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# An E2-guided E3 Screen Identifies the RNF17-UBE2U Pair as Regulator of the Radiosensitivity, Immunodeficiency, Dysmorphic Features, and Learning Difficulties (RIDDLE) Syndrome Protein RNF168*

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**Protein ubiquitination has emerged as a pivotal regulatory reaction that promotes cellular responses to DNA damage. With a goal to delineate the DNA damage signal transduction cascade, we systematically analyzed the human E2 ubiquitin- and ubiquitin-like-conjugating enzymes for their ability to mobilize the DNA damage marker 53BP1 onto ionizing radiation-induced DNA double strand breaks. An RNAi-based screen identified UBE2U as a candidate regulator of chromatin responses at double strand breaks. Further mining of the UBE2U interactome uncovered its cognate E3 RNF17 as a novel factor that, via the radiosensitivity, immunodeficiency, dysmorphic features, and learning difficulties (RIDDLE) syndrome protein RNF168, enforces DNA damage responses. Our screen allowed us to uncover new players in the mammalian DNA damage response and highlights the instrumental roles of ubiquitin machineries in promoting cell responses to genotoxic stress.**

Radiosensitivity, immunodeficiency, dysmorphic features, and learning difficulties (RIDDLE)[4] syndrome is an immunodeficiency and radiosensitivity disorder that is manifested by, at cellular levels, hypersensitivity to ionizing radiation, cell cycle checkpoint abnormalities, and impaired end joining in the recombined switch regions (1). Mutations in the E3 ubiquitin ligase RNF168 were identified as the culprit responsible for the cellular deficits observed in RIDDLE cells (2) and suggested that RNF168 may play an important role in mounting cellular responses to DNA damage. Indeed, RNF168 has emerged as a core intermediate in the ubiquitin-driven DSB signal transduction cascade. By depositing ubiquitin adducts onto DSB-flanking chromatin domains, RNF168 assembles DSB response proteins, including 53BP1, BRCA1, and RAD18 (2–4), to drive DNA damage responses (DDRs). Accordingly, RNF168-null mice exhibit immunodeficiency, increased radiosensitivity, and impaired spermatogenesis, highlighting pivotal roles of the E3 ubiquitin ligase in DSB responses and organismal development (5).

Cell exposure to ionizing radiation (IR) triggers the phosphorylation of the histone variant H2AX (also known as γH2AX) (6), a histone mark that initiates a cascade of signaling events at the local chromatin to mobilize DSB response proteins into punctate structures commonly referred to as IR-induced foci (IRIF) (7). Among the expanding list of DSB response proteins that concentrate at the damaged chromatin, RNF168 occupancy at IRIF requires its ability to recognize ubiquitin adducts, a property attributable to its ubiquitin binding motifs that display preference for different ubiquitin species (2, 3, 8, 9). The ability of RNF168 to dock at DSBs also underlies its key role in propagating ubiquitin-mediated DSB signals (2, 3) as mutations that inactivate either its ubiquitin binding motifs or its E3 ubiquitin ligase activity impaired DNA damage-induced ubiquitylation and compromised retention of 53BP1 and BRCA1 at DSBs (2, 3, 10). Although the molecular basis for the RNF168-dependent recruitment of BRCA1 onto DSBs remains to be defined, RNF168 catalyzes H2A ubiquitination at $Lys^{13/15}$ (11), a biochemical reaction regulated by a conserved acidic patch distal to lysine residues on the H2A/H2B surface (12, 13), to effectively dock 53BP1 at DSBs (14, 15).

Intriguingly, the observations that DSBs are decorated by $Lys^{63}$-based ubiquitin species (16) and that depletion of either RNF168 or UBC13 suppressed $Lys^{63}$-ub at IRIF led to the original proposal that RNF168 may pair with the E2 ubiquitin-conjugating enzyme UBC13 to catalyze $Lys^{63}$-ub reactions (2, 3), although subsequent studies suggested otherwise (11, 17–21). Thus, given the dynamic nature of DSB-associated ubiquitylation (22), together with the complexity of ubiquitin topologies at play (19, 23–26), the identities and roles of each of the human ubiquitin machineries in shaping the ubiquitin landscape at DSBs remain to be defined. To this end, we used an RNA interference (RNAi)-based approach designed to target ubiquitin and ubiquitin-like E2 enzymes and have uncovered UBE2U as a new E2 enzyme important in driving DDRs. Further analysis of E3-E2 interaction networks led to the identification of the

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[4] The abbreviations used are: RIDDLE, radiosensitivity, immunodeficiency, dysmorphic features, and learning difficulties; IR, ionizing radiation; DSB, double strand break; DDR, DNA damage response; IRIF, IR-induced foci; ub, ubiquitin; UBC, ubiquitin-conjugating; PIWI, P-element-induced wimpy testis; piRNA, PIWI-interacting RNA; Gy, gray.

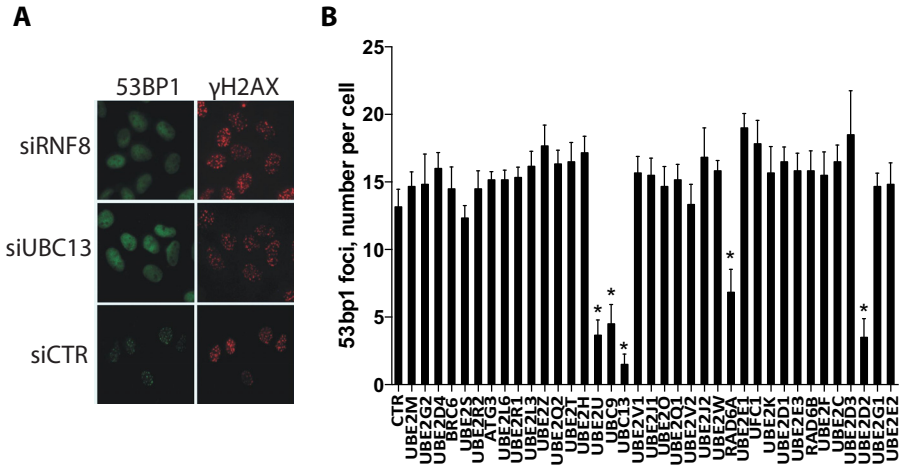


FIGURE 1. **RNAi screen identifies novel E2 players in the DDR.** *A* and *B*, HeLa cells transfected with non-targeting siRNAs (*siCTR*), pooled siRNAs designed to target each of the human E2s (see Table 1), or those that targeted RNF8 (*siRNF8*) were irradiated (10 Gy). 4 h post-IR treatment, cells were fixed, permeabilized, and immunostained with antibodies against 53BP1 and γH2AX. Representative images are shown for cells depleted of RNF8 or UBC13 (*siUBC13*; *A*), and the number of 53BP1 foci per cell was quantified and plotted (*B*). Data represent the mean of two independent experiments, and at least 200 nuclei were counted each time. *Error bars* represent S.D. *n.s.*, no significance; *, $p < 0.05$ *versus* control (*CTR*) cells.

RNF17-UBE2U pair as a novel module that promotes DSB signal transduction via the RIDDLE syndrome protein RNF168.

## Results

*RNAi Screen Identifies UBE2U as a Novel Regulator of 53BP1 Accumulation at DSBs*—Ubiquitylation entails a cascade of ATP-driven reactions that involve an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin ligase. By pairing with their E3 partners, E2 enzymes not only serve "ubiquitin carrier" functions but also determine the topology and length of ubiquitin chains, which in turn control the ultimate fate of its substrates (27). Among the dozens of human E2s, UBC13 encodes the only known $Lys^{63}$-ub promoting activity and is arguably the most studied ubiquitin-conjugating enzyme in the DDR network (28). Indeed, UBC13 plays established roles in propagating ubiquitin signals to assemble 53BP1 and BRCA1 at DSBs (29–32). Given the critically important roles of regulatory ubiquitylation in DSB signal transduction and repair processes (33), we were compelled to define the ubiquitylating activities in the DDR network. Because the human genome encodes only a few dozens of E2 ubiquitin- and ubiquitin-like-conjugating enzymes and given the availability of E2-E3 interacting protein networks (34–36), we reasoned that a primary screen tailored to target E2 enzymes may allow us to more efficiently isolate E2-E3 pairs as modules that regulate cellular responses to DNA damage.

We took advantage of the fact that IR treatment mobilizes DNA damage mediator protein 53BP1 into discernable focal structures, which would allow us to readily single out E2 candidates for further in-depth study. We used an RNAi approach for this purpose. We targeted each E2 candidate using a pool of four siRNAs and assessed IR-induced 53BP1 focus formation in U2OS cells 48 h following siRNA transfection (Fig. 1*A*). In support of the validity of the screen, a number of E2 enzymes with reported roles in the DSB signal transduction pathway were identified, including UBC13 (29–32), UBC9 (37, 38), RAD6A (20), and UBE2D2 (39) (Fig. 1*B*). Notably, our screen also led to the identification of UBE2U as a candidate regulator of mammalian DDRs.

*UBE2U Is Required for DSB Accumulation of DNA Damage Mediator Proteins*—UBE2U encodes a largely uncharacterized E2 ubiquitin-conjugating enzyme and is composed of a typical UBC domain at its N terminus (40) with an extension at its C terminus (Fig. 2*A*). Cancer-derived UBE2U mutations, many of which target its catalytic domain, have been catalogued (cBioPortal). We deconvoluted the pooled siRNAs and found that two independent UBE2U-targeting siRNAs suppressed expression of HA-tagged UBE2U as determined by Western blotting analysis (Fig. 2*B*). More importantly, resembling those depleted of UBC13, cells pretreated with the two UBE2U siRNAs also showed a substantial reduction of 53BP1 IRIF (Fig. 2*C*). Our attempt to detect endogenous UBE2U was not successful, although our rabbit polyclonal anti-UBE2U antibodies that were raised against GST-UBE2U fusion proteins as well as those from a commercial source (Novus catalogue number H00148581-B01) specifically recognized overexpressed UBE2U (Fig. 2*B*).

