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PRINCIPAL INVESTIGATOR: Junjie Chen, Ph.D.

CONTRACTING ORGANIZATION: M.D. Anderson Cancer Center
Houston, TX 77030

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14. ABSTRACT We are continuing our investigation of mechanisms underlying the maintenance of genomic stability and breast cancer development. Our analyses on BRCA1 and DNA damage response have resulted in the identification of several new components involved in DNA damage signaling pathways and revealed how these pathways act together to ensure genomic stability in response to DNA damage. In addition, we have performed a series of studies focusing on replication checkpoint control, which help us to understand how stalled replication forks are protected in vivo for the maintenance of genomic integrity. We have already published some of these exciting discoveries. We hope that the ongoing studies will continue to provide new insights into breast cancer etiology and identify new targets for cancer therapy.					
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Introduction:

Cancer is a disease caused by genomic instability. Our genetic material is continuously challenged by genotoxic stress. DNA damage can arise during normal cellular metabolic processes such as DNA replication, from endogenous sources like free radicals, or from exogenous agents such as UV light and ionizing radiation. To ensure genome stability, cells have evolved the ability to sense DNA damage, activate cell cycle checkpoint, and initiate DNA repair. These events guard the integrity of our genetic material and are called collectively as “DNA damage response”. The initiation and progression of carcinogenesis require the activation of many oncogenic signals and the inactivation of numerous tumor suppression functions. These scores of genetic alternations can only occur when normal DNA damage response and cell cycle checkpoints become defective. Indeed, numerous cancer-predisposing clinical syndromes are attributed to mutations in components involved in cellular processes that counteract genotoxic stress and ensure normal cell cycle progression. One of the best examples is hereditary breast cancer, since human genetic studies demonstrate that many genes involved in DNA damage response and DNA repair, including p53, BRCA1 (Breast cancer susceptibility gene 1) and BRCA2 (Breast cancer susceptibility gene 2), are frequently mutated and responsible for the development of familial breast and ovarian cancers. The goal of our research is to understand how DNA damage response normally operate in the cell and how the disruption of this DNA damage response influences tumorigenesis and anti-cancer therapy. We also study mitotic checkpoints, which are important for the prevention of another form of genomic instability, named chromosomal instability. The focus of my research program is to uncover the signaling networks that control genomic integrity in humans and how deregulation of these pathways promote tumorigenesis.

Body:

The Specific Aims are:

Specific Aim 1: Develop biomarkers for early detection of breast cancers.

The objective of this specific aim is to understand early genetic alternations that would eventually lead to the development of malignant breast cancers. We are continuing to identify new components involved in DNA damage pathways that would act with BRCA1 and contribute to the maintenance of genomic stability and tumor suppression. In the last few years, we have discovered several key DNA damage checkpoint proteins and demonstrated that the proper DNA damage response depends not only on damage-activated protein kinases but also on a group of regulators or mediator proteins, which facilitate the transduction of DNA damage signals. More importantly, our ongoing studies on BRCA1 have provided new mechanistic insights into the regulation of DNA damage signaling pathways.

FAN1 Acts with FANCI-FANCD2 to Promote DNA Interstrand Cross-Link (ICL) Repair

As we described in our previous report, the well-studied H2AX/MDC1 pathway relays the DNA damage signals to RNF8. RNF8 then initiates an ubiquitin-dependent signal

transduction pathway that regulates the accumulation of many DNA damage repair proteins including BRCA1 and RAD18. As for RAD18, we showed previously that this ubiquitin-dependent recruitment is mediated by RAD18 ubiquitin-binding zinc finger (UBZ) domain (Huang et al., 2009). To identify additional ubiquitin-binding proteins that may be involved in the transduction of these ubiquitin-dependent signals, we performed BLAST search using the UBZ domain of RAD18 and identified KIAA1018, a previously uncharacterized protein, which contains a UBZ domain at its N-terminus and a VRR-nuclease domain at its C terminus (**Figure 1A**; Kinch et al., 2005). As will be discussed below, this protein turned out to act in Fanconi Anemia (FA) pathway and thus it was named as FAN1 (Fanconi anemia associated nuclease 1).

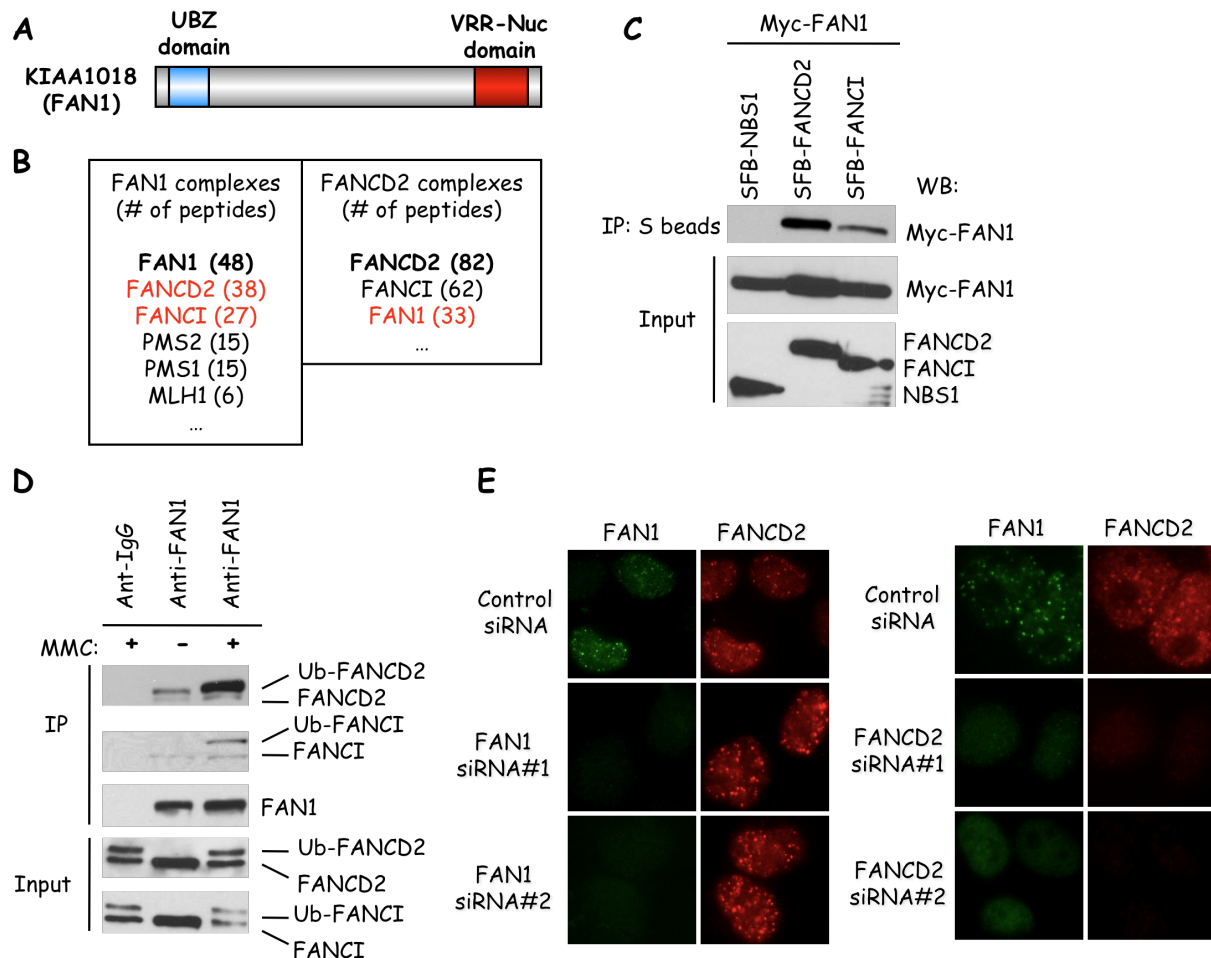


Figure 1. Identification of FAN1 as a FANCI-FANCD2 binding protein. (A) Schematic representation of domain architecture of FAN1 protein. (B) Tables are summaries of proteins identified by mass spectrometry analysis. (C) Ectopically expressed FAN1 interacts with FANCD2/FANCI. (D) The interaction of FAN1 with FANCD2 or FANCI before and after MMC treatment. (E) FANCD2 is required for FAN1 foci formation after MMC treatment.

To specify the DNA damage-responsive or DNA repair pathways that FAN1 is involved in, we generated a human 293T-derivative cell line stably expressing a triple-tagged FAN1 to identify potential FAN1-interacting proteins. We repeatedly found FANCI-FANCD2 complex (ID complex) as major FAN1-associated proteins (**Figure 1B**). To

further confirm that FANCI-FANCD2 exists in the same complex with FAN1, we generated cells stably expressing triple-tagged FANCD2. Notably, mass spectrometry analyses of FANCD2-associated protein complexes revealed peptides corresponding to FAN1 (**Figure 1B**), suggesting that these proteins likely form a complex *in vivo*.

We first confirmed the interaction between FAN1 and the ID complex. As shown in **Figure 1C**, FAN1 interacted with FANCD2 and, to a less degree, FANCI. More interestingly, although FAN1 could interact with unmodified FANCD2 or FANCI, its association with FANCD2 or FANCI was greatly enhanced after treatment with Mitomycin C (MMC), which coincided with FANCD2 or FANCI monoubiquitination (**Figure 1D**). These data suggest that FAN1 is recruited to DNA damage sites, probably via its association with mono-ubiquitinated FANCD2 and FANCI. As a matter of fact, we showed that while FAN1 depletion does not affect MMC-induced FANCD2 foci formation, FANCD2 depletion abolished FAN1 foci formation (**Figure 1E**). Taken together, these data indicate that FAN1 acts downstream of FANCD2.

Next, we sought to identify the region or regions within FAN1 that are important for its translocation to damage-induced foci. As shown in **Figure 2A**, only the UBZ domain deletion (Δ UBZ) mutant of FAN1 totally lost foci-forming ability, whereas wild-type FAN1 and the nuclease domain deletion (Δ NUC) mutant of FAN1 still localized to nuclear foci following MMC treatment. Moreover, the N-terminal UBZ domain of FAN1 was sufficient for foci formation following MMC treatment (**Figure 2A**). This observation is similar to that of damage-induced foci formation for RAD18 (Huang et al., 2009), which is also mediated by its UBZ domain. Indeed, just like with RAD18, wild-type and the Δ NUC mutant of FAN1, but not the Δ UBZ mutant of FAN1, specifically interacted with Ubiquitin-GST fusion protein *in vitro* (**Figure 2B**).

In addition, Ubiquitin-GST fusion protein pulled down the N-terminal fragment of FAN1 containing the UBZ domain (**Figure 2B**). These observations prompted us to speculate that monoubiquitination of FANCD2 may be the upstream signal that targets FAN1 to DNA damage-induced foci. Supporting this hypothesis, whereas wild-type FANCD2 could restore FAN1 foci formation in FANCD2-deficient PD20 cells, a monoubiquitination mutant of FANCD2 (K561R) failed to do so (**Figure 2C**). These results, together with the enhanced association between FAN1 and mono-ubiquitinated FANCD2 described above (**Figure 1D**), strongly indicate that monoubiquitinated FANCD2 acts to facilitate FAN1 accumulation at sites of DNA damage.

FA pathway is important for interstrand cross-link (ICL) repair *in vivo*. An important step in ICL repair is nucleolytic cleavage at, or near the site of an ICL, which produces a suitable substrate that can be subsequently repaired by homologous recombination repair (HRR) pathway. Because FAN1 contains a highly conserved VRR-nuclease domain at its C-terminus, we first sought to confirm that FAN1 is a bona fide nuclease. We purified FAN1 (**Figure 2D**) and demonstrated that FAN1 displayed endonuclease activity on a 5'-flap DNA substrate (**Figure 2E** and data not shown). To confirm that the nuclease activity we observed is intrinsic to FAN1, we generated FAN1 mutations at two highly conserved residues in its nuclease domain (D960A and K977A). Both of these

mutants abolished the 5'-flap endonuclease activity of FAN1 (**Figure 2E**). Furthermore, deletion of FAN1 nuclease domain abolished this nuclease activity (data not shown), but deletion of its UBZ domain did not affect FAN1 nuclease activity (**Figure 4E**). To further determine the physiological relevance of this highly conserved nuclease domain and the UBZ domain of FAN1 in ICL repair, we knocked down FAN1 expression in cells using FAN1-specific siRNA and reintroduced siRNA-resistant full-length FAN1, the Δ UBZ or the nuclease domain mutants of FAN1 into these siRNA-treated cells. Clonogenic survival assays indicated that reconstitution of FAN1 depleted cells by wild-type FAN1, but not the Δ UBZ or the nuclease domain mutants, restored cell survival following Mitomycin C (MMC) treatment (**Figure 2F**), suggesting that both the nuclease and UBZ domains of FAN1 are important for FAN1 function in promoting cell survival following MMC treatment.

