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RNF126 promotes homologous recombination via regulation of E2F1-mediated BRCA1 expression

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Abstract

RNF126 is an E3 ubiquitin ligase. The deletion of RNF126 gene was observed in a wide range of human cancers and is correlated with improved disease-free and overall survival. These data highlights the clinical relevance of RNF126 in tumorigenesis and cancer therapy. However, the specific functions of RNF126 remain largely unknown. Homologous recombination (HR)-mediated DNA double-strand break repair is important for tumor suppression and cancer therapy resistance. Here, we demonstrate that RNF126 facilitates HR by promoting the expression of

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BRCA1, in a manner independent of its E3 ligase activity but depending on E2F1, a well-known transcription factor of BRCA1 promoter. In support of this result, RNF126 promotes transactivation of BRCA1 promoter by directly binding to E2F1. Most importantly, an RNF126 mutant lacking 11 amino acids that is responsible for the interaction with E2F1 has a dominant-negative effect on BRCA1 expression and HR by suppressing E2F1-mediated transactivation of BRCA1 promoter and blocking the enrichment of E2F1 on BRCA1 promoter. Lastly, RNF126 depletion leads to the increased sensitivity to ionizing radiation (IR) and poly (ADP-ribose) polymerase (PARP) inhibition. Collectively, our results suggest a novel role of RNF126 in promoting HR-mediated repair through positive regulation on BRCA1 expression by direct interaction with E2F1. This study not only offers novel insights into our current understanding of the biological functions of RNF126 but also provides a potential therapeutic target for cancer treatment.

Keywords

Homologous Recombination; RNF126; E2F1; BRCA1

Introduction

DNA double-strand breaks (DSBs) are the most cytotoxic forms of DNA damage. DSBs can not only be caused by replication stress but also be the principle cytotoxic lesions for ionizing radiation (IR) and chemotherapeutic drugs. Homologous recombination (HR) is one major repair pathway required for repairing DSBs. Defects in HR not only promote cancer due to the increased genomic instability, but also lead to an increased sensitivity to radiotherapy and chemotherapy.

HR is an error-free repair pathway and requires homologous donor template. Thus, in mammalian cells, HR predominantly occurs in S phase or G2 phase when the DNA template of the sister chromatid is available. Given the importance of HR in repair of DSBs during DNA replication, a deficiency in HR can cause cell proliferation defect ([1–3]). BRCA1 is believed to be important for HR via promoting single strand DNA (ssDNA) resection by association with CtIP or acts as a recombination mediator/comediator ([4]). Thus, BRCA1 is required for the recruitment of recombinase protein RAD51. In general, the function of BRCA1 in HR is considered to be one of the major mechanisms contributing to its tumor suppressor activity ([5, 6]), and the cause of hypersensitivity to PARP inhibitors when BRCA1 is defective ([7]). BRCA1 is not only mutated in familial breast and ovarian cancer patients but also exhibits low expression in sporadic cancers including breast and ovarian cancers ([8–10]). A growing consensus has emerged suggesting that a large percentage of sporadic, non-inherited breast cancers are associated with altered transcriptional regulation of the BRCA1 gene ([11, 12]), suggesting that the control of BRCA1 transcription might be important to tumorigenesis. While several groups have demonstrated that the *BRCA1* promoter is regulated by transcriptional co-activators and co-repressors ([13–15]), as well as methylation of specific CpG islands ([16]), the mechanism regulating BRCA1 expression remains largely unknown.

E2F1 is a member of the E2F gene family of transcription factors, which regulates the expression of genes involved in a wide range of cellular processes including cell-cycle progression, DNA replication, DNA repair, differentiation, and apoptosis. E2F1 can act either as an oncogene or a tumor suppressor, depending on tissue type and experimental conditions ([17]). Emerging data suggest that, in addition to having a prominent role in apoptosis, E2F1 may function as a tumor suppressor by promoting DNA damage repair and maintenance of genomic stability ([18–20]). E2F1 promotes DNA repair in transcription-dependent or -independent mechanisms ([21]). First, E2F1 transcriptional activity leads to high expression of several DNA repair genes, including BRCA1, RAD51 and RAD52 ([14, 22–26]). Second, E2F1 enables the formation of multicomponent protein complexes at sites of damage or recruitment of protein factors ([19, 27, 28]). Thus, while it is clear that E2F1 plays a direct role in regulating BRCA1 expression ([22]), the mechanistic details are currently an enigma.

In an siRNA screen targeting 555 individual E3 ubiquitin ligases, RNF126 was identified as one of the fourteen proteins required for cell growth through targeting p21 for ubiquitination dependent degradation ([29]). Other reports have shown that RNF126 also targets EGFR ([30]) and AID for ubiquitylation ([31]). Together, these data demonstrate multiple functions of RNF126 associated with a diverse set of cellular processes. The RNF126 gene is mapped to chromosome 19 p13.3 which is a commonly deleted region in ovarian cancer ([32–34]). In addition, high-resolution 19p13.2-13.3 allele typing of breast carcinomas demonstrates frequent loss of heterozygosity ([35]). The RNF126 E3 ubiquitin ligase contains a RING finger and a Zinc finger domain, both of which are common in a variety of functionally distinct proteins and domains that participate in protein-protein and protein-DNA interactions. In addition, RNF126 proteins contain a serine-rich array that is evolutionarily conserved and is similar to that found in transcriptional activators present at RNAPII-dependent promoters ([31]). Taken together, recently emerging data hint at an eminent role of RNF126 in transcriptional regulation.

In this report we demonstrate that RNF126 promotes HR. Mechanically, RNF126 enhances transcription of HR protein *BRCA1* by binding directly to E2F1. We show that disruption of the RNF126-E2F1 interaction impairs HR and BRCA1 expression by interfering E2F1-mediated transactivation of BRCA1 promoter. Additionally, we demonstrate that RNF126 is required for the resistance to PARP inhibitor and IR. Cumulatively, our results define a direct link between RNF126 and HR repair via regulating E2F1-mediated BRCA1 expression. Furthermore, our data demonstrating a direct, functional interaction between E2F1 and RNF126 could provide a novel therapeutic target for cancer therapy.

RESULTS

1. RNF126 promotes HR

We first determined the role of RNF126 in HR by using MCF7-pDRGFP, a cell line with a chromosomally integrated HR plasmid substrate *DR-GFP* as described previously ([36–40]). In this system, a DSB is generated by expressing the I-SceI endonuclease, whose 18-bp recognition site has been integrated into the GFP gene such that it disrupts the gene. Repair of the cleaved I-SceI site by HR gives rise to a functional GFP gene when the template used

for repair is a truncated GFP fragment located downstream in the plasmid, and HR activity is measured by flow cytometric analysis of the number of GFP+ cells following I-SceI expression. Using two shRNAs targeting different regions in the RNF126 transcript (Fig. 1A), it was found that RNF126 was involved in HR (Fig. 1B). Consistent with the magnitude of decrease in protein levels, a greater decrease in HR frequency was observed in cells infected with RNF126 shRNA #3 compared to those infected with RNF126 shRNA #1 (Fig. 1B). These results provide direct evidence that RNF126 promotes DSB-induced HR. The impaired HR in cells depleted of RNF126 was not caused by alteration of the cell cycle profile, because almost identical cell cycle profiles were observed in MCF7 cells with or without RNF126 depletion (Fig. 1C). The effect of RNF126 on HR was further confirmed in U2OS cells with the chromosomal integration of HR substrate DR-GFP (Fig. 1D, E). The knockdown of RNF126 by shRNA #3 (Fig. 1D) led to a decrease in I-SceI induced HR (Fig. 1E). In summary, the results in Fig. 1 suggest that RNF126 promotes HR. Because RNF126 depletion by virus infection of shRNA #3 cause more severe RNF126 knockdown compared to #1, we focused on the use of shRNA #3.

2. RNF126 facilitates BRCA1 gene expression, independent of its E3 ligase activity

To test if the decreased HR was due to decreased levels of HR proteins, we initially screened the majority of proteins required for HR. BRCA1 protein levels were reduced in MCF7 cells depleted of RNF126 (Fig. 2A, left panel). Of note, although the RAD51 level was also slightly decreased the magnitude is much less profound than those observed for BRCA1. However, other screened proteins were unchanged, indicating that RNF126 specifically regulates BRCA1 protein expression. The effect of RNF126 on BRCA1 protein expression was further confirmed in a second cell line, MDA-MB 231 (Fig. 2A right panel). In support of the hypothesis that RNF126 promotes HR via regulation of the BRCA1 protein level, overexpression of BRCA1 restored the HR deficiency caused by RNF126 depletion (Fig. 2B, C). In contrast, depletion of RNF126 did not lead to a significant decrease in HR in BRCA1-deficient cells (Fig. S1). In addition, consistent with the role of RNF126 in BRCA1 expression, RNF126 depletion decreased the amplitude of IR-induced foci (IRIF) of BRCA1 by immunostaining (Fig. 2E, left panel). Similarly, RNF126 depletion reduced the percentage of cells with RAD51 foci (Fig. 2E, right panel), which is consistent with the report that BRCA1 regulates HR by promoting RAD51 recruitment ([41]). In addition, the lower RAD51 expression could also contribute to the impaired RAD51 IRIF. Representative IRIF of BRCA1 and RAD51 in cells depleted of RNF126 are shown in Fig. 2E. A similar result was obtained in a second cell line MDA-MB-231 (data not shown), indicating the lower BRCA1 protein expression leads to the impaired BRCA1 recruitment.