Given the positive roles of UBE2U in supporting IR-induced 53BP1 focus formation, we tested whether UBE2U might regulate the DSB accumulation of other DDR proteins, including MDC1, RNF8, RNF168, and BRCA1. Interestingly, although UBE2U depletion compromised BRCA1 and RNF168 IRIF formation (Fig. 2, *D* and *F*), knockdown of UBE2U had no measurable effect on the ability of MDC1 or RNF8 to relocalize to IR-induced DSBs (Fig. 2*E*), suggesting that UBE2U operates in the canonical H2AX-dependent DSB signal transduction pathway and that it may regulate chromatin responses at DSBs at the RNF168 level (Fig. 2*G*).

To explore the role of UBE2U in DNA damage responses, we further examined its requirement in checkpoint control following cell exposure to IR. We found that UBE2U-depleted cells, much like those depleted of UBC13, failed to properly arrest at the $G_2$/M checkpoint following IR challenge (Fig. 3, *A* and *B*). Given our observations that UBE2U may regulate DSB

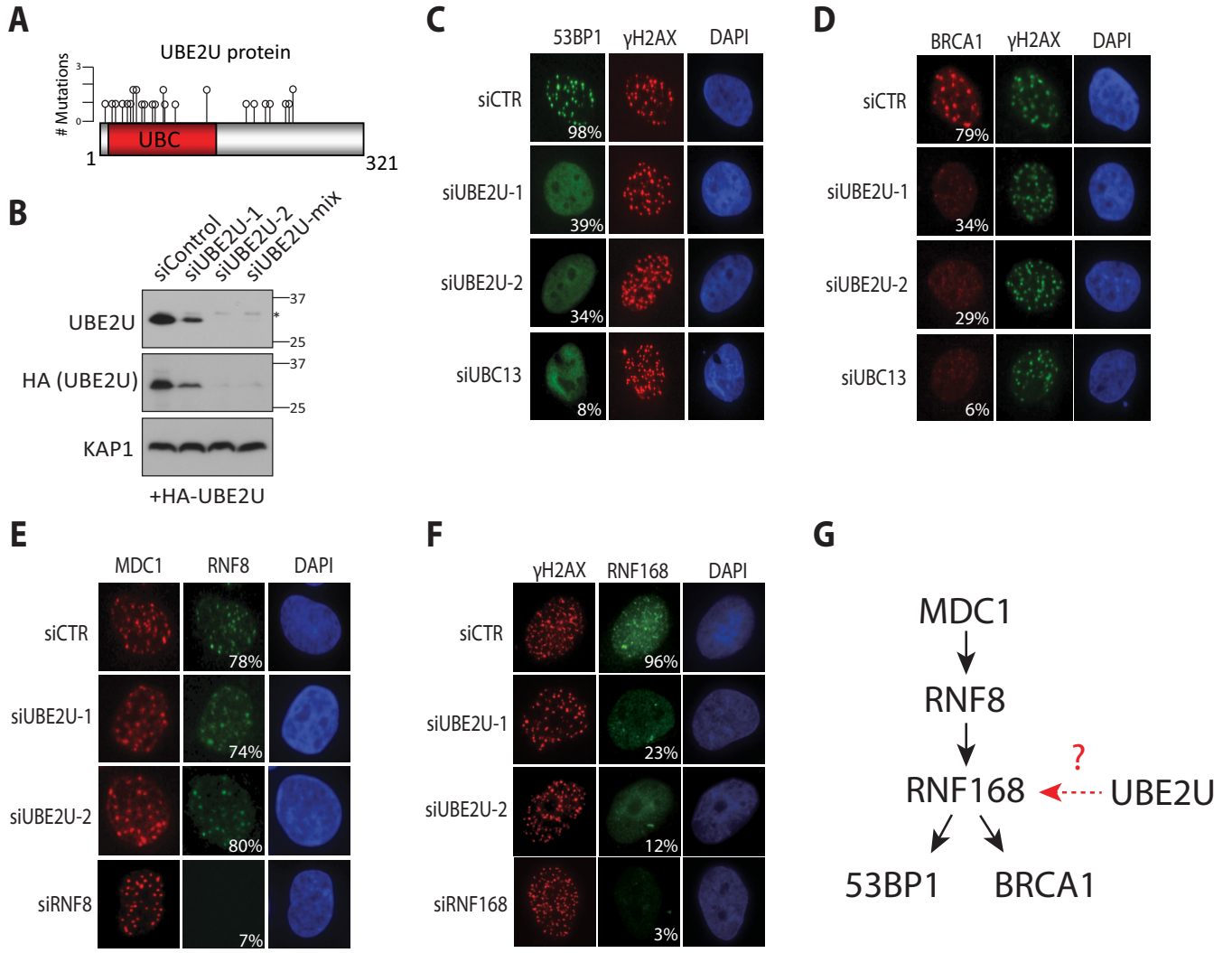


FIGURE 2. **UBE2U is a novel factor in DNA damage responses.** *A*, schematic illustration of UBE2U protein domain organization and cancer-associated mutations on the UBE2U coding sequence (adapted from cBioPortal). *B*, Western blotting analysis of RNAi-mediated UBE2U silencing. Cells were transfected with control siRNAs (*siCTR*) or UBE2U-specific siRNAs either separately (*siUBE2U-1* and *siUBE2U-2*) or as a pool (*siUBE2U-mix*) together with an expression construct that encodes HA-UBE2U. 48 h post-transfection, cells were lysed, and proteins were subjected to SDS-PAGE and Western blotting experiments using the indicated antibodies. *Asterisk* denotes non-specific bands. *C* and *D*, UBE2U is required for 53BP1 and BRCA1 IRIF. U2OS cells pretreated with two individual UBE2U-specific siRNAs were irradiated and processed for immunostaining experiments. Cells were labeled with antibodies against 53BP1 or BRCA1 and γH2AX as a DSB marker. siRNA-mediated depletion of UBC13 was used as positive control. *E*, UBE2U is not required for MDC1 or RNF8 IRIF. U2OS cells transfected with siRNAs that targeted UBE2U (*siUBE2U-1* and *siUBE2U-2*), RNF8 (*siRNF8*), or non-targeting siRNAs (*siCTR*) were irradiated (10 Gy). 4 h post-IR treatment, cells were fixed, permeabilized, and immunostained with antibodies against MDC1 and RNF8. Nuclei were visualized with DAPI. *F*, UBE2U promotes RNF168 IRIF. U2OS cells pretreated with siRNAs that targeted UBE2U or RNF168 (*siRNF168*) were processed essentially as in *E*. *G*, schematic depicting the canonical DSB signal transduction pathway where UBE2U may play a role at the RNF168 level (see text). The percentage of cells positive for IRIF is shown and represents the mean from at least two independent experiments (see "Experimental Procedures").

responses at the RNF168 level (Fig. 2*G*) and that UBE2U depletion led to marked reduction in RNF168 expression (Figs. 2*F* and 3*C*), we reasoned that if UBE2U enforces DNA damage responses solely via RNF168 then the partial reduction in RNF168 protein level in UBE2U-silenced cells should correlate with milder phenotypic deficits when compared with RNF168 deficiency. To this end, we inactivated RNF168 or UBE2U using the RNAi approach (Fig. 3*C*), evaluated cell sensitivity to IR using the clonogenic survival assay, and assessed the integrity of the $G_2$/M checkpoint by measuring cell arrest at the $G_2$/M border following exposure to IR-induced DNA damage. Intriguingly, although RNF168-depleted cells were much more sensitive to IR treatment (Fig. 2*D*), UBE2U silencing consistently impaired the $G_2$/M checkpoint to greater extents than that in RNF168 knockdown cells (Fig. 2*E*). Together, these lines of evidence suggest that UBE2U may promote DNA damage responses via both RNF168-dependent and RNF168-independent pathways.

*RNAi-based Screen of UBE2U-binding E3s Identifies New Regulators of DSB Responses*—E2 ubiquitin-conjugating enzymes pair with E3 ubiquitin ligases to confer substrate specificity. To better understand how UBE2U participates in DDRs, we set out to identify UBE2U-interacting E3 partners. We took advantage of the fact that previous E2-E3 interaction studies have identified a total of 65 putative E3 partners of UBE2U (Fig. 4*A*) (34–36). To systematically examine whether any of these E3s may participate in DSB responses, we again designed a library of siRNAs that targeted these E3 ubiquitin ligases and

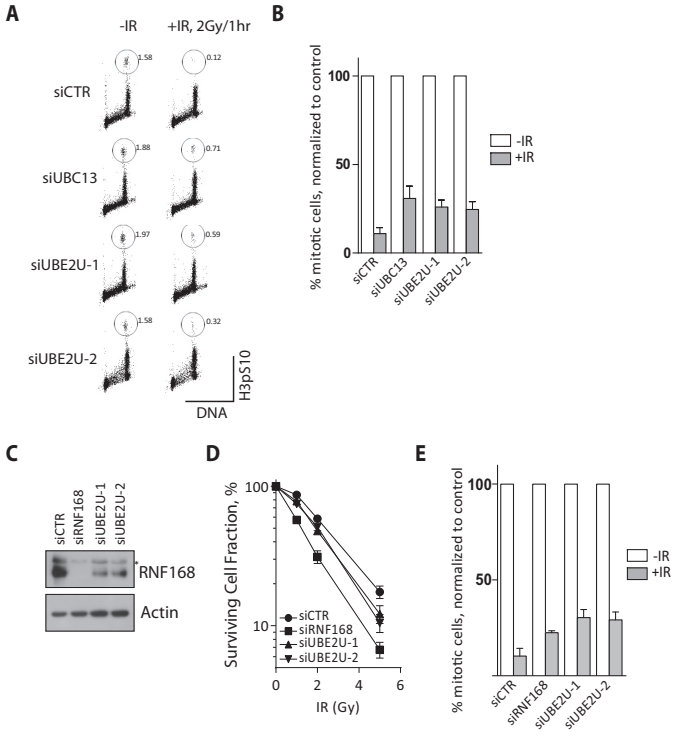


FIGURE 3. **UBE2U promotes cell responses to IR.** *A* and *B*, U2OS cells transfected with the indicated siRNAs were either left untreated or irradiated (2 Gy). Cells were fixed 1 h post-IR and immunolabeled with anti-H3-Ser(P)[10] antibodies. Cells were subjected to flow cytometry-based analyses (*A*), and means of three independent experiments are plotted (*B*). *Error bars* represent S.D. *C–E*, U2OS cells were transfected with control siRNAs (*siCTR*) or those that targeted RNF168 (*siRNF168*) or UBE2U (*siUBE2U-1* and *siUBE2U-2*). Cells were subsequently lysed and processed for Western blotting analysis using the indicated antibodies (*C*), plated onto 60-mm dishes for the clonogenic survival assay (*D*), or processed as in *A* to determine the integrity of the $G_2$/M checkpoint (*E*). For the clonogenic survival assay, cells were allowed to recover, and the number of colonies that formed after 14 days was counted. Data represent the mean of three independent experiments, each performed in triplicates. *Error bars* represent S.D.

assessed whether any of the E3s may also be required for IR-induced 53BP1 foci. We scored the percentage of 53BP1 focus-positive cells following IR treatment and analyzed the z score for each RNAi event as described previously (32) (Fig. 4*B*). Interestingly, this approach led to the identification of a number of candidates that were required for 53BP1 IRIF formation, including RNF17, TRIM34, TRIM65, and RNF10 (Fig. 4*C*). As a control, inactivation of RNF168 resulted in marked reduction of 53BP1 IRIF (Fig. 4*C*).