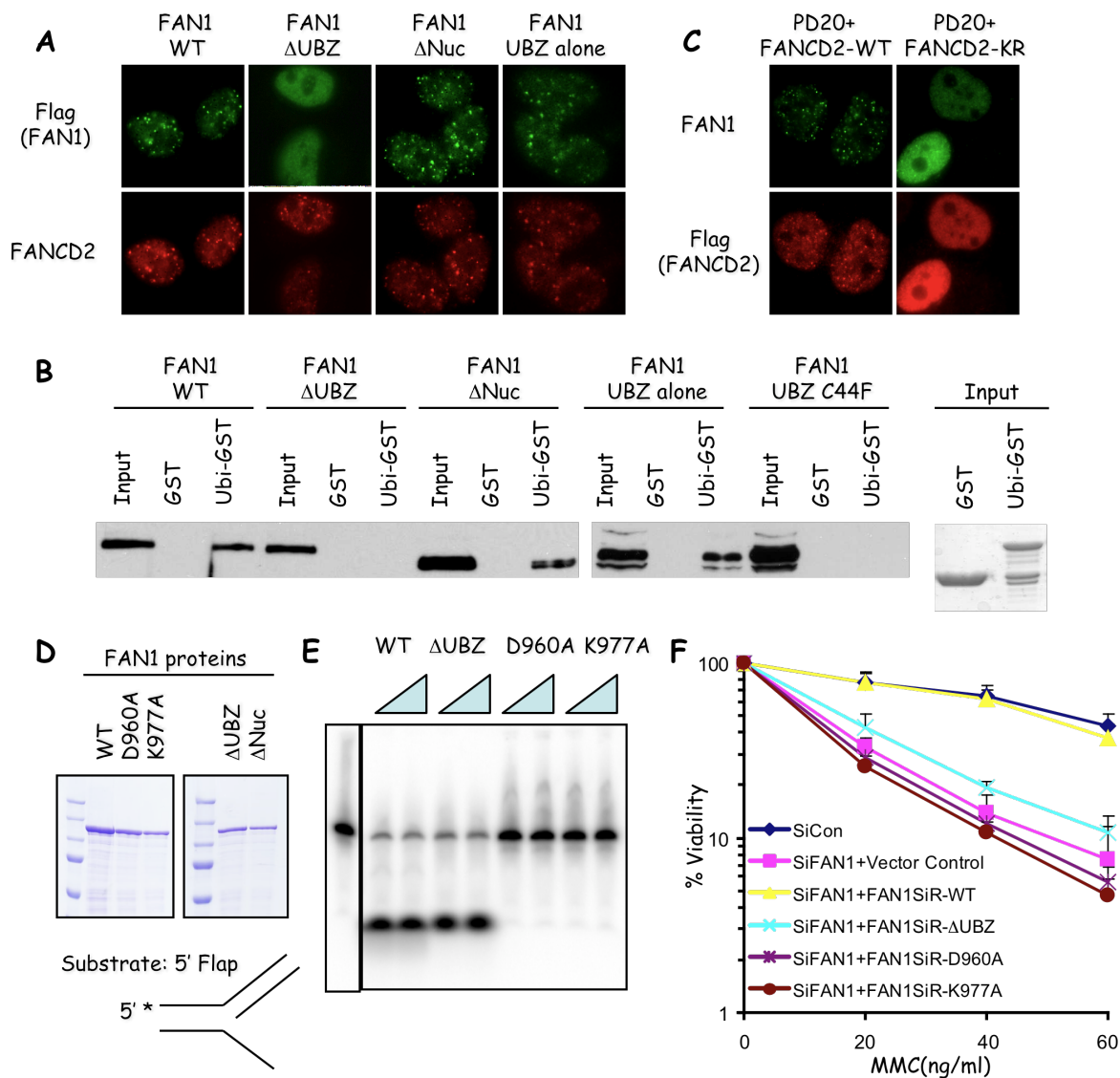


Figure 2. FAN1 is a nuclease that acts downstream of FANCD2 and participates in ICL repair. (A) The UBZ domain of FAN1 targets its localization to MMC-induced foci. **(B)** The UBZ domain of FAN1 is essential and sufficient for binding to ubiquitin in vitro. **(C)** Dependence of DNA damage-induced FAN1

foci formation on mono-ubiquitination of FANCD2. **(D)** SDS-PAGE profile of purified wild type and mutants of FAN1. **(E)** FAN1 displays 5' Flap Endonuclease activity. **(F)** Both the UBZ domain and the Nuclease domain of FAN1 are required for restoring cellular resistance to MMC.

In summary, we identified a nuclease FAN1 that associates with monoubiquitinated FANCI/FANCD2. The cytological and biochemical characterization of this protein, described herein, strongly suggests that FAN1 acts downstream from FANCD2 in FA pathway and participates in cell survival following MMC treatment. I am delighted to report here that the manuscript describing these findings was published recently (Liu et al., 2010). Three other groups also reported similar findings (Kratz et al., 2010; MacKay et al., 2010; Smogorzewska et al., 2010). Together, these studies uncovered a nuclease that acts downstream of FANCD2 and participates in interstrand cross-link (ICL) repair. Since ICL repair is critically important for cell survival following cisplatin, further characterization of FAN1 in breast cancer cell lines is still ongoing in the laboratory.

MDC1 collaborates with TopBP1 in DNA replication checkpoint control

There are two main cell cycle checkpoint pathways that operate following DNA damage. One is the DNA damage checkpoint pathway, which is primarily activated in response to DNA double-strand breaks. This pathway functions throughout the cell cycle and regulates many cell cycle transitions. This pathway requires ATM kinase and many ATM-dependent signaling events. The other pathway is the replication checkpoint pathway, which is also called replication stress pathway. Many types of DNA lesions would result in stalled or stressed replication forks in S phase. These replication stresses activate an ATR/Chk1 dependent pathway, which mainly acts in S/G2 phase of the cell cycle. As a critical player involved in DNA damage responses, BRCA1 has been implicated in both DNA damage checkpoint and replication checkpoint pathways. While the ATM-dependent DSB-induced DNA damage-signaling pathway is well studied, the ATR-dependent replication checkpoint pathway still needs further investigation. Especially, early steps involved in the activation of ATR-dependent replication checkpoint pathway remain elusive.

Topoisomerase II binding protein 1 (TopBP1) is a key regulator involved in ATR activation. The question we are addressing now is how TopBP1 accumulation at stalled replication forks is regulated *in vivo*. We showed previously that the fifth BRCT domain (BRCT5) of TopBP1 is required for its focus localization following DNA damage (Yamane et al., 2002). The upstream regulator that would bind to the BRCT5 domain of TopBP1 and accumulate TopBP1 at stalled replication forks was not identified.

We carried out tandem affinity purification using lysate prepared from cells stably expressing triple-tagged (S-protein, FLAG and streptavidin binding peptide; dubbed as SFB tag) BRCT4/5 domain of TopBP1. Interestingly, mass spectrometry analysis identified Mediator of Damage checkpoint protein 1 (MDC1) as the major TopBP1-associated protein (data not shown), indicating that MDC1 may be involved in TopBP1 accumulation at stalled replication forks. Indeed, as shown in **Figure 3A**, TopBP1 foci formation was greatly reduced in MDC1^{-/-} MEFs, indicating that the HU-induced focus localization of TopBP1 requires MDC1. Similarly, we also observed diminished TopBP1 focus formation in H2AX deficient cells, suggesting that the H2AX/MDC1 pathway is

involved in the accumulation of TopBP1 following replication stress. On the other hand, normal TopBP1 focus localization was observed in RNF8^{-/-} MEFs (**Figure 3A**), suggesting that RNF8-dependent ubiquitination cascade is not involved in this process.

We confirmed that endogenous TopBP1 associated with MDC1 and the interaction between TopBP1 and MDC1 requires the 5th BRCT domain of TopBP1 (data not shown). To define the TopBP1 binding region on MDC1, we used a series of internal deletion mutants of MDC1 (**Figure 3B**) and showed that the interaction between MDC1 and TopBP1 was significantly diminished by D3 (**Figure 3C**), which is the deletion of a region of MDC1 that is enriched for Ser-Asp-Thr-Asp (SDTD) repeats. We and others showed previously that the SDTD repeats of MDC1 are involved in its interaction with NBS1 (Chapman and Jackson, 2008; Melander et al., 2008; Spycher et al., 2008; Wu et al., 2008). These SDTD repeats are phosphorylated by CK2 kinase (Chapman and Jackson, 2008; Melander et al., 2008; Spycher et al., 2008; Wu et al., 2008). If TopBP1 binds to these phosphorylated repeats on MDC1, we would expect that a 12A mutant of MDC1, in which the Ser/Thr residues in all six SDTD repeats were changed to Alanine, would abolish the MDC1/TopBP1 interaction. Indeed, this is the case (**Figure 3D**). Together, these data indicate that TopBP1 associates with MDC1 via its conserved SDTD motifs.

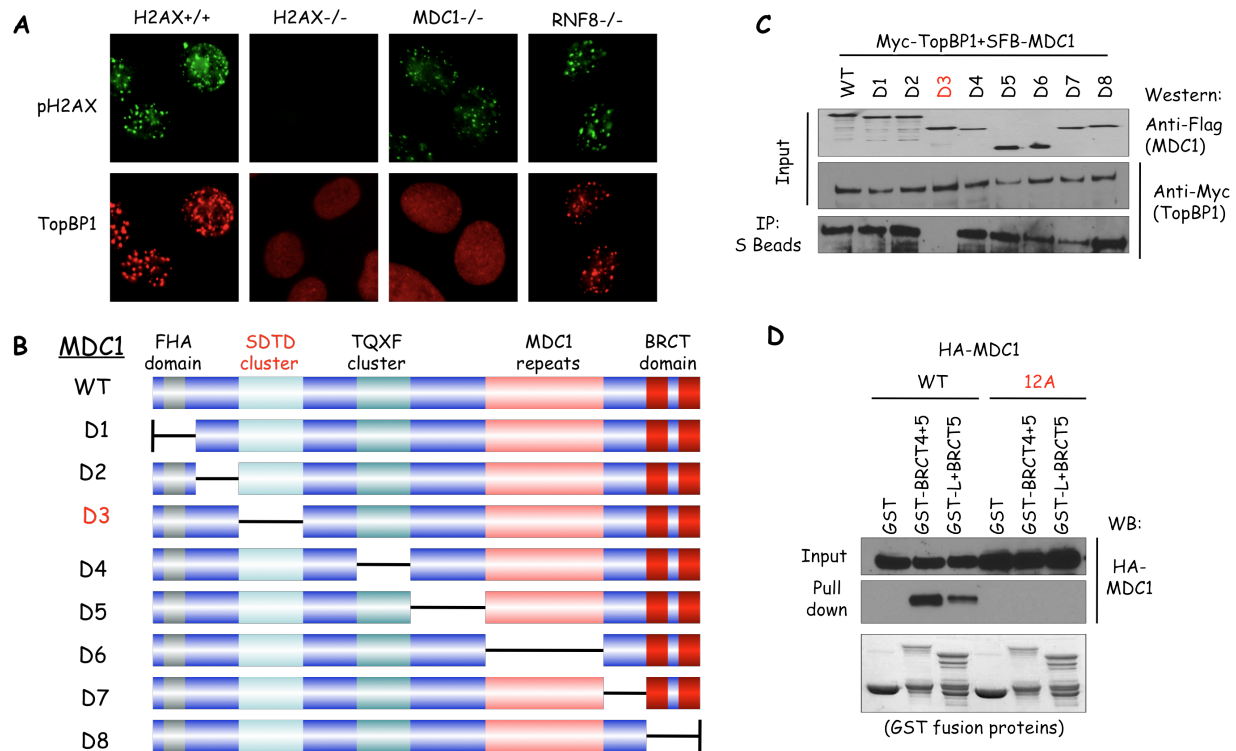


Figure 3. (A) TopBP1 foci formation depends on H2AX/MDC1, but not RNF8. Cells deficient for H2AX, MDC1, RNF8 and their respective wild-type counterparts were treated with HU and immunostaining experiments were performed using anti-TopBP1 and anti-pH2AX antibodies. **(B)** Schematic diagram of wild-type and deletion mutants of MDC1 used in this study. **(C)** Cells were transfected with plasmids encoding Myc-tagged TopBP1 together with plasmids encoding wild-type or deletion mutants of SFB-tagged MDC1. Precipitation reactions were performed using S-protein beads and then subjected to

Western blot analyses using antibodies as indicated. **(D)** Extracts prepared from cells expressing HA-tagged wild-type (WT) or 12A mutant of MDC1 were incubated with glutathione agarose beads coated with GST, GST-BRCT4+5 or GST-BRCT5 fusion proteins. The amount of MDC1 that bound specifically to TopBP1 BRCT domain was evaluated by immunoblotting using anti-HA antibody.

In support of the idea that a physical interaction between TopBP1 and MDC1 is required for the MDC1-dependent recruitment of TopBP1, we showed that HU-induced focus formation of TopBP1 was only observed in cells expressing wild-type MDC1, but not in those expressing D3 mutant or 12A mutant of MDC1 (**Figure 4A**). More importantly, TopBP1 is required for Chk1 activation following replication stress [(Burrows and Elledge, 2008); also see **Figure 4B**]. While the expression of siRNA-resistant wild-type TopBP1 completely restored Chk1 activation in cells depleted of endogenous TopBP1, reconstitution with TopBP1 mutant deleted of its fifth BRCT domain failed to rescue HU-induced Chk1 phosphorylation (**Figure 4B**). Similarly, knockdown MDC1 expression impaired Chk1 phosphorylation following HU treatment (**Figure 4C**). While the expression of siRNA-resistant wild-type MDC1 fully rescued Chk1 activation in MDC1 depleted cells, the expression of siRNA-resistant D3 mutant or 12A mutant of MDC1 failed to do so (**Figure 4C**). Together, these data indicate that the TopBP1/MDC1 interaction plays an important role in Chk1 activation following replication stress. A manuscript summarizing these data was submitted and revised for publication.

