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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.

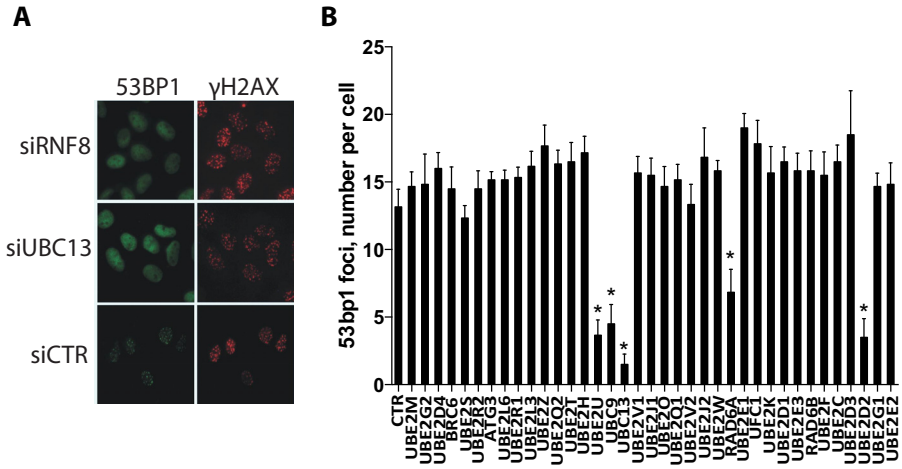

FIGURE 1. **RNAi screen identifies novel E2 players in the DDR.** *A* and *B*, HeLa cells transfected with non-targeting siRNAs (*siCTR*), pooled siRNAs designed to target each of the human E2s (see Table 1), or those that targeted RNF8 (*siRNF8*) were irradiated (10 Gy). 4 h post-IR treatment, cells 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