*E3 Ubiquitin Ligase RNF17 Is Required for DSB Accumulation of 53BP1 and BRCA1*—We performed a thorough literature search and found that none of the E3 ubiquitin ligases have documented roles in DDRs. Assuming that the E3s promoted 53BP1 in concerted efforts with UBE2U, one would expect that silencing of the candidate E3s may phenocopy UBE2U inactivation (Fig. 5*A*). Given the possible link between RNF17 and RNF168 (see below), we decided to experimentally test whether RNF17 also promotes DSB responses. Having confirmed the RNF17-UBE2U interaction (Fig. 5*B*), we irradiated U2OS cells following siRNA-mediated depletion of RNF17 and examined whether the E3 ubiquitin ligase is required for docking of DSB response factors at DNA breaks. Reminiscent to those seen in UBE2U-depleted cells, RNF17 inactivation did not noticeably affect DNA damage-induced focal accumulation of MDC1 and RNF8 (Fig. 5*C*). Notably, not only was RNF17 required for RNF168 IRIF (Fig. 5*D*) but inactivation of RNF17 compromised 53BP1, BRCA1, and ubiquitin conjugate accumulation at DSBs (Fig. 5, *E–G*). Altogether, we concluded that RNF17 promotes DSB ubiquitylation and enforces DSB accumulation of 53BP1 and BRCA1.

*E3 Ubiquitin Ligase RNF17 Regulates mRNA Level of RNF168*—Having established an important role of RNF17 in facilitating DSB accumulation of tumor suppressors 53BP1 and BRCA1, we set out to explore the underlying mechanism. RNF17 is a very large protein with 1623 amino acids and consists of an N-terminal RING finger motif and five Tudor domains that are distributed along the length of the protein (Fig. 6*A*). RNF17 was originally identified in mouse as Mmip-2 (Mad member-interacting protein-2) where it interacts with transcriptional repressor Mad family proteins and regulates the transcriptional activity of c-Myc (41, 42). Intriguingly, RNF17 inactivation by gene targeting resulted in a complete arrest in round spermatids, indicating a key role of RNF17 in regulating spermiogenesis (43). More importantly, a recent study showed that mouse RNF17 might be involved in the PIWI-interacting RNA (piRNA) pathway and may post-transcriptionally regulate expression of a cohort of genes, including RNF168 (44).

Our observations that the cellular deficits arising from RNF17 depletion closely resembled those in RIDDLE cells are in support of the idea that RNF17 plays integral roles in the H2AX-dependent DSB signal transduction pathway by regulating RNF168 expression (see "Discussion"). Prompted by this possibility, we further tested whether RNF17 knockdown may affect RNF168 expression by Western blotting analysis. Our effort in detecting endogenous RNF17 was hampered by the lack of good antibodies either from a commercial source (Fig. 6, *A* and *B*) or those that were raised against bacterially expressed and purified RNF17 protein fragments (Fig. 6, *A* and *C*). Regardless, we found that RNAi-mediated depletion of UBE2U or RNF17 led to marked reduction of RNF168 proteins (Fig. 6, *D* and *E*) but did not affect RNF8, an upstream E3 ubiquitin ligase in the DSB signal transduction pathway. This lends credence to the idea that UBE2U and RNF17 specifically regulate RNF168. To test whether UBE2U or RNF17 promotes RNF168 gene expression, we next quantified the RNF168 mRNA expression level by real time PCR. Intriguingly, UBE2U and RNF17 depletion reproducibly resulted in marked reduction in the RNF168 mRNA level (Fig. 6*F*). Taken together, we speculate that UBE2U and/or RNF17 promotes RNF168 gene expression at the mRNA level, which in turn enforces functions of RNF168 in DDRs.

To further explore whether roles of UBE2U or RNF17 in regulating DDRs are effected via RNF168, we undertook a functional bypass approach to experimentally examine whether reduction of RNF168 may account for the phenotypic deficits associated with RNF17 inactivation. Noting the possibility that the RNF17-UBE2U module may promote DDRs in both RNF168-dependent and -independent manners, we decided to evaluate the more immediate RNF168-dependent DSB responses. We reasoned that if UBE2U or RNF17 promotes 53BP1 IRIF via RNF168 then by reintroducing RNF168 in

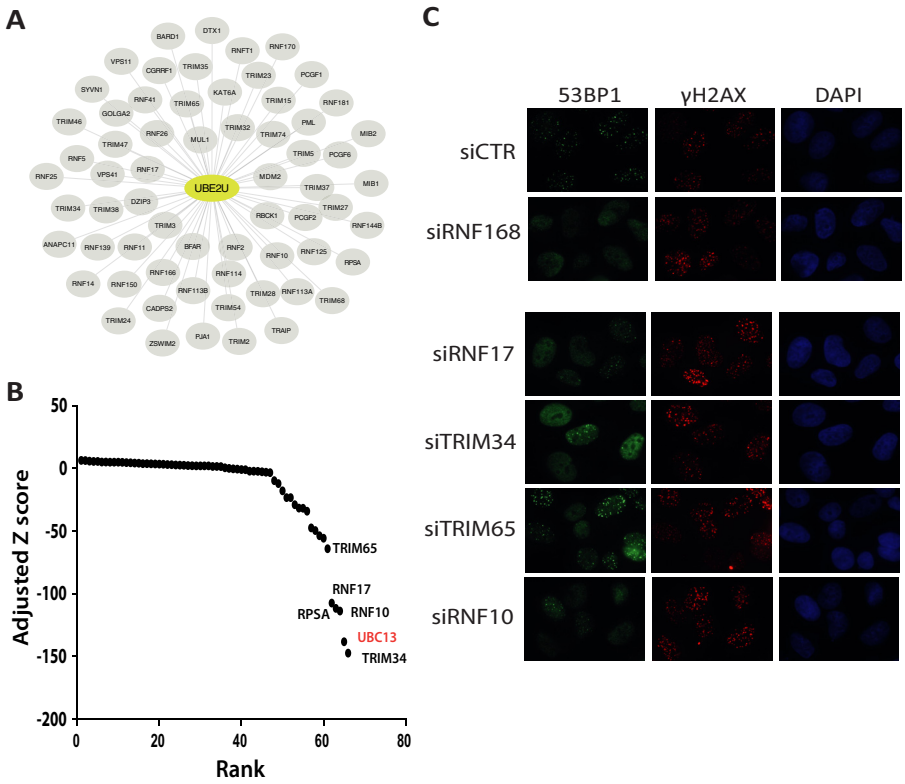


FIGURE 4. **Screen of UBE2U-interacting E3s identifies novel regulators of DSB responses.** *A*, network illustration of interactions between UBE2U and its potential E3 partners (35, 36). *B* and *C*, U2OS cells transfected with an E3-targeting siRNA library or the indicated siRNAs were irradiated (10 Gy). 4 h post-IR treatment, cells were fixed, permeabilized, and immunostained with antibodies against 53BP1 and γH2AX, and the percentage of 53BP1 focus-positive cells (>10 foci per cell) was quantified. Ranking by z score of all samples was plotted as described under "Experimental Procedures." Representative images following RNAi-mediated depletion of RNF17, TRIM34, TRIM65, or RNF10 are shown (*C*). Non-targeting siRNAs (*siCTR*) or RNF168-targeting siRNAs (*siRNF168*) were used as negative and positive controls, respectively.

UBE2U- or RNF17-depleted cells one should restore 53BP1 IRIF. To this end, we transiently expressed RNF168 in UBE2U- or RNF17-silenced cells and scored cells positive for 53BP1 focus formation after IR treatment. Most notably, although depletion of RNF168, RNF17, and UBE2U impaired 53BP1 IRIF, re-expression of FLAG-tagged RNF168 efficiently alleviated defective IRIF formation of 53BP1 in both UBE2U- and RNF17-depleted cells (Fig. 6, *G* and *H*), indicating that reduction of RNF168 accounts for, at least in part, the defective DSB responses seen with UBE2U and RNF17 deficiencies.

## Discussion

Cell responses to DSBs entail a sophisticated ubiquitin-driven DDR network (45). By utilizing an siRNA library designed to target 37 known and predicted E2s, we systematically screened for a novel E2 that promotes chromatin responses at DSBs and have successfully identified UBE2U as a positive regulator of 53BP1 IRIF formation. Further mining of the UBE2U interactome uncovered RNF17, among other candidate E3 ubiquitin ligases, as a novel regulator of DSB responses. Together, our E2-guided screen approach not only uncovered the UBE2U-RNF17 pair as a new E2-E3 module important in driving mammalian DDRs but also highlights how such an approach may be adaptable to studying and identifying ubiquitin components in other biological processes.