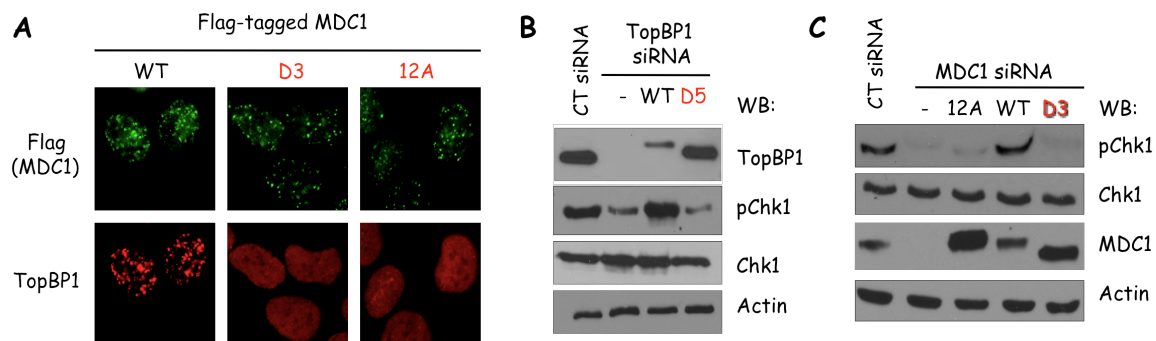


Figure 4. The TopBP1/MDC1 interaction is required for replication checkpoint control. (A) The SDTD repeats of MDC1 is required for TopBP1 focus formation in response to HU. Cells were transfected with constructs encoding FLAG-tagged siRNA-resistant wild-type, D3 mutant or 12A mutant of MDC1, and with MDC1 siRNA twice at 24-hour time intervals. Cells were then treated with HU. Immunostaining experiments were performed using anti-Flag and anti-TopBP1 antibodies. **(B, C)** The interaction between TopBP1 and MDC1 is required for Chk1 activation. Cells stably expressing siRNA-resistant wild-type or D5 deletion mutant of TopBP1 were transfected with TopBP1 siRNA **(B)**. Alternatively, cells were transfected with constructs encoding Flag-tagged siRNA-resistant wild-type, D3 mutant or 12A mutant of MDC1, and together with MDC1 siRNA twice at 24-hour time intervals **(C)**. Cells were treated with HU and cell lysates were immunoblotted with antibodies as indicated.

The HARP domain dictates the annealing helicase activity of HARP/SMARCA1.

The extension of ssDNA regions is critical for the activation of ATR-dependent replication checkpoint pathway. However, this has to be tightly controlled in the cell, since ssDNA regions, even if they are bound and protected by RPA, are still prone for nucleolytic digestion by various nucleases *in vivo* and may give rise to DNA double-strand breaks. We showed recently that an annealing helicase HARP may be involved

in the stabilization of stalled replication forks (Yuan et al., 2009). HARP/SMARCAL1 (HepA-related protein, also called SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin subfamily a-like 1) is an SWI/SNF-like helicase. HARP/SMARCAL1 mutation is responsible for Schimke immunoosseous dysplasia (SIOD), an autosomal recessive disorder characterized by skeletal dysplasia, renal failure, and T cell immunodeficiency. Interestingly, HARP was also reported to have unique annealing helicase activity (Yusufzai and Kadonaga, 2008). We showed that Replication protein A (RPA) binds directly to HARP and recruits HARP to stalled replication forks. Like many other proteins involved in DNA damage and replication stress-responsive pathways, HARP is phosphorylated following replication stress. In addition, HARP-depletion cells displayed increased DNA damage and G2/M arrest, suggesting that HARP may be involved in the protection of stalled replication forks (Yuan et al., 2009).

Continuing our study on HARP, we addressed why this particular SWI/SNF family member, but not the others, has this unique annealing helicase activity (Yusufzai and Kadonaga, 2008). When we compared the sequence of HARP with the sequences of other SWI/SNF-related proteins, we found that HARP has two specific regions that are evolutionarily conserved. These are the N-terminal RPA-binding domain and the repeated HARP motifs located in the middle of the protein (**Figure 5A**).

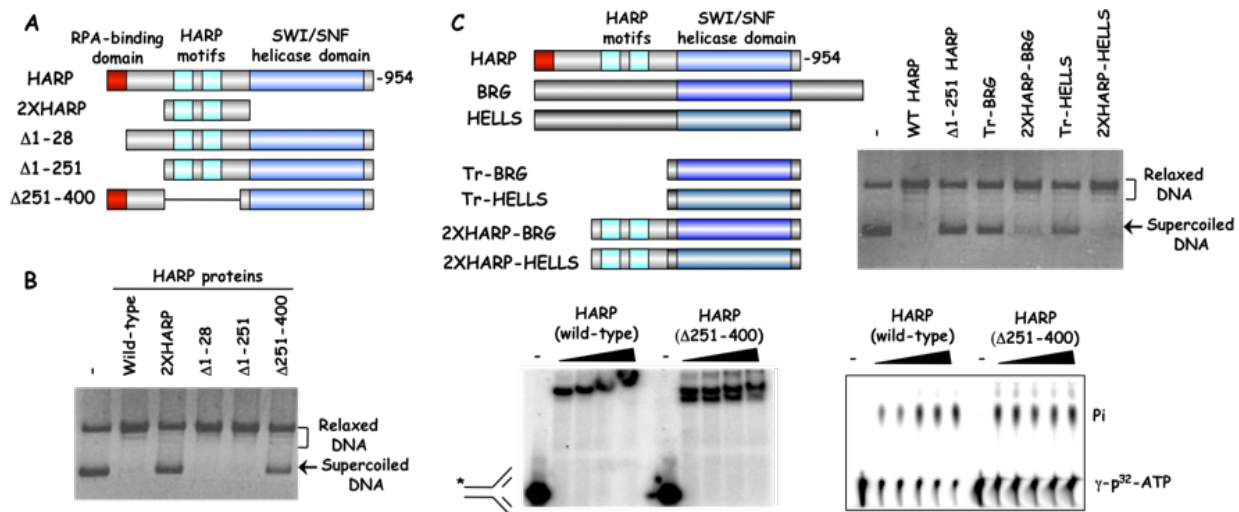


Figure 5. (A) Schematic diagrams of wild-type and mutant HARP used in these experiments. **(B)** HARP motifs are specifically required for HARP annealing helicase activity *in vitro*. Annealing helicase assay was conducted as previously published. The DNA binding activity and ATPase activity of wild-type and mutant HARP were also assessed. **(C)** HARP motif-fusion proteins have *in vitro* annealing helicase activity.

The functional significance of HARP motifs was not known. Thus, we examined whether these unique HARP motifs could account for its annealing helicase activity. We established an *in vitro* annealing helicase assay, as previously reported (Yusufzai and Kadonaga, 2008). Using this assay, we showed that the HARP motifs were indeed required for HARP's annealing helicase activity, but were dispensable for either the

DNA binding activity or the ATPase activity of HARP (**Figure 5B**). Moreover, while two other SWI/SNF-related proteins, BRG1/SMARCA4 and HELLS/SMARCA6, normally do not have annealing helicase activity (data not shown), their corresponding HARP motif fusion proteins possessed annealing helicase activity (**Figure 5C**). Together, these data indicate that the annealing helicase activity of HARP is determined by its unique HARP motifs. We are now determining whether these fusion proteins are fully functional *in vivo* as annealing helicases.

Human C9orf119/C10orf78 Complex Participates in Homologous Recombination Repair

In the previous annual report, we described the identification of PALB2 as the protein that bridges the BRCA1/BRCA2 interaction (Sy et al., 2009b) and our analyses of PALB2 in homologous recombination repair (Sy et al., 2009a; Sy et al., 2009c). We have now further investigated the regulation of homologous recombination repair *in vivo*. RAD51 is an evolutionarily conserved DNA repair protein (Sung et al., 2003) and an essential component of the HR pathway in humans. We established a cell line stably expressing SBP/S/F-tagged RAD51 and performed tandem affinity purification to isolate RAD51-associated proteins. As expected, we identified many known RAD51-associated proteins, including BRCA2, PALB2, RAD54L, RAD54B, XRCC3, RAD51C, and RAD51AP1 (**Figure 6A**). Excitingly, we were also able to identify several novel RAD51-associated proteins, including C9orf119 and C10orf78, in our purification (**Figure 6A**). We are particularly interested in these two proteins, C9orf119 and C10orf78, since they share extensive sequence homology with yeast Mei5 and Sae3, respectively (**Figure 6B**). Budding yeast Mei5 and Sae3 are meiotic proteins (Ferrari et al., 2009; Hayase et al., 2004; Okada and Keeney, 2005; Tsubouchi and Roeder, 2004). They form a stable complex and are specifically required for the loading of DMC1 (RAD51 ortholog in meiotic cells) during meiosis (Ferrari et al., 2009; Hayase et al., 2004). These proteins are evolutionary conserved. Budding yeast Sae3 is homologous to fission yeast Swi5, and budding yeast Mei5 has at least two homologues in fission yeast, Swi2 and Sfr1 (Akamatsu et al., 2003; Haruta et al., 2008; Haruta et al., 2006). Unlikely their counterparts in budding yeast, fission yeast Mei5/Swi5 complexes are involved in both mitotic and meiotic recombination. Because of this evolutionary conservation, we decided to study the role of these two proteins in HR repair in human cells. Indeed, we confirmed that C9orf119 and C10orf78 are expressed in mitotic cells (data not shown). Moreover, these two proteins form a stable complex (**Figure 6C**), just like their yeast homologues. We hypothesize that this protein complex (C9orf119 and C10orf78) may be involved in the regulation of RAD51 loading, similar to their functions in yeast.

Indeed, depletion of C9orf119 or C10orf78 led to reduced RAD51 foci formation and diminished HR repair efficiency (**Figure 6D and 6E**). Interestingly, depletion of C9orf119 or C10orf78 did not affect IR-induced RPA foci formation (**Figure 6D**), suggesting that this protein complex is likely to act downstream of RPA, but is specifically required for RAD51 loading following DNA damage. We will further study: 1) how these proteins promote RAD51-dependent strand exchange and other biochemical activities *in vitro*; 2) how this complex works with RAD51 paralog complexes and BRCA1/PALB2/BRCA2 complex, e.g. do they operate in the same or different sub-pathways? We hope that the

answer to these questions will reveal a previously unknown regulatory pathway involved in homologous recombination repair. We would also like to determine whether either of these two genes are mutated or dysregulated in sporadic breast cancers by screening the breast cancer cell lines we have accumulated over the years.

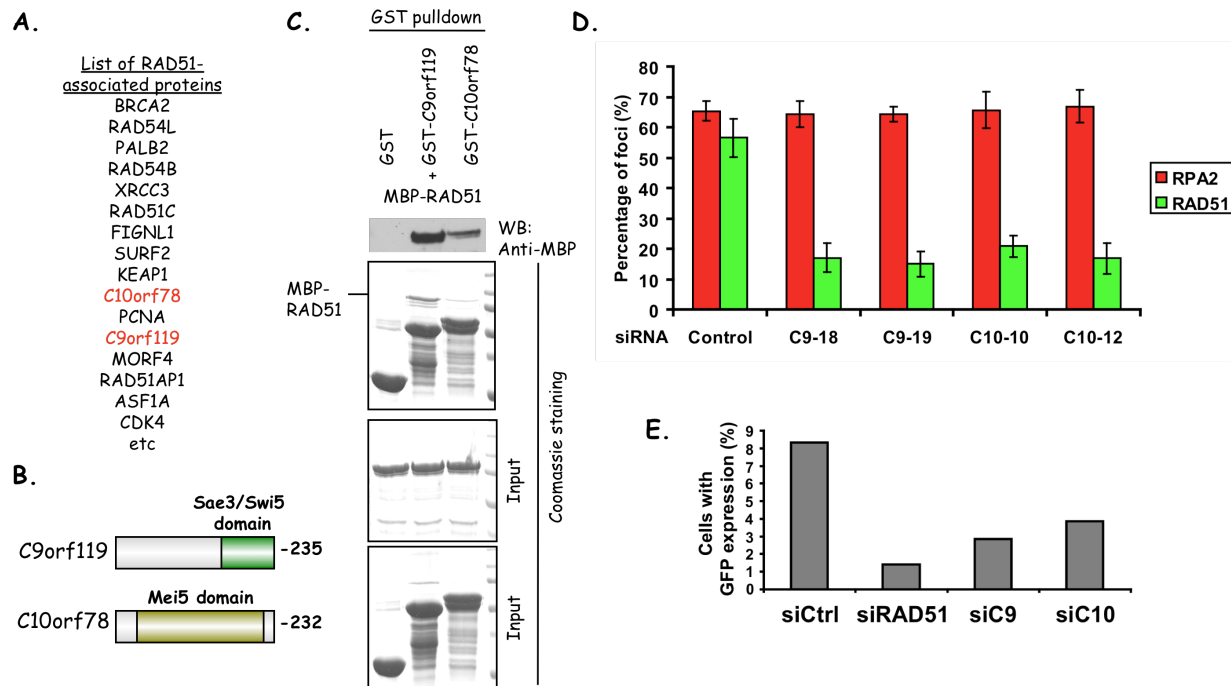


Figure 6. (A) Identification of RAD51-associated proteins by mass spectrometry analysis. **(B)** C9orf119 and C10orf78 are respectively homologues of yeast Sae3/Swi5 and Mei5. **(C)** GST pull down experiments confirmed a direct interaction between C9orf119 (or C10orf78) with RAD51. **(D)** Cells were transfected with control, C9orf119 (C9-18 and C9-19) or C10orf78 (C10-10 and C10-12) specific siRNAs. RPA and RAD51 foci formation were determined after ionizing radiation (IR) and quantified. **(E)** Cells with DR-GFP reporter were transfected with control, RAD51, C9orf119 or C10orf78 specific siRNAs. Percentages of GFP positive cells (indicative of HR efficiency) were determined.

Specific Aim 2: Explore Chfr/Aurora pathway for breast cancer development and treatment.

Besides DNA damage responsive pathways, we also study mitotic progression especially how the dysregulation of proper mitotic control would lead to chromosomal instability and tumorigenesis. We have previously studied how an E3 ligase Chfr may control the expression of several key mitotic regulators and thus ensure genome integrity especially during mitotic transitions. We are now taking a cell biology approach and attempt to identify new microtubule binding proteins that would be involved in the regulation of mitotic progressions and chromosomal segregation. We are now transferring all available ORFs to our SBP-Flag-S triple tagged Gateway-compatible vector and to then perform high throughput screening for their colocalization with microtubules, especially in mitosis. The goal is to screen for the localization of ~16,000 full-length human ORFeome clones (Open Biosystems) that are currently available in the lab. These ORFeome clones in pDonor vectors have already been purified and

individually arrayed on 96-well plates and are ready for transferring into the viral-based SBP-Flag-S Destination vectors through Gateway cloning. Viruses generated from these vectors will be used to infect human cells to produce ~16,000 cell lines expressing different human ORFs tagged with SBP-Flag-S triple tags. Cells will be fixed and immunostained with anti-microtubule and anti-FLAG antibodies to identify proteins that colocalize with microtubules, especially in mitotic cells. This large-scale Omics screening is currently ongoing in the laboratory.

