



Title	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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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 pro-

teins, 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 γ H2AX) (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 Lys⁶³-based ubiquitin species (16) and that depletion of either RNF168 or UBC13 suppressed Lys⁶³-ub at IRIF led to the original proposal that RNF168 may pair with the E2 ubiquitin-conjugating enzyme UBC13 to catalyze Lys⁶³-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.

Role of the RNF17-UBE2U Axis in DNA Damage Responses

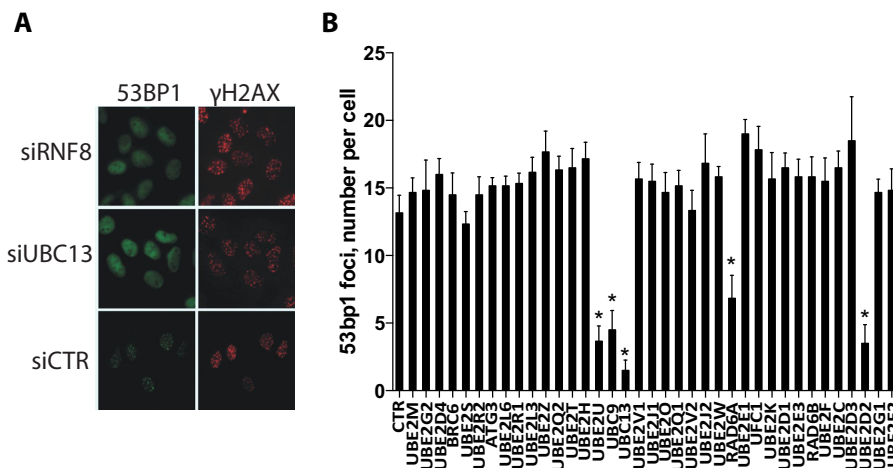


FIGURE 1. RNAi screen identifies novel E2 players in the DDR. *A* and *B*, HeLa cells transfected with non-targeting siRNAs (*siCTR*), pooled siRNAs designed to target each of the human E2s (see Table 1), or those that targeted RNF8 (*siRNF8*) were irradiated (10 Gy). 4 h post-IR treatment, cells were fixed, permeabilized, and immunostained with antibodies against 53BP1 and γ H2AX. 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 Lys⁶³-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 G₂/M checkpoint following IR challenge (Fig. 3, *A* and *B*). Given our observations that UBE2U may regulate DSB

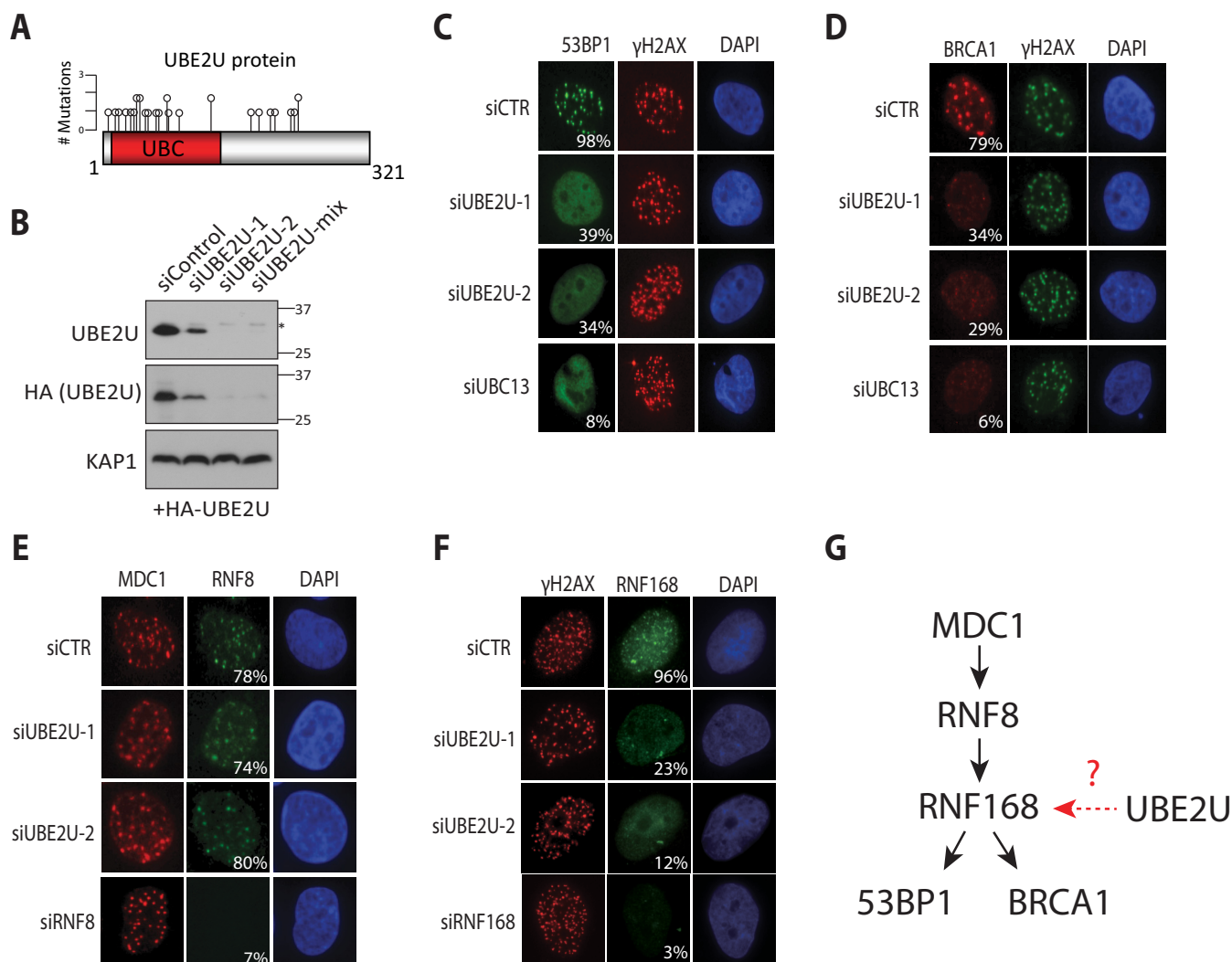


FIGURE 2. UBE2U is a novel factor in DNA damage responses. *A*, schematic illustration of UBE2U protein domain organization and cancer-associated mutations on the UBE2U coding sequence (adapted from cBioPortal). *B*, Western blotting analysis of RNAi-mediated UBE2U silencing. Cells were transfected with 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 γ H2AX 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. 2G) and that UBE2U depletion led to marked reduction in RNF168 expression (Figs. 2F and 3C), 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. 3C), evaluated cell sensitivity to IR using the clonogenic survival assay, and assessed the integrity of the G₂/M checkpoint by measuring cell arrest at the G₂/M border following exposure to IR-induced DNA damage. Intriguingly, although RNF168-depleted cells were much more sensitive to IR treatment (Fig. 2D), UBE2U silencing consistently impaired the G₂/M checkpoint to greater extents than that in