Next, we determined how RNF126 regulates BRCA1 expression by measuring BRCA1 mRNA levels. RNF126 likely regulate BRCA1 at the transcriptional level because RNF126 depletion resulted in a decrease in the BRCA1 mRNA level (Fig. 2F). The effect of RNF126 on BRCA1 expression was further supported by an overexpression experiment in which Flag-RNF126 overexpression led to increased mRNA level of BRCA1 compared to control cells (Fig. 2G,H). Most importantly, the E3 ligase activity of RNF126 appears to be dispensable for the regulation of BRCA1 expression, because the expression of a validated RNF126 E3 ligase mutant (RNF126 C229A/C232A) ([29]) retained the ability to increase

BRCA1 protein expression (Fig. 2I). In summary, the results in Fig. 2 suggest that RNF126 promotes recruitment of BRCA1/RAD51 and HR due to its regulation on BRCA1 mRNA expression.

3. The role of RNF126 in promoting BRCA1 expression and BRCA1 recruitment depends on E2F1

Given that E2F1 is a well-known transcription factor of BRCA1 promoter, we next examined whether E2F1 is involved in RNF126-mediated regulation of BRCA1 expression. A cell clone with a stable E2F1 knockdown (MCF7-E2F1sh) was generated by BS/U6-E2F1 RNAi transfection and subsequent selections. Effective E2F1 knockdown was verified by western blot (Fig. 3A). Depletion of RNF126 considerably decreased the BRCA1 mRNA level in control MCF7 cells but RNF126 depletion causes a much less decrease of BRCA1 mRNA in MCF7-E2F1sh cells (Fig. 3B). In support of this result, the decreased protein level of BRCA1 due to RNF126 depletion was partially abrogated in cells with a stable E2F1 knockdown (Fig. 3C). Moreover, the dependence of E2F1 was further confirmed by immunostaining assay, indicating that depletion of RNF126 had no obvious effect on IRIF of BRCA1 although a reduced percentage of BRCA1 IRIF was observed in control cells (Fig. 3D). Therefore, we conclude that RNF126 regulated BRCA1 expression through E2F1.

4. RNF126 promotes E2F1-mediated transactivation of the BRCA1 promoter

The requirement of E2F1 for RNF126-induced BRCA1 expression promoted us to investigate if the E2F1 transactivation activity for BRCA1 promoter is facilitated by RNF126. We first tested this hypothesis by using a luciferase reporter driven by a BRCA1 promoter ([22]). Indeed, RNF126 overexpression enhanced the luciferase activity in cells with E2F1 co-expression (Fig. 4A, B,C left panel) although no significant increase was observed when sole RNF126 is overexpressed (Fig. 4C, right panel).

We further determined the occupancy of E2F1 on the BRCA1 promoter by chromatin immunoprecipitation (ChIP) as described previously ([42]), in the presence or absence of RNF126 overexpression. A much more profound increase in E2F1 on BRCA1 promoter was detected when Flag-RNF126 and HA-E2F1 are co-overexpressed (Fig. 4D, left panel) whereas a less enrichment of E2F1 on BRCA1 promoter was found in cells overexpressing only E2F1 (Fig. 4D, right panel). In addition, no obvious recruitment of RNF126 protein on BRCA1 promoter was observed (Fig. 4E). In further support of our hypothesis, RNF126 depletion reduced the enrichment of E2F1 on BRCA1 promoter (Fig. 4F). Taken together, Figure 4 suggested that RNF126 promotes transactivation activity of E2F1 via facilitating the binding of E2F1 to BRCA1 promoter.

5. RNF126 directly binds to E2F1

In order to understand the mechanisms by which RNF126 promotes E2F1-mediated transactivation in detail, we next determined if RNF126 interacts with E2F1 by co-IP. Indeed, an interaction between endogenous RNF126 and E2F1 was detected by reciprocal co-immunoprecipitation in MCF7 cells (Fig. 5A). This result was further confirmed by exogenous overexpression assay. In brief, Flag-RNF126 was overexpressed in MCF7 cells. E2F1 was found in the anti-Flag immunoprecipitates (Fig. 5B upper panel). The Flag-

RNF126 protein levels were confirmed by re-probing with an anti-Flag antibody (Fig. 5B bottom panel). The association of RNF126 and E2F1 was supported by GST pull-down assay (Fig. 5C) via incubating glutathione S-transferase (GST)-RNF126 fusion proteins with lysates prepared from the cells overexpressing HA-E2F1. The absorbate to the GST column was probed with Anti-HA antibody which revealed that a GST-RNF126 retained E2F1 protein (Fig. 5C, upper blot). The membrane was reprobed with Anti-GST antibody for a control purpose (Fig. 5C lower blot). Most importantly, an in vitro GST pull down assay showed a further direct binding between RNF126 and His-E2F1 (Fig. 5D). Following incubation of GST-RNF126 and His-E2F1 prepared from bacteria, GST-RNF126 pulled down by the GST column was probed with an antibody to His and revealed that a GST-RNF126 retained His-E2F1 protein (Fig. 5D, upper lane), indicating that the interaction of these two proteins was direct. The membrane was reprobed with antibodies against RNF126 (middle panel) or GST for a control purpose (third panel).

In order to map the E2F1-binding domain in RNF126, a series of RNF126 mutants were generated as indicated in Fig. 5E (left panel). Purified GST-E2F1 efficiently pulled down Flag-RNF126-WT and the Flag-RNF126-A, B, D, G mutants but not RNF126-C, E, F mutants (Fig. 5E, right panel), indicating that the residues of RNF126 from 40-228 is important for interaction, because this region is deleted in all three RNF126 mutants C, E, F. However, there was a likelihood that the lack of interaction could be caused by lower protein expression because no detectable proteins expression was found for Flag-RNF126-C, E, F (Fig. 5F, upper panel) even if the film was exposed for a longer period of time (Fig. 5F, bottom panel). In order to test this possibility, we further generated a series of RNF126 deletion mutants (Fig. 5G, left panel). We narrowed down the interaction region to 11aa in RNF126 from 185-195 that is critical for the interaction with E2F1 because GST-E2F1 failed to pull-down RNF126-Δf that lacks the 11 aa from 185-195 (Fig. 5G, right panel), although a comparable protein expression was observed in the cells expressing Flag-RNF126-WT or RNF126-Δf (Fig. 5H). Of note, a much lower expression of RNF126-Δb and RNF126-Δe was observed compared to RNF126-WT. Because both mutants lack residues aa130-140, indicating these 11aa is likely important for RNF126 stability (Fig. 5H). However, an interaction of GST-E2F1 and Flag-RNF126-Δb/Δe was still observed (Fig. 5G, right panel), indicating that these residues are important for RNF126 stability without affecting the interaction with E2F1. Although future investigation is warranted illustrating the mechanisms causing the degradation of Flag- RNF126-Δb/Δe is out of the scope of the current study. Collectively, these results in Figure.5 suggested that the 11aa located in RNF126 protein from 185-195 is critical for the interaction with E2F1.