Specific Aim 3: Identify novel druggable targets for the development of anti-cancer agents.

My lab is interested in signaling networks, especially how protein modifications are involved in the regulation of various pathways. As a long term goal, we would like to establish a large panel of tagged protein kinases, phosphatases, E3 ubiquitin ligases, deubiquitinating enzymes, protein acetylases, deacetylases and others purified from human cells for *in vivo* and *in vitro* studies. These reagents will not only help our studies of the physiological functions of these enzymes, but also provide essential tools for developing and validating any specific inhibitors we and others may develop in the future.

The Lys 63-specific deubiquitinating enzyme BRCC36 is regulated by two scaffold proteins localizing in different subcellular compartments.

As we reported last year, we found that the JAMM domain-containing deubiquitinating enzymes BRCC36 exist in two different complexes *in vivo*. One is the nuclear complex that contains RAP80, CCDC98/Abraxas, BRCC45/BRE and MERIT40/NBA1 (Feng et al., 2009; Shao et al., 2009; Wang et al., 2009). The other is a cytoplasmic complex contains BRCC45/BRE, MERIT40/NBA1 and a new component KIAA0157, which shares significant similarity with CCDC98/Abraxas. The major difference between CCDC98 and KIAA0157 is that KIAA0157 lacks the pSXXF motif at its very C-terminus, which is the motif that mediates the interaction between CCDC98 and BRCA1 (Kim et al., 2007; Liu et al., 2007; Wang et al., 2007).

BRCC36 expressed and purified from insect cells was catalytically inactive (**Figure 7A**). However, the BRCC36 complexes isolated from human cells were proficient in cleaving K63-linked ubiquitin chains (**Figure 7B**), suggesting that BRCC36 deubiquitinating (DUB) activity is likely to be regulated by its associated proteins. To demonstrate that, we purified BRCC36 alone, BRCC36/KIAA0157 complex or BRCC36/CCDC98 complex from bacteria. While BRCC36 alone was catalytically inactive, the BRCC36/KIAA0157 complex showed robust DUB activity (**Figure 7C**). Surprisingly, a similar CCDC98/BRCC36 complex was catalytically inactive (**Figure 7C**). Only the five-subunit complex containing RAP80, CCDC98, BRCC45, MERIT40 and BRCC36 displayed *in vitro* DUB activity (**Figure 7A**). Together, these data suggest that these two BRCC36-containing complexes are regulated differently and may have distinct functions in the cell.

While KIAA0157 mainly localizes in cytosol, CCDC98 and RAP80 are nuclear proteins (data not shown). Interestingly, when co-expressed with CCDC98, Flag-tagged BRCC36 predominantly localized in nuclei (**Figure 7D**). In contrast, co-expression with KIAA0157 induced cytoplasmic location of BRCC36 (**Figure 7D**). These data indicate that there are two cellular pools of BRCC36. KIAA0157/BRCC36 complex mainly exist in cytosol and may regulate cytoplasmic function of BRCC36, while CCDC98 determines the nuclear localization of BRCC36 and they form a nuclear complex with three additional components RAP80, BRCC45 and MERIT40, which is important for nuclear function of BRCC36, especially involved in DNA damage response.

These two complexes seem to communicate with each other. Depletion of KIAA0157 destabilized BRCC36 and led to an over-all reduction of BRCC36 expression in the cell (**Figure 7E**). However, the loss of KIAA0157 actually enhanced the interaction between endogenous CCDC98 with RAP80 and BRCC36 (**Figure 7E**), indicating that the loss of cytoplasmic BRCC36 complex could promote the assembly of nuclear BRCC36 complex. The manuscript describing these findings was published recently (Feng et al., 2010).

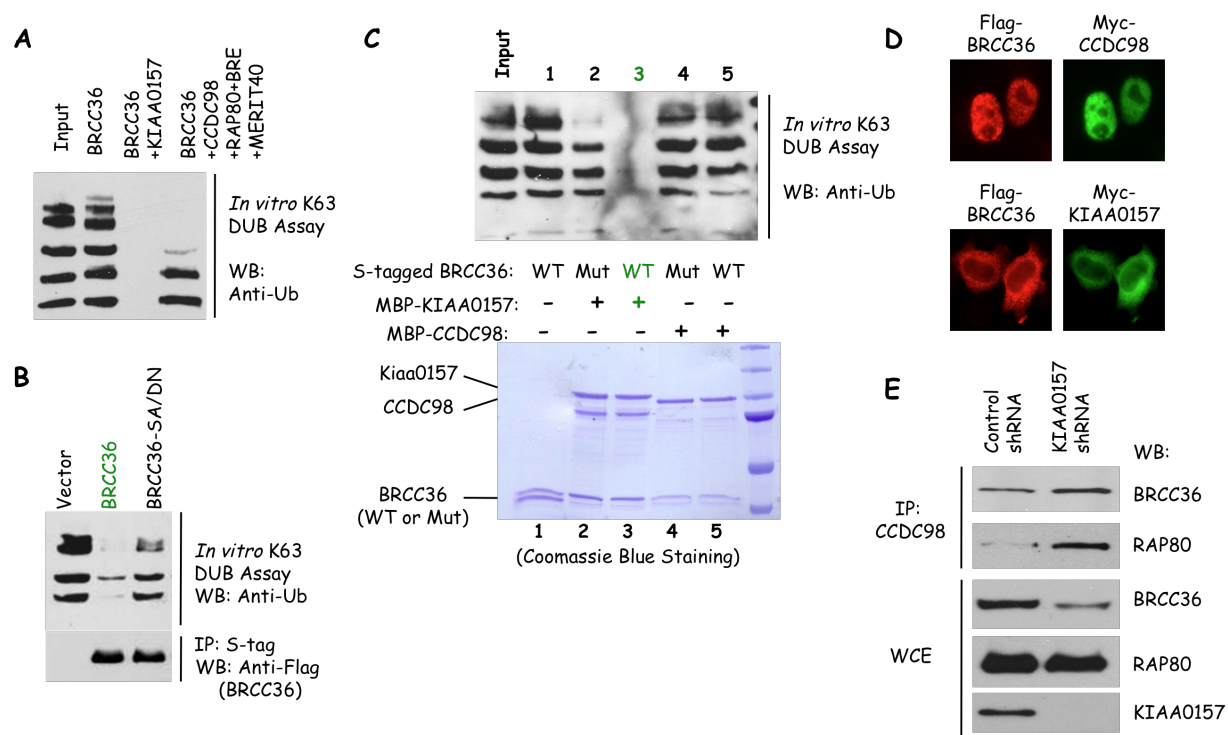


Figure 7. (A) An *in vitro* DUB assay was conducted using K63 ubiquitin chains as substrate and insect cell-expressed BRCC36, the BRCC36/KIAA0157 complex, or the five-subunit RAP80/BRCC36A complex as enzyme sources. **(B)** An *in vitro* DUB assay was performed as outlined in (A) except that immunoprecipitated WT BRCC36 or catalytically inactive mutant (SA/DN) of BRCC36 was used as enzyme source. **(C)** *In vitro* DUB assay using bacterially expressed S-tagged BRCC36 alone, BRCC36 or its inactive mutant (S132A/D135N) coexpressed and co-purified with MBP-tagged KIAA0157 or MBP-tagged CCDC98. DUB reactions were performed similar to that described in (A). BRCC36 and its associated proteins were visualized by Coomassie blue staining. **(D)** KIAA0157 and CCDC98 determine the subcellular localization of BRCC36. Cells were transfected with constructs encoding indicated

constructs and immunostaining was conducted using anti-Flag and anti-Myc antibodies. **(E)** Cells transfected with empty vector or Kiaa0157-specific shRNAs were harvested. Cell lysates were immunoprecipitated with anti-CCDC98 antibody. Immunoblotting was conducted using antibodies as indicated.

Training potential for the PI:

This training award gives us opportunities to explore new ideas and directions. As mentioned above, this award has allowed us to initiate many new projects and several large-scale studies focusing on understanding breast cancer etiology and treatment.

This award also gives me the flexibility to train and promote junior scientists to pursue a career in breast cancer research. As we indicated in the previous annual report, we successfully trained six junior faculty, who left my laboratory and established their own independent research groups in the past. I am happy to report here that two additional fellows left the lab and became independent researchers during the last funding period. Dr. Zheng Fu worked on Chfr and mitotic regulation. She recently moved to Virginia Commonwealth University as a tenure-track assistant professor. Dr. Jun Huang worked on ubiquitination-dependent DNA damage signaling pathways in my lab. He recently moved back to China and is now a group leader and professor in Zhejiang University. I am confident that they will develop their own research programs and continue to contribute to breast cancer research.

Key Research Accomplishments:

- Identified a new nuclease FAN1, which acts downstream of FANCD2/FANCI and participates in ICL repair.
- Discovered that the H2AX/MDC1 pathway is required for TopBP1 focus formation and replication checkpoint control.
- Demonstrated that the conserved HARP domain of HARP/SMARCAL1 determines its unique annealing helicase activity.
- Isolated a new protein complex that interacts with RAD51 and promotes homologous recombination repair.
- Uncovered two distinct BRCC36-containing proteins complexes involved in the cleavage of Lys 63-specific ubiquitin chains.

Reportable Outcomes:

Manuscripts:

Feng L, Wang J, Chen J. The Lys 63-specific deubiquitinating enzyme BRCC36 is regulated by two scaffold proteins localizing in different subcellular compartments. J Biol Chem. 285(40):30982-8, 2010.

Liu T, Ghosal G, Yuan J, Chen J, Huang J. FAN1 Acts with FANCI-FANCD2 to Promote DNA Interstrand Cross-Link Repair. Science 329(5992):693-6, 2010.

Abstracts and Presentations: None

Patents and Licenses: None

Development of Cell lines, tissue or serum repositories: None

Animal models and databases: None

Funding applied for: Applied for an NIH grant to support the continuation of FAN1 studies (CA157448).

Employment or Research opportunities applied for: None

Conclusions:

We are continuing to explore new directions involved in genomic maintenance. Our recent discovery of KIAA1018/FAN1 in FA pathway and ICL repair indicates that this protein may play a role in cellular response to chemotherapeutic agents like cisplatin. We have applied for additional funding to further explore the roles of FAN1 and FA pathway in the etiology and treatment of human cancers. Our studies on replication checkpoint point out an intriguing balance of checkpoint activation and collapse of stalled replication forks controlled at the step of generating ssDNA regions. These studies highlighted that the importance to understand how a balance of cellular process is achieved *in vivo* and how the disruption of such balance would lead to genomic instability and tumorigenesis. Further studies will focus on the enzymes that may be directly involved in the collapse of replication forks and how their activities may be controlled by checkpoint signaling. In addition, we identify several new components involved in homologous recombination repair. How these new components act with BRCA1/BRCA2 will be explored further. In the remaining of this proposal, we will also study the maintenance of chromosomal stability via the regulation of microtubule dynamics and how a number of posttranslational modifications are involved in various signaling networks that are important for cell survival and tumor suppression.

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Appendices:

Manuscript 1 Feng L, Wang J, Chen J. The Lys 63-specific deubiquitinating enzyme BRCC36 is regulated by two scaffold proteins localizing in different subcellular compartments. J Biol Chem. 285(40):30982-8. 10/2010. e-Pub 7/2010.

Manuscript 2 Liu T, Ghosal G, Yuan J, Chen J, Huang J. FAN1 Acts with FANCI-FANCD2 to Promote DNA Interstrand Cross-Link Repair. Science 329(5992):693-6, 8/2010. e-Pub 7/2010.

The Lys⁶³-specific Deubiquitinating Enzyme BRCC36 Is Regulated by Two Scaffold Proteins Localizing in Different Subcellular Compartments*

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Lin Feng, Jiadong Wang, and Junjie Chen¹

From the Department of Experimental Radiation Oncology, University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

BRCC36 is a member of the JAMM/MPN⁺ family of zinc metalloproteases that specifically cleaves Lys 63-linked polyubiquitin chains *in vitro*. We and others showed previously that BRCC36 is a component of the BRCA1-A complex, which consists of RAP80, CCDC98/ABRAXAS, BRCC45/BRE, MERIT40/NBA1, BRCC36, and BRCA1. This complex participates in the regulation of BRCA1 localization in response to DNA damage. Here we provide evidence indicating that BRCC36 regulates the abundance of Lys⁶³-linked ubiquitin chains at chromatin and that one of its substrates is diubiquitinated histone H2A. Moreover, besides interacting with CCDC98 within the BRCA1-A complex, BRCC36 also associates with another protein KIAA0157, which shares significant sequence homology with CCDC98. Interestingly, although CCDC98 functions as an adaptor of BRCC36 and regulates BRCC36 activity in the nucleus, KIAA0157 mainly localizes in cytosol and activates BRCC36 in the cytoplasm. Moreover, these two complexes appear to exist in fine balance *in vivo* because reduction of KIAA0157 expression led to an increase of the BRCA1-A complex in the nucleus. Together, these results suggest that scaffold proteins not only participate in the regulation of BRCC36 activity but also determine its subcellular localization and cellular functions.

Ubiquitin (Ub)² is a protein of 76 residues that is highly conserved from yeast to humans. Conjugation of Ub needs a cascade of reactions that involve E1, E2, and E3 enzymes, which ultimately lead to the formation of an isopeptide bond between C-terminal Gly of Ub and a Lys residue on the substrate. Ubiquitin contains seven lysine residues (at positions 6, 11, 27, 29, 33, 48, and 63), and polyubiquitin chain assembly can occur at any of these lysine residues (1). Lys⁴⁸-linked polyubiquitination of proteins is quite common and normally targets substrates for proteolysis by 26 S proteasome, whereas Lys⁶³-linked polyubiquitination

is not typically associated with protein degradation (2, 3). Instead, Lys⁶³-linked ubiquitination modification is often a signaling event and has been shown to participate in diverse cellular functions, including endocytosis, autophagy, NF- κ B activation, and DNA damage repair (4–7).