RNF168 knockdown cells (Fig. 2E). 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. 4A) (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

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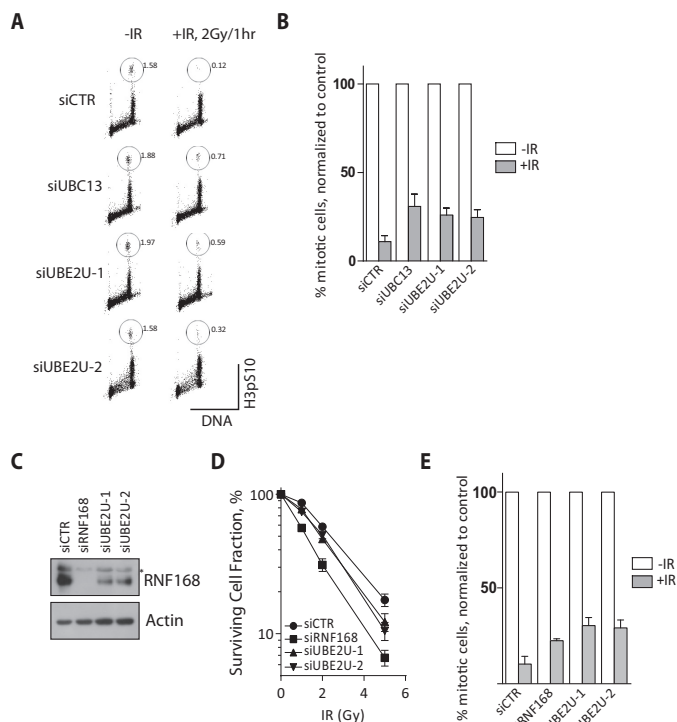


FIGURE 3. UBE2U promotes cell responses to IR. *A* and *B*, U2OS cells transfected with the indicated siRNAs were either left untreated or irradiated (2 Gy). Cells were fixed 1 h post-IR and immunolabeled with anti-H3-Ser(P)¹⁰ antibodies. Cells were subjected to flow cytometry-based analyses (*A*), and 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 G₂/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 Mmp-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

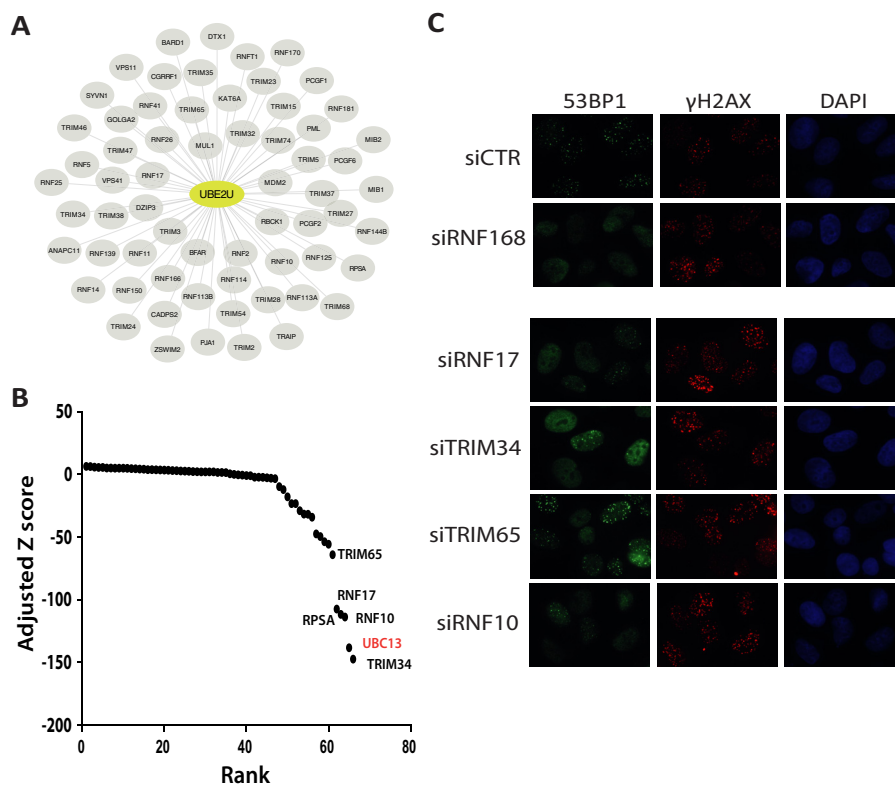


FIGURE 4. **Screen of UBE2U-interacting E3s identifies novel regulators of DSB responses.** *A*, network illustration of interactions between UBE2U and its potential E3 partners (35, 36). *B* and *C*, U2OS cells transfected with an E3-targeting siRNA library or the indicated siRNAs were irradiated (10 Gy). 4 h post-IR treatment, cells were fixed, permeabilized, and immunostained with antibodies against 53BP1 and γ H2AX, 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

