linked. We have previously shown that the formation of non-DNA damage-induced hSNM1B/Apollo foci, which predominantly colocalize with TRF1 and TRF2,
depends on its physical interactor TRF2 (18), and this accords with rapid degradation of hSNM1B/Apollo when unbound to TRF2 (36). TRF2 is known to bind to FANCP/SLX4; however, the functional relevance of this interaction remains elusive (7). In the present study, we found a clear reduction of cellular TRF2 levels as a consequence of siRNA-mediated FANCP/SLX4 knockdown. TRF2 was also reduced in the FA-P cell line analyzed, although carrying only one FANCP/SLX4 null mutation and expressing a partial function- al FANCP/SLX4 from the other allele (4), underscoring the FANCP/SLX4 effect on the cellular TRF2 level. While FANCP/SLX4 stability does not depend on TRF2 (unpublished data), it is reasonable to assume that hSNM1B/Apollo foci formation does not directly depend on FANCP/SLX4, but rather on TRF2. The shelterin complex, of which TRF2 is a member, is required for telomere length regulation and protection of telomeres against degradation and fusion (37). TRF2 has also been demonstrated to have a crucial function in the repair of non-telomeric DSBs (38,39), including its requirement for DSB repair by HR (40). Some aspects of the phenotype of cellular TRF2 deficiency might be explained by the dependence of hSNM1B/Apollo stability on TRF2 (36); however, TRF2 has been found to stimulate strand inva- sion in telomeric and non-telomeric DNA (41), suggesting that TRF2 itself has a direct role in the DNA repair process and cooperates with hSNM1B/Apollo in processing certain types of DNA substrates.

Our finding of a reduced cellular amount of TRF2 as a consequence of FANCP/SLX4 deficiency is also interesting with respect to the considerable number of chromosome end fusions found in cells from FA patients. Callén et al. (42) investigated TRF2 binding to telomeres in FA patient cells by IF analysis and detected clear telomeric TRF2 signals. From these results, they concluded that end fusions in FA are TRF2-independent. Our experience is that siRNA-mediated reduction of TRF2 to <20% (18) does not result in a reduced IF signal identifiable by microscopic inspection. The results presented here reopen the question of the TRF2 de- pendency of chromosome end fusions in FA. A detailed analysis of FA cells from the different complementation groups and their complemented counterparts will be the subject of further investigations in order to clarify this question.

Our earlier studies suggested that hSNM1B/Apollo functions within the FA/BRCA pathway, with further evidence for such a role coming from other studies, including the present analysis (11,13,19). Here we present data showing that FANCP/SLX4 and hSNM1B/Apollo function epistatically in the cellular response to MMC-induced ICLs. This extends recent findings on the epistatic function of hSNM1B/Apollo and FANC D2 (13) and establishes, together with the earlier findings and the interaction of hSNM1B/Apollo with FANCP/SLX4 as a known member of this pathway (this study), hSNM1B/Apollo as a molecule of the FA/BRCA pathway. We also found that FANCP/SLX4 depletion resulted in IR hypersensitivity in U2OS cells, which is in line with a report on a similar analysis of HEK293 cells (6). This is in contrast to what was found in other species, mouse and chicken (DT40), where IR hypersensitivity was not a pheno- type observed as a consequence of a knockout of the FANCP/SLX4 orthologs (8,20). Interestingly, SNM1B/Apollo knockout in DT40 cells also failed to increase IR sensitivity (23), suggesting that the spectrum of substrates processed by vertebrate hSNM1B/Apollo orthologs, coordinated by FANCP/SLX4, might differ between species.

Following double knockdown of FANCP/SLX4 and hSNM1B/Apollo, we found no difference in IR sensitivity when compared with cells depleted for either of the two pro- teins alone. This indicates that both genes have also epistatic function in the repair of DSBs, which is in agreement with the idea that both proteins are members of the FA/BRCA pathway participating in an early repair step involving HR. We and others previously found that monoubiquitination of FANCD2 in response to MMC-induced DNA damage is independent of hSNM1B/Apollo, also pointing to a function of hSNM1B/Apollo within the FA/BRCA pathway downstream of DNA damage detection and signaling (11,13). This broadens the functional spectrum of hSNM1B/Apollo, since our previous studies implicated this molecule in the early DNA damage response, accumulating at sites of DNA damage within seconds and stimulating autophosphorylation of the central player in maintenance of genome integrity, ATM (18).