6. RNF126 promotes E2F1-mediated transactivation of BRCA1 via interaction with E2F1

Next, we attempted to determine if the RNF126-Δf mutant lacking the association with E2F1 loses the functions of RNF126. RNF126-Δf overexpression failed to induce a significant increase in luciferase activity driven by the BRCA1 promoter in the cells co-expressing E2F1 whereas overexpression of RNF126-WT leads to an increased luciferase activity in the same condition (Fig. 6A). In contrast, RNF126-Δf expression reduced the luciferase activity compared to controls cells (Fig. 6A), indicating that RNF126-Δf expression has a dominant-negative effect which may interfere the function of endogenous

RNF126. This result was further supported by a ChIP assay showing that RNF126 overexpression enhanced the enrichment of E2F1 on BRCA1 promoter but RNF126-Δf overexpression resulted in a significant decrease in E2F1 protein binding to BRCA1 promoter (Fig. 6B). The dominant negative effect of RNF126-Δf was further supported by Fig. 6C, D showing that the decreased expression of BRCA1 in both mRNA and protein levels were observed in cells expressing RNF126-Δf compared to controls cells whereas the increased BRCA1 mRNA and proteins expression was found in cells expressing RNF126-WT as described in Fig. 2. Last, dominate negative effect of RNF126-Δf expression on HR was also observed as indicated in Fig. 6E.F. However, we did not see an obvious increased in HR when the RNF126-WT and RNF126 E3 ligase mutant overexpressed (Fig. 6F). This could be caused by the following two reasons. First, the HR activity is saturated although RNF126 or BRCA1 protein levels are increased. Second, the slight toxicity was observed when RNF126-WT or RNF126-RNF126 C229A/C232A overexpressed, which is consistent with the well-known biological toxicity caused by BRCA1 over-expression ([43]) that may prevent us from seeing the increased HR frequency in cells overexpression RNF126. To test this hypothesis, we overexpressed RNF126-Δf in cells treated with siRNA targeting UTR region of RNF126, and we found that overexpression of RNF126-Δf failed to restore the decreased HR due to RNF126 depletion, although expression of RNF126-WT successfully restored HR activity (Fig. 6G). Taken together, the results described in Fig. 6 strongly suggested that via the interaction with E2F1, RNF126 promotes E2F1-mediated BRCA1 expression and HR. In addition, expression of the RNF126 mutant lacking the interaction with E2F1 has a dominant negative effect on BRCA1 expression.

7. RNF126 is required for the resistance to PARP inhibitor and IR

Defect in HR leads to the enhanced sensitivity to chemotherapeutic drugs as well as IR. To test the hypothesis that RNF126 depletion may sensitize cells to PARP inhibitor due to its role in HR, we determined if RNF126 has a role in the resistance to the PARP inhibitor ABT-888. The survival of cells depletion of RNF126 was reduced in MCF7 cells (Fig. 7A). Also, an enhanced sensitivity to IR was observed in MCF7 depleted of RNF126 (Fig. 7B), suggesting that RNF126 is required for cell survival after PARP inhibitor treatment and IR exposure. However, the magnitude of decrease in cell survival following PARP inhibitor treatment and IR exposure is less than that observed in BRCA1-deficient cells (Data not show). We speculated that targeted inhibition of RNF126 sensitized cells to PARP inhibition and IR exposure due to an effect on apoptosis reduce given that it has been shown that E2F1 also regulates several pro-apoptotic genes.

In order to test the hypothesis, we assayed its effect on the expression of apoptotic gene p73 in 293T cells, in which apoptosis has been shown to be specifically dependent on E2F1 ([44], [45]). In support of the idea that RNF126 is important for E2F1-mediated p73 expression, we found overexpression of RNF126 leads to an increase in level of p73 mRNA when E2F1 is co-overexpressed (Fig. S2A). In order to test the occupancy of E2F1 on p73 promoter, we performed chromatin immunoprecipitation (ChIP) in 293T cells. We found an increased HA-E2F1 protein occupancy on p73 promoter when RNF126 and E2F1 are co-overexpressed, indicating that RNF126 enhanced the binding of E2F1 to p73 promoter (Fig. S2B). In addition, a similar result was seen for caspase 7, another E2F1-target apoptosis gene. We

found that RNF126 enhanced the occupancy of E2F1 on caspase 7 promoter (Fig. S2C). These data suggested a role for RNF126 in promoting expression of apoptosis genes regulated by E2F1. Of note, we are not pursuing to investigate if an RNF126 depletion cause a decreased apoptosis because of the role of RNF126 in HR repair may counteracts its action in promotion of E2F1- mediated apoptosis. Together, we concluded that RNF126 is important for resistance to IR and PARP1 inhibitor resistance although RNF126 also regulates other E2F1 target genes involved in apoptosis.

Discussions

Germ-line mutations in the *BRCA1* tumor suppressor gene contribute to hereditary breast and ovarian cancer syndrome. In addition, decreased expression of the *BRCA1* gene is common in sporadic breast and ovarian tumors, and the magnitude of the decrease correlates with disease progression. Identify how these regulators controlling BRCA1 expression could lead to a better understanding of sporadic breast/ovarian cancer etiology and the generation of novel therapeutic strategies targeting sporadic breast/ovarian tumors. In our study, we identify a novel role of RNF126 in HR via regulation of E2F1-dependent BRCA1 expression (Fig. 7C).

HR-mediated DSBs repair is a key mechanism required for tumor suppression by maintaining genomic stability. The finding that RNF126 promotes HR via regulating BRCA1 expression (Fig. 1, 2) suggested a potential mechanism regulating *BRCA1* gene expression. The question as to whether RNF126 could function as a tumor suppressor by functioning in BRCA1-mediated HR and whether a subtype breast/ovarian cancer display a lower BRCA1 expression due to RNF126 deficiency need to be determined in clinical settings.

We identified a new function of RNF126 in transcriptional regulation of BRCA1 gene by acting as a regulator of E2F1 (Fig. 3–6). The tandem array of serines at the very C terminus of RNF126 is homologous to similar serine stretches found in transcriptional activators present at RNAPII-dependent promoters ([31]). Thus, the existence of serine array in RNF126 suggested that RNF126 may act as a transcriptional activator by the corporation with other transcriptional factors. Our results strongly support this hypothesis by demonstrating that RNF126 facilitates the transacriptional activity of E2F1 on BRCA1 promoter via a direct interaction with E2F1. Nevertheless, the nature of interaction of RNF126 and E2F1 is not clear. Given that E2F1 is important for the chromatin remodeling ([46]), it would be very interesting to determine if chromatin remodeling is regulated by RNF126 via interaction with E2F1, which could be a potential mechanism promoting BRCA1 expression. Of note, E3 ligase activity of RNF126 appears to be not important for RNF126-mediated BRCA1/HR regulation (Fig. 2I, Fig. 6E). However, we cannot exclude the possibility that E3 ligase activity is required for ubiquitination of multiple proteins which oppositely affects BRCA1 expression/HR. Thus, no obvious effect on HR/BRCA1 expression was observed in cells expressing E3 ligase mutant.

Consistent with its role in HR, RNF126 contributes to the resistance to IR and PARP inhibitor (Fig. 7). Given the fact that cisplatin is the initial treatment for 90% ovarian cancer

patients, and HR is important for repairing DNA damage caused by platinum drugs this result could suggest that RNF126 deficiency could be a predicative marker for the better outcome of cancer therapy. Interestingly, The Cancer Genome Atlas (TCGA) data suggest that homozygous and/or heterozygous deletion of RNF126 gene is observed in a broad spectrum of human tumors ([47], [48]). For instance, the large majority of ovarian serous cystadenocarcinoma which accounts for the largest proportion of malignant ovarian tumours display either homozygous or heterozygous deletion of the RNF126 gene (Fig. S3A)([47], [48]). Intriguingly, tumors with decreased expression of RNF126 correlated with improved disease-free ($p<000001$) (Fig. S3B) and overall survival ($p<0.0001$) (Fig. S3C)([47], [48]). In addition to deletion and low mRNA expression the mutation of RNF126 are also observed in a variety of human tumors, indicating the RNF126 inactivation may broadly exist in human tumor ([47], [48]). Therefore, it could be possible that RNF126 are inactive in cancer patients due to deletion or mutation or low mRNA expression. Overall, this data highlights the clinical relevance of RNF126 and indicates that RNF126 may serve as a relevant therapeutic target for novel therapies for serous ovarian cancer patients.

In addition to BRCA1, other E2F1 target genes such as apoptosis genes are also regulated by RNF126 (Fig. S2). It is conceivable that RNF126 deficiency on one hand impaired DSBs repair pathway and on the other hand impairs apoptosis. Thus, the alteration in the expression of apoptosis gene could counteract to the cell death caused by the defect of DNA repair. In addition, the proteins regulating anti-apoptosis could also be a target of RNF126. For instance, EGFR1, a protein required for the anti-apoptosis protein expression, can be targeted for degradation by RNF126 ([30]). The further work should focus on how to identify the population which dominated control by RNF126 mediated DNA repair pathway instead of its effect on apoptosis activity or vice versa. This information would be help to identify the special group with a deregulated RNF126 expression to be specifically targeted by radiation therapy and PARP inhibitor related therapy.

In addition to pRb family members which have long been considered to be the major regulators of E2F1, TOPBP1 and MCPH1 are recently found to be a new class of regulators for E2F1. However, these two proteins have opposite role in apoptosis ([28], [42]). Our studies apparently provide the strong evidence suggesting that RNF126 is a novel factor regulating E2F1 dependent transcriptional activities. Additional work will be required to determine the composition of different RNF126/E2F1-containing complexes and their unique functions in E2F1-mediated transcriptional regulation.