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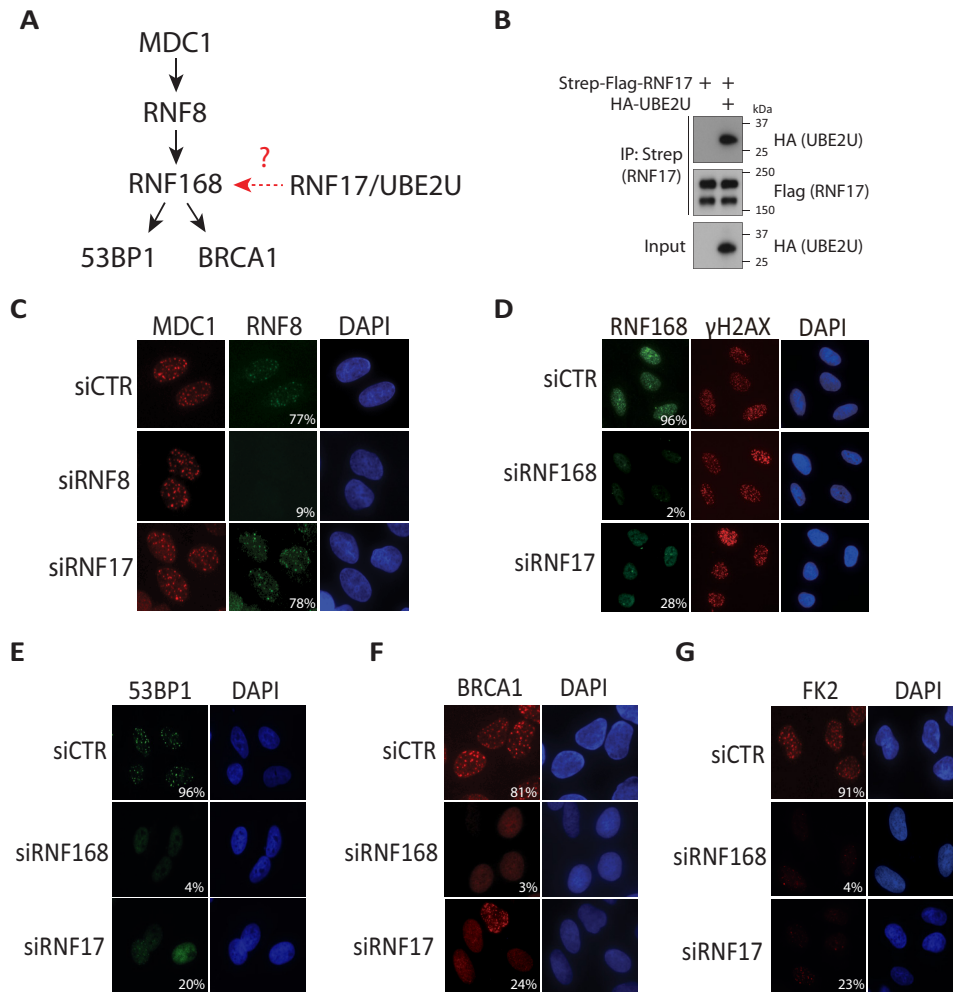


FIGURE 5. RNF17 is required for DSB retention of BRCA1 and 53BP1. *A*, schematic depicting the canonical DSB signal transduction pathway where the RNF17-UBE2U pair may play a role at the RNF168 level (see text). *B*, UBE2U interacts with RNF17. 293T cells were transfected with constructs encoding HA-UBE2U 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 γ H2AX (*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

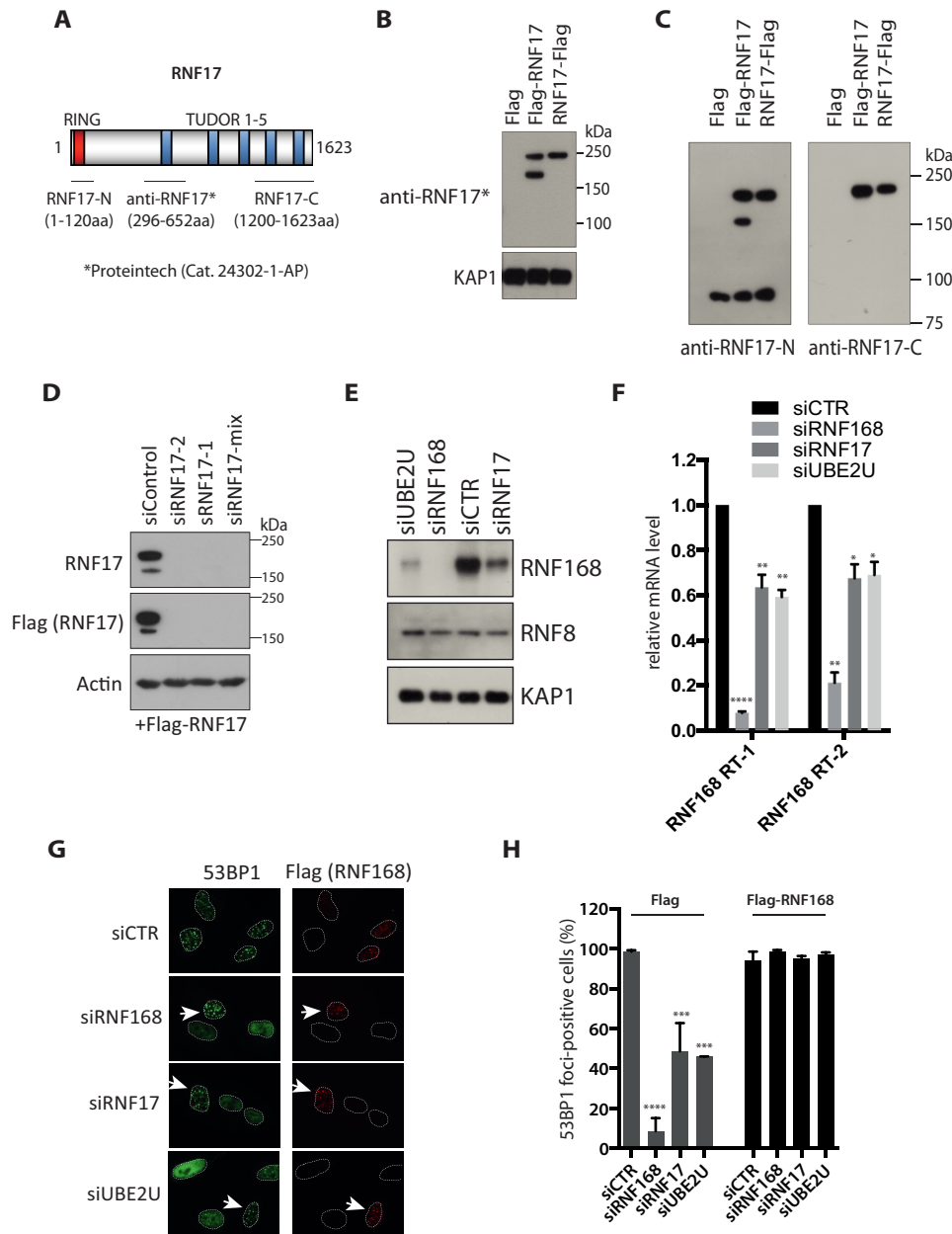


FIGURE 6. RNF17 regulates RNF168 expression. *A*, schematic depicting RNF17 protein domain organization and the epitopes that are recognized by anti-UBE2U antibodies. *B* and *C*, Proteintech or in-house anti-UBE2U antibodies specifically recognized ectopically expressed RNF17. *D*, validating RNAi-mediated 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 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 biol-