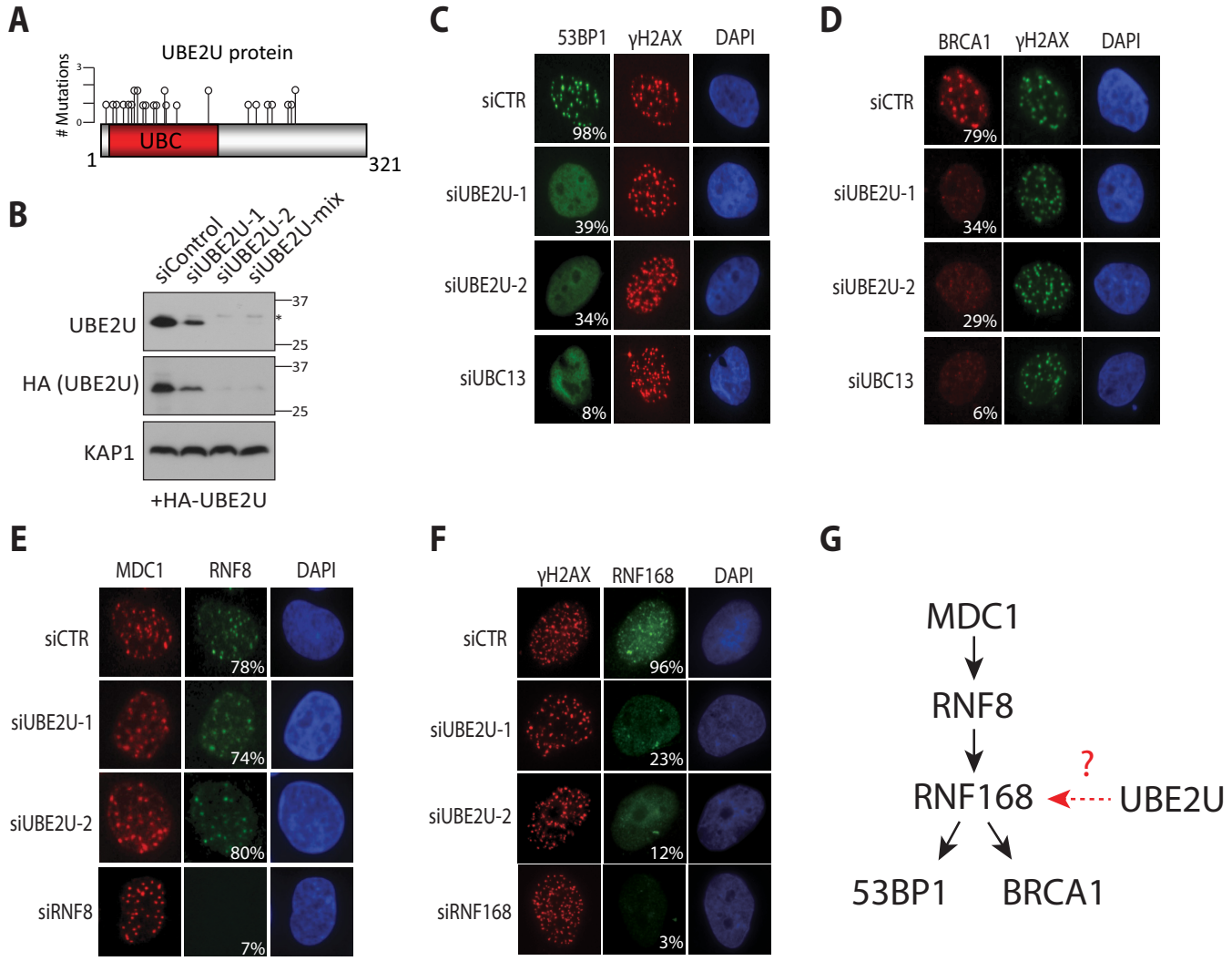

FIGURE 2. **UBE2U is a novel factor in DNA damage responses.** *A*, schematic illustration of UBE2U protein domain organization and cancer-associated mutations on the UBE2U coding sequence (adapted from cBioPortal). *B*, Western blotting analysis of RNAi-mediated UBE2U silencing. Cells were transfected 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

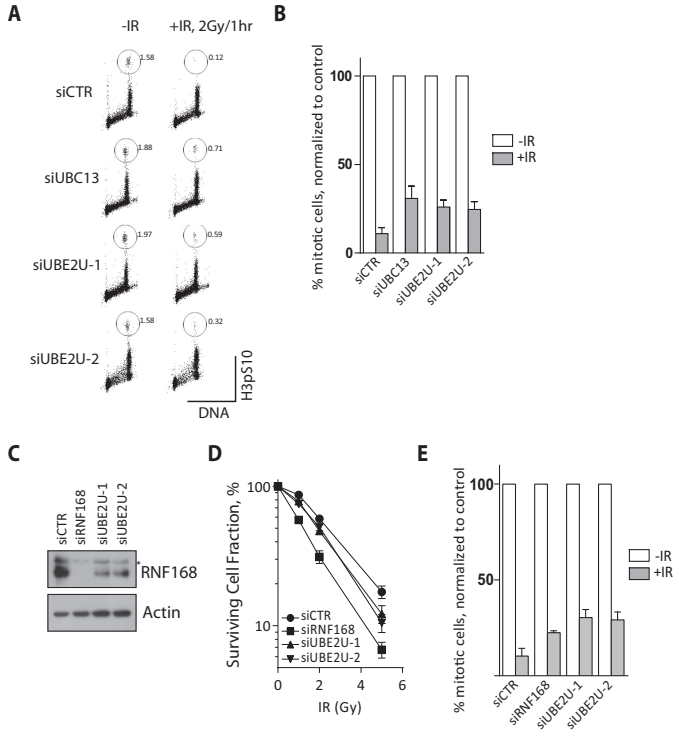

FIGURE 3. **UBE2U promotes cell responses to IR.** *A* and *B*, U2OS cells transfected with the indicated siRNAs were either left untreated or irradiated (2 Gy). Cells were fixed 1 h post-IR and immunolabeled with anti-H3-Ser(P)[10] antibodies. Cells were subjected to flow cytometry-based analyses (*A*),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