Opposing the functions of E3 ligases that promote protein ubiquitination, deubiquitinating enzymes (DUBs) are proteases that specifically remove ubiquitin moieties from substrates. Although there are around 600 E3 ligases in humans, there are only ~80 DUBs, implying that DUB activities may be regulated by their associated proteins. These DUBs can be divided into five subfamilies: UCH (ubiquitin C-terminal hydrolase), USP (ubiquitin-specific protease), OUT (ovarian tumor protease), Josephin, and JAMM/MPN⁺ (Josephin and JAB1/MPN/MOV34 metalloenzyme) families (8–10). Except for the JAMM/MPN⁺ family of DUBs that are zinc metalloproteases, all of the other DUBs are cysteine proteases.

The JAMM domain is found in all three major kingdoms of life, bacteria, archaea, and eukarya, although bacteria do not have deubiquitinating activity. Therefore, it is suggested that JAMM domain may have adopted a new function as a protease during evolution. At least five JAMM/MPN⁺ domain-containing DUBs have been reported. These include the 26 S proteasome-associated POH1 (a human PAD1 homolog, also known as Rpn11 in yeast) (11, 12), CSN5 (COP9 signalosome subunit 5) (13), the ESCRT machinery-associated DUBs AMSH and AMSH-LP (14), and BRCC36 (BRCA1-BRCA2-containing complex subunit 36) (15). These five DUBs have distinct cellular functions. POH1 cleaves at or near the proximal end of the polyubiquitin chain and is required for proteasome integrity (11, 12), whereas CSN5 removes Ub-like protein Nedd8 from Cullin1 (13). Incorporation into large protein complexes is required for the activation of POH1 and CSN5 enzymatic activities, but this is not the case for AMSH and AMSH-LP. These two have intrinsic Lys⁶³-specific DUB activity, which is determined by their abilities to specifically recognize Lys⁶³-linked ubiquitin chains over other linkages (14, 16, 17).

The fifth member of this family is BRCC36. BRCC36 is a component of the BRCA1-A complex, which also contains a ubiquitin-binding motif (UIM) domain-containing protein (RAP80), a coiled-coil domain-containing protein (CCDC98/ABRAXAS), BRCC45/BRE, MERIT40/NBA1, and BRCA1. This complex is responsible for the stable accumulation of BRCA1 at sites of DNA breaks and thus plays a role in DNA damage response (15, 18–21). However, exactly how this com-

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¹ Recipient of Department of Defense Era of Hope Scholar Award W81XWH-05-1-0470. To whom correspondence should be addressed: Dept. of Experimental Radiation Oncology, University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030. Tel.: 713-792-4863; Fax: 713-794-5369; E-mail: jchen8@mdanderson.org.

² The abbreviations used are: Ub, ubiquitin; DUB, deubiquitinating enzyme; UIM, ubiquitin-binding motif; DSB, double strand break; SFB, S-tag, FLAG tag, and streptavidin-binding peptide tag.

plex works *in vivo* remains elusive. Especially, it is not yet clear how BRCC36 activity is controlled in the cell. To gain further insight into BRCC36 function *in vivo*, we generated cell line with stable BRCC36 knockdown and observed accumulation of chromatin-associated Lys⁶³-linked Ub chains in these cells, indicating that BRCC36 is normally involved in the regulation of Lys⁶³-linked ubiquitin chain formation in the nucleus. Interestingly, besides the BRCC36-containing BRCA1-A complex, we also found that BRCC36 associates with KIAA0157, a protein that shares extensive sequence homology with CCDC98/ABRAXAS. Our further analyses of these two BRCC36-containing complexes suggest that BRCC36 activity and its localization are highly regulated in the cell.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—RAP80, CCDC98, and BRCC36 polyclonal antibodies were generated as described previously (18, 22, 23). KIAA0157 polyclonal antibody was raised by immunizing rabbits with GST-KIAA0157 fusion protein (residue 1–196 and 296–419) and was subsequently affinity-purified. Additional antibodies used in this study are as follows: anti- β -actin antibody (Sigma), anti-FLAG antibody M2 (Sigma), anti-Myc antibody 9E10 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-ubiquitin antibody P4D1-A11 (Upstate Biotechnology Inc.), anti-ubiquitinated-H2A antibody E6C5 (Millipore), anti-BRCA1 antibody (D-9, Santa Cruz Biotechnology, Inc.), anti-H3 antibody (Upstate Cell Signaling), and anti-BRCC36 antibody (ProSci Inc.). RAP80 UIM-agarose beads, poly-Ub Lys⁶³-linked chains (Ub2 to -7), and poly-Ub Lys⁴⁸-linked chains (Ub2 to -7) (Boston Biochem). BRCC36 shRNA-resistant constructs were generated by introducing silent mutations (5'-CCAACAGCATTACAAAGAGCT-3') in the shBRCC36 targeting sequence.

The BRCC36 inactive mutant (S132A/D135N) was generated using the QuikChange site-directed mutagenesis kit (Stratagene) and verified by sequencing. Plasmid encoding SFB-Lys⁶³ only was a gift from Michael S. Y. Huen (Hong Kong University of Science and Technology), and plasmids encoding His-Ub was a gift from Richard Baer (Columbia University).

Cell Culture, Transfection, and Establishment of Stable Cell Lines—HeLa cells were maintained in RPMI/1640 medium containing 10% bovine serum and penicillin/streptomycin. Transient transfection was performed with the polyethylenimine (25 kDa) method. Stable knockdown cell lines were established by transfecting HeLa with pLKO.1 empty vector or shRNAs (Open Biosystems) that specifically target BRCC36 (5'-CCAACAGCATTTCAGGAATT-3'), CCDC98 (5'-GCATGTCTGAACAACCTGGGTT-3'), or KIAA0157 (5'-CAGAGCCTTCTAATAGTGAAT-3'). Puromycin (2 μ g/ml)-resistant clones were selected, and down-regulation of targeted genes was verified by Western blotting. Puromycin was withdrawn during subsequent culture.

Chromatin Fractionation and Pull-down Assay—HeLa cells were harvested, resuspended in high salt NETN buffer (20 mM Tris, pH 8.0, 500 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA) supplemented with protease inhibitor and 5 mM NEM, and then incubated on ice for 30 min. Pellets were washed twice using the same buffer and extracted with 5 volumes of 0.2 N HCl

on ice for 30 min. The extracted chromatin fractions were neutralized with 1 volume of 1 M Tris (pH 8.8). 10 volumes of normal NETN buffer supplemented with protease inhibitor and 5 mM NEM were added for the pull-down assay.

Cytoplasmic and Nuclear Fractionation—The cell pellet was resuspended in 10 volumes of cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1 mM DTT) containing protease inhibitor and allowed to swell on ice for 30 min. Nonidet P-40 was then added to a final concentration of 0.2%. After vortexing continuously for 5 s, the homogenate was spun for 5 min at 3000 rpm. The supernatant containing the cytoplasmic fraction was transferred to a new tube, and the concentration of NaCl was adjusted to 200 mM with an equal volume of buffer C (20 mM HEPES, pH 7.9, 0.4 M NaCl, 0.4% Triton X-100, 1 mM DTT). The crude nuclear pellet was washed once in buffer A and then suspended in buffer C with protease inhibitor, vigorously vortexed, and put on ice for 30 min. The homogenate was centrifuged again at high speed. The clarified supernatant containing the nuclear fraction was transferred to a new tube, and the concentration of NaCl was adjusted to 200 mM by adding an equal volume of buffer A.

In Vitro Deubiquitination Assay—Purified proteins were incubated with 250 ng of Lys⁶³-linked ubiquitin chains in DUB reaction buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM DTT) at 37 °C. Reactions were stopped at the indicated time by the addition of SDS sample buffer. Samples were resolved on 12% SDS-polyacrylamide gels and blotted with anti-ubiquitin antibody.

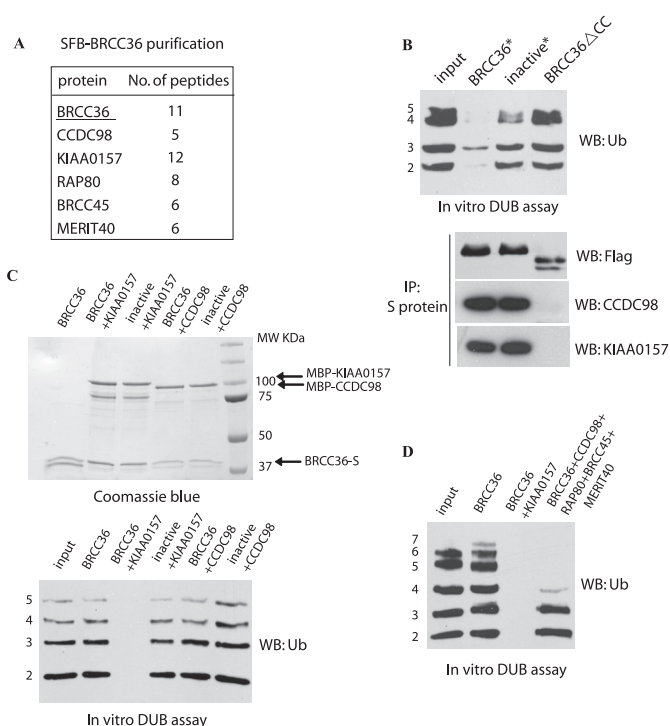
Detection of H2A Ubiquitination in Vivo—HeLa cells were transfected with various constructs as indicated and harvested 48 h later. Cells were lysed in phosphate/guanidinium buffer (6 M guanidinium HCl, 0.1 M phosphate, pH 8.0, 1 mM DTT, and 5 mM imidazole) with sonication. The ubiquitinated proteins were affinity-purified on nickel affinity gel (Sigma), eluted with SDS sample buffer, and immunoblotted with anti-Myc antibody.

Baculoviral Expression and Purification—We generated baculoviruses encoding the following proteins: GST-RAP80, SFB-BRCC36, SFB-KIAA0157, SFB-CCDC98, His₆-BRCC45, and untagged MERIT40. Sf9 cells were co-infected with various baculovirus stocks and harvested 48 h later. Cells were lysed in NETN buffer supplemented with protease inhibitor and centrifuged to remove insoluble material. Supernatants were subjected to sequential affinity chromatography using streptavidin-Sepharose and S-protein-agarose beads (18).

Tandem Affinity Purification (TAP), Irradiation, Immunostaining, and Immunoprecipitation—All of these procedures were performed as described previously (18).

RESULTS

BRCC36 Regulates Lys⁶³-linked Ubiquitin Conjugates in Chromatin Fractions—Earlier studies have already established that BRCC36 only cleaves Lys⁶³-linked polyubiquitin chains (2, 24, 25), and it localizes at sites of DNA double strand breaks (DSBs) (20). These observations suggest that this highly specified DUB may modulate Lys⁶³-linked polyubiquitin chains that are known to be involved in DNA damage response. Thus, we tested whether the down-regulation of BRCC36 would enhance



Next we wanted to determine whether or not H2A/H2AX are substrates of BRCC36. Histone H2A and H2AX are targets of E3 ligases RNF8 and RNF168. For example, RNF8 is known to be responsible for increased H2AX diubiquitination upon IR (26). RNF168 interacts with diubiquitinated H2A through its MIU domains (28). Moreover, a Lys⁶³-specific E2 enzyme UBC13 functions together with RNF8 and RNF168 (26–29). These data suggest that histones H2A and H2AX are substrates of RNF8/RNF168, which probably promote Lys⁶³-dependent ubiquitination events. Based on these findings, we reasoned that the same Lys⁶³-linked H2A might

polyubiquitin chain formation in the chromatin fraction. We used high salt condition to remove all soluble fractions and proteins that only loosely associate with chromatin. Western blotting with anti-Ub antibody indicated that the most abundant ubiquitinated proteins in chromatin fraction are proteins of ~ 22 kDa, which correspond to monoubiquitinated histones (see below). The abundance of these monoubiquitinated histone species did not change after DNA damage. However, another ubiquitinated band of ~ 30 kDa quickly appeared following IR treatment (Fig. 1A, *lanes 1 and 2*), which is likely to be