A recent study showed that RNF17 might be involved in the piRNA pathway (44). PIWI-interacting RNAs are a class of small RNAs with length from 24 to 31 nucleotides. These small RNAs interact with PIWI proteins to form an RNA-induced silencing complex to repress transposons as well as other protein-coding genes. The PIWI-piRNA pathway has been extensively studied in transposon silencing in germ lines. Although the somatic function of PIWI proteins has been documented, whether somatic piRNAs exist remains obscure (46, 47). Notably, RNF17 associates with PIWI (48) and was reported to suppress the biogenesis of secondary piRNAs (44). Consequently, upon loss of RNF17, there was a >700-fold increase in secondary piRNAs that mapped to protein-coding genes. Coincidentally, one of these candidate targets included RNF168, suggesting that RNF17 inactivation might trigger RNF168 gene silencing through the piRNA pathway (44). Although the mechanistic basis that details the RNF17-dependent regulation of RNF168 expression remains to be defined, our observation that silencing of RNF17 and UBE2U correlated with substantial reduction in RNF168 mRNA and protein expression is in support of such a relationship. Because ectopically expressed RNF168 restored 53BP1 IRIF in RNF17- and UBE2U-depleted cells, we propose that the RNF17-UBE2U module promotes DSB signal transduction, at least in part, via regulating RNF168 expression.

The E3 ubiquitin ligase RNF168 was originally identified as the RIDDLE syndrome protein (1) where RNF168 deficiency correlated with phenotypic defects in ubiquitin-mediated DSB

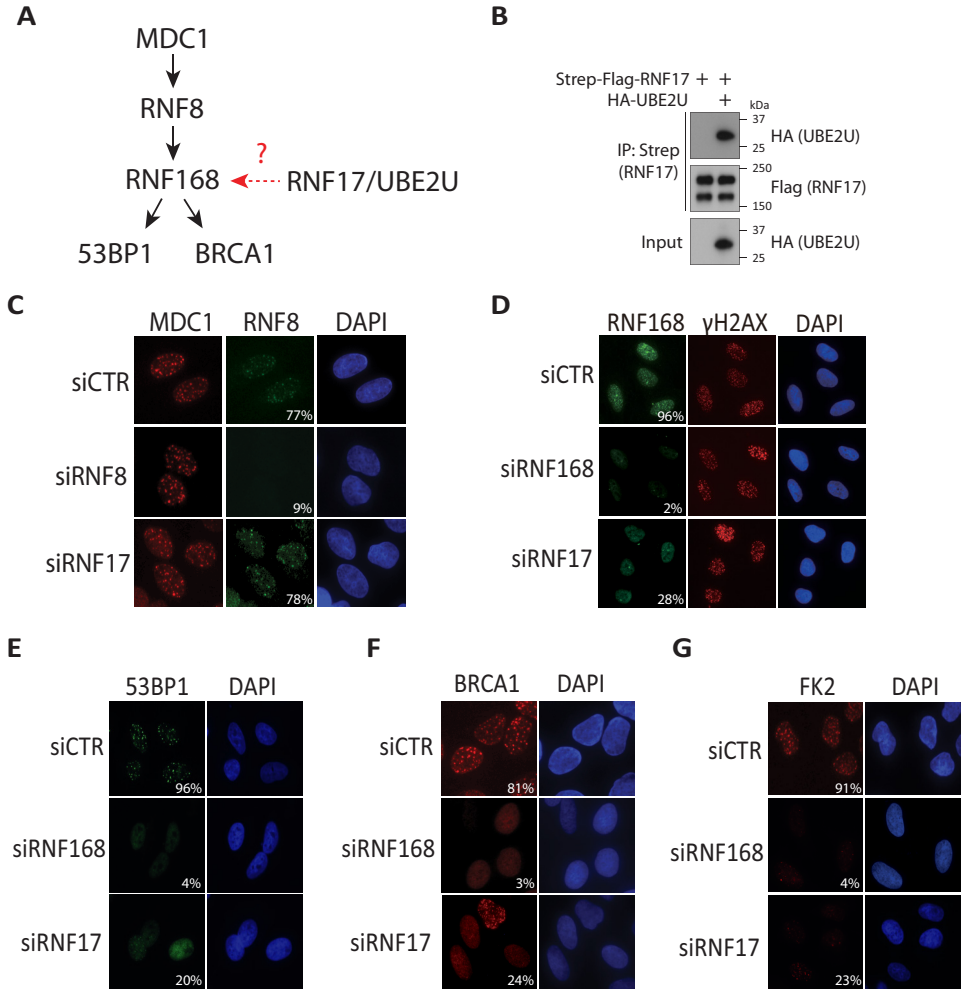


FIGURE 5. **RNF17 is required for DSB retention of BRCA1 and 53BP1.** *A*, schematic depicting the canonical DSB signal transduction pathway where the RNF17-UBE2U pair may play a role at the RNF168 level (see text). *B*, UBE2U interacts with RNF17. 293T cells were transfected with constructs encoding HA-UBE2U or streptavidin binding peptide-FLAG-tagged (*Strep-FLAG*) RNF17. Lysates were subjected to immunoprecipitation using streptavidin beads. RNF17-co-purifying proteins were subjected to SDS-PAGE and Western blotting analysis using the indicated antibodies. *C–G*, representative images showing IR-induced focus formation of MDC1 and RNF8 (*C*), RNF168 and γH2AX (*D*), 53BP1 (*E*), BRCA1 (*F*), or ubiquitin conjugates (*FK2*; *G*) following RNF17 silencing. U2OS cells pretreated with RNF17-targeting siRNAs were irradiated (10 Gy) and processed for immunostaining experiments using the indicated antibodies. Nuclei were visualized by DAPI staining. siRNA-mediated depletion of RNF8 or RNF168 was used as a positive control, and non-targeting siRNAs (*siCTR*) were used as a negative control. The percentage of cells positive for IRIF is shown and represents the mean from at least two independent experiments (see "Experimental Procedures").

signal transduction (2). As a key intermediate that propagates DSB signals to orchestrate DDRs, RNF168 is also regulated by many different factors to ensure that its function is executed with spatiotemporal control. Indeed, although a number of ubiquitin machineries, including TRIP12 and USP34, have been reported to regulate RNF168 turnover (49–51), others fine-tune RNF168 output by targeting its substrates (52–54). Importantly, our identification of the UBE2U-RNF17 pair as regulator of RNF168 expression represents an unprecedented strategy to stringently keep RNF168 in check. Although we cannot exclude the possibility that RNF17 may also target other components to effect DDRs because forced expression of RNF168 restored 53BP1 IRIF in RNF17-depleted cells, we favor a model in which RNF17, in concert with UBE2U, suppresses secondary piRNA biogenesis to facilitate RNF168 expression (Fig. 7). Together, these regulatory and proteolytic mechanisms act to regulate RNF168 functions.

Although we envisage that RNF17-UBE2U encodes an intrinsic RNF168 regulator, it is highly probable that the E3-E2 pair also regulates other cellular factors, especially given the documented role of RNF17 in the piRNA pathway (44). Indeed, although silencing of RNF17 or UBE2U led to partial reduction of RNF168 expression, checkpoint control at the $G_2$/M border was consistently compromised to greater extents in cells depleted of UBE2U. These data point to the exciting possibility that the RNF17-UBE2U module may enforce DDRs in both RNF168-dependent and -independent manners. Further work will be necessary to comprehensively understand how the RNF17-UBE2U pair regulates mammalian cellular responses to genotoxic stress.

Although UBE2U remains to be characterized biochemically, the fact that it associates with many E3 ubiquitin ligases points to the possibility that it may serve as a catalytic activity in driving protein ubiquitylation (35, 36, 55). The observation that