hSNM1B/Apollo mutations have not been found in FA patients until now and, considering that knockout of hSnm1b/Apollo in the mouse is not compatible with life (43), it is conceivable that the gene is essential in humans as well. A splice variant of hSNM1B/Apollo was detected in a patient with a severe subtype of dyskeratosis congenita, Hoyeraal-Hreidarsson syndrome (HH), characterized by premature aging, bone marrow failure and immunodeficiency. The genetic basis of this splice variant remained unidentified, since no mutation was found within the hSNM1B/Apollo gene. The protein resulting from the variant transcript, however, lacked ~100 C-terminal amino acids. Interestingly, the phenotype of cultured cells from the patient could be attributed to the telomeric function of hSNM1B/Apollo, but not to its function in the cellular response to non-telomeric DNA damage, as indicated by the lack of MMC sensitivity (44). Therefore, alterations in the portion of hSNM1B/ Apollo not affected in the described HH patient might be associated with an FA-like phenotype.

The identification of the hSNM1B/Apollo interaction with a member of the FA/BRCA pathway, FANCP/SLX4, the epistatic function of both proteins in the cellular response to ICLs and DSBs in the context of other recent reports on the function of these proteins, establishes hSNM1B/Apollo as a molecule of the FA/BRCA pathway. It will be important to further investigate hSNM1B/Apollo’s role in human disease.

MATERIALS AND METHODS

Cell lines and culture

HEK293T and U2OS human cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml strepto- mycin (Life Technologies). Lymphoblastoid cell lines, 457–2 (FA-P) (4) and Con (normal control), were grown in RPMI 1640 with 10% fetal calf serum and antibiotics. Cells were grown in a humidified 5% CO2 incubator at 37°C.
hSNM1B expression constructs

Generation of the plasmids pCMV–Tag2B–hSNM1B (Flag-hSNM1B), allowing the expression of hSNM1B/Apollo with an N-terminally fused Flag-tag and pEGFP-NI-hSNM1B/Apollo encoding hSNM1B/Apollo fused C-terminally to the green fluorescent protein, were described previously (11,18).

Partial hSNM1B/Apollo cDNAs were amplified using pT7T319U-hSNM1B (11) as a PCR template with primer pairs hSNM1B-Psfl F: 5′–AAACTGCAGATGAATGGGGTTCTGATCC/hSNM1B 897R': 5′–ACGCCCTGAGCTAACATTACAATGGGCCACACCCT (for expression of amino acids 1–299); hSNM1B-Psfl F/ hSNM1B 1203 R-XhoI: 5′-ACGCCCTGAGCTAGAACAACGCTGTTCTTGTACCC (for expression of amino acids 1–401) followed by cloning into the Psfl/XhoI sites of the expression plasmid pCMV–Tag2B (Stratagene), in-frame with a Flag-tag at its 5′ end. fragment encoding the C-terminal amino acids 268–532 was amplified with primers hSNM1B del1–267-Psfl F: 5′–AAACTGCAGATGAATGGGGTTCTGATCC/hSNM1B 897R': 5′–ACGCCCTGAGCTAACATTACAATGGGCCACACCCT (for expression of amino acids 1–299) end. fragment encoding the C-terminal amino acids 268–532 was amplified with primers hSNM1B del1–267-Psfl F: 5′–AAACTGCAGATGAATGGGGTTCTGATCC/hSNM1B 897R': 5′–ACGCCCTGAGCTAACATTACAATGGGCCACACCCT (for expression of amino acids 1–299); hSNM1B-Psfl F/ hSNM1B 1203 R-XhoI: 5′-ACGCCCTGAGCTAGAACAACGCTGTTCTTGTACCC (for expression of amino acids 1–401) followed by cloning into the Psfl/XhoI sites of the expression plasmid pCMV–Tag2B (Stratagene), in-frame with a Flag-tag at its 5′ end. After plasmids were generated in frame with EGFP at its 3′ end. The cloned cDNAs were subsequently sequence-verified.

siRNAs, transfections

siRNAs specific for hSNM1B/Apollo and FANCP/SLX4 have been described before (5,11) and were purchased from Dharmacon Research (Lafayette, CO, USA). AllStars negative control siRNA from Qiagen (Hilden, Germany) was used. A total of 1.5 × 105 U2OS cells in 800 μl of DMEM without antibiotics were plated 24 h before transfection in wells of a 6-well plate (on coverslips for IF experiments). Then, 7.4 μl of the siRNA-duplexes (20 μm) were diluted in Opti-MEM1 medium (Life Technologies) to a final volume of 185 μl (for double knockdown, 7.4 μl of either siRNA was used). In a separate tube, 3 μl of Oligofectamine transfection reagent (Invitrogen, Carlsbad, CA, USA) was mixed with 12 μl of Opti-MEM and incubated for 5 min at room temperature. The diluted siRNAs were combined with the Oligofectamine mixture, incubated for 20 min at room temperature and then added to the cells without changing the medium. After 6 h incubation at 37°C, the transfection medium was replaced by DMEM without antibiotics. siRNA transfection was repeated 18 h later as described for the first transfection. The medium was changed the next day (DMEM + antibiotics) and cells were used for experiments 66 h after start of the first round of transfection.