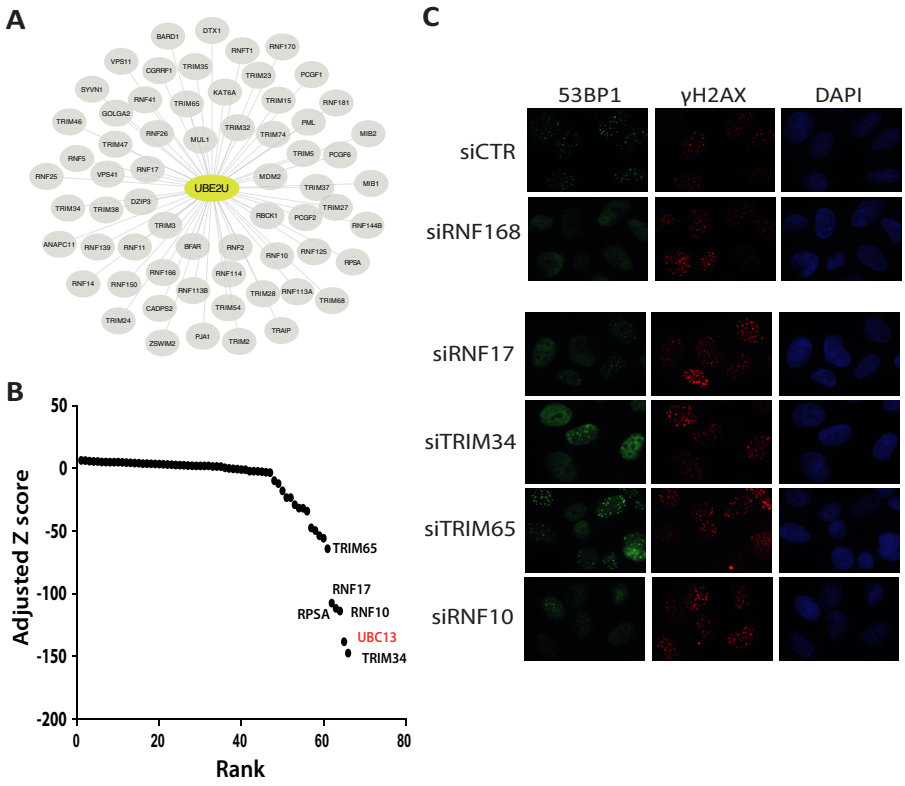

FIGURE 4. **Screen of UBE2U-interacting E3s identifies novel regulators of DSB responses.** *A*, network illustration of interactions between UBE2U and its potential E3 partners (35, 36). *B* and *C*, U2OS cells transfected with an E3-targeting siRNA library or the indicated siRNAs were irradiated (10 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