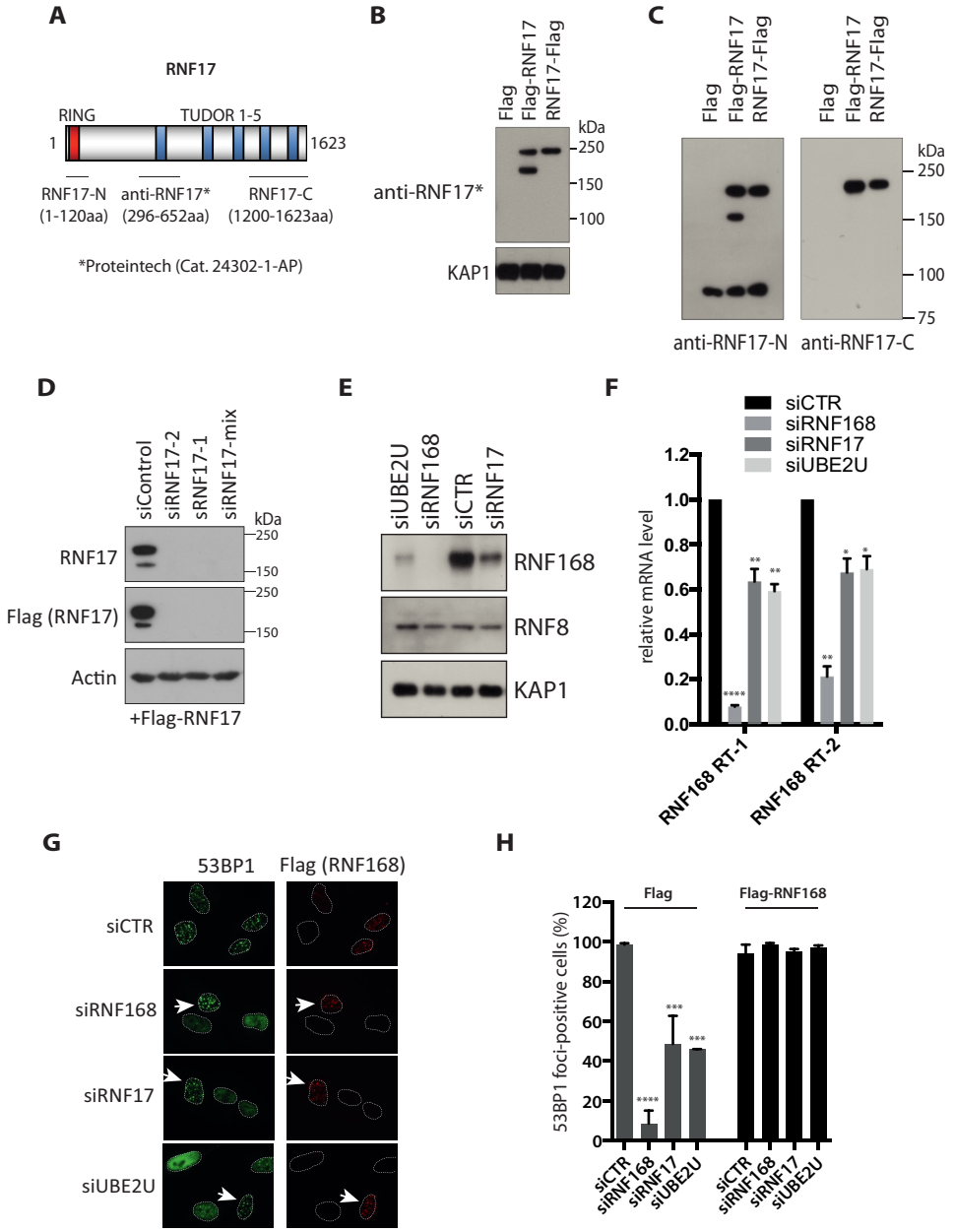


FIGURE 6. **RNF17 regulates RNF168 expression.** *A*, schematic depicting RNF17 protein domain organization and the epitopes that are recognized by anti-UBE2U antibodies. *B* and *C*, Proteintech or in-house anti-UBE2U antibodies specifically recognized ectopically expressed RNF17. *D*, validating RNAi-mediated knockdown of RNF17. U2OS cells were co-transfected with FLAG-RNF17 and the indicated siRNAs. Cells were harvested, and lysates were separated by SDS-PAGE. Western blotting experiments were performed using the indicated antibodies. *E*, depletion of RNF17 or UBE2U down-regulated RNF168 protein expression. U2OS cells were treated with the indicated siRNAs twice at 24-h intervals. 48 h after the second siRNA transfection, cells were lysed for Western blotting analysis using the indicated antibodies. *F*, RNF17 or UBE2U silencing correlated with reduction in RNF168 mRNA levels. The experiment was performed as described under "Experimental Procedures." RNAi-mediated knockdown of RNF168 was used as a positive control. *G* and *H*, ectopically expressed RNF168 restored 53BP1 IRIF in RNF17- and UBE2U-inactivated cells. Cells were depleted of RNF17 or UBE2U by the RNAi approach. 24 h after the second siRNA transfection, cells were transiently transfected with FLAG-tagged RNF168-expressing constructs or empty vector. 53BP1 IRIF were analyzed by co-staining with anti-FLAG antibodies 4 h after cell exposure to 10 Gy IR. Representative images are shown (*G*). The percentage of cells positive for 53BP1 IRIF following reintroduction of RNF168 is plotted (*H*). As a control, 53BP1 IRIF-positive cells were scored when foci numbered >10 per nuclei following RNAi. For FLAG-RNF168 re-expression experiments, 53BP1 IRIF were scored only in FLAG-positive cells. Results represent the mean of three independent experiments. At least 100 nuclei were counted each time. *Arrows* indicate cells that ectopically express Flag-RNF168. *Error bars* represent S.D. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ *versus* control. *siCTR*, control siRNAs.

multiple cancer-associated mutations have been identified on the UBE2U coding sequence, in particular its UBC domain, argues in favor of a critically important role of the E2 in cell proliferation and homeostasis. Although we are beginning to understand how the UBE2U-RNF17 pair may promote DSB responses, much remains to be elucidated in the UBE2U biology because a number of other putative UBE2U-interacting E3s were also isolated in our E3 screen for their ability to promote 53BP1 IRIF formation. It would be of significant interest to delineate precisely how the UBE2U network may have evolved to innervate the DSB signal transduction pathways. In sum, our RNAi-based screen for ubiquitin machineries important in

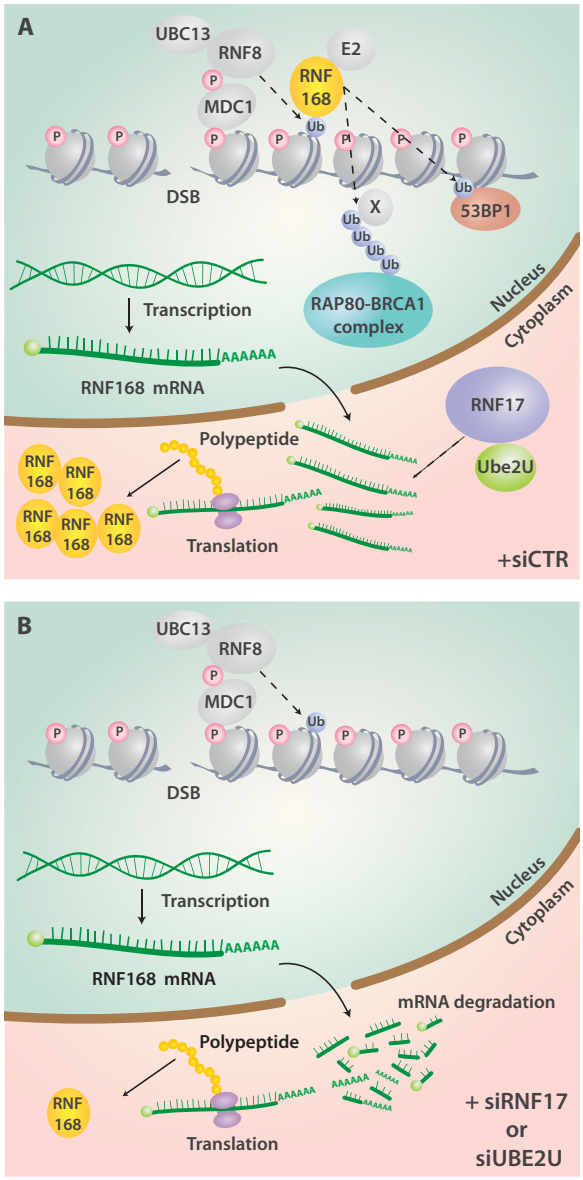


FIGURE 7. **Working model depicting the role of UBE2U and RNF17 in RNF168-dependent DSB responses.** *A*, RNF17 and UBE2U may suppress biogenesis of secondary piRNAs. RNF168 is expressed and catalyzes chromatin ubiquitylation at DSBs to assemble 53BP1 and BRCA1. *B*, silencing of RNF17 or UBE2U leads to down-regulation of RNF168 expression. Ubiquitin-mediated DSB signals are not propagated, and cells do not efficiently support 53BP1 and BRCA1 IRIF. *siCTR*, control siRNAs.

DSB signal transduction events has uncovered a list of novel ubiquitin components and highlights the important regulatory role of ubiquitin machineries in genome integrity protection.

## Experimental Procedures

*Cell Culture*—U2OS, 293T, and HeLa cell lines from ATCC were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in 5% $CO_2$.

*Plasmids and siRNAs*—The expression construct of FLAG epitope-tagged RNF168 was described previously (4). For RNAi experiments, cells were transfected twice with target siRNAs (Dharmacon) using Oligofectamine according to the manufacturer's instructions (Invitrogen). The siRNA libraries target-