ogy 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

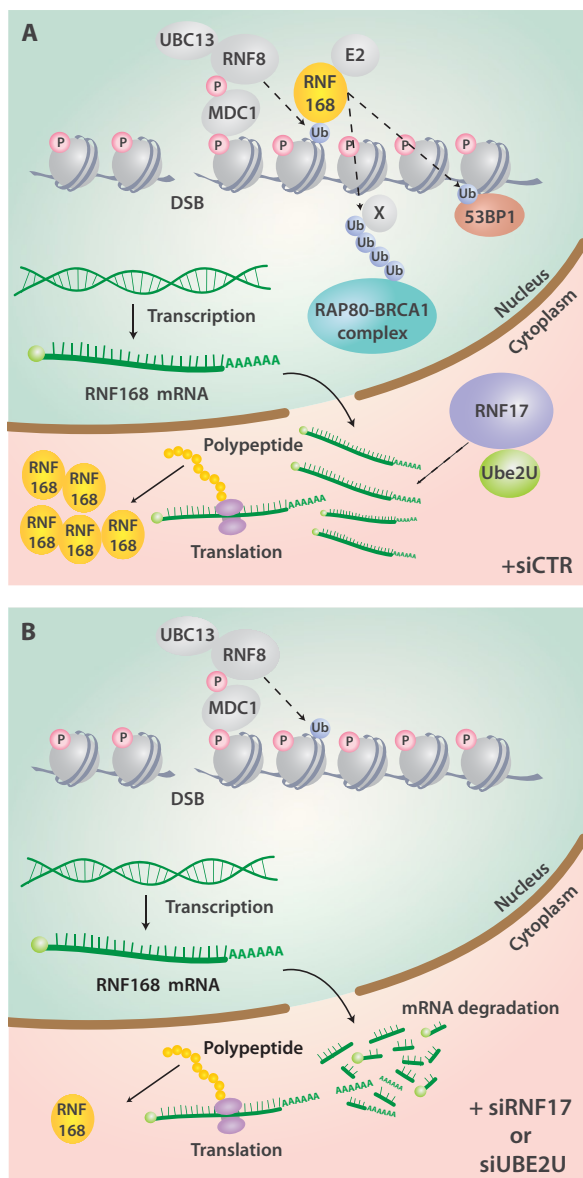


FIGURE 7. Working model depicting the role of UBE2U and RNF17 in RNF168-dependent DSB responses. A, RNF17 and UBE2U may suppress biogenesis of secondary piRNAs. RNF168 is expressed and catalyzes chromatin ubiquitylation at DSBs to assemble 53BP1 and BRCA1. B, silencing of RNF17 or UBE2U leads to down-regulation 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% CO₂.

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

E2s	Sense (5'–3')
UBE2A-homo-257	CGUCCGAGAACAACAUAUUTT
UBE2A-homo-374	CACCUACAGUUAGAUUUGUTT
UBE2A-homo-468	CCAACCUAUGAUGUGUCUUTT
UBE2A-homo-569	GGAGAACAACCGGAAUAUUTT
UBE2B-homo-517	GCAGUGGAAUGCAGUUUAUATT
UBE2B-homo-447	GGGAUUUCAAGCGGUUACATT
UBE2B-homo-794	GCACAGCUUUUACAGGAAATT
UBE2B-homo-668	GGUAGCAUAUGUUUAGAUATT
UBE2C-homo-204	CAUGAUGUCUGGCGAUAAATT
UBE2C-homo-574	CUCGAAGAAACCUACUCAATT
UBE2C-homo-490	CCAAGAUUGCUUUCACAATT
UBE2C-homo-282	GGAGCAGCUGGAACAGUAUUTT
UBE2D1-homo-229	CCUGAAGAGGAUUCAGAAATT
UBE2D1-homo-416	CCAAGAUUGCUUUCACAATT
UBE2D1-homo-504	CCAGCUCUGACUGUAUCAATT
UBE2D1-homo-579	GUACCAGAUUUUGCACAATT
UBE2D2-homo-868	GGCAGCAUUUGCUUUGAUATT
UBE2D2-homo-642	CCACAAGGAAUUGAAUGAUUTT
UBE2D2-homo-1048	GGACUCAGAAGUAUGCGAUUTT
UBE2D2-homo-756	CCCUUUCAGGGGAGUAUUTT
UBE2D3-homo-486	GGCGCUGAAACGGAUUAUUTT
UBE2D3-homo-727	GGCAGCAUUUGUCUGAUUUTT
UBE2D3-homo-599	GACCUAAUGACAGCCCAUUTT
UBE2D3-homo-869	CAGACAGAGUAAGUACAATT
UBE2D4-homo-483	CCGACAGAGAGAAUGACAATT
UBE2D4-homo-342	GCAGCAUCUGCCUUGAUUUTT
UBE2D4-homo-213	GUCCUUACCAAGGAGGUGUTT
UBE2D4-homo-2653	GGGACUGAUUCAUGUJUUTT
UBE2E1-homo-444	GGCGAUAAUCAUCUAUGAAUUTT
UBE2E1-homo-738	CCACUCAGUAUAUGACCAATT
UBE2E1-homo-645	GGAGUCCAGCACUAACCAUUTT
UBE2E1-homo-556	GCCUCCAAGGUUACAUIUUTT
UBE2E2-homo-510	CACCAGACUAUCCGUUUAUUTT