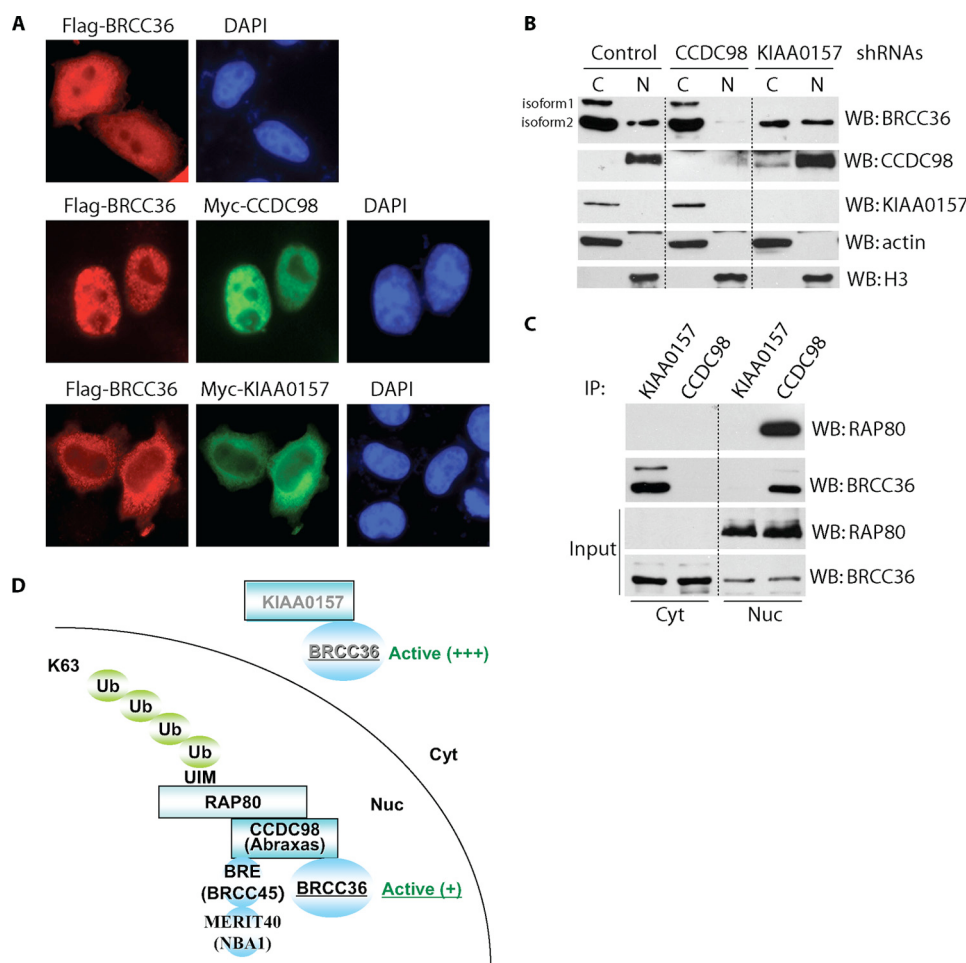


FIGURE 3. KIAA0157 and CCDC98 determine the subcellular localization of BRCC36. *A*, HeLa cells were transfected with constructs encoding SFB-tagged BRCC36 alone or together with constructs encoding Myc-tagged KIAA0157 or Myc-tagged CCDC98. Immunostaining was conducted using antibodies as indicated. *B*, HeLa cells stably transfected with empty vector, KIAA0157, or CCDC98-specific shRNAs were used for cellular fractionation experiments. Equal amounts of cytoplasmic (C) or nuclear (N) proteins were loaded and blotted (WB) with antibodies as indicated. Please note that BRCC36 has two splicing isoforms. *C*, cytoplasmic and nuclear fractions were subjected to immunoprecipitation (IP) using anti-KIAA0157 or CCDC98 antibodies and immunoblotted with antibodies as indicated. *D*, a model of BRCC36 in two distinct protein complexes.

also be the substrates of BRCC36. Indeed, BRCC36 knock-down was accompanied by an increase in the level of diubiquitinated H2A, which is ~30 kDa (Fig. 1*B*). To further confirm that the 30-kDa H2A is Lys⁶³-polyubiquitinated H2A, we took advantage of the fact that the two UIM domains of RAP80 have preferential binding to Lys⁶³-linked Ub chains (30, 31). Thus, we used RAP80 UIM agarose beads as affinity matrix to pull down Lys⁶³-linked ubiquitinated chains from chromatin fractions. As expected, the ~30-kDa band was recognized by anti-uH2A antibody (Fig. 1*C*). Moreover, higher molecular weight ubiquitinated H2A species were also enriched in BRCC36-deficient cells (Fig. 1*D*). These results suggest that diubiquitinated and poly-Lys⁶³-ubiquitinated H2A are BRCC36 substrates *in vivo*. To further confirm these results, HeLa cells were transfected with plasmids encoding Myc-tagged H2A together with plasmids encoding His₆-tagged Ub, RNF8, or BRCC36. Nickel column affinity chromatography was performed, followed by Western blotting using anti-Myc antibody. As reported previously (26, 27), RNF8 induced di- and polyubiquitination of H2A (Fig.

1*E*, lane 2), which was further enhanced with the knockdown of BRCC36 (Fig. 1*D*, lane 3). In addition, reconstitution using shRNA-resistant BRCC36 restored normal levels of H2A ubiquitination (Fig. 1*E*, lane 4), indicating that BRCC36 antagonizes RNF8-mediated H2A ubiquitination. These results suggest that at least one of the substrates of BRCC36 is ubiquitinated H2A.

We also observed higher molecular weight bands following ionizing radiation or in BRCC36-deficient cells (Fig. 1*A*, *long exp.*). The pattern of these ubiquitinated bands is very similar in control cells following ionizing radiation with that observed in BRCC36-deficient cells, suggesting that BRCC36 is the major DUB involved in the removal of ubiquitin conjugates in chromatin fractions. Using a RAP80 UIM-agarose bead pull-down assay similar to that shown in Fig. 1*C*, Lys⁶³-specific ubiquitin conjugates increased quickly upon IR in control cells (Fig. 1*E*, lanes 1 and 2); however, such an increase was not observed in BRCC36-depleted cells (Fig. 1*E*, lanes 3 and 4). The major difference between control and BRCC36-depleted cells is at the basal levels of Lys⁶³-specific ubiquitin conjugates in non-irradiated cells (Fig. 1*E*, lanes 1 and 3). These data suggest that BRCC36 negatively regulates the chromatin-associated, Lys⁶³-linked ubiquitin chain formation *in vivo*. To confirm that this involvement of BRCC36 in Lys⁶³-linked ubiquitin chain formation requires the enzymatic activity of BRCC36, we introduced the shRNA-resistant wild-type BRCC36 or its inactive mutant S132A/D135N (32) into BRCC36-deficient cells. Only wild-type BRCC36 and not its catalytic inactive mutant decreased the ubiquitin conjugates that associated with RAP80 UIM-agarose beads (Fig. 1*F*), validating that BRCC36 DUB activity is needed for the regulation of chromatin-associated Lys⁶³-linked ubiquitin chains.

Only BRCC36-containing Complexes and Not BRCC36 Alone Have DUB Activity—Besides the BRCA1-A complex including RAP80, CCDC98/Abraxas, BRCC45/BRE, and MERIT40/NBA1, we also identified another protein, KIAA0157, as a BRCC36-associated protein (Fig. 2*A*). KIAA0157 is 39% identical to CCDC98 at its N-terminal region, which contains the JAMM/MPN⁺ domain and a coiled-coil domain (33, 34). Previous studies suggest that the JAMM/MPN⁺ domain binds to BRCC45/BRE, whereas the coiled-coil domain is responsible for its interaction with BRCC36 (18, 19, 21, 33). The major

BRCC36 Is Regulated by Two Scaffold Proteins

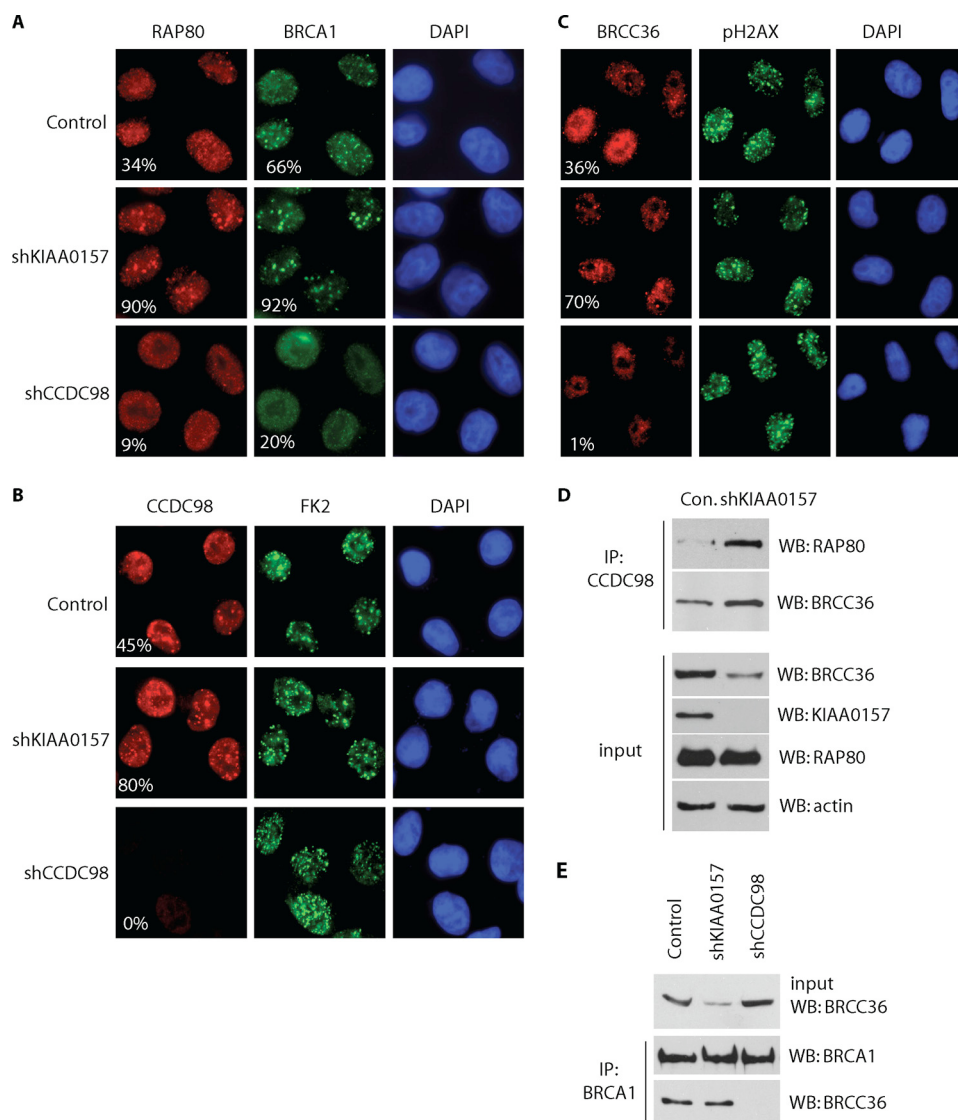


FIGURE 4. Loss of cytoplasmic scaffold protein KIAA0157 enhances the formation and focus localization of BRCA1-A complex. A and B, HeLa cells stably transfected with empty vector, KIAA0157, or CCDC98-specific shRNAs were irradiated (10 grays). 2 h later, cells were fixed and immunostained with anti-RAP80 and BRCA1 antibodies (A), anti-CCDC98 and anti-conjugated ubiquitin FK2 antibodies (B), or anti-BRCC36 antibody (C). For the visualization of BRCC36 in nuclear foci (C), cells were pre-extracted with 0.5% Triton before fixation. The staining of BRCC36 in CCDC98 knockdown cells was dim because CCDC98 is responsible for nuclear localization of BRCC36. More than 150 cells in each sample were counted to evaluate the percentage of IRIF-positive nuclei (cells with more than five foci). D, HeLa cells stably transfected with empty vector, KIAA0157, or CCDC98-specific shRNAs were harvested. Cell lysates were immunoprecipitated (IP) with anti-CCDC98 antibody and immunoblotted (WB) with indicated antibodies. E, CCDC98, but not KIAA0157, is required for BRCC36/BRCA1 interaction. Lysates prepared from HeLa cells stably transfected with empty vector, KIAA0157, or CCDC98-specific shRNAs were immunoprecipitated with anti-BRCA1 antibody and immunoblotted with antibodies as indicated.

difference between CCDC98 and KIAA0157 is that KIAA0157 lacks the pSXXF motif at its very C terminus, which is the motif that mediates the interaction between CCDC98 and BRCA1 (23, 34).

A previous study (15) indicated that BRCC36 expressed and purified from insect cells was catalytically inactive. However, the BRCC36 complexes isolated from HeLa cells were proficient in cleaving Lys⁶³-linked ubiquitin chains (Fig. 2B, top), suggesting that BRCC36 DUB activity is likely to be regulated by its associated proteins. Indeed, the coiled-coil domain deletion mutant of BRCC36, which still contains the intact JAMM/

MPN+ domain, failed to associate with either CCDC98 or KIAA0157 and did not display any DUB activity *in vitro* (Fig. 2B). To determine how BRCC36 activity is regulated by its binding partners, we purified BRCC36 alone, BRCC36-KIAA0157 complex, or BRCC36-CCDC98 complex from bacteria. In agreement with a recent report (15, 24), although BRCC36 alone was catalytically inactive, the BRCC36-KIAA0157 complex showed robust DUB activity (Fig. 2C). Surprisingly, a similar CCDC98-BRCC36 complex was catalytically inactive (Fig. 2C). In addition, we failed to detect DUB activity in either the RAP80-CCDC98-BRCC36 subcomplex or the CCDC98-BRCC36-BRCC45-MERIT subcomplex (data not shown). Only the five-subunit complex containing RAP80, CCDC98, BRCC45, MERIT40, and BRCC36 displayed *in vitro* DUB activity (Fig. 2C). This scenario is very similar to POH1 and CSN5, which also need to be assembled into multisubunit protein complexes like proteasome (11, 12) or COP9 signalosome (13) to exhibit their DUB activities.

The Two Scaffold Proteins KIAA0157 and CCDC98 Determine the Subcellular Localization of BRCC36—Although both RAP80 and CCDC98 are nuclear proteins (20, 22, 23, 34, 35), the KIAA0157-BRCC36 complex was recently isolated from S100 fraction (24), indicating that KIAA0157 may exist in cytoplasm. Indeed, epitope-tagged KIAA0157 mainly localized in cytosol (Fig. 3A). Interestingly, ectopically expressed SFB-tagged BRCC36 showed both cytoplasmic and nuclear localization; however, co-transfection of BRCC36 with CCDC98 resulted in

predominant nuclear localization of BRCC36. In contrast, co-transfection with KIAA0157 promoted cytoplasmic translocation of BRCC36 (Fig. 3A). Similarly, Western blot analysis indicated that endogenous BRCC36 existed in both cytosol and nucleus, and knockdown of endogenous CCDC98 resulted in a dramatic reduction of nuclear BRCC36 (Fig. 3B). Although the reduction of KIAA0157 expression decreased the cytoplasmic pool of BRCC36, it did not affect the abundance of BRCC36 in nucleus (Fig. 3B). Co-immunoprecipitation experiments further confirmed that KIAA0157 only interacted with BRCC36 in cytosol, whereas CCDC98 (and RAP80) associated with

BRCC36 in nuclear fractions (Fig. 3C). Together, these data indicate that there are two cellular pools of BRCC36. The KIAA0157-BRCC36 complex mainly exists in cytosol and may regulate cytoplasmic function of BRCC36, whereas CCDC98 determines the nuclear localization of BRCC36, and they form a nuclear complex with three additional components, RAP80, BRCC45, and MERIT40, which is important for nuclear function of BRCC36, especially in response to DNA damage (Fig. 3D).