**TABLE 1**
**Sequences of siRNAs**

| | Sense (5′–3′) |
|---|---|
| **E2s** | |
| UBE2A-homo-257 | CGUCCGAGAACAACAUAAUTT |
| UBE2A-homo-374 | CACCUACAGUUAGAUUUGUTT |
| UBE2A-homo-468 | CCAACCUAUGAUGUGUCUUTT |
| UBE2A-homo-569 | GGAGAACAAACGGGAAUAUTT |
| UBE2B-homo-517 | GCAGUGGAAUGCAGUUAUATT |
| UBE2B-homo-447 | GGGAUUUCAAGCGGUUACATT |
| UBE2B-homo-794 | GCACAGCUUUAUCAGGAAATT |
| UBE2B-homo-668 | GGUAGCAUAUGUUUAGAUATT |
| UBE2C-homo-204 | CAUGAUGUCUGGCGAUAAATT |
| UBE2C-homo-574 | CUGCAAGAAACCUACUCAATT |
| UBE2C-homo-490 | GCCUUCUAGGAGAACCCAATT |
| UBE2C-homo-282 | GGAGCAGCUGGAACAGUAUTT |
| UBE2D1-homo-229 | GCUGAAGAGGAUUCAGAAATT |
| UBE2D1-homo-416 | CCAAAGAUUGCUUUCACAATT |
| UBE2D1-homo-504 | CCAGCUCUGACUGUAUCAATT |
| UBE2D1-homo-579 | GUACCAGAUAUUGCACAAATT |
| UBE2D2-homo-868 | GGCAGCAUUUGUCUUGAUATT |
| UBE2D2-homo-642 | CCACAAGGAAUUGAAUGAUTT |
| UBE2D2-homo-1048 | GGACUCAGAAGUAUGCGAUTT |
| UBE2D2-homo-756 | CCCUAUCAGGGUGGAGUAUTT |
| UBE2D3-homo-486 | GGCGCUGAAACGGAUUAAUTT |
| UBE2D3-homo-727 | GGCAGCAUUUGUCUCGAUATT |
| UBE2D3-homo-599 | GACCUAAUGACAGCCCAUATT |
| UBE2D3-homo-869 | CAGACAGAGAUAAGUACAATT |
| UBE2D4-homo-483 | CCGACAGAGAGAAGUACAATT |
| UBE2D4-homo-342 | GCAGCAUCUGCCUUGAUAUTT |
| UBE2D4-homo-213 | GUCCUUACCAAGGAGGUGUTT |
| UBE2D4-homo-2653 | GGGACUGAUUCACAUGUUATT |
| UBE2E1-homo-444 | GGCGAUAACAUCUAUGAAUTT |
| UBE2E1-homo-738 | CCACUCAGUAUAUGACCAATT |
| UBE2E1-homo-645 | GGAGUCCAGCACUAACCAUTT |
| UBE2E1-homo-556 | GCCUCCAAAGGUUACAUUUTT |
| UBE2E2-homo-510 | CACCAGACUAUCCGUUUAATT |
| UBE2E2-homo-623 | CCGGCUUUAACUAUUUCUATT |
| UBE2E2-homo-325 | CGCUGCUAAAUUGUCAACUTT |
| UBE2E2-homo-713 | CCACACAGUACAUGACCAATT |
| UBE2E3-homo-845 | CCCGCUUUGACUAUUUCAATT |
| UBE2E3-homo-583 | GAAGGAGCUAGCUGAAAUATT |
| UBE2E3-homo-985 | GACCAAGAGAUACGCAACATT |
| UBE2E3-homo-1089 | GGACUUCUGUGUAUAUGUUTT |
| UBE2F-homo-482 | GUGCCUCCCAAAGUGAAAUTT |
| UBE2F-homo-205 | GCUAACGCUAGCAAGUAAATT |
| UBE2F-homo-290 | GGGUUUCUGUGAGAGACAATT |
| UBE2F-homo-711 | GGAGGACUUCCGGAAUAAATT |
| UBE2G1-homo-487 | GCCCUCCAGAUACACUUUATT |
| UBE2G1-homo-593 | GCACCCAAAUGUUGAUAAATT |
| UBE2G1-homo-395 | GCUGGCAGAACUCAACAAATT |
| UBE2G1-homo-695 | CCUAUCCACACUGUGGAAATT |
| UBE2G2-homo-267 | CCACUUGAUUACCCGUUAATT |
| UBE2G2-homo-470 | GCAGAGCCCAAUGACGAAATT |
| UBE2G2-homo-112 | GCUGAUGGCCGAGUACAAATT |
| UBE2G2-homo-345 | GGAGAGUCUGCAUUUCCAUTT |
| UBE2H-homo-545 | GGCGGAGUAUGGAAAGUUATT |
| UBE2H-homo-730 | GCCUCAGUUAUUGGCCUAUTT |
| UBE2H-homo-896 | GCUCAUCGGAGAGCUCUAUTT |
| UBE2H-homo-481 | GAUCCUGGGAGGACUUAAUTT |
| UBE2I-homo-550 | GAGGCCUACACGAUUUACUTT |
| UBE2I-homo-257 | GCACGAUGAACCUCAUGAATT |
| UBE2I-homo-325 | GCUUGUUUAAACUACGGAUTT |
| UBE2I-homo-194 | GAGGAAAGCAUGGAGGAAATT |
| UBE2J1-homo-636 | CAGCCUUCGUGGAGUAUAATT |
| UBE2J1-homo-1034 | GCAUCCUUUCAUCAACCUATT |
| UBE2J1-homo-868 | CUAGGCAAAUAAGCUUUAATT |
| UBE2J1-homo-421 | CGCAGCCUUUAGAGGAUAATT |
| UBE2J2-homo-440 | CCAGAGAAUUUCCUUUCAATT |
| UBE2J2-homo-237 | GCUGAAGCAGGACUACCUUTT |
| UBE2J2-homo-662 | GCAGUGCAGAGUUUAGCAUTT |
| UBE2J2-homo-754 | GCACAAGACGAACUCAGUATT |
| UBE2K-homo-498 | GGUCCGGUUUAUCACUAAATT |
| UBE2K-homo-857 | CUGCAACAGAAUUGCUUCUTT |
| UBE2K-homo-3572 | GCAGUUAGUGGACCCAAAUTT |
| UBE2K-homo-604 | GCACGGUAUUAUUGUCAUUTT |
| UBE2L3-homo-369 | CACCGAAGAUCACAUUUAATT |
| UBE2L3-homo-250 | CCAGGUUGAUGAAGCUAAUTT |
| UBE2L3-homo-437 | CCAGUAAUUAGUGCCGAAATT |
| UBE2L3-homo-2934 | GGCAGAGAAUAGGCUUUCUTT |
| UBE2L6-homo-424 | GCUGGUGAAUAGACCGAAUTT |
| UBE2L6-homo-287 | CCUCCCAUGAUCAAAUUCATT |
| UBE2L6-homo-1161 | CUCAGACUGUGAAGUAUAUTT |

**TABLE 1—*continued***

| | Sense (5′–3′) |
|---|---|
| UBE2L6-homo-165 | GAACCUGUCCAGCGAUGAUTT |
| UBE2M-homo-954 | CCAGUCCUUACGAUAAACUTT |
| UBE2M-homo-875 | GUGUGAGACAAUGGUCUAUTT |
| UBE2M-homo-918 | GCAACGUCUGCCUCAACAUTT |
| UBE2M-homo-723 | CCCAAGACGUGUGAUAUCATT |
| UBE2N-homo-877 | CCUCUUUGUUUGCAUUUAATT |
| UBE2N-homo-613 | GGGAAGAAUAUGUUUAGAUTT |
| UBE2N-homo-2109 | GUCCUCAGUUAAUGAUUCUTT |
| UBE2N-homo-2516 | CUGGUAUCCUUCCAAAUAATT |
| UBE2O-homo-962 | GCAGGUUGUAGAGUUGAAATT |
| UBE2O-homo-2001 | GCUGACCACCCUGACUUUATT |
| UBE2O-homo-3064 | GCCUCUACUUGUUUGACAUTT |
| UBE2O-homo-3750 | CCACCCAGUGUGAAACCAATT |
| UBE2Q1-homo-679 | GGACACAGAAGACUUAGAUTT |
| UBE2Q1-homo-1000 | CCAGAUCCUCAAAGAGAAATT |
| UBE2Q1-homo-1185 | CCAUAGAGUCAGUGAUCAUTT |
| UBE2Q1-homo-493 | GCUGGUGGACAUAAAGAAATT |
| UBE2Q2-homo-677 | GCAAUUGAAGUGGUUGAUATT |
| UBE2Q2-homo-1358 | GCAAAUAAAUGCCACCUUATT |
| UBE2Q2-homo-734 | GGAUGUUGAGAUGCUAGAUTT |
| UBE2Q2-homo-576 | CCAUCUUCUUCACCGAUAUTT |
| UBE2R1-homo-435 | CCAAGAUGUGGCACCCUAATT |
| UBE2R1-homo-550 | GCAGAACGUCAGGACCAUUTT |
| UBE2R1-homo-675 | GGGAGUACACAGACAUCAUTT |
| UBE2R1-homo-1400 | CUGCUUUGGUUUGUUUGAATT |
| UBE2R2-homo-610 | CCUGAUGCUCGAGCUGAAATT |
| UBE2R2-homo-741 | GCUACUUCAAGGCGCAUAUTT |
| UBE2R2-homo-930 | GUGAGGACUAUCCUAUUAATT |
| UBE2R2-homo-1158 | GUGCCUUCCAAUGACAACATT |
| UBE2S-homo-451 | CCGAUGGCAUCAAGGUCUUTT |
| UBE2S-homo-536 | GAGGUCUGUUCCGCAUGAATT |
| UBE2S-homo-598 | GCUACUUCCUGACCAAGAUTT |
| UBE2S-homo-700 | ACGUACUGCUGACCAUCAATT |
| UBE2T-homo-684 | CCAGUCAGCUAGUAGGCAUTT |
| UBE2T-homo-233 | CCUGCGAGCUCAAAUAUUATT |
| UBE2T-homo-738 | CCUGGUUCAUCUUAGUUAATT |
| UBE2T-homo-510 | GCUGACAUAUCCUCAGAAUTT |
| UBE2U-homo-440 | GCUCCUCCAGUUGUGAAAUTT |
| UBE2U-homo-604 | GCUAGAGAAUCCAGUGAAUTT |
| UBE2U-homo-815 | GCCACAGAAUACUACAGAATT |
| UBE2U-homo-898 | GCAUCAGAAAGAAUGGAAUTT |
| UBE2V1-homo-751 | GCGCCUAAUGAUGUCUAAATT |
| UBE2V1-homo-492 | GACGAAGACAUGACACUUATT |
| UBE2V1-homo-1409 | GGCCGAAGCAUAGAUUGUATT |
| UBE2V1-homo-799 | GACAGUGUUACAGCAAUUATT |
| UBE2V2-homo-155 | GGUGGACAGGCAUGAUUAUTT |
| UBE2V2-homo-430 | CCACCAGAAGGACAAACAUTT |
| UBE2V2-homo-249 | CUCCGUCAGUUAGAUUUGUTT |
| UBE2V2-homo-1201 | GACUGUGCCAUUUCUAUUATT |
| UBE2W-homo-215 | GGUGCACCAGGUACCUUAUTT |
| UBE2W-homo-323 | CCUGUUCAUCCUCAUGUUUTT |
| UBE2W-homo-484 | GCGAACAUGUAACAAGAAUTT |
| UBE2W-homo-100 | GCAGAAACGACUACAGAAATT |
| UBE2Z-homo-683 | GCAAUGGGAAAGUCUGCUUTT |
| UBE2Z-homo-755 | CCUCAGUGCUCAUCUCUAUTT |
| UBE2Z-homo-450 | GGGAUAUCAUGUCCAUUUATT |
| UBE2Z-homo-1111 | GAGGCUCCAUAAUGAGAAUTT |
| ATG3-homo-424 | GCGGAUGGGUAGAUACAUATT |
| ATG3-homo-545 | GGAAGAAGAUGAAGAUGAATT |
| ATG3-homo-254 | CCACUGUCCAACAUGGCAATT |
| ATG3-homo-176 | CCUCAAGGAAUCAAAGUUUTT |
| BIRC6-homo-1428 | GCAAUUGUACAACAGCUUATT |
| BIRC6-homo-2777 | GCAGUUAACCUCAAAGAAUTT |
| BIRC6-homo-6793 | GCAGUAGCCUUGAUAGAUUTT |
| BIRC6-homo-10755 | GCUGUUGACAGUCUACUUUTT |
| UFC1-homo-470 | CCAUGACCUCCUGAAAUAUTT |
| UFC1-homo-601 | GCCUGACGGAUCAUUUCAATT |
| UFC1-homo-376 | GGUAUGUGGAGAACAACAATT |
| UFC1-homo-722 | GGGCGUCAUCCAACACAAATT |
| **E3s** | |
| ANAPC11-homo-278 | CAUGGAGAAAGCAUUUCUATT |
| ANAPC11-homo-466 | GACCAGUUCACUACUCAGATT |
| ANAPC11-homo-409 | GUCUAGGGAAGAGUCUUCUTT |
| BARD1-homo-1282 | GUCCGAUGAAUUCAUUAGUTT |
| BARD1-homo-1795 | GCUAGCCACUGCUCAGUAATT |
| BARD1-homo-541 | GCAGGAAACAAGAAGAAUUTT |
| BFAR-homo-1460 | GGAGCCAUUUCUGGAAAGUTT |
| BFAR-homo-1002 | GGAGCUAGAACGUGUCAAATT |
| BFAR-homo-351 | CCCUCAGAUUUCUGUUAGUTT |
| CADPS2-homo-1022 | GCUCAUGGAUAGCCAAAUATT |
| CADPS2-homo-2028 | GCAGCUUGAAGGCUAUACUTT |