UBE2E2-homo-623	CCGGCUUUAAACUAUUUCUUTT
UBE2E2-homo-325	CGCUGCUAAAUAUGUCAUUTT
UBE2E2-homo-713	CCACACAGUACAUGACCAATT
UBE2E3-homo-845	CCCGCUUUAGACUAUUUCAATT
UBE2E3-homo-583	GAAGGAGCUGAGCUGAAUUTT
UBE2E3-homo-985	GACCAAGAGAUACGCAACATT
UBE2E3-homo-1089	GGACUUCUGUGUAUAUGUUTT
UBE2F-homo-482	GUGCCUCCAAGUGAAUUTT
UBE2F-homo-205	GCUAACGCUAGCAAGUAAUUTT
UBE2F-homo-290	GGGUUUCUGUGAGAGACAATT
UBE2F-homo-711	GGAGGACUCCGGAUUAUUTT
UBE2G1-homo-487	GCCUUCAGAUACACUUUUAUTT
UBE2G1-homo-593	GCACCCAAUUGUUAUUAUUTT
UBE2G1-homo-395	CGUGGCAGAACUCAACAATT
UBE2G1-homo-695	CCUAUCCACACUGUGGAAUUTT
UBE2G2-homo-267	CCACUUGAUUACCCGUUAUUTT
UBE2G2-homo-470	GCAGAGCCCAAUGACGAAUUTT
UBE2G2-homo-112	GCUGAUGCCGAGUAACAATT
UBE2G2-homo-345	GGAGAGUCUGCAUUUCAUUTT
UBE2H-homo-545	GGCGGAGUAUGGAAUGUUTT
UBE2H-homo-730	GCCUCAGUUUAUGGCUUUAUTT
UBE2H-homo-896	GCUCAUCGAGAGCUCUAUUTT
UBE2H-homo-481	GUAUCCUGGAGGACUUAUUTT
UBE2I-homo-550	GAGGCCUACACGAUUUACUUTT
UBE2I-homo-257	GCAGGAUGAACCUAUGAAUUTT
UBE2I-homo-325	GCUUUGUUAAACUACGGAUUTT
UBE2I-homo-194	GAGGAAAGCAUGGAGGAAUUTT
UBE2J1-homo-636	CAGCCUUCGUGGAGUAUUAUTT
UBE2J1-homo-1034	GCAUCCUUUCAUACACCUAUTT
UBE2J1-homo-868	CUAGGCAAAUAAGCUUUAUUTT
UBE2J1-homo-421	CGCAGCCUUUAGAGGAUUAUTT
UBE2J2-homo-440	CCAGAGAAUUUCCUUUCAUUTT
UBE2J2-homo-237	GCUGAAGCAGGACUACCUUUTT
UBE2J2-homo-662	GCAGUGCAGAGUUUAGCAUUTT
UBE2J2-homo-754	GCACAAGACGAACUCAGUAUUTT
UBE2K-homo-498	GGUCCGGUUUAUCACUAUUAUTT
UBE2K-homo-857	CUGCAACAGAAUUGCUUCUUTT
UBE2K-homo-3572	GCAGUUUAGUGGACCCAAUUTT
UBE2K-homo-604	GCACGGUAUUUAUUGCAUUTT
UBE2L3-homo-369	CACCGAAGAUCACAUUUAUUTT
UBE2L3-homo-250	CCAGGUUGAUGAAGCUAAUUTT
UBE2L3-homo-437	CAGUAUUUAUGGCGGAAUUTT
UBE2L3-homo-2934	GGCAGAGAAUAGGCUUUCUUTT
UBE2L6-homo-424	GCUGUGGAAUAGACCGAAUUTT
UBE2L6-homo-287	CCUCCGAUGAUCAAUUAUUTT
UBE2L6-homo-1161	CUCAGACUGUGAAGUAUUAUTT