In summary, we have identified a novel function of RNF126 in HR. In addition, our study links RNF126 to BRCA1, one of the most important tumor suppressor genes in breast and ovarian cancers, by demonstrating that RNF126 promotes E2F1-dependent BRCA1 expression via a direct interaction with E2F1 (Fig. 7C). Our study offers novel insights into our current understanding of biological function of RNF126 and provides a potential target for cancer therapy.

MATERIALS AND METHODS

Cell lines, infections and transfections

MCF-7, MDA-MB-231, 293T and U2OS cells were cultured in Dulbecco modified Eagle medium (DMEM, Invitrogen) supplemented with 10% bovine calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C, 5% CO_2. The short hairpins (shRNA) of RNF126 were purchased from Sigma-Aldrich. Targeting region of RNF126 shRNA126 ([1]) and shRNA ([3]) are TGCATGGTTTGTGGCGGAAGA and GCAACGAGAACGCCACATGGT, respectively. The control siRNA and RNF126 siRNA were obtained directly from IDT (Integrated DNA Technologies). RNF126 siRNA targets the untranslated region of RNF126 messenger RNA, and target region is CTCTGTCTAACCTCACCCTCTAAAC. The full-length wild-type (WT) RNF126 and RING domain mutated (C229A/C232A) RNF126 were described previously([29]). HA-E2F1 and GST-E2F1 were kindly offered by Dr. Matthew Summers (Cleveland Clinic, Cleveland, OH) and Dr. Weei-Chin Lin (Baylor College of Medicine, Houston, Texas), respectively. The His-E2F1 was a gift from Maria Alvarado-Kristensson (Skåne University Hospital, SE-20502 Malmö, Sweden). The adenoviral I-SceI endonuclease Ad-SceI-NG expression vector was obtained from Kristoffer Valerie (78). The BRCA1 promoter reporter was a gift from David G. Johnson (University of Texas MD Anderson Cancer Center). U2OS cells with the chromosomal integration of HR substrate DR-GFP was provided by Zhongsheng You (Washington University School of Medicine). All the DNA plasmid transfections were performed using Lipofectamine 2000 according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA).

To generate Flag-RNF126 construct, the full length RNF-126 fragment was amplified by PCR from pGEX-6P-1 vector expressing RNF126 ([29]), and subcloned into vector pCMV-3Tag-1A (3xFlag-tagged) using the *EcoRI* and *XhoI, and the* primer sets P1 (5′-AAAAGAATTCGCCGAGGCGTCGCCGCATCC-3′) and P2 (5′-AAAACTCGAGTCACGAGTTGCTTGTGGCGTTCTCG-3′); To generate GST-RNF126 construct, the full length RNF-126 was subcloned into vector pCMV-3Tag-1A (GST-tagged) using the *BamHI* and *XhoI, and the* primer sets P1 (5′-AAAAGGATCCGCCGAGGCGTCGCCGCATCC-3′) and P2 (5′-AAAACTCGAG TCACGAGTTGCTTGTGGCGTTCTCG-3′).

To generate a series of deletional mutant RNF126-B,C,D,E,F, QuikChange® II Site-Directed Mutagenesis Kit (Cat # 200523, Stratagene) was used. To generate a series of deletional mutant RNF126-Δa, b,c,d,e,f, QuikChange Lightning Site-Directed Mutagenesis Kit (Cat # 210518, Stratagene) was used. The primers used to generate RNF126 mutation will be available upon request.

To generate cells line with E2F1 stable knockdown, MCF-7 cells were transfected with empty BS/U6 RNAi vector or with BS/U6-E2F1 RNAi vector together with pcDNA-3, which encodes a neomycin-selectable marker. BS/U6 RNAi vector and BS/U6-E2F1 RNAi vector are gifts from Cress WD (The H. Lee Moffitt Cancer Center and Research Institute, Tampa, Florida). The details was described in our previous publication ([37]).

Homologous recombination assays

HR was measured in MCF7-pDR-GFP cells by flow cytometry as described previously ([39]).

Cell Cycle Analysis

Cell Cycle Analysis was conducted as we described previously ([39]).

Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

qRT-PCR was conducted as our previous publication([39]). BRCA1 forward primer: GAA ACCGTGCCAAAAGACTTC. BRCA1 reverse primer: TCACAAGCAGCCAATTCAATGT. p73 forward primer and reverse primer are described in a previous publication ([42]).

Immunofluorescence analysis

Immunofluorescence assays were performed as published ([39]).

Immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblotting was conducted as described previously ([37], [49]).

GST fusion proteins and pull-down assay

GST pull-down assay was performed according to our previous publication ([39]).

Dual luciferase assays

Dual luciferase assays was conducted as we described previously ([50]).

Chromatin immunoprecipitation (ChIP) assay

ChIP assay were performed as previously described ([42]).

Clonogenic survival assays

Clonogenic survival assays were performed according to our previous publications ([37], [39]).

Antibodies

For WB, the following conditions are used. Anti-RNF126 (Clone C-1; 1:200; Santa Cruz Technology); Anti-RNF126 (Clone 1B5; 1:1000; Abcam); Anti-BRCA1 (Clone D-9; 1:200; Santa Cruz Technology); Anti-RAD51 (Clone H92; 1:200; Santa Cruz Technology); Anti-BRCA2 (clone 5.23; 1:500; EMD Millipore); Anti-RAD52 (Clone 5H9; 1:200; GeneTex); Anti-RPA1 (Clone NA13; 1:100; Calbiochem/EMD Millipore) and anti-RPA2 (Clone NA18; 1:100; Calbiochem/EMD Millipore); Anti-53BP1(Clone 1B9; 1:1000; Novus biologicals); Anti-FLAG M2 (Clone M2; 1:1000; Sigma-Aldrich); Anti-E2F1(Clone KH95; 1:200; Santa Cruz Technology); Anti-HA (Clone 16B12; 1:1000; Covance); Anti-His (Clone H-15; 1:200; Santa Cruz Technology); Anti-GST (Clone B-14; 1:200; Santa Cruz Technology); Anti-Filamin (Clone FLMN01; 1:1000; Pierce); Anti-β-Actin (Clone AC-74; 1:10000; Sigma-Aldrich). Secondary antibodies were goat-anti-mouse IgG–HRP conjugated and goat-anti-rabbit IgG–HRP conjugated both at 1:5 000 dilutions for immune blotting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Sonoda E, Sasaki MS, Buerstedde JM, Bezzubova O, Shinohara A, Ogawa H, et al. Rad51-deficient vertebrate cells accumulate chromosomal breaks prior to cell death. The EMBO journal. 1998; 17(2):598–608. Epub 1998/02/28. [PubMed: 9430650]
2. Andreassen PR, Ho GP, D'Andrea AD. DNA damage responses and their many interactions with the replication fork. Carcinogenesis. 2006; 27(5):883–92. Epub 2006/02/24. [PubMed: 16490739]
3. Evers B, Jonkers J. Mouse models of BRCA1 and BRCA2 deficiency: past lessons, current understanding and future prospects. Oncogene. 2006; 25(43):5885–97. Epub 2006/09/26. [PubMed: 16998503]
4. Zhang J. The role of BRCA1 in homologous recombination repair in response to replication stress: significance in tumorigenesis and cancer therapy. Cell & bioscience. 2013; 3(1):11. Epub 2013/02/08. [PubMed: 23388117]
5. Moynahan ME, Jasin M. Mitotic homologous recombination maintains genomic stability and suppresses tumorigenesis. Nature reviews Molecular cell biology. 2010; 11(3):196–207. Epub 2010/02/24. [PubMed: 20177395]
6. Helleday T. Homologous recombination in cancer development, treatment and development of drug resistance. Carcinogenesis. 2010; 31(6):955–60. Epub 2010/03/31. [PubMed: 20351092]
7. Farmer H, McCabe N, Lord CJ, Tutt AN, Johnson DA, Richardson TB, et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. Nature. 2005; 434(7035):917–21. Epub 2005/04/15. [PubMed: 15829967]
8. Thompson ME, Jensen RA, Obermiller PS, Page DL, Holt JT. Decreased expression of BRCA1 accelerates growth and is often present during sporadic breast cancer progression. Nature genetics. 1995; 9(4):444–50. Epub 1995/04/01. [PubMed: 7795653]
9. Russell PA, Pharoah PD, De Foy K, Ramus SJ, Symmonds I, Wilson A, et al. Frequent loss of BRCA1 mRNA and protein expression in sporadic ovarian cancers. International journal of cancer Journal international du cancer. 2000; 87(3):317–21. Epub 2000/07/18. [PubMed: 10897034]
10. Zheng W, Luo F, Lu JJ, Baltayan A, Press MF, Zhang ZF, et al. Reduction of BRCA1 expression in sporadic ovarian cancer. Gynecologic oncology. 2000; 76(3):294–300. Epub 2000/02/24. [PubMed: 10684699]
11. Turner NC, Reis-Filho JS, Russell AM, Springall RJ, Ryder K, Steele D, et al. BRCA1 dysfunction in sporadic basal-like breast cancer. Oncogene. 2007; 26(14):2126–32. Epub 2006/10/04. [PubMed: 17016441]
12. Wilson CA, Ramos L, Villasenor MR, Anders KH, Press MF, Clarke K, et al. Localization of human BRCA1 and its loss in high-grade, non-inherited breast carcinomas. Nature genetics. 1999; 21(2):236–40. Epub 1999/02/13. [PubMed: 9988281]
13. Mueller CR, Roskelley CD. Regulation of BRCA1 expression and its relationship to sporadic breast cancer. Breast cancer research : BCR. 2003; 5(1):45–52. Epub 2003/02/01. [PubMed: 12559046]