Loss of KIAA0157 Expression Enhances the Assembly of Nuclear BRCC36-containing Complex—Next we asked whether KIAA0157-BRCC36 and CCDC98-BRCC36 are two independent complexes or if they can communicate with each other. We did not observe any clear cytoplasmic to nuclear translocation of BRCC36 following ionizing radiation (data not shown). However, we noticed that IRIF of the BRCA1-A complex was enhanced in KIAA0157-depleted cells, as suggested by immunostaining using antibodies recognizing RAP80, BRCA1, CCDC98, or BRCC36 (Fig. 4, A–C). On the other hand, loss of CCDC98 greatly reduced the foci formation of RAP80, BRCA1, and BRCC36, as previously reported (18, 20, 21, 23, 34). These results indicate that the loss of cytoplasmic BRCC36 complex could promote the assembly of nuclear BRCC36 complex.

As shown in Fig. 4D, although depletion of KIAA0157 destabilized BRCC36 and led to an overall reduction of BRCC36 expression in the cell, the loss of KIAA0157 actually enhanced the interaction between endogenous CCDC98 with RAP80 and BRCC36 (Fig. 4D). We also examined BRCC36/BRCA1 interaction in the absence of either KIAA0157 or CCDC98. Consistent with previous reports (18, 21), knockdown of CCDC98 abolished the interaction between BRCC36 and BRCA1. In contrast, although KIAA0157 depletion greatly decreased total level of BRCC36, the BRCC36/BRCA1 interaction was not affected (Fig. 4D).

DISCUSSION

In this study, we provide evidence indicating that BRCC36 is a Lys⁶³ chain-specific DUB that acts to modulate chromatin-associated ubiquitin chain formation. Besides BRCC36, another DUB USP3 has also been implicated in DNA damage response (36). USP3 belongs to the USP family, and its depletion enhances monoubiquitination of H2A and H2B (36). USP3 does not seem to localize to DNA break sites, which may be explained by the transient nature of its interaction with its chromatin substrates (36). On the contrary, BRCC36 is readily detected at DSB sites and its foci formation depends on RAP80, which contains tandem UIMs that specifically bind to Lys⁶³-linked polyubiquitinated chains (20, 22, 34). The paradox is that although BRCC36 depletion leads to the accumulation of chromatin-associated Lys⁶³-linked ubiquitin chains (Fig. 1), it compromises RAP80 IRIF formation (18, 19, 21). Our *in vitro* DUB assay showed that the RAP80-CCDC98-BRCC36-BRCC45-MERIT40 complex is not a very active DUB, and the disassembly of the Lys⁶³ chain is incomplete even after long term incubation (Fig. 2D). Thus, we speculate that the main function of this five-subunit BRCC36-containing nuclear complex is not just to remove all Lys⁶³-linked ubiquitin chains at DSB sites. Instead, it may act with BRCA1 and promote BRCA1-depen-

dent non-canonical K6-linked protein ubiquitination (37, 38). Further studies are needed to address whether the DUB activity of BRCC36 in the RAP80-CCDC98-containing complex would facilitate BRCA1 E3 ligase activity and more importantly whether a ubiquitin chain editing event occurs at DSB sites. One can imagine that with the help of a nuclear BRCC36-containing complex, the initial Lys⁶³-linked ubiquitin chains formed at DSB sites may be gradually switched to BRCA1-dependent Lys⁶-linked ubiquitin chains for certain yet-to-be-identified functions in DNA damage response.

Another unexpected observation is that depletion of BRCC36 mainly affects the formation of ubiquitin conjugates in non-irradiated cells (Fig. 1), implying that a key aspect of BRCC36 function is to diminish the basal level of chromatin-associated ubiquitin chains. It is likely that this function of BRCC36 is to prevent premature activation of DNA damage response. This negative role of BRCC36 in ubiquitin chain formation can be overcome following DNA damage by the specific recruitment of E3 ligases RNF8 and RNF168 to sites of DNA damage and thus allow the proper activation of ubiquitin-dependent DNA damage signaling pathways. As we discussed above, the exact task of BRCC36 at DNA damage foci remains to be determined.

Besides nuclear BRCC36, there is also a fraction of BRCC36 existing in the cytoplasm, which is activated by a CCDC98-like protein KIAA0157. Although the binding of KIAA0157 to BRCC36 is sufficient to activate BRCC36, the association of CCDC98 with BRCC36 is not. These observations clearly indicate that these two scaffold proteins differentially regulate BRCC36 catalytic activities. In addition, within the JAMM/MPN⁺ family, only AMSH and AMSH-LP have intrinsic Lys⁶³-specific DUB activity because they have two unique insertions at their JAMM domain, which are absent in BRCC36, POH1, or CSN5 (17). These unique insertions allow AMSH and AMSH-LP to bind specifically to Lys⁶³-linked ubiquitin chains, which may be responsible for its specificity toward Lys⁶³-linked ubiquitin chains (17). However, KIAA0157 binds to both Lys⁴⁸ and Lys⁶³ chains (24, 33), and thus the chain specificity of KIAA0157-BRCC36 complex is not due to the selective binding of this complex to Lys⁶³-linked ubiquitin chain. Further structural studies are needed to explore the molecular mechanism of linkage selectivity of these DUB complexes.

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In this report, we demonstrate that the lincRNA HOTAIR can link a histone methylase and a demethylase by acting as a modular scaffold (fig. S11). Other lincRNAs may also contain multiple binding sites for distinct protein complexes that direct specific combinations of histone modifications on target gene chromatin. Some lincRNAs may be “tethers” that recruit several chromatin modifications to their sites of synthesis (2) while other lincRNAs can act on distantly located genes as “guides” to affect their chromatin states (2). On the basis of their dynamic patterns of expression (28), specific lincRNAs can potentially direct complex patterns of chromatin states at specific genes in a spatially and temporally organized manner during development and disease states.

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FAN1 Acts with FANCI-FANCD2 to Promote DNA Interstrand Cross-Link Repair

Ting Liu,^{1*} Gargi Ghosal,^{2*} Jingsong Yuan,² Junjie Chen,^{2†} Jun Huang^{1†}

Fanconi anemia (FA) is caused by mutations in 13 *Fanc* genes and renders cells hypersensitive to DNA interstrand cross-linking (ICL) agents. A central event in the FA pathway is mono-ubiquitylation of the FANCI-FANCD2 (ID) protein complex. Here, we characterize a previously unrecognized nuclease, Fanconi anemia-associated nuclease 1 (FAN1), that promotes ICL repair in a manner strictly dependent on its ability to accumulate at or near sites of DNA damage and that relies on mono-ubiquitylation of the ID complex. Thus, the mono-ubiquitylated ID complex recruits the downstream repair protein FAN1 and facilitates the repair of DNA interstrand cross-links.

Fanconi anemia (FA) is characterized by congenital malformations, bone marrow failure, cancer, and hypersensitivity to DNA interstrand cross-linking (ICL) agents (1–3). Resistance to DNA ICL agents probably requires all FA proteins (4, 5). Eight FA proteins (A, B, C, E, F, G, L, and M) are assembled into the nuclear FA core complex that mono-ubiquitylates its two substrates, FANCI and FANCD2 (4–10), which, in turn, form DNA damage-induced nuclear foci together with other key DNA damage-response proteins (1, 4). Failure to mono-ubiquitylate FANCI

and FANCD2 results in highly decreased efficiency of DNA cross-link repair (4). The mono-ubiquitylated FANCI-FANCD2 (ID) complex might promote the recognition and subsequent removal of DNA lesions through nucleolytic cleavage of DNA strands by recruitment of ubiquitin-binding proteins that are important for this repair process (11–16). DNA damage-response and –repair proteins can be recruited to sites of DNA damage via their ubiquitin-binding domains (17–20). We identified a protein, KIAA1018, that contains a single ubiquitin-binding zinc finger (ZNF) domain at its N terminus and a virus-type replication-repair (VRR)–nuclease domain (or DUF994 domain) (21, 22) at its C terminus (fig. S1, A and B). KIAA1018 relocated to damage-induced foci after mitomycin C (MMC) treatment (fig. S1, C to F), suggesting that this protein is involved in DNA damage response. Because of

the functional analyses performed below, we designated this protein as Fanconi anemia-associated nuclease 1 (FAN1).

Proteins associated with FAN1 were identified by mass spectrometry in a human embryonic kidney 293T–derivative cell line stably expressing a triple-tagged FAN1 (23). We repeatedly found the ID complex as major FAN1-associated proteins (fig. S2A). Mass spectrometry analyses of triple-tagged FANCD2-associated protein complexes revealed peptides that corresponded to FAN1 (fig. S2B), indicating that these proteins probably form a complex in vivo. Immunoprecipitation (IP) confirmed the interaction of FAN1 with FANCD2 and weakly with FANCI (Fig. 1A). Although FAN1 could interact with the unmodified ID complex, its association with the ID complex was greatly enhanced after MMC treatment (Fig. 1B), which coincides with FANCI-FANCD2 mono-ubiquitylation (6–8). Their association was further confirmed by in vivo colocalization experiments (fig. S2, C and D).

Upon reduction of endogenous FAN1 expression, we still detected MMC-induced mono-ubiquitylation and foci formation of FANCI-FANCD2 (Fig. 1C and fig. S3, A and B). Upon depletion of FANCD2 or FANCI, we failed to observe FAN1 foci after MMC treatment and saw a substantially reduced chromatin accumulation of FAN1 after MMC treatment (Fig. 1, D and E, and fig. S3C). Knockdown of FAN1 caused a significant increase in MMC but not camptothecin sensitivity (Fig. 1, F and G, and fig. S3D), increased levels of MMC-induced chromosome instability (fig. S4, A and B), and profound G₂/M-phase arrest (fig. S4C), all typical of FA cells (1–5). Double knockdown of FAN1 with FANCD2 or FANCA did not lead to any further increase in these phenotypes (Fig. 1F and

¹Life Sciences Institute, Zhejiang University, Hangzhou, Zhejiang 310058, China. ²Department of Experimental Radiation Oncology, University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA.

*These authors contributed equally to this work.

†To whom correspondence should be addressed. E-mail: jchen8@mdanderson.org (J.C.), jhuang@zju.edu.cn (J.H.)

fig. S4). Altogether, FAN1 promotes ICL repair downstream of the ID complex and does so through a common pathway. The ZNF domain deletion mutant (Δ ZNF) of FAN1 lost its foci-formation ability, whereas the nuclease domain mutant (Δ NUC) still localized to nuclear foci after MMC treatment (fig. S5, A to C). An N-terminal fragment, which contains the intact ZNF domain, but not a ZNF domain-disrupting point mutant (ZNF-C44F), is sufficient for foci formation after MMC treatment (fig. S5, A to C). Wild-type (WT) or the Δ NUC mutant of FAN1, but not the FAN1 mutant that lacks its ZNF domain (Δ ZNF), specifically interacted with a ubiquitin-glutathione S-transferase fusion protein (Ubi-GST) in vitro (Fig. 2A). In addition,

Ubi-GST pulled down the N-terminal fragment of FAN1 containing the ZNF domain, but not the ZNF-C44F mutant (Fig. 2A). WT FAN1, but not FAN1 mutant that lacks its ZNF domain (Δ ZNF), interacts with the ID complex, and this interaction is greatly enhanced after MMC treatment (Fig. 2B). WT FANCD2/FANCI interacted strongly with FAN1, whereas the ubiquitylation-deficient point mutants showed only moderate or residual binding (Fig. 2C). Moreover, the N-terminal fragment of FAN1 containing the intact ZNF domain, but not its corresponding ZNF-C44F mutant or the ZNF domain of RAD18 (RAD18-ZNF), interacted with FANCD2 (Fig. 2D). WT FANCD2 could restore FAN1 foci formation in FANCD2-deficient PD20 cells, but the mono-ubiquitylation mutant

Lys⁵⁶¹ $\rightarrow$ Arg⁵⁶¹ (K561R) (24) of FANCD2 did not (Fig. 2E and fig. S5D). Foci formation of the FAN1 ZNF domain alone also depends on FANCD2 mono-ubiquitylation (Fig. 2F and fig. S5E). The mono-ubiquitylation mutant (K523R) of FANCI partially complemented FAN1 foci formation in FANCI-depleted cells (Fig. 2G and fig. S5F), as mono-ubiquitylation of FANCI is not critical for the function of the FA pathway (8, 25). Thus, mono-ubiquitylated FANCD2 (and FANCI) acts to facilitate FAN1 accumulation at sites of DNA damage.

ICL repair involves nucleolytic cleavage at or near the site of ICL to produce a suitable substrate that can subsequently be repaired by homologous recombination (HR) (1–4). Purified

Fig. 1. FAN1 acts in ICL repair downstream of FANCD2/I. **(A)** Ectopically expressed FAN1 interacts with FANCD2/FANCI. 293T cells were cotransfected with plasmids encoding Myc-tagged FAN1 and S, FLAG, and, streptavidin-binding peptide tag (SFB)-tagged FANCD2 or FANCI. IP reactions were done using the antibodies as indicated. **(B)** Interaction between FAN1 and FANCD2/I before and after MMC treatment was monitored by IP with an antibody to FAN1 (anti-FAN1) and detected on SDS-polyacrylamide gel electrophoresis (PAGE) gels with the indicated antibodies (top three panels). IgG, immunoglobulin G. **(C)** FAN1 is not required for FANCD2/I mono-ubiquitylation. Soluble and chromatin fractions prepared from mock-treated or MMC-treated HeLa cells and immunoblotting experiments were performed using the indicated antibodies. W, whole-cell extracts; SiCon, control siRNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H3, histone H3. **(D)** FANCD2/I is required for FAN1 foci formation. HeLa cells were transfected with either FANCD2/I siRNAs or control siRNA and then treated with 1 μ M MMC for 24 hours before immunostaining experiments were performed. DAPI, 4',6'-diamidino-2-phenylindole. **(E)** FANCD2/I is required for FAN1 chromatin recruitment. Chromatin fractions were isolated, and immunoblotting experiments were performed using the indicated antibodies. **(F and G)** FAN1-depleted cells display increased MMC sensitivity. These experiments were performed in triplicate, and the results were the average of three independent experiments. The SD is shown for different doses of MMC or irradiation.