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TABLE 1—continued

	Sense (5'–3')
UBE2L6-homo-165	GAACCUGUCCAGCGAUGAUAATT
UBE2M-homo-954	CCAGUCCUUACGAUAAACUATT
UBE2M-homo-875	GUGUGAGACAAUGGUCUAUATT
UBE2M-homo-918	GCAACGUCUGCCUCAACAUAATT
UBE2M-homo-723	CCCAAGACGUGUGUAUAUACATT
UBE2N-homo-877	CCUCUUUGUUUGCAUUUAATT
UBE2N-homo-613	GGGAAGAAUAUGUUUAGAUAATT
UBE2N-homo-2109	GUCCUCAGUUAAUGAUUUCUATT
UBE2N-homo-2516	CUGGUAUCCUCCAAUAUAATT
UBE2O-homo-962	GCAGGUUGUAGAGUUGAAUATT
UBE2O-homo-2001	GCUAGACCACCCUGACUUUAATT
UBE2O-homo-3064	GCCUCUACUUGUUUGACAUAATT
UBE2O-homo-3750	CCACCCAGUGUGAAACCAUATT
UBE2Q1-homo-679	GGACACAGAAGACUUAGAUAATT
UBE2Q1-homo-1000	CCAGAUCUCAAAGAGAAUATT
UBE2Q1-homo-1185	CCAUAAGAGUCAGUGAUCAUATT
UBE2Q1-homo-493	GCUUGGGACAUAAAGAAUATT
UBE2Q2-homo-677	GCAAUUGAAGUGGUUGAUATT
UBE2Q2-homo-1358	GCAAAUAAAUGCCACCUUATT
UBE2Q2-homo-734	GGAUGUUGAGAUGCUAGAUAATT
UBE2Q2-homo-576	CCAUCUUCACCCGAAUUAATT
UBE2R1-homo-435	CCAAGAUGUGGCCACCUAATT
UBE2R1-homo-550	GCAGAACGUCAGGACCAUUAATT
UBE2R1-homo-675	GGAGUACACAGACAUAUAATT
UBE2R1-homo-1400	CUGCUUUGGUUUUGUAUAATT
UBE2R2-homo-610	CCUGAUGCUCGAGCUGAAUATT
UBE2R2-homo-741	GCUAUCUCAAAGGCGCAUAUATT
UBE2R2-homo-930	GUGAGGACUAUCCUAUAUAATT
UBE2R2-homo-1158	GUGCCUUCCAAUGACAACUATT
UBE2S-homo-451	CCGAUGGCAUCAAGGUCUUAATT
UBE2S-homo-536	GAGGUCUGUUCGCAUGAUAATT
UBE2S-homo-598	GCUACUUCUGACCAAGAUAATT
UBE2S-homo-700	ACGUAUCUGCUGACCAUAUATT
UBE2T-homo-684	CCAGUCAGCUAGUAGGCAUUAATT
UBE2T-homo-233	CCUGCGAGCUCAAAUAUAUUAATT
UBE2T-homo-738	CCUGGUUCAUCUUAGUUUAATT
UBE2T-homo-510	GCAGUCAUAUCCUCAGAAUUAATT
UBE2U-homo-440	GCUCCUCCAGUUGUGAAUAUUAATT
UBE2U-homo-604	GCUAAGAAUCCAGUGAAUUAATT
UBE2U-homo-815	GCACAGAAUACUACAAUAUUAATT
UBE2U-homo-898	GCAUCAGAAAGAAUGGAAUUAATT
UBE2V1-homo-751	GCGCCUAAUGAUGUCUAAUUAATT
UBE2V1-homo-492	GACGAAGCAUGACACUAUUAATT
UBE2V1-homo-1409	GGCCGAAGCAUAGAUUGUAUUAATT
UBE2V1-homo-799	GACAGUGUUACAGCAAUUAUUAATT
UBE2V2-homo-155	GGUGGACAGGCAUGAUUAUUAATT
UBE2V2-homo-430	CCACCAGAAGGACAAACAUAUUAATT
UBE2V2-homo-249	CUCCGUCAGUUAGAUUUUGUUAATT
UBE2V2-homo-1201	GACUGGCCAUUUUCUAUAUUAATT
UBE2W-homo-215	GGUGCACCAGGUACCUUAUUAATT
UBE2W-homo-323	CCUGUUAUCCUCAUGUUUAUUAATT
UBE2W-homo-484	GCGAAUAUGUAACAAGAAUUAATT
UBE2W-homo-100	GCAGAAACGACUACAGAAUUAATT
UBE2Z-homo-683	GCAAUUGGAAAGUCUGCUUAUUAATT
UBE2Z-homo-755	CCUCAGUGCUCUUCUUAUUAATT
UBE2Z-homo-450	GGGAUAUCAUGUCCAUAUUAATT
UBE2Z-homo-1111	GAGGCUCAUAAUGAGAAUUAATT
ATG3-homo-424	GCGGAUGGGUAGAUACAUAUUAATT
ATG3-homo-545	GGAAGAAGAUGAAGAUAUUAATT
ATG3-homo-254	CCACUGUCCAACAUGGCAUUAATT
ATG3-homo-176	CCUCAAGGAAUCAAAAGUUUAATT
BIRC6-homo-1428	GCAAUUGUACAACAGCUUAUUAATT
BIRC6-homo-2777	GCAGUUAACCCUCAAAGAAUUAATT
BIRC6-homo-6793	GCAUAGCCUUGAUAGAUUAUUAATT
BIRC6-homo-10755	GCUGUUGACAGUCUACUUAUUAATT
UFC1-homo-470	CCAUGACCUCUGAAUAUAUUAATT
UFC1-homo-601	GCCUGACGGUAUCAUUUAUUAATT
UFC1-homo-376	GGUAUGUGGAGAACAACAUAUUAATT
UFC1-homo-722	GGGGUCAUCCAACAACAUAUUAATT
E3s	
ANAPC11-homo-278	CAUGGAGAAAGCAUUUCUUAATT
ANAPC11-homo-466	GACCAGUUCACUACUCAGATT
ANAPC11-homo-409	GUCUAGGGAAGAGUCUUAUUAATT
BARD1-homo-1282	GUCGGAUGAAUUCUUUAUUAATT
BARD1-homo-1795	GCUAGCCACUGCUCAGUAUUAATT
BARD1-homo-541	GCAAGAAACAAGAAUAUUAATT
BFAR-homo-1460	GGAGCAUUUCUGGAAUUAUUAATT
BFAR-homo-1002	GGAGCUAGAACGUGUCAAAUUAATT
BFAR-homo-351	CCUCAGAUUUUCUGUAUUAUUAATT
CADPS2-homo-1022	GCCUAGGUAUGCCAAUAUUAATT
CADPS2-homo-2028	GCAGCUUGAAGGCUAUAUUAATT