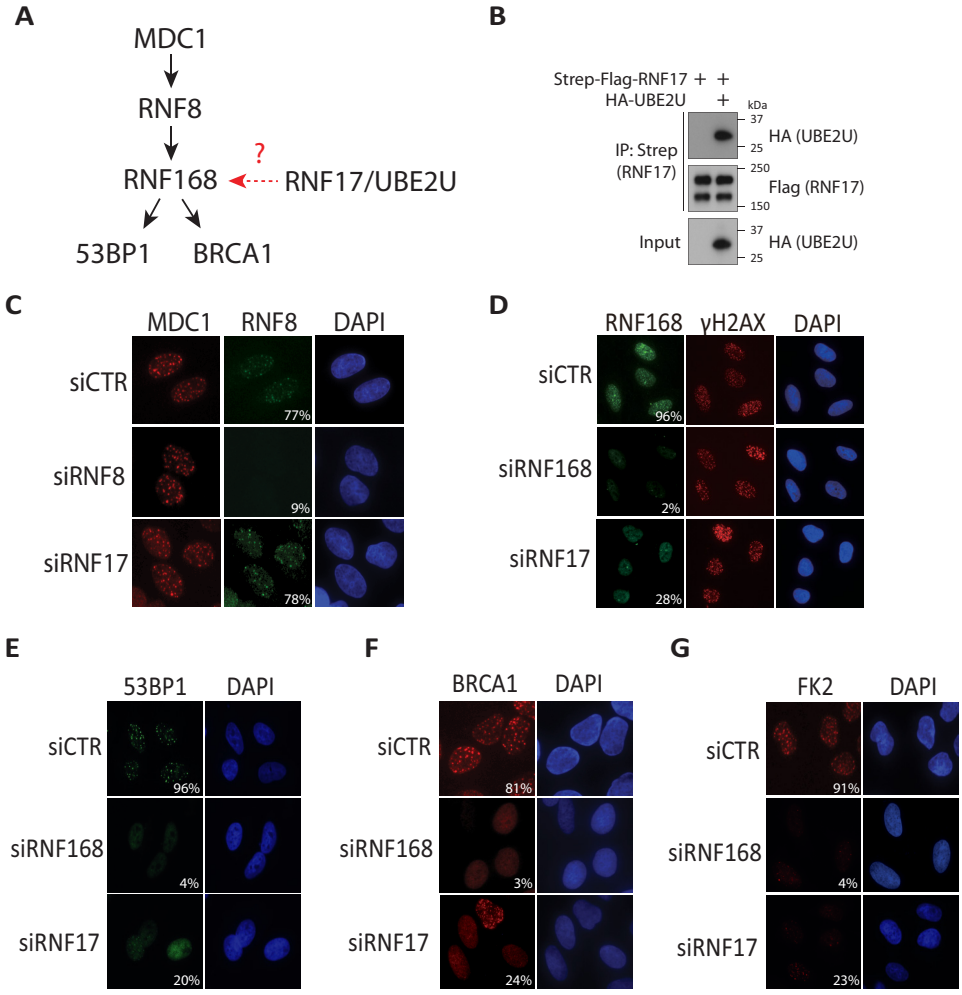

FIGURE 5. **RNF17 is required for DSB retention of BRCA1 and 53BP1.** *A*, schematic depicting the canonical DSB signal transduction pathway where the RNF17-UBE2U pair may play a role at the RNF168 level (see text). *B*, UBE2U interacts with RNF17. 293T cells were transfected with constructs encoding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