**TABLE 1—*continued***

| | Sense (5′–3′) |
|---|---|
| CADPS2-homo-2183 | CCACAGGUCAAUCAUAUAATT |
| CGRRF1-homo-740 | GGCUCAAGGUCAAUUUCAUTT |
| CGRRF1-homo-639 | GACCGGGAAAUUUAUGAUATT |
| CGRRF1-homo-545 | CCAGUUACCAAGAGAUACUTT |
| DTX1-homo-2127 | CUAGCUACCUAGACAACGUTT |
| DTX1-homo-564 | GCAGUCCAUGCACCAGUUUTT |
| DTX1-homo-1906 | GGGAAGAAGUUCACCGCAATT |
| DZIP3-homo-1823 | GCCUCCUGUUAGCUCUUAUTT |
| DZIP3-homo-1529 | GACCGAAGAUCAGUUUAAATT |
| DZIP3-homo-2582 | GCCUGGAUGAAUUGCAUAUTT |
| GOLGA2-homo-1163 | GUCGGUUAGACAACUACAATT |
| GOLGA2-homo-683 | GAUCCUCGUAUCAGAGAAATT |
| GOLGA2-homo-519 | CUGGACUCCAGCUAUGUAATT |
| KAT6A-homo-1397 | GCGCUAUACUAAUCCAAUATT |
| KAT6A-homo-4972 | CACCCUCUAUGCAGAACAUTT |
| KAT6A-homo-3756 | GCUGAUGACACUCCUAUCUTT |
| MDM2-homo-475 | GGCCAGUAUAUUAUGACUATT |
| MDM2-homo-912 | GGAGAUAUGUUGUGAAAGATT |
| MDM2-homo-1489 | CAGCCAUCAACUUCUAGUATT |
| ZSWIM2-homo-1520 | CAGGUAUCUUCAAGAUUUATT |
| ZSWIM2-homo-729 | GGGAUUCCCUGUAAUAACUTT |
| ZSWIM2-homo-1787 | GAGCACAGCUAAACUUAGUTT |
| MIB1-homo-883 | GGGCAUGUCUGAUCUGAAATT |
| MIB1-homo-883 | CCGAGUACAACAGAUUUAUTT |
| MIB1-homo-883 | GACCUGAGCAUUCGAAAUATT |
| MIB2-homo-2150 | GUGCCAAACAUCGAUGUUATT |
| MIB2-homo-2226 | CGCUAGCUGUGAGAAAGAUTT |
| MIB2-homo-1486 | GGUGGUGAAAGUGUUUGGATT |
| MUL1-homo-340 | GCGUGCCUUAUGCUGUUAUTT |
| MUL1-homo-801 | GGCAUGCAGUACUAUCUAATT |
| MUL1-homo-451 | GCACAAGAUGGUGUGGAAUTT |
| PCGF1-homo-647 | GUCACCGCUUGAUGCUAAATT |
| PCGF1-homo-503 | CCCACUACUAUCGCUAUGATT |
| PCGF1-homo-706 | CCUGAUCACAUGACAAUGATT |
| PCGF2-homo-839 | GAGCCACUGAAGGAAUACUTT |
| PCGF2-homo-774 | CCAAGUUUCUCCGCAACAATT |
| PCGF2-homo-636 | GCCUCUCCAUCGAAUUCUATT |
| PCGF6-homo-837 | GGAGUUCAUUGGUGCUAAUTT |
| PCGF6-homo-426 | GCGCCUGAUUAAUCUCUCUTT |
| PCGF6-homo-1005 | GGAGCAGUAUCAAACUCUATT |
| PJA1-homo-1368 | GACCAAGUGAAACCAGAAATT |
| PJA1-homo-2165 | CAUCUGCUGUAGCGAAUAUTT |
| PJA1-homo-807 | GGCAAGUUUAAAGAUGAUATT |
| PML-homo-682 | CCCGCAAGACCAACAACAUTT |
| PML-homo-884 | GGAGCAGGAUAGUGCCUUUTT |
| PML-homo-1534 | GAGGAUGUCUCCAAUACAATT |
| RBCK1-homo-1150 | GGUGCACCUUCAUCAACAATT |
| RBCK1-homo-2070 | CUGCCACUGAGCUAAAGAUTT |
| RBCK1-homo-1900 | AGAUCGUGGUACAGAAGAATT |
| RNF10-homo-879 | CUGGUCCUAAGAAGAUCAATT |
| RNF10-homo-1036 | CUGCCAAUUUGUGGUGUCUTT |
| RNF10-homo-2776 | CAGCCAAGCUAUUGAAGCATT |
| RNF11-homo-808 | GGGACCCAAUUCGAUUUCUTT |
| RNF11-homo-595 | CGCCAUAUCAGGAACAAGUTT |
| RNF11-homo-693 | GCUCAAAGAAUAGGUCUUATT |
| RNF113A-homo-1222 | GAGGAUGCAAUUCCCAUUATT |
| RNF113A-homo-497 | GUCUCGGCGUGGUUUAUAATT |
| RNF113A-homo-396 | GACCCACAAUCCAAUGAUATT |
| RNF113b-homo-597 | GGGAUUACCAGCCUGACAUTT |
| RNF113b-homo-703 | GGAGAUUGAACGGGAGCUUTT |
| RNF113b-homo-837 | CCAAGUGCAGGCAUUAUUUTT |
| RNF114-homo-353 | GUGGCUACUUGUUCCAAAUTT |
| RNF114-homo-503 | GUGGAACACUGCAAAUUAUTT |
| RNF114-homo-401 | GCCACCAUUAAGGAUGCAUTT |
| RNF125-homo-839 | GCUGGAUCAUUGUAUUACUTT |
| RNF125-homo-719 | GGCACAUAUUCGGACUUGUTT |
| RNF125-homo-934 | GUGGCAGUUUAAUAAGACATT |
| RNF139-homo-1951 | GGAGCCGCUUACAAGAAAUTT |
| RNF139-homo-1162 | GGGACCUCAUUUGCAAUCUTT |
| RNF139-homo-1271 | GGCCUUUAUUGGAUCAACUTT |
| RNF14-homo-861 | CCCUAGCAUACUUGAAUAUTT |
| RNF14-homo-1682 | GGAACUCCCAUAGAGAAAUTT |
| RNF14-homo-587 | CCACAGAAUUUCAAGAUAUTT |
| RNF144b-homo-815 | GAGACAGUCAGCCUAUUGUTT |
| RNF144b-homo-542 | CUGACAUGGUGUGCCUAAATT |
| RNF144b-homo-976 | GGUACUGCCUCCAGAACUUTT |
| RNF150-homo-1351 | GAGGUUUCGAUAUGCAAAUTT |
| RNF150-homo-1269 | GCACUUCGGUUGUGUUUGUTT |
| RNF150-homo-1605 | GCAAGAUGAACAUUCUUAATT |
| RNF166-homo-363 | GCAGCUCUCAUCCUACAAATT |
| RNF166-homo-411 | GACCCUGGCAAAGAUGAGATT |