TABLE 1—continued

	Sense (5'–3')
CADPS2-homo-2183	CCACAGGUCAAUCAUAUAUUAATT
CGRRF1-homo-740	GGCUCAAAGGUCAAUUUAUUAATT
CGRRF1-homo-639	GACCCGGAAUUUUUAUGUAUUAATT
CGRRF1-homo-545	CCAGUUACCAAGAGUAUAUUAATT
DTX1-homo-2127	CUAGCUACCUAGACAACGUAUUAATT
DTX1-homo-564	GCAGUCCAUAGCACCAGUUUAATT
DTX1-homo-1906	GGGAAGAAGUUCACCGCAUUAATT
DZIP3-homo-1823	GCCUCCUGUAGCUCUUUAUUAATT
DZIP3-homo-1529	GACCGAAGAUCAGUUUAUUAATT
DZIP3-homo-2582	GCCUGGAUGAAUUGCAUAUUAATT
GOLGA2-homo-1163	GUCGGUUAGACAACUAUUAATT
GOLGA2-homo-683	GAUCCUCGUAUCAGAGAAUUAATT
GOLGA2-homo-519	CUGGACUCCAGCUAUGUAUUAATT
KAT6A-homo-1397	GCGCUAUAUUAUUAUUAATT
KAT6A-homo-4972	CACCCUCUAUGCAGAAUAUUAATT
KAT6A-homo-3756	GCUGAUGACACUCCUAUUAATT
MDM2-homo-475	GGCCAGUAUUAUUAUUAATT
MDM2-homo-912	GGAGUAUUGUUGAAAGUAUUAATT
MDM2-homo-1489	CAGCCAUAACUUAUUAUUAATT
ZSWIM2-homo-1520	CAGGUAUCUUAAGAUUAUUAATT
ZSWIM2-homo-729	GGGAUUCUUAUUAUUAUUAATT
ZSWIM2-homo-1787	GAGCAUCCUUAUUAUUAUUAATT
MIB1-homo-883	GGGCAUGUCUGAUCUGAAUUAATT
MIB1-homo-883	CCGUAACAACAGAUUAUUAATT
MIB1-homo-883	GACGUGAUCUUAUUAUUAATT
MIB2-homo-2150	GUGCCAAACAUCGAUGUAUUAATT
MIB2-homo-2226	CGCUAGCUGUGAGAAUAUUAATT
MIB2-homo-1486	GGUGGUUAAAGUUGUAUUAATT
MUL1-homo-340	GCGUGCCUUAUGCUGUAUUAATT
MUL1-homo-801	GGCAUGCAGUAUUAUUAUUAATT
MUL1-homo-451	GCACAAGAUGGUGUAUUAUUAATT
PCGF1-homo-647	GUCACCGCUUGAUGCUAAUUAATT
PCGF1-homo-503	CCCACUAUCAUCGCUAUAUUAATT
PCGF1-homo-706	CCUGUACAUAGCAUAUUAATT
PCGF2-homo-839	GAGCCACUGAAGGAUAUUAATT
PCGF2-homo-774	CCAAGUUUCUCCGCAACAUAUUAATT
PCGF2-homo-636	CCGCUUUAUUAUUAUUAATT
PCGF6-homo-837	GGAGUUAUUGGUGCUAAUUAATT
PCGF6-homo-426	GCGCCUGAUUAUUAUUAUUAATT
PCGF6-homo-1005	GGAGCAUUAUUAUUAUUAATT
PJA1-homo-1368	GACCAAGUGAAACCAGAAUUAATT
PJA1-homo-2165	CAUCUGCUGAUGCAUAUUAATT
PJA1-homo-807	GGCAAGUUUAAGAUAUUAATT
PML-homo-682	CCCGCAAGCAACAACAUAUUAATT
PML-homo-884	GGAGCAGUAUUGCCUUAUUAATT
PML-homo-1534	GAGGAGUCUCCAUAUUAATT
RBCK1-homo-1150	GGUGCACCUCUAUUAUUAATT
RBCK1-homo-2070	CUGCCACUGAGCUAAAGUAUUAATT
RBCK1-homo-1900	AGAUCUGGUACAGAGAUAUUAATT
RNF10-homo-879	CUGGUCCUUAAGAAGUAUUAATT
RNF10-homo-1036	CUGCCAAUUUGGUGUCUUAATT
RNF10-homo-2776	CAGCCAAGCUUAUGAAGUAUUAATT
RNF11-homo-808	GGGACCAAAUUCGAUUUAUUAATT
RNF11-homo-595	CGCCAUUAUCGGAACAUAUUAATT
RNF11-homo-693	GCUCAAAGAAUAGGUCUUAUUAATT
RNF113A-homo-1222	GAGGAUGCAAAUUCUUAUUAATT
RNF113A-homo-497	GUCUCGGCGUGUUUAUUAATT
RNF113A-homo-396	GACCCAAUUAUUAUUAUUAATT
RNF113b-homo-597	GGGAUUAUUAUUAUUAUUAATT
RNF113b-homo-703	GGAGAUUUAACGGGAGCUUAUUAATT
RNF113b-homo-837	CCAAGUUGCAGGCAUUUAUUAATT
RNF114-homo-353	GUGGCUAUCUUAUUAUUAUUAATT
RNF114-homo-503	GUGGAACACUGCAAUAUUAATT
RNF114-homo-401	GCCACAAUUAAGGUAUUAUUAATT
RNF125-homo-839	GCUGGAUUAUUAUUAUUAUUAATT
RNF125-homo-719	GGCACAUAUUCGGACUUAUUAATT
RNF125-homo-934	GUGGCGAUUUUAUUAUUAUUAATT
RNF139-homo-1951	GGAGCCCUUAACAAGAAUUAATT
RNF139-homo-1162	GGGACCUAUAUUAUUAUUAATT
RNF139-homo-1271	GGCCUUUAUUAUUAUUAUUAATT
RNF14-homo-861	CCCUAUGCAUUAUUAUUAUUAATT
RNF14-homo-1682	GGAACUCCAUAGAGAAUUAATT
RNF14-homo-587	CCACAGAAUUUAUUAUUAUUAATT
RNF144b-homo-815	GAGACAGUCAGCCUUAUUAATT
RNF144b-homo-542	CUGACAUGGUGGCCUUAUUAATT
RNF144b-homo-976	GGUACUCCUCCAGAAUUAATT
RNF150-homo-1351	GAGGUUUUGAUAUUAUUAATT
RNF150-homo-1269	GCACUUCGGUUGUUAUUAATT
RNF150-homo-1605	GCAAGAUAACAUAUUAUUAATT
RNF166-homo-363	GACGUCUCAUUAUUAUUAATT
RNF166-homo-411	GACCCUGGCAAGAUAUUAATT