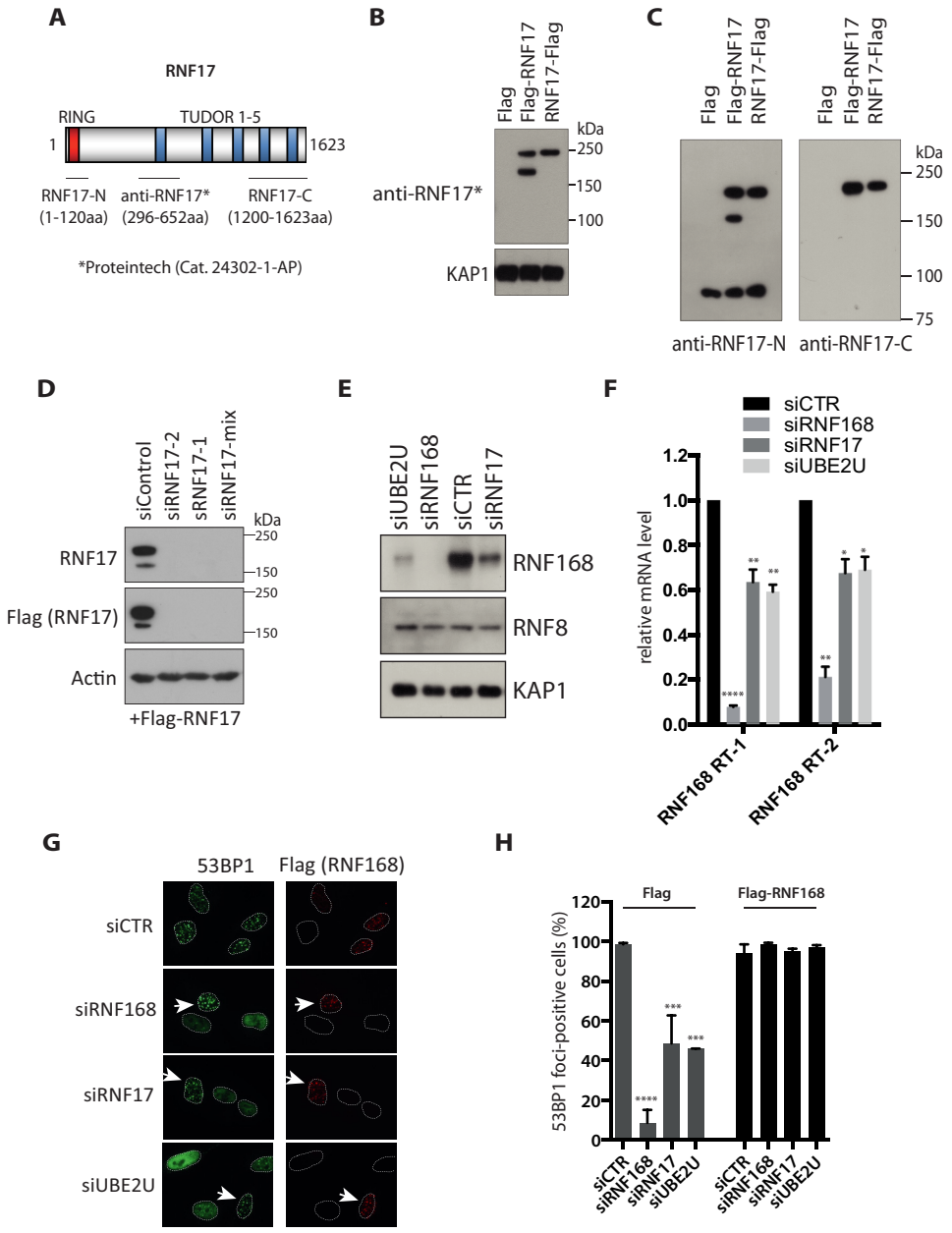

FIGURE 6. **RNF17 regulates RNF168 expression.** *A*, schematic depicting RNF17 protein domain organization and the epitopes that are recognized by anti-UBE2U antibodies. *B* and *C*, Proteintech or in-house anti-UBE2U antibodies specifically recognized ectopically expressed RNF17. *D*, validating RNAi-mediated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

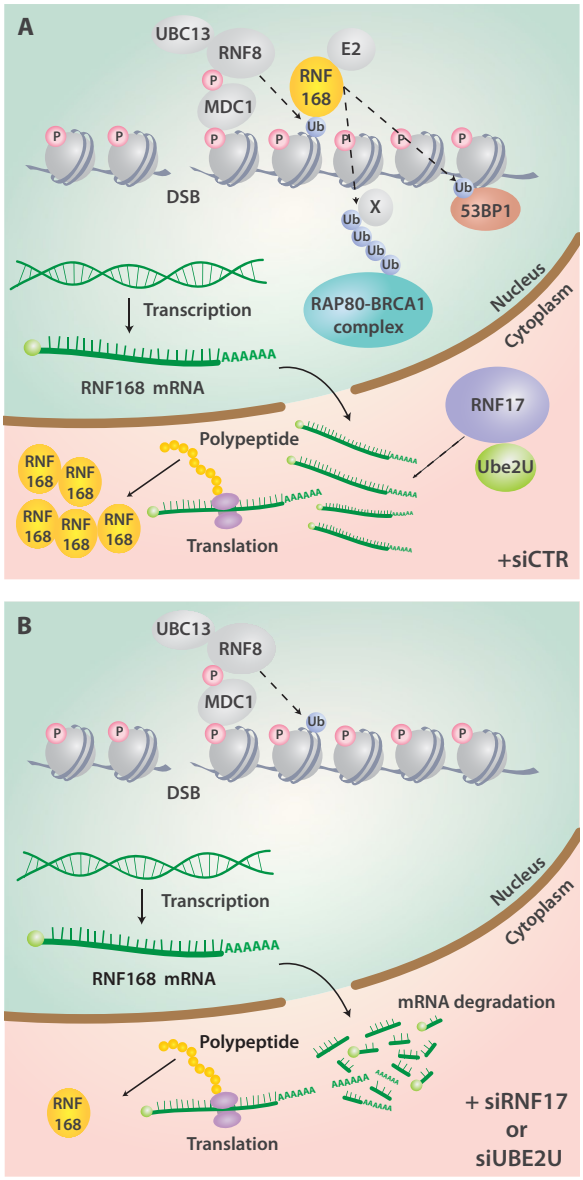

FIGURE 7. **Working model depicting the role of UBE2U and RNF17 in RNF168-dependent DSB responses.** *A*, RNF17 and UBE2U may suppress biogenesis of secondary piRNAs. RNF168 is expressed and catalyzes chromatin ubiquitylation at DSBs to assemble 53BP1 and BRCA1. *B*, silencing of RNF17 or UBE2U leads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

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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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