For plasmid transfections, 5 × 105 HEK293T cells were plated in 2 ml of medium without antibiotics in 6-wells. Cells were transfected the next day with 1.5 μg plasmid DNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions and analyzed ∼24 h later as described below.

Colony survival assays

The sensitivity of U2OS cells to increasing concentrations of DNA-damaging agents following a transfection with siRNAs was determined by measuring their ability to form colonies. Cells were siRNA-transfected as described above, trypsinized and plated at a density between 1000 and 10 000 cells/100 mm tissue culture dish. Four hours after plating, MMC (Sigma-Aldrich, St Louis, MO, USA) was added to the final concentrations (in duplicates, and triplicates for untreated cells). After 1 h, the drug-containing media were removed and cells were washed twice with PBS, followed by addition of fresh media. To determine IR sensitivity, cells were irradiated (Cs-137 γ radiator, OB29/4; STS, Braunschweig, Germany) before plating in 100 mm dishes. After 10–12 days of incubation, colonies were fixed, stained and counted. Survival assays were repeated at least two times.

Immunoblotting and IF

Total cell extracts were prepared as described (11) and were electrophoresed using the NuPage system (Invitrogen) in 4–12% Bis–Tris gradient gels. Following electrophoresis, proteins were transferred to Invitronol PVDF membranes (Invitrogen). Membranes were blocked for at least 1 h in 10% non-fat milk in Tris-buffered saline, pH 7.6, with 0.1% Tween-20 (TBS-T). Incubation with primary and secondary antibodies was performed in 5% non-fat milk in TBS-T. All washing steps were carried out using TBS-T. Immunoblots were probed with the following primary antibodies: FANCp (Bethyl Laboratories, Inc.), TRF2 (Imgenex), Flag-tag (Sigma-Aldrich), GFP and actin (Abcam). Primary antibodies were detected with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (BD Pharmingen, San Diego, CA, USA). Chemiluminescence was developed using Western Lightning (PerkinElmer Life Sciences, Boston, MA, USA). To quantify signals, band intensities were determined using Image Quant Version 5.2.

Immunoprecipitates were prepared by lysing transfected cells in 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM EDTA and 0.3% Triton X-100 containing a protease inhibitor mixture (Roche Applied Science). Lysates were immunoprecipitated with the FANCp antibody (Bethyl Laboratories, Inc.) and Dynabeads Protein G (Invitrogen) for 3 h. Immunoprecipitates were washed four times with lysis buffer and proteins were eluted from the beads by boiling for 5 min. Immunoblotting was performed as described above.

For indirect IF analysis, cells were grown and siRNA-treated on glass coverslips in 6-wells in parallel with the cells used for preparing cell lysates and for survival experiments. Cells were fixed after 15 min with 4% paraformaldehyde–0.1% Triton X-100 and were blocked overnight in 10% fetal calf serum in phosphate-buffered saline. Cells were stained with antibodies specifically recognizing hSNM1B (VMRC10) (11,18). The primary antibodies were detected with goat anti-rabbit IgG coupled to Alexa 488 (Molecular Probes, Eugene, OR, USA). Analysis was performed using the Zeiss Axiophot microscope equipped with a CCD camera (SensiCam) and using the Zeiss filter set 13 (excitation 470, emission 505–530). Fluorescent signals were pseudo-colored by the AxioVision software and optimized for contrast.

For quantification of hSNM1B/Apollo foci-positive nuclei, slides were coded and >500 nuclei were assessed for the presence of foci (three or more for evaluation of knockdown...
efficiency (siRNA) and without a threshold in the other experiments), using DAPI stain to count total nuclei. Statistical analysis was done by Fisher’s exact test (two-tailed) using the GraphPad QuickCalcs internet tools (http://www.graphpad.com/quickcalcs/).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

Conflict of Interest statement. The authors have no conflicts of interest to declare.

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