14. Bindra RS, Gibson SL, Meng A, Westermark U, Jasin M, Pierce AJ, et al. Hypoxia-induced down-regulation of BRCA1 expression by E2Fs. Cancer research. 2005; 65(24):11597–604. Epub 2005/12/17. [PubMed: 16357170]
15. De Siervi A, De Luca P, Byun JS, Di LJ, Fufa T, Haggerty CM, et al. Transcriptional autoregulation by BRCA1. Cancer research. 2010; 70(2):532–42. Epub 2010/01/14. [PubMed: 20068145]
16. Rice JC, Massey-Brown KS, Futscher BW. Aberrant methylation of the BRCA1 CpG island promoter is associated with decreased BRCA1 mRNA in sporadic breast cancer cells. Oncogene. 1998; 17(14):1807–12. Epub 1998/10/20. [PubMed: 9778046]
17. Johnson DG, Degregori J. Putting the Oncogenic and Tumor Suppressive Activities of E2F into Context. Current molecular medicine. 2006; 6(7):731–8. Epub 2006/11/15. [PubMed: 17100599]
18. Biswas AK, Johnson DG. Transcriptional and nontranscriptional functions of E2F1 in response to DNA damage. Cancer research. 2012; 72(1):13–7. Epub 2011/12/20. [PubMed: 22180494]
19. Chen J, Zhu F, Weaks RL, Biswas AK, Guo R, Li Y, et al. E2F1 promotes the recruitment of DNA repair factors to sites of DNA double-strand breaks. Cell Cycle. 2011; 10(8) Epub 2011/03/31.
20. Biswas AK, Mitchell DL, Johnson DG. E2F1 Responds to Ultraviolet Radiation by Directly Stimulating DNA Repair and Suppressing Carcinogenesis. Cancer research. 2014 Epub 2014/04/18.
21. Putzer BM, Engelmann D. E2F1 apoptosis counterattacked: evil strikes back. Trends in molecular medicine. 2013; 19(2):89–98. Epub 2012/12/12. [PubMed: 23219173]
22. Wang A, Schneider-Broussard R, Kumar AP, MacLeod MC, Johnson DG. Regulation of BRCA1 expression by the Rb-E2F pathway. The Journal of biological chemistry. 2000; 275(6):4532–6. Epub 2000/02/08. [PubMed: 10660629]
23. Iwanaga R, Komori H, Ohtani K. Differential regulation of expression of the mammalian DNA repair genes by growth stimulation. Oncogene. 2004; 23(53):8581–90. Epub 2004/10/07. [PubMed: 15467751]
24. Bindra RS, Glazer PM. Repression of RAD51 gene expression by E2F4/p130 complexes in hypoxia. Oncogene. 2007; 26(14):2048–57. Epub 2006/09/27. [PubMed: 17001309]
25. Stanelle J, Stiewe T, Theseling CC, Peter M, Putzer BM. Gene expression changes in response to E2F1 activation. Nucleic acids research. 2002; 30(8):1859–67. Epub 2002/04/09. [PubMed: 11937641]
26. Ren B, Cam H, Takahashi Y, Volkert T, Terragni J, Young RA, et al. E2F integrates cell cycle progression with DNA repair, replication, and G(2)/M checkpoints. Genes & development. 2002; 16(2):245–56. Epub 2002/01/19. [PubMed: 11799067]
27. Guo R, Chen J, Zhu F, Biswas AK, Berton TR, Mitchell DL, et al. E2F1 localizes to sites of UV-induced DNA damage to enhance nucleotide excision repair. The Journal of biological chemistry. 2010; 285(25):19308–15. Epub 2010/04/24. [PubMed: 20413589]
28. Liu K, Lin FT, Ruppert JM, Lin WC. Regulation of E2F1 by BRCT domain-containing protein TopBP1. Molecular and cellular biology. 2003; 23(9):3287–304. Epub 2003/04/17. [PubMed: 12697828]
29. Zhi X, Zhao D, Wang Z, Zhou Z, Wang C, Chen W, et al. E3 ubiquitin ligase RNF126 promotes cancer cell proliferation by targeting the tumor suppressor p21 for ubiquitin-mediated degradation. Cancer research. 2012 Epub 2012/10/03.
30. Smith CJ, Berry DM, McGlade CJ. The E3 ubiquitin ligases RNF126 and Rabring7 regulate endosomal sorting of the epidermal growth factor receptor. Journal of cell science. 2013; 126(Pt 6):1366–80. Epub 2013/02/19. [PubMed: 23418353]
31. Delker RK, Zhou Y, Strikoudis A, Stebbins CE, Papavasiliou FN. Solubility-based genetic screen identifies RING finger protein 126 as an E3 ligase for activation-induced cytidine deaminase. Proceedings of the National Academy of Sciences of the United States of America. 2013; 110(3): 1029–34. Epub 2013/01/02. [PubMed: 23277564]
32. Wang ZJ, Churchman M, Campbell IG, Xu WH, Yan ZY, McCluggage WG, et al. Allele loss and mutation screen at the Peutz-Jeghers (LKB1) locus (19p13.3) in sporadic ovarian tumours. British journal of cancer. 1999; 80(1–2):70–2. Epub 1999/07/02. [PubMed: 10389980]

33. Yanaihara N, Okamoto A, Matsufuji S. A commonly deleted region in ovarian cancer on chromosome 19p13.3, not including the OAZ1 gene. International journal of oncology. 2003; 23(3):567–75. Epub 2003/07/31. [PubMed: 12888889]
34. Amfo K, Neyns B, Teugels E, Lissens W, Bourgain C, De Sutter P, et al. Frequent deletion of chromosome 19 and a rare rearrangement of 19p13.3 involving the insulin receptor gene in human ovarian cancer. Oncogene. 1995; 11(2):351–8. Epub 1995/07/20. [PubMed: 7624149]
35. Yang TL, Su YR, Huang CS, Yu JC, Lo YL, Wu PE, et al. High-resolution 19p13.2-13.3 allelotyping of breast carcinomas demonstrates frequent loss of heterozygosity. Genes, chromosomes & cancer. 2004; 41(3):250–6. Epub 2004/08/31. [PubMed: 15334548]
36. Litman R, Peng M, Jin Z, Zhang F, Zhang J, Powell S, et al. BACH1 is critical for homologous recombination and appears to be the Fanconi anemia gene product FANCJ. Cancer cell. 2005; 8(3):255–65. Epub 2005/09/13. [PubMed: 16153896]
37. Shi W, Feng Z, Zhang J, Gonzalez-Suarez I, Vanderwaal RP, Wu X, et al. The role of RPA2 phosphorylation in homologous recombination in response to replication arrest. Carcinogenesis. 2010; 31(6):994–1002. Epub 2010/02/05. [PubMed: 20130019]
38. Chen H, Ma Z, Vanderwaal RP, Feng Z, Gonzalez-Suarez I, Wang S, et al. The mTOR inhibitor rapamycin suppresses DNA double-strand break repair. Radiation research. 2011; 175(2):214–24. Epub 2011/01/28. [PubMed: 21268715]
39. Zhang J, Ma Z, Treszezamsky A, Powell SN. MDC1 interacts with Rad51 and facilitates homologous recombination. Nature structural & molecular biology. 2005; 12(10):902–9. Epub 2005/09/28.
40. Pierce AJ, Johnson RD, Thompson LH, Jasin M. XRCC3 promotes homology-directed repair of DNA damage in mammalian cells. Genes & development. 1999; 13(20):2633–8. Epub 1999/10/29. [PubMed: 10541549]
41. Scully R, Chen J, Plug A, Xiao Y, Weaver D, Feunteun J, et al. Association of BRCA1 with Rad51 in mitotic and meiotic cells. Cell. 1997; 88(2):265–75. [PubMed: 9008167]
42. Yang SZ, Lin FT, Lin WC. MCPH1/BRIT1 cooperates with E2F1 in the activation of checkpoint, DNA repair and apoptosis. EMBO reports. 2008; 9(9):907–15. Epub 2008/07/29. [PubMed: 18660752]
43. Chen J, Zhu F, Weaks RL, Biswas AK, Guo R, Li Y, et al. E2F1 promotes the recruitment of DNA repair factors to sites of DNA double-strand breaks. Cell Cycle. 2011; 10(8):1287–94. Epub 2011/04/23. [PubMed: 21512314]
44. Wang B, Liu K, Lin FT, Lin WC. A role for 14-3-3 tau in E2F1 stabilization and DNA damage-induced apoptosis. The Journal of biological chemistry. 2004; 279(52):54140–52. Epub 2004/10/21. [PubMed: 15494392]
45. Pediconi N, Ianari A, Costanzo A, Belloni L, Gallo R, Cimino L, et al. Differential regulation of E2F1 apoptotic target genes in response to DNA damage. Nature cell biology. 2003; 5(6):552–8. Epub 2003/05/27. [PubMed: 12766778]
46. Guo R, Chen J, Mitchell DL, Johnson DG. GCN5 and E2F1 stimulate nucleotide excision repair by promoting H3K9 acetylation at sites of damage. Nucleic acids research. 2011; 39(4):1390–7. Epub 2010/10/26. [PubMed: 20972224]
47. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer discovery. 2012; 2(5):401–4. Epub 2012/05/17. [PubMed: 22588877]
48. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Science signaling. 2013; 6(269):pl1. Epub 2013/04/04. [PubMed: 23550210]
49. Feng Z, Zhang J. A dual role of BRCA1 in two distinct homologous recombination mediated repair in response to replication arrest. Nucleic acids research. 2012; 40(2):726–38. Epub 2011/09/29. [PubMed: 21954437]
50. Zhuang J, Zhang J, Willers H, Wang H, Chung JH, van Gent DC, et al. Checkpoint kinase 2-mediated phosphorylation of BRCA1 regulates the fidelity of nonhomologous end-joining. Cancer research. 2006; 66(3):1401–8. Epub 2006/02/03. [PubMed: 16452195]