**TABLE 1—*continued***

| | Sense (5′–3′) |
|---|---|
| RNF166-homo-677 | GCUACAAGAGCGCCAACUUTT |
| RNF17-homo-2793 | GCCUGUGCAUAUCUGUAAUTT |
| RNF17-homo-4174 | GAGGAACAAUGGGAAAUAATT |
| RNF17-homo-4491 | GCCUUGCCUUGCAGAAUAUTT |
| RNF170-homo-1020 | GGCAUGCAUUCAGGGAAAUTT |
| RNF170-homo-800 | GCCUGCAUUAUUGCUUACUTT |
| RNF170-homo-478 | GGCCAAAUAUCAAGGUGAATT |
| RNF181-homo-51 | GGCGUCCUAUUUCGAUGAATT |
| RNF181-homo-440 | GACGAGAUAAGGCUCGAAATT |
| RNF181-homo-363 | CCCUGGGCUAAGCAAGACAATT |
| RNF2-homo-842 | GGCUAGAGCUUGAUAAUAATT |
| RNF2-homo-1131 | CCAGUUCACUGUAUUAAAUTT |
| RNF2-homo-620 | GGAUCAACAAGCACAAUAATT |
| RNF25-homo-251 | GGACCAGGAUUCACAGUAUTT |
| RNF25-homo-208 | CACCAUGGGAGAUCUACAUTT |
| RNF25-homo-1021 | CCCAGCACAUAUGUGAGAATT |
| RNF26-homo-1286 | GCUUUGUGCUUGUCAAUCUTT |
| RNF26-homo-1048 | CUGGUGGCUUAUGUGAUCATT |
| RNF26-homo-800 | GAGUCUUGCUUUCAUUGCUTT |
| RNF41-homo-468 | GGCACCUCAUUGUGAACAUTT |
| RNF41-homo-1184 | GACGCUACUAUGAGAACUATT |
| RNF41-homo-881 | GAGACAUCCAGCUGCUAAATT |
| RNF5-homo-194 | GACCUUCGAAUGUAAUAUATT |
| RNF5-homo-308 | GCAAGAGUGUCCAGUAUGUTT |
| RNF5-homo-904 | GGAGGAUGGAUUGAGAGAATT |
| RNFT1-homo-1112 | GGGCAUCUGAGAACUUUCATT |
| RNFT1-homo-1284 | GCAUGACCUUAUGGUUUAATT |
| RNFT1-homo-371 | GCAAGCUCCAGAAAUAUAATT |
| RPSA-homo-755 | GGAGGAAUUUCAGGGUGAATT |
| RPSA-homo-163 | GCACCAAUCUUGACUUCCATT |
| RPSA-homo-586 | CUCACUCAGUGGGUUUGAUTT |
| SYVN1-homo-652 | CCAUCUUCAUCAAGUAUGUTT |
| SYVN1-homo-1726 | GGAGACUGCCACUACAGUUTT |
| SYVN1-homo-943 | GCUCCAGGCAAUGGACAAUTT |
| TRAIP-homo-602 | GGAGCAGAUUGAGCUUCUATT |
| TRAIP-homo-1101 | CAGCAUGGUUACUACGAAATT |
| TRAIP-homo-813 | CAGACAGUCUACUCUGAAUTT |
| TRIM15-homo-893 | CGGAGAGAGAUGAGAUUGATT |
| TRIM15-homo-1247 | CUCCUGACCUUGUCAAGAATT |
| TRIM15-homo-956 | CUCAGAUCGAAAGCAAGAATT |
| TRIM2-homo-390 | GCGCUCCAGAACAAUUUCUTT |
| TRIM2-homo-689 | GCUCCCAGAAAUAGAUUCUTT |
| TRIM2-homo-1637 | GGUAGCUGCAUCUACAAAUTT |
| TRIM23-homo-299 | GCCCAUUUGAUCGACAAGUTT |
| TRIM23-homo-1377 | GCCCAUUCCAACAAUUGGUTT |
| TRIM23-homo-168 | GCUAGAGUGUGGAGUUUGUTT |
| TRIM24-homo-1261 | GCUGGACUCUCUAAACAAUTT |
| TRIM24-homo-779 | GAGCUCAUCAGAGGGUAAATT |
| TRIM24-homo-2095 | GACUGUUCAAGUACUAUUATT |
| TRIM27-homo-1251 | GCAGUCAGAUAUGGAGAAATT |
| TRIM27-homo-1578 | GGCAGUGUCUUUGUGGUAUTT |
| TRIM27-homo-918 | GCAGCUGUAUCACUCCUUATT |
| TRIM28-homo-1828 | CUGAGGACUACAACCUUAUTT |
| TRIM28-homo-1624 | GCAGGAAGGCUAUGGCUUUTT |
| TRIM28-homo-2385 | CCAACCAGCGGAAAUGUGATT |
| TRIM3-homo-2589 | CUGGCAACCACUGCUUUAATT |
| TRIM3-homo-1534 | CCACAAGAAUGGCACAUAUTT |
| TRIM3-homo-2311 | GGACUUCCAUAACCAUUCATT |
| TRIM32-homo-687 | CCUUCAGGCAAGGUAUAAATT |
| TRIM32-homo-1296 | GACCAGUCAAGGUGAAGUATT |
| TRIM32-homo-403 | GUGCUAAAGAUCAUUGAUATT |
| TRIM34-homo-629 | GGAAGCUGAGAAGCUGGAATT |
| TRIM34-homo-728 | GCUUAGAAGCAUCCUAAAUTT |
| TRIM34-homo-1060 | GGGUGGAUGUCACACUGAATT |
| TRIM35-homo-965 | GCUUGCAUCUGUGGAAUCUTT |
| TRIM35-homo-646 | GCAGGAGUUUGAUAAGCUUTT |
| TRIM35-homo-1529 | GCACAUCAGUGUCAAGGAATT |
| TRIM37-homo-1712 | GCUGCACAGACUAGUUAUATT |
| TRIM37-homo-619 | GCUACGAGAACUAGUAAAUTT |
| TRIM37-homo-1030 | GCUGAAGAAUAAGCUUAUATT |
| TRIM38-homo-1064 | GUCCACAGCAAUGCGAAUATT |
| TRIM38-homo-1937 | CCAGGUUUAUCAAUAUUCUTT |
| TRIM38-homo-775 | GGGCUCCAUUUCAUAUGGATT |
| TRIM46-homo-492 | GCUGCUUAAGUCAGGCUUUTT |
| TRIM46-homo-579 | CCAAGGUGAUGUGGAGCUUTT |
| TRIM46-homo-903 | CCAACGCCUGGUAUGUCAATT |
| TRIM47-homo-1351 | GCUGUUUGGAACCAAAGGUTT |
| TRIM47-homo-1092 | GGAGCUCAGCUUCACCAAATT |
| TRIM47-homo-1610 | GCUUCUCCGUCUGGUUUCATT |
| TRIM5-homo-1442 | CCUGAUGCAAUGUGUAAUATT |
| TRIM5-homo-1349 | GGCUCUCAAAGUAUCACAUTT |

**TABLE 1—*continued***

| | Sense (5′–3′) |
|---|---|
| TRIM5-homo-1298 | GGGACAAGAUACCAGACAUTT |
| TRIM54-homo-854 | GGCACAAACACAACUUACUTT |
| TRIM54-homo-915 | CCUCGCAGAACACUAAGAUTT |
| TRIM54-homo-1236 | CCAUGGAAGAGCCACAAAUTT |
| TRIM65-homo-1044 | GGCAGAAUUAUCGCAAUCUTT |
| TRIM65-homo-1083 | CCAACCGUCACUUCUAUCUTT |
| TRIM65-homo-627 | GCUACAGGCCCUGGAAAUATT |
| TRIM68-homo-1058 | GCUGGAUGUUGCAGGAUAUTT |
| TRIM68-homo-619 | GAGGAUGUCUUGAUAAUGUTT |
| TRIM68-homo-731 | CCCUCGAACAUCUGAAGAATT |
| TRIM74-homo-1016 | GGUUCCUCCAUUCAGCUUATT |
| TRIM74-homo-1057 | CCUAUAGCUGGCUCUAUAATT |
| TRIM74-homo-1173 | GCAGAAGUUCACUCAAGCUTT |
| VPS11-homo-622 | GGGCAACUAUCCUGUAACUTT |
| VPS11-homo-909 | GAGGCUACCUUAUCAUUGUTT |
| VPS11-homo-535 | GGCCAUUGGUUUCACAGAUTT |
| VPS41-homo-1308 | CUGGGAAUAUGAAGUUUAUTT |
| VPS41-homo-1453 | GCCACAUUGAUCCGAGAAUTT |
| VPS41-homo-2349 | GCACCGAACUCAAAUGAAATT |

ing 37 E2s and 64 UBE2U-interacting E3s were purchased from GenePharma (Table 1). siRNAs for cell depletion of UBC13, RNF8, and RNF168 were described previously (31, 56). Sequences for siRNAs that target UBE2U were as follows: siUBE2U-1, 5′-GCUCCUCCAGUUGUGAAAUTT-3′; siUBE2U-2, 5′-GCCACAGAAUACUACAGAATT-3′. Sequences of siRNAs that targeted RNF17 were as follows: siRNF17-1, 5′-GAGGAACAAUGGGAAAUAATT-3′; siRNF17-2, 5′-GAACAUAGCAAGAGCAUUAUU-3′.

*Antibodies*—Antibodies against γH2AX, FK2, UBC13, RNF8, RNF168, and MDC1 were described previously (4, 31, 57). Anti-FLAG (M2) and anti-actin were from Sigma. Anti-BRCA1 (D9) was purchased from Santa Cruz Biotechnology. Anti-HA antibodies were from Covance. The phosphospecific antibody against histone H3-Ser(P)$^{10}$ was purchased from Cell Signaling Technology. Anti-KAP1 was from BD Biosciences.

*Immunofluorescence Staining*—To detect IRIF, cells were irradiated (10 Gy) 48 h post-siRNA transfection. Cells were subsequently fixed with 3% paraformaldehyde at room temperature for 15 min. Permeabilization was performed using 0.5% Triton X-100 solution for 1 min prior to standard immunostaining procedures. Images were acquired using an Olympus BX51 fluorescence microscope. The number of 53BP1 foci per cell was counted using Imaris software.

*$G_2$/M Checkpoint Assay*—U2OS cells were treated with the indicated siRNAs twice and subjected to IR treatment (2 Gy) or left untreated. 1 h post-IR, cells were trypsinized and fixed with 70% ice-cold ethanol. To analyze the mitotic cell population, cells were stained with primary antibody against histone H3-Ser(P)$^{10}$ followed by incubation with FITC-conjugated goat anti-rabbit antibodies (Jackson ImmunoResearch Laboratories). DNA content was measured by propidium iodide staining, and percentage of histone H3-Ser(P)$^{10}$-positive cells was determined by flow cytometric analysis on a BD Biosciences LSR Fortessa analyzer.

*RNA Isolation and Real Time PCR Analyses*—U2OS cells were treated with the indicated siRNAs twice. 48 h after the second transfection, cells were collected by trypsinization. Total RNA was isolated using TRIzol reagent (Invitrogen, Life Technologies) following the manufacturer's protocol. The concentration and purity of RNAs were measured by NanoDrop

2000 (Thermo Scientific) and agarose gel electrophoresis, respectively. 1 $\mu$g of RNA for each sample was reverse transcribed into cDNA using SuperScript® II reverse transcriptase (Invitrogen, Life Technologies) following the manufacturer's protocol. Expression of RNF168 mRNAs was determined by real time PCR. cDNA samples were amplified using iQ™ SYBR® Green Supermix on a Bio-Rad iQ5 following the manufacturer's protocol. Results were derived from three independent experiments, each performed in triplicates. Real time PCR primers for RNF168 and GAPDH (control) were as follows: RNF168 RT1-forward, 5′-AATCTCAGTTTGGGTCAGCC-3′; RNF168 RT1-reverse, 5′-TGGAGAAAGTGTCGGCAT-ATC-3′; RNF168 RT2-forward, 5′-AGTTCGTCTGCTCAGT-AAACC-3′; RNF168 RT2-reverse, 5′-CTTCTTCCTCCTC-TGCCAAC-3′; GAPDH-forward, TGATGACATCAAGAAG-GTGGTGAAG-3; GAPDH-reverse, 5′-TCCTTGGAGGCCA-TGTAGGCCAT-3′.

*Clonogenic Survival Assay*—U2OS cells were transfected with siRNAs against UBE2U or RNF168 or control siRNA twice. 48 h after the second transfection, 500 cells were plated onto 60-mm dishes and treated with different dosages of IR. Cells were allowed to grow for 14 days and subjected to Coomassie Brilliant Blue staining before colony counting.

*Author Contributions*—M. S. Y. H. and S. M. H. S. conceptualized and supervised the project. Y. G. and L. A. performed all the experiments with help from H.-M. N. and S. M. H. S. M. S. Y. H., S. M. H. S., and Y. G. analyzed the data. Y. G. and M. S. Y. H. drafted the manuscript. H.-M. N. designed the schematic illustration. All authors reviewed the results and approved the final version of the manuscript.

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