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A Proteomic Analysis of Ataxia Telangiectasia-mutated (ATM)/ ATM-Rad3-related (ATR) Substrates Identifies the Ubiquitin-Proteasome System as a Regulator for DNA Damage Checkpoints*[S]

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ATM (ataxia telangiectasia-mutated) and ATR (ATM-Rad3-related) are proximal checkpoint kinases that regulate DNA damage response (DDR). Identification and characterization of ATM/ATR substrates hold the keys for the understanding of DDR. Few techniques are available to identify protein kinase substrates. Here, we screened for potential ATM/ATR substrates using phospho-specific antibodies against known ATM/ATR substrates. We identified proteins cross-reacting to phospho-specific antibodies in response to DNA damage by mass spectrometry. We validated a subset of the candidate substrates to be phosphorylated in an ATM/ATR-dependent manner *in vivo*. Combining with a functional checkpoint screen, we identified proteins that belong to the ubiquitin-proteasome system (UPS) to be required in mammalian DNA damage checkpoint control, particularly the G_1 cell cycle checkpoint, thus revealing protein ubiquitylation as an important regulatory mechanism downstream of ATM/ATR activation for checkpoint control.

DNA damage response (DDR)[3] coordinates cell cycle checkpoints, DNA repair and apoptosis. G_1, intra-S, and G_2/M checkpoints operate to block cells at different cell cycle stages (1). G_1 checkpoint prevents G_1 cells from entering S phase with damaged DNA (2, 3), which operates in two distinct phases in response to ionizing radiation (IR): an initial rapid response, typically within 4 h after IR (4) and a sustained response that operates from 4 to 24 h after IR (2). While the sustained G_1 arrest is mediated by p53-dependent transcription, the rapid G_1 arrest is p53-independent and is poorly understood. While many proteins that regulate intra-S and G_2/M checkpoints have been identified, the number of proteins known to participate in G_1 checkpoint is very limited.

Central to DDR are the two checkpoint kinases, ATM and ATR, that phosphorylate the effector kinases Chk1 and Chk2 and other downstream effectors (5). ATM/ATR substrates execute DDR directly or indirectly and are the keys for the understanding of this response. The identification of p53, NBS1, BRCA1, Chk1/Chk2, and SMC1 as substrates of ATM/ATR has significantly advanced our understanding of DDR (6–12). A thorough understanding of DDR in mammalian cells awaits the identification of new ATM/ATR substrates. Unfortunately, few techniques are available today to systematically identify *in vivo* substrates of protein kinase.

ATM and ATR kinases preferentially phosphorylates the S/TQ motif as a consensus sequence (13). It has been observed that a phospho-specific antibody raised against a phosphopeptide is not strictly site or sequence specific; it also recognizes other proteins (cross-reacting proteins) with similar sequences flanking the phosphorylation site. It has been recently realized that these cross-reacting proteins could also be substrates of the same kinase (14–17). Thus, the phospho-antibodies can be used to find potential kinase substrates.

EXPERIMENTAL PROCEDURES

See supplemental material I for "Experimental Procedures" (18–20).

RESULTS AND DISCUSSION

To screen for putative ATM/ATR substrates, we used phospho-antibodies raised against known ATM/ATR substrates that recognize pSQ motifs to immunoprecipitate potential new substrates. HeLa nuclear extracts were prepared from cycling cells and from those treated with IR and hydroxyurea (HU), conditions that activate ATM and ATR, respectively. Each lane of Coomassie Blue-stained gels was divided into 12 regions and proteins in each region were identified with mass spectrometry (MS). As shown as an example in Fig. 1*a*, structure maintenance of chromosomes 1 and 3 (SMC1/3) migrating at 170 and 150 kDa, respectively, are the most abundant proteins recognized by the ATM-pS1981 antibody (21). Many proteins were identified from cycling cells and IR- or HU-treated cells (supplemental Table I). Similar experiments were carried out with antibodies against phospho-BRCA1 (pS1387, pS1423, and pS1457), and the results are summarized in supplemental Table I.

It is possible that some proteins identified using this approach were co-precipitated by association with phosphorylated proteins. To minimize precipitation of associated proteins, we used radioimmunoprecipitation buffer containing high detergent concentration to wash the immunoprecipitates

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Material I and Table I.

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[3] The abbreviations used are: DDR, DNA damage response; ATM, ataxia telangiectasia-mutated; ATR, ATM-Rad3-related; HU, hydroxyurea; MS, mass spectrometry; siRNA, small interfering RNA; UPS, ubiquitin-proteasome system; Gy, gray; CldU, chlorodeoxyuridine; IdU, iododeoxyuridine.

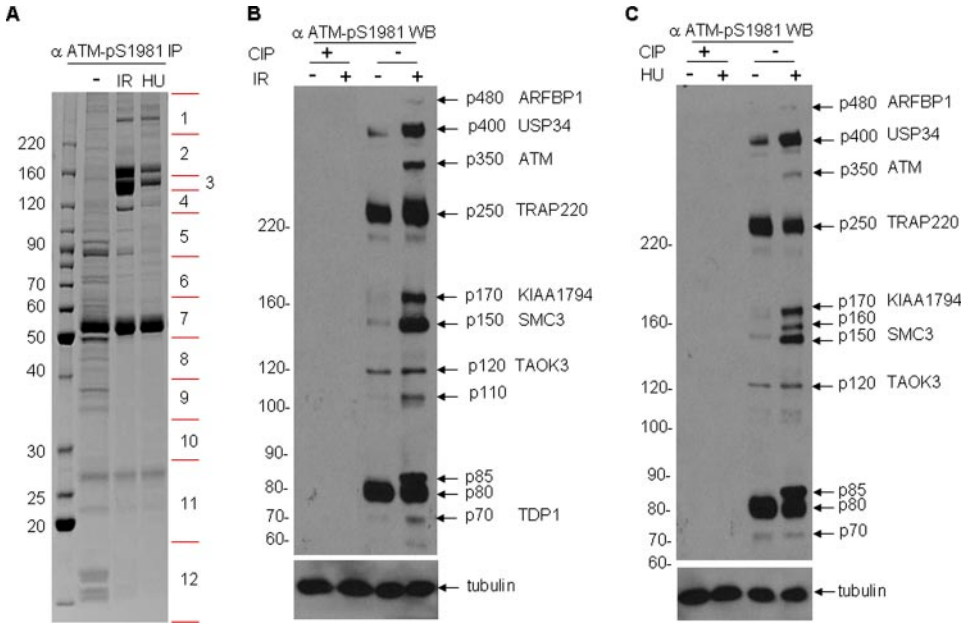

FIGURE 1. **Identification of potential ATM/ATR substrates by immunoprecipitation of proteins cross-reacting to anti-ATM-pS1981.** *A*, HeLa nuclear extracts made from cycling, IR-treated (10 Gy, 2-h recovery), or HU-treated (1 mM for 16 h) cells were immunoprecipitated (*IP*) with an antibody against ATM-pS1981. The immunoprecipitates were separated on a 4–20% of gradient SDS-PAGE gel and stained with Coomassie Blue. Each lane was divided into 12 regions and proteins were in-gel-digested by trypsin, and the resulting peptides were analyzed with mass spectrometry. *B* and *C*, Western blotting of HeLa nuclear extract prepared as described for *A*. An aliquot of each sample was also treated with 1 unit of calf intestine phosphatase (*CIP*) to de-phosphorylate the proteins. Fifty μg of protein extracts per lane were resolved on an 8% SDS-PAGE gel. Western blotting (*WB*) of tubulin was used as loading controls.