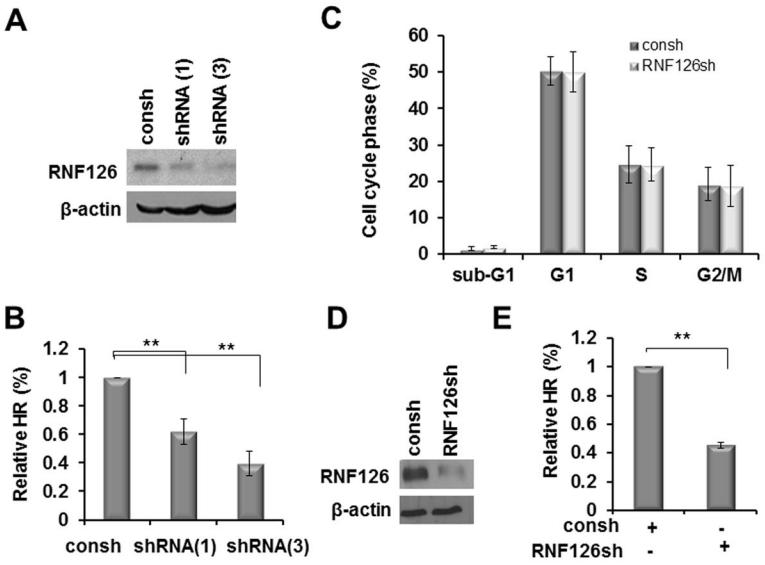

Fig. 1. Impairment of I-Sce-I induced HR in RNF126-deficient cells

(**A**) RNF126 knockdown via shRNA targeting RNF126 (RNF126sh) in MCF7 cells. (**B**) RNF126-deficient cells show significant reductions in HR mediated by short track gene conversion. HR induced by I-Sce-I was measured by dual-color flow cytometric detection of GFP-positive cells. The relative HR frequencies in cells depleted of RNF126 are shown vs. cells with intact RNF126 expression. (**C**) Cell cycle distribution of cells with or without RNF126 depletion. Subconfluent MCF-7 cells with control or RNF126sh infection were collected and analyzed for DNA content by propidium iodide staining and flow cytometry. (**D**) RNF126 knockdown via #3 shRNA in U2OS cells. (**E**) RNF126-deficient cells show significant reductions in HR mediated by short track gene conversion in U2OS cells. Results are means from three independent experiments, with standard errors in Figs. 1 B, C, E (T test, ** p <0.01).

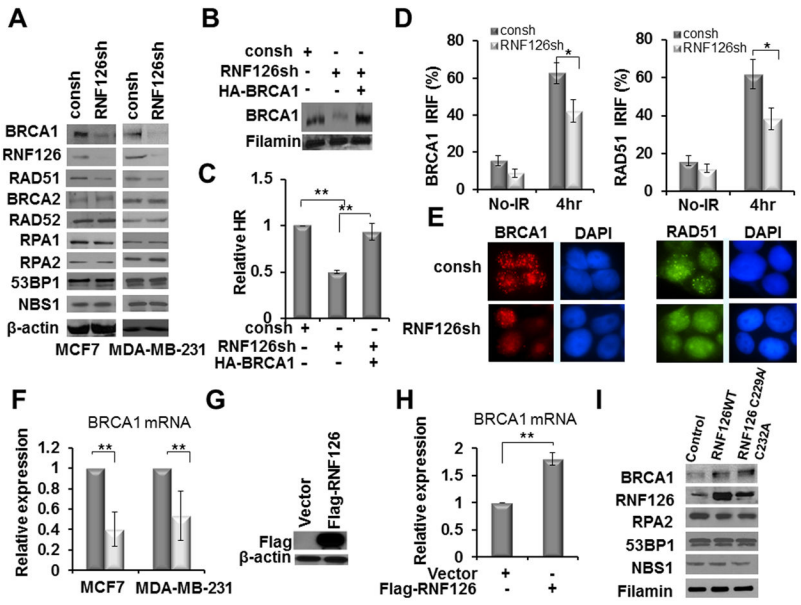

Fig. 2. RNF126 promotes BRCA1 mRNA expression

(**A**) The levels of proteins required for HR in MCF7 and MDA-MB-231 cells with or without RNF126 shRNA infection. (**B**) BRCA1 expression in the indicated cells with RNF126 down regulation and subsequent BRCA1 overexpression. (**C**) Overexpression BRCA1 restored the HR deficiency due to RNF126 depletion. MCF7 DR-GFP cells with or without RNF126 knockdown were transfected with or without HA-BRCA1 and then HR was detected as described in Figure 1.B. P-values were calculated by T-test. Error bars represent the SD of three independent experiments. (**D**) Exponentially growing MCF7 cells with or without RNF126sh infection were untreated or irradiated (8 Gy) and then were fixed 4 hr later. The IR-induced foci (IRIF) of BRCA1 and RAD51 were detected by immunostaining. The Y axis represents the percentage or cells with positive foci of BRCA1 (left panel) or RAD51 (right panel). Cells were scored positive when 10 nuclear foci were visible. (**E**) The IR-induced foci (IRIF) of BRCA1 and RAD51 were detected by immunostaining. Representative foci of BRCA1 (left panel) and RAD51 (right panel) are indicated. (**F**) BRCA1 mRNA was down-regulated when the RNF126 is depleted in MCF7 and MDA-MB-231 cells. BRCA1 mRNA was amplified by RT-PCR and the results are presented as a relative value compared to control cells infected with a control shRNA (consh). Data were collected from three independent experiments, with standard errors (T-test, ** p<0.01). (**G**) Exogenous Flag-RNF126 expression was detected by immunoblotting. (**H**) The levels of BRCA1 mRNA in MCF7 cells with or without exogenous Flag- RNF126 expression. (**I**) MCF7 cells were infected with RNF126-WT, control vector or ligase-deficient RNF126 (RNF126-C229A/C232A) retrovirus. Protein levels in cell lysates were measured by immunoblotting.