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TABLE 1—continued

	Sense (5'–3')
RNF166-homo-677	GCUACAAGAGCGCCAACUUTT
RNF17-homo-2793	GCCUGUGCAUAUCUGUAAUUTT
RNF17-homo-4174	GAGGAACAAUUGGAAAUAATT
RNF17-homo-4491	GCCUUGCCUUGCAGAAUAUUTT
RNF170-homo-1020	GGCAUGCAUUCAGGGAAAUUTT
RNF170-homo-800	GCCUGCAUUUAUUGCUUACUUTT
RNF170-homo-478	GGCCAAUAUCAAGGUGAATT
RNF181-homo-51	GGCGUCCUAUUUCGAUGAATT
RNF181-homo-440	GACGAGAUAAAGGCUCGAAATT
RNF181-homo-363	CCCUGGCCUAAAGCAAGACAATT
RNF2-homo-842	GGCUAGAGCCUUGAUAAAUAATT
RNF2-homo-1131	CCAGUUCACUGUAUUAAAUUTT
RNF2-homo-620	GGAUCAACAAGCACAAUAATT
RNF25-homo-251	GGACCAGGAUUUCACAGUAUUTT
RNF25-homo-208	CACCAUUGGGAGAUUCAUUTT
RNF25-homo-1021	CCCAGCACAUUUGUGAGAATT
RNF26-homo-1286	GCUUUGUGCUUUGCAAUCUUTT
RNF26-homo-1048	CUGGUGCCUUAUGUGAUCATT
RNF26-homo-800	GAGUCUUGCUUUCUUGCUTT
RNF41-homo-468	GGCACCUCUUAUGUGAACAUUTT
RNF41-homo-1184	GACGCUACUUAUGAGAUAUUTT
RNF41-homo-881	GAGACAUCAGCUGCUAAAATT
RNF5-homo-194	GACCUUCGAAUGUAAAUAUUTT
RNF5-homo-308	GCAAGAGUGUCCAGUAUGUUTT
RNF5-homo-904	GGAGGAUGGAUUGAGAGAATT
RNFT1-homo-1112	GGGCAUCUGAGAACUUUUCATT
RNFT1-homo-1284	GCAUGACCUUAUGGUUUUAATT
RNFT1-homo-371	GCAAGCUCCAGAAAUAUAATT
RPSA-homo-755	GGAGGAAUUUCAGGGUGAATT
RPSA-homo-163	GCCACCAUCUUGACUUCATT
RPSA-homo-586	CUCACUCAGUGGGUUUGAUTT
SYVN1-homo-652	CCAUCUUCUAAGAUAUGUUTT
SYVN1-homo-1726	GGAGACUGCCACUACAGUUTT
SYVN1-homo-943	GCUCACGGCAAUGGACAATT
TRAIIP-homo-602	GGAGCAGAUUGAGCUUCUATT
TRAIIP-homo-1101	CAGCAUGGUUACUACGAAATT
TRAIIP-homo-813	CAGACAGUCUACUCUGAUAUUTT
TRIM15-homo-893	CGGAGAGAGAUGAGAUUGATT
TRIM15-homo-1247	CUCUGACCUCUUGUCAAGAATT
TRIM15-homo-956	CUCAGAUUCGAAAGCAAGAATT
TRIM2-homo-390	GCGCUCCAGAACAAUUUCUUTT
TRIM2-homo-689	GCUCCCAGAAAUAUGAUUCUUTT
TRIM2-homo-1637	GGUAGCUGCAUCUACAAAATT
TRIM23-homo-299	GCCCAUUUGAUCGACAAGUUTT
TRIM23-homo-1377	GCCCAUUCACAAUUUGGUUTT
TRIM23-homo-168	GCUAGAGUGUGGAGUUUGUUTT
TRIM24-homo-1261	GCUGGACUCUCUAAAACAATT
TRIM24-homo-779	GAGCUAUCAGAGGGUAAUUTT
TRIM24-homo-2095	GACUGUUCAGUAUUAUUAATT
TRIM27-homo-1251	GCAGUCAGAUUUGGAGAAATT
TRIM27-homo-1578	GGCAGUGUCUUUGUGGUAUUTT
TRIM27-homo-918	GCAGUCUGUAUCACUUCUUAATT
TRIM28-homo-1828	CUGAGGACUACAACUUUAUUTT
TRIM28-homo-1624	GCAGGAAGGCUAUGGCUUUTT
TRIM28-homo-2385	CCAACCAGCGGAAAUGUGATT
TRIM3-homo-2589	CUGGCAACCCACUGCUUUAATT
TRIM3-homo-1534	CCACAAGAAUGGCACAUUAUUTT
TRIM3-homo-2311	GGACUUCUUAACCAUUCATT
TRIM32-homo-687	CCUUCAGGCAAGGUUAUAATT
TRIM32-homo-1296	GACCAGUCAAGGUGAAGUATT
TRIM32-homo-403	GUGCUAAAGAUCUUAUGAUAUUTT
TRIM34-homo-629	GGAAUCUGAGAAGCUGGAATT
TRIM34-homo-728	GCUUAGAAGCAUCCUAAAATT
TRIM34-homo-1060	GGGUGGAUGUCACACUGAATT
TRIM35-homo-965	GCUUGCAUCUGUGGAAUCUUTT
TRIM35-homo-646	GCAGGAGUUUGAUAGCUUUTT
TRIM35-homo-1529	GCACUACAGUGUCAAGGAATT
TRIM37-homo-1712	GCUCACAGACUAGUUAUUAATT
TRIM37-homo-619	GCUACGAGAACUAGUAAAATT
TRIM37-homo-1030	GCUGAAGAAUUAAGCUUAUUAATT
TRIM38-homo-1064	GUCCACAGCAAUGCGAAUATT
TRIM38-homo-1937	CCAGGUUUUAUCAUUAUUCUUTT
TRIM38-homo-775	GGGCUCCAUUUCAUUAUGGATT
TRIM46-homo-492	GCUGCUUUAAGUCAGGCUUUTT
TRIM46-homo-579	CCAAGGUGAUGUGGAGCUUUTT
TRIM46-homo-903	CCAACGCGUGGUAUGCAUUAATT
TRIM47-homo-1351	GCUGUUUGGAACCAAAAGGUTT
TRIM47-homo-1092	GGAGCUCAGCUUCACCAAAATT
TRIM47-homo-1610	GCUUCUCCGUCUGGUUUUUAATT
TRIM5-homo-1442	CCUGAUGCAAUGGUAAAUAATT
TRIM5-homo-1349	GGCUCUCAAAAGUAUCAUUAATT

TABLE 1—continued

	Sense (5'–3')
TRIM5-homo-1298	GGGACAAGAUACCAGACAATT
TRIM54-homo-854	GGCACAACACAACUUAUUAATT
TRIM54-homo-915	CCUCGCAAGAACACUAAGAUUTT
TRIM54-homo-1236	CCAUGGAAGAGCCACAAAATT
TRIM65-homo-1044	GGCAGAAUUUAUCGCAAUCUUTT
TRIM65-homo-1083	CCAACCGUCACUUCUAUUAATT
TRIM65-homo-627	GCUACAGGCCUGGAAAUAATT
TRIM68-homo-1058	GCUGGAUGUUGCAGGAUAUUAATT
TRIM68-homo-619	GAGGAUGUCUUGAUAAAUGUUTT
TRIM68-homo-731	CCCUGCAACAUCUGAAGAATT
TRIM74-homo-1016	GGUUCUCCAUUCAGCUUUAATT
TRIM74-homo-1057	CCUAUAGCUGGCUCUUAUAATT
TRIM74-homo-1173	GCAGAAGUUCACUCAAGCUUUTT
VPS11-homo-622	GGGCAACUUAUCCUGUAUUAATT
VPS11-homo-909	GAGGCUACCUUUAUCAUUAATT
VPS11-homo-535	GGCCAUUGGUUUCACAGAUUTT
VPS41-homo-1308	CUGGGAAUAUGAAGUUUAUUAATT
VPS41-homo-1453	GCCACAUGAUCGAGAAUUAATT
VPS41-homo-2349	GCACCGAACUCAAAUUAUUAATT

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'-GCUCCUCCAGUUGUGAAAATT-3'; siUBE2U-2, 5'-GCCACAGAAUACUACAGAATT-3'. Sequences of siRNAs that targeted RNF17 were as follows: siRNF17-1, 5'-GAGGA-ACAAUGGGAAAUAATT-3'; siRNF17-2, 5'-GAACA-AGCAAGAGCAUUUAU-3'.

Antibodies—Antibodies against γ H2AX, 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.

G₂M 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 μ 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 iQTM 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'-TGGAGAAAGTGTCCGCAT-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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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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