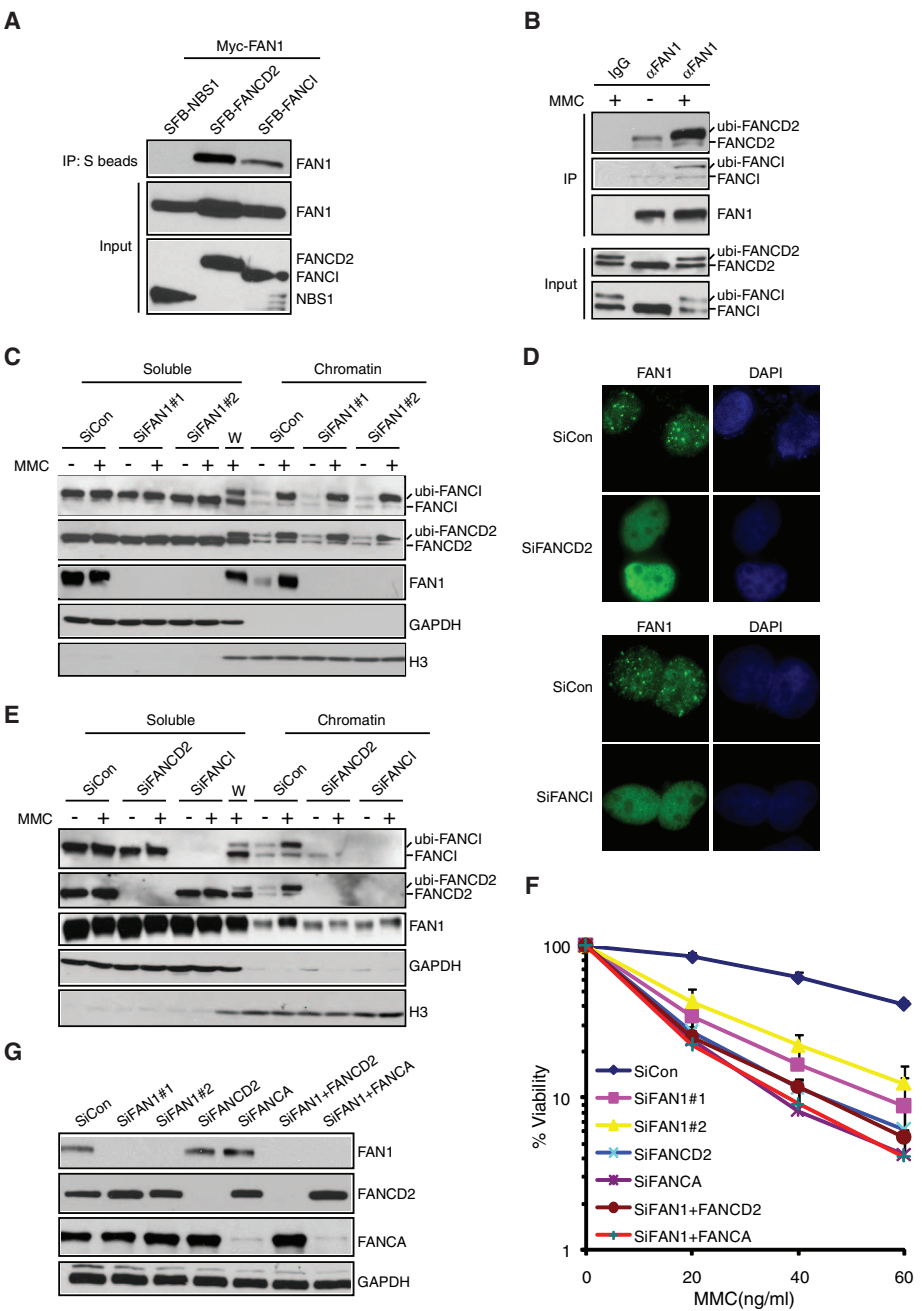
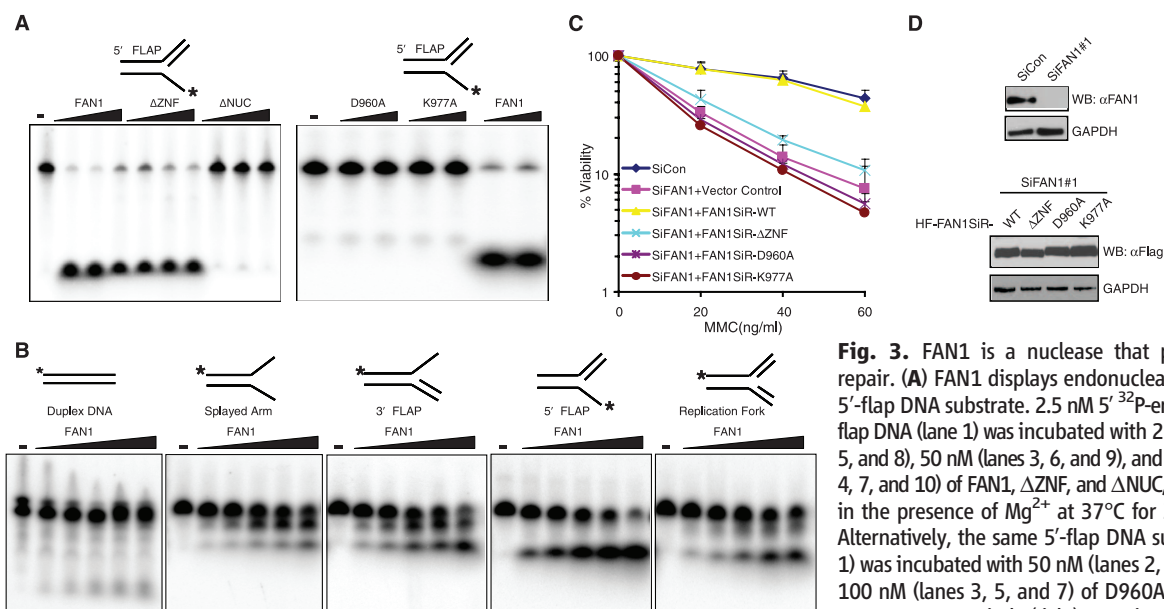
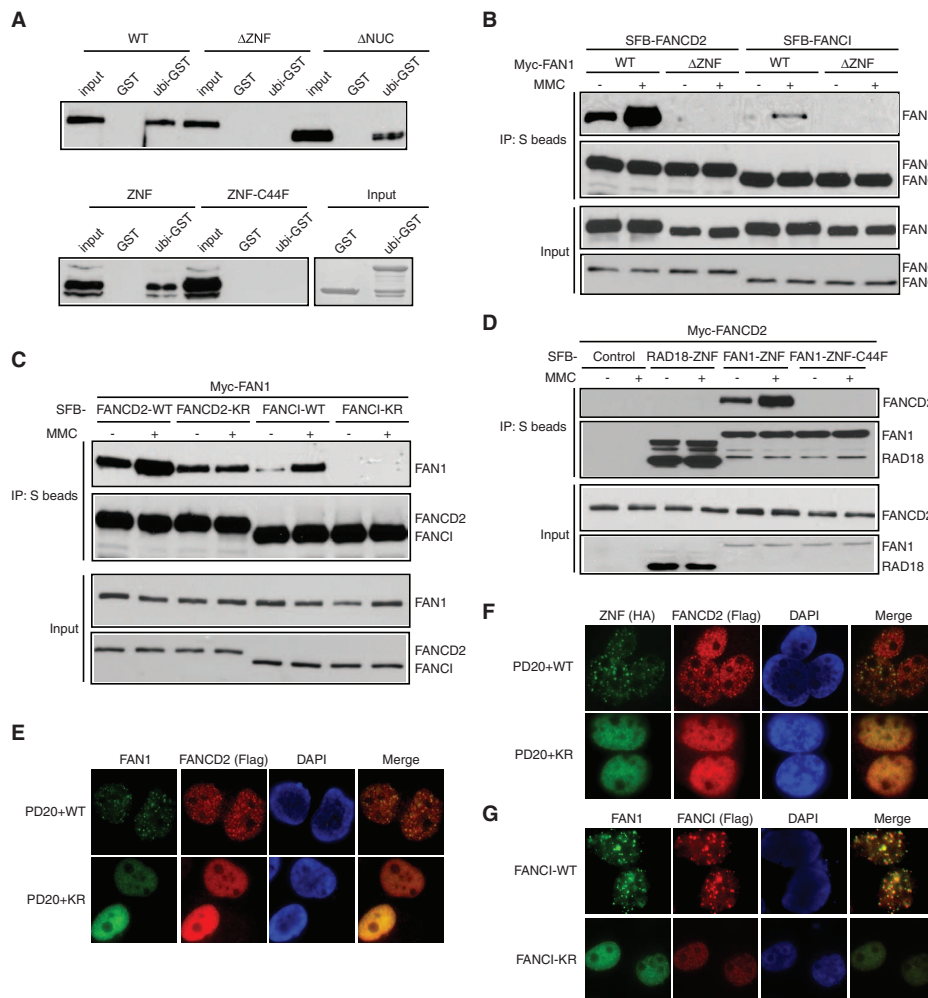


Fig. 2. Focus-localization of FAN1 depends on its ZNF domain and mono-ubiquitylation of the ID complex. **(A)** The ZNF domain of FAN1 is essential and sufficient for binding to ubiquitin in vitro. **(B)** The ZNF domain of FAN1 is required for binding to FANCD2/FANCI. 293T cells stably expressing SFB-tagged FANCD2 or FANCI were transfected with plasmids encoding Myc-tagged WT or ZNF domain–deletion mutant of FAN1. Cells were mock treated or treated with MMC before IP reactions were performed. **(C)** Mono-ubiquitylation of FANCD2/FANCI is required for binding to FAN1. **(D)** The ability to bind the mono-ubiquitylated form of FANCD2 is specific to the ZNF domain of FAN1. IP reactions were performed as described in (B). **(E and F)** Dependence of DNA damage–induced FAN1 foci formation on mono-ubiquitylation of FANCD2. PD20 cells, expressing SFB-tagged WT FANCD2 or the K561R mutant (FANCD2-KR), were treated with MMC (E) or transfected with plasmids encoding hemagglutinin (HA)–tagged FAN1 ZNF domain before MMC treatment (F). Immunostaining experiments were performed as indicated. **(G)** Partial dependence of damage-induced FAN1 foci formation on mono-ubiquitylation of FANCI. HeLa cells depleted of endogenous FANCI were infected with viruses encoding siRNA-resistant HA-Flag-tagged WT or K523R mutant of FANCI (FANCI-KR). Cells were treated with MMC and immunostained as indicated.



analyzed on denaturing PAGE. Asterisks indicate 5' 32 P-end label. **(B)** FAN1 is a structure-specific endonuclease. Indicated 2.5 nM 5' 32 P-end-labeled DNA substrates (lane 1) were incubated with 1, 5, 10, 25, and 50 nM FAN1 (lanes 2 to 6) in the presence of Mg^{2+} for 30 min at 37°C. Reaction products were analyzed on denaturing PAGE. **(C and D)** The ZNF domain and nuclease activity of FAN1 are required for restoring cellular resistance to MMC. HeLa-derivative cell lines stably expressing siRNA-resistant HA-Flag-tagged WT (FAN1SiR-WT), Δ ZNF mutant (FAN1SiR- Δ ZNF), D960A mutant (FAN1SiR-D960A), and K977A mutant (FAN1SiR-K977A) of FAN1 were generated. FAN1 expression was confirmed by immunoblotting with the use of Flag antibody, and extracts were prepared from cells transfected with FAN1 siRNA#1 (D). These experiments were performed in triplicate, and the results were the average of three independent experiments (C). The SD is shown for different doses of MMC.

FAN1 (fig. S6, A and B) was incubated with 5'-flap DNA substrate and displayed nuclease domain-dependent endonuclease activity (Fig. 3A). FAN1 could also cleave branched DNA structures (such as splayed-arm, 3'-flap, 5'-flap, or replication-fork structures), but not duplex DNA (Fig. 3B), indicating that FAN1 is a structure-specific endonuclease. To confirm that the nuclease activity we observed is intrinsic to FAN1, we generated FAN1 mutations at two highly conserved residues within its nuclease domain (D960A and K977A). Both of these mutants abolished the endonuclease activity of FAN1 on 5'-flap DNA substrate or other branched DNA substrates (Fig. 3A and fig. S6C).

To explore the physiological relevance of this highly conserved nuclease domain and the ZNF domain of FAN1 in ICL repair, we knocked down FAN1 in HeLa cells using siFAN1#1 [FAN1-specific small interfering RNA (siRNA) 1] and reintroduced siRNA-resistant full-length FAN1, Δ ZNF, or the nuclease-inactivating mutants (D960A and K977A) of FAN1. Clonogenic assays indicated that reconstitution with WT FAN1, but not its ZNF deletion (D960A or K977A mutant), restored cell survival after MMC treatment (Fig. 3, C and D), suggesting that both the nuclease activity and the ZNF domain of FAN1 are important for FAN1 function in promoting cell survival after MMC treatment.

FAN1 is a nuclease that associates with mono-ubiquitylated FANCI-FANCD2, mutations that may be responsible for FA in a subset of human patients. FAN1 is a structure-specific endonuclease that may act together with other repair proteins to mediate endonucleolytic digestion of cross-linked DNA structures and, thus, generate ends that can serve as substrates for HR repair.

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23. Materials and methods are available as supporting material on Science Online.
24. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Supporting Online Material

www.sciencemag.org/cgi/content/full/science.1192656/DC1

Materials and Methods

SOM Text

Figs. S1 to S8

References

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