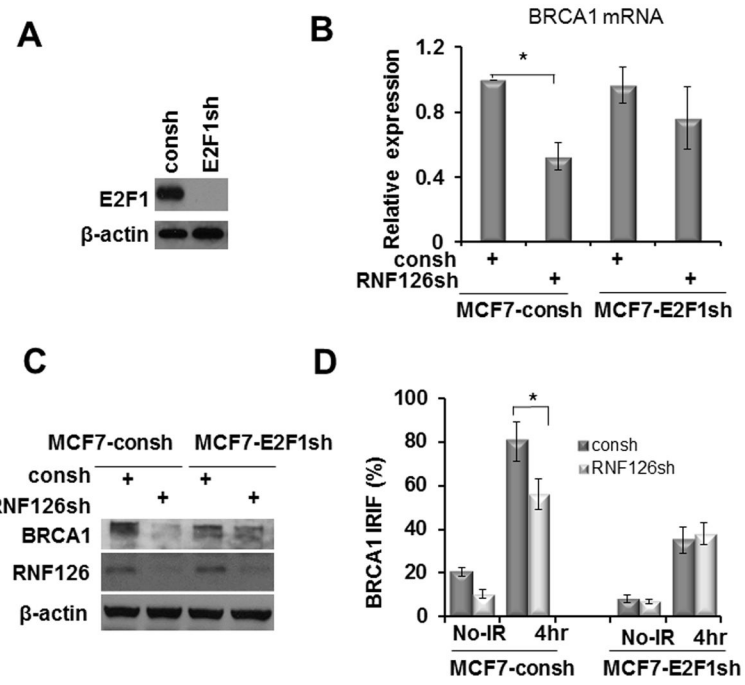

Fig. 3. The roles of RNF126 in promotion of BRCA1 mRNA expression/IRIF depend on E2F1

(**A**) Characterization of a G418-selected MCF7 cell single clone (MCF7-E2F1sh) with stable E2F1 knockdown. (**B**) BRCA1 mRNA decrease induced by RNF126 depletion requires E2F1. In brief, MCF7 cells with or without a stable depletion of E2F1 were further infected with RNF126sh or consh. BRCA1 mRNA levels were measured 48 hr later by RT-PCR, and the results are presented as a relative value compared to cells infected with consh. (**C**) BRCA1 protein decrease induced by RNF126 depletion requires E2F1. In brief, MCF7 cells with or without stable depletion of E2F1 were further infected with RNF126sh or consh. BRCA1 protein levels were measured 48 hr later by immunoblotting. (**D**) The promotion of IR-induced recruitment of BRCA1 requires E2F1. Exponentially growing MCF7-consh or MCF7-E2F1sh cells with or without RNF126sh infection were irradiated (8 Gy) and then were fixed 4 hr later. The Y axis represents the percentage of cells with positive foci of BRCA1. Results are means from three independent experiments, with standard errors shown (T-test, *p<0.05).

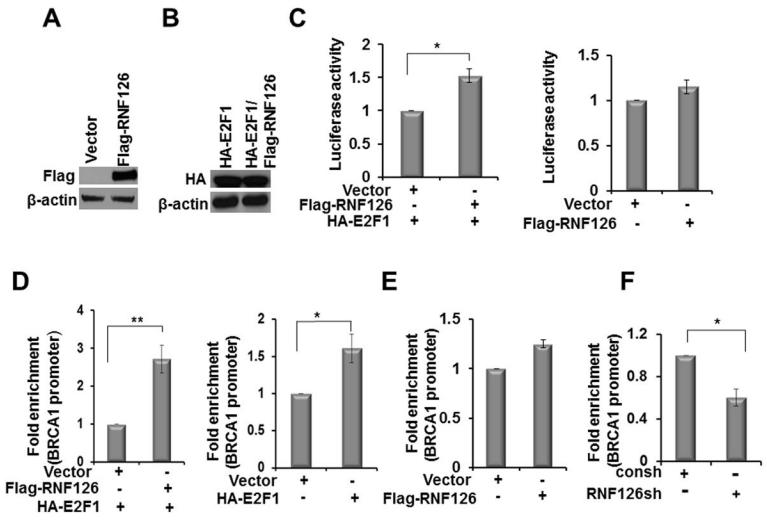

Fig. 4. RNF126 promotes transactivation activity of E2F1 on BRCA1 promoter

(**A, B**) Flag-RNF126 and HA-E2F1 expression detected by immunoblotting in 293T cells. (**C**) RNF126 overexpression enhances E2F1-mediated transactivation on BRCA1 promoter in cells co-overexpressing E2F1 (left panel). 293T cells were transfected with HA-E2F1 or Flag-RNF126 or both, then cells were transfected with luciferase BRCA1 promoter reporter or promoterless control vector with Renilla luciferase plasmid pRL-SV40. pRL-SV40 was used as internal control plasmid. Luciferase activities were assayed using the dual luciferase assay system 24hr after transfection. Luciferase activities were presented relative to the levels of Renilla luciferase activity. Each sample was tested in triplicate. The results are the average of three independent experiments, with standard errors shown (T-test, * p<0.05). No substantial induction of luciferase activity was observed when cells overexpressed RNF126 alone in 293Tcells lines (right panel). (**D**) The occupation of E2F1 on BRCA1 promoter in the presence (left panel) and absence of RNF126 overexpression (right panel). Cells were co-transfected with HA-E2F1 and control vector or HA-E2F1 and Flag-RNF126. ChIP assay was performed in 293T cells using HA antibody (left panel) or E2F1 antibody (right panel) against E2F1. Enrichment of E2F1 for the BRCA1 promoter was assessed by quantitative PCR. Fold enrichment represents the enrichment of E2F1 proteins on BRCA1 promoter. Data points represent an average of three independent repeated experiments with standard errors shown (T-test, * p<0.05;** p <0.01). (**E**) No substantial recruitment of RNF126 on BRCA1 promoter was observed by Chip assay. (**F**) RNF126 depletion results in the reduced recruitment of E2F1 on BRCA1 promoter (* p<0.05).

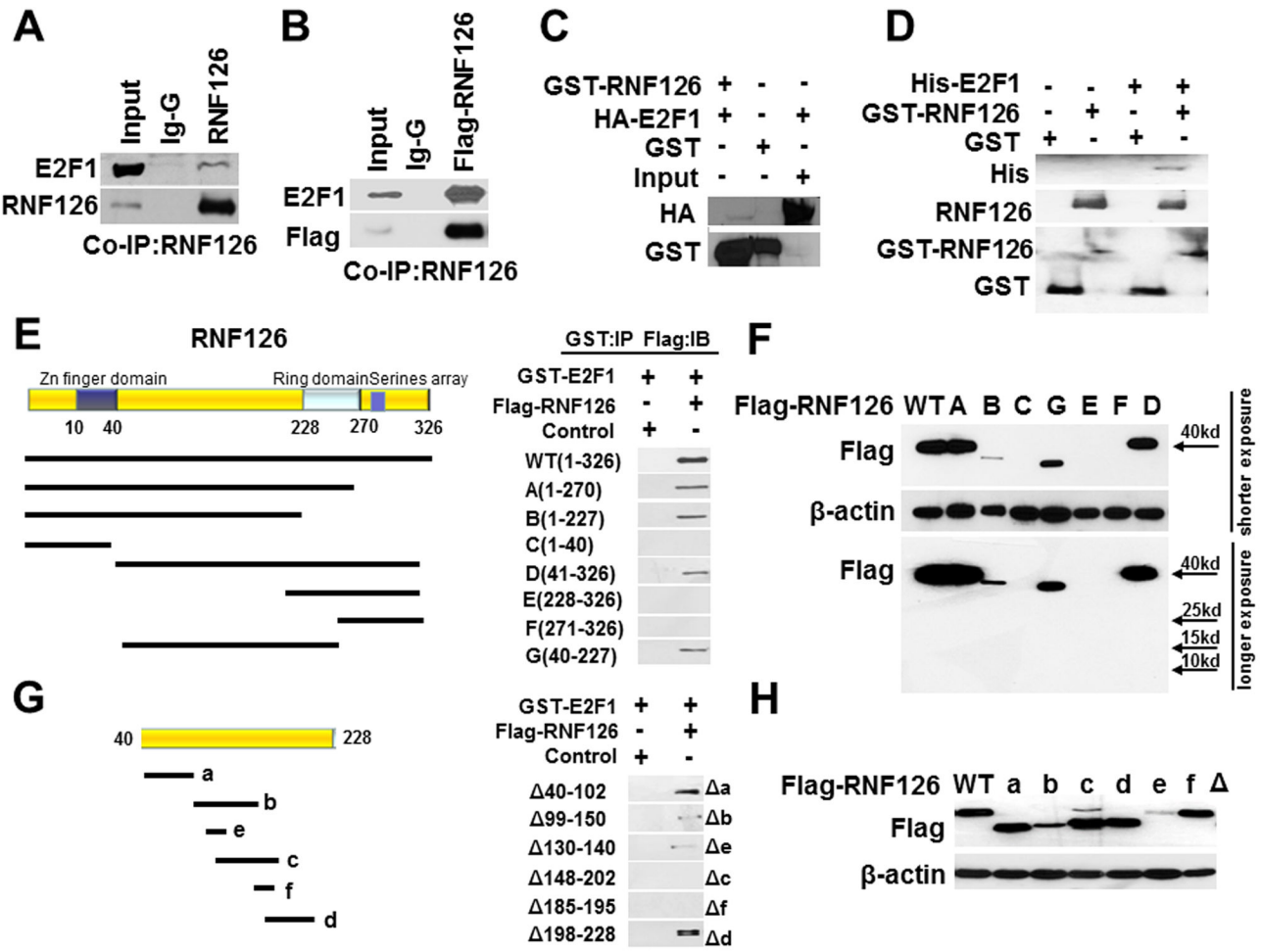

Fig. 5. RNF126 interacts with E2F1

(**A**) Endogenous RNF126 forms a complex with endogenous E2F1. MCF7 cells were immunoprecipated by antibody against E2F1 (upper panel) and then probed with anti-E2F1 (lower panel). IgG served as a negative control. (**B**) Flag-RNF126 is associated with the E2F1. MCF7 cells were transfected with Flag-RNF126 or control. Exogenous Flag-RNF126 was immunoprecipitated with Flag-specific antibody. The membrane was probed with anti E2F1 antibody (upper panel) and then probed with anti-FLAG antibody (lower panel). (**C**) Glutathione S-transferase (GST) -RNF126 is associated with HA-E2F1 *in vivo*. 293T cells were transfected with HA-E2F1 or control. GST-RNF126 were generated from bacterial and incubated with HA-E2F1 lysates. The immunoprecipitates were analyzed by immunoblotting with an antibody against HA (upper panel) and a subsequent antibody against GST (lower panel). (**D**) RNF126 directly binds to E2F1. GST-RNF126 were incubated with purified His-E2F1 prepared from bacteria. The immunoprecipitates were analyzed by immunoblotting with an antibody against His (upper panel) and a subsequent antibody against RNF126 (middle panel) and GST (lower panel). Lanes 1, 2 and 3 are input of GST lysate, GST-RNF126 lysates, and HA-E2F1 lysate, as controls. (**E**) The region from 40-227aa in RNF126 is important for either the association between RNF126 with E2F1 or the degradation of RNF126. The left panel is the schematic map for a series deletional

mutant of Flag-RNF126. The right panel shows the association of Flag-RNF126 and GST-E2F1. In brief, GST-E2F1 were generated from bacterial and incubated with Flag-RNF126 lysates. The immunoprecipitates were analyzed by immunoblotting with an antibody against Flag. (**F**) The expression of Flag-RNF126-WT and a series deletional Flag- RNF126 mutant (upper panel). (**G**) The schematic map for RNF126 mutant with the region of 40-227aa in RNF126 (left panel). RNF126-Δf mutant lacking the 11 aa of RNF126 from 185-195 and RNF126- Δc mutant lacking the region from 148-202aa impaired the association with E2F1 (right panel). (**H**) The expression of Flag-RNF126-WT and a series deletional mutant as indicated.

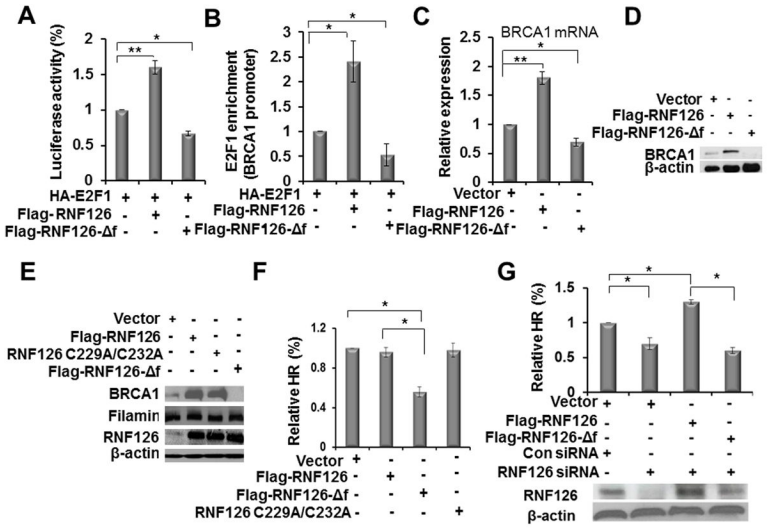

Fig. 6. Expression of RNF126-Δf inhibits BRCA1 expression and HR by interfering the binding of E2F1 to BRCA1 promoter

(**A**) RNF126 -Δf expression suppresses E2F1-mediated transactivation on BRCA1 promoters. E2F1 transactivation activity was measured with a BRCA1 promoter-luciferase activity in MCF7 cells as described in Fig. 4C. Results are means from three independent experiments, with standard errors (T test, * p <0.05; ** p <0.01). (**B**) Enrichment of E2F1 for the BRCA1 promoter was assessed by quantitative PCR. 293T cells with indicated protein expression were subjected to ChIP assay using a mouse HA antibody, or control normal rabbit IgG. Input DNA and protein-bound DNA fragments were amplified with BRCA1 primer pairs. Results are means from three independent experiments, with standard errors (T test, * p <0.05; ** p <0.01). (**C**) RNF126 -Δf expression suppress BRCA1 mRNA expression in 293 T cells. The RT-PCR was performed as described in Fig. 2F. (**D**) RNF126 -Δf expression suppresses BRCA1 protein expression in MCF7 cells. (**E**) BRCA1 expression in the cells with RNF126-WT or RNF126 -Δf or RNF126-C229A/C232A overexpression. (**F**) The relative HR frequencies in cells expressing RNF126 -Δf are shown vs. cells expressing RNF126-WT or RNF126-C229A/C232A. HR was performed as described in Fig. 1. Results are means from three independent experiments, with standard errors (T-test,*p<0.05). (**G**) Flag-RNF126-Δf expression failed to restore the impaired HR due to RNF126 depletion. In brief, silencing of endogenous RNF126 was accomplished by a siRNA targeting RNF126 3′ untranslated region of messenger RNA. Then the exogenous RNF126 was introduced into cells. HR was determined as described in Fig. 1. Error bars show standard errors for the means from three independent experiments (T-test,*p<0.05). RNF126 expression was monitored by western blot.

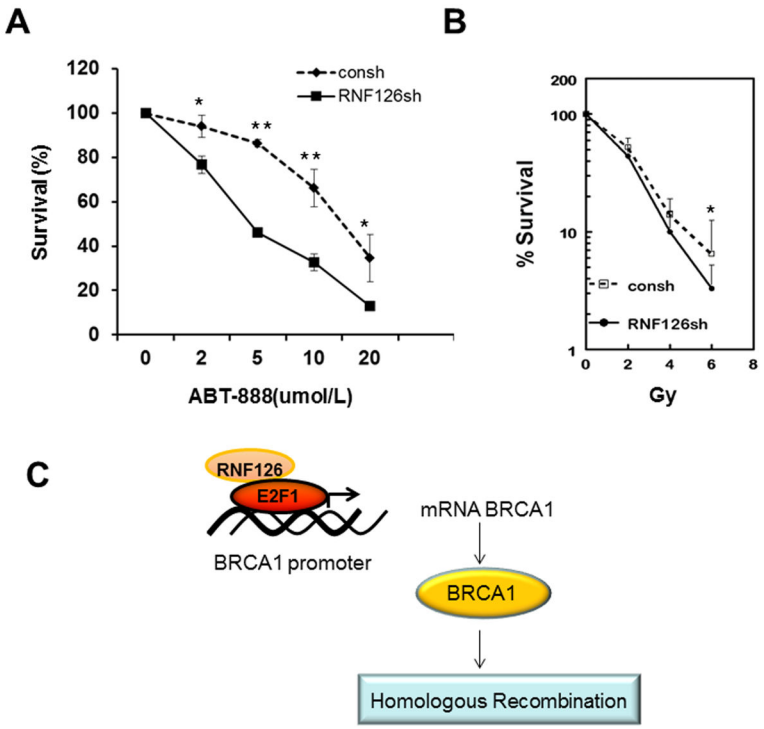

Fig. 7. RNF126 depletion leads to an enhanced sensitivity to PARP inhibitor and IR

(**A**) Clonogenic survival in MCF7 cells with or without depletion of RNF126 after ABT-888 treatment (T-test, *p <0.05, ** p <0.01). (**B**) Clonogenic survival following IR in MCF7 cells with or without depletion of RNF126. Survival experiments were repeated three times and the error bars in the graphs depicting the SE (T-test, *p <0.05). (**C**) Schematic hypothetical model for the mechanism by which RNF126 controls BRCA1 transcription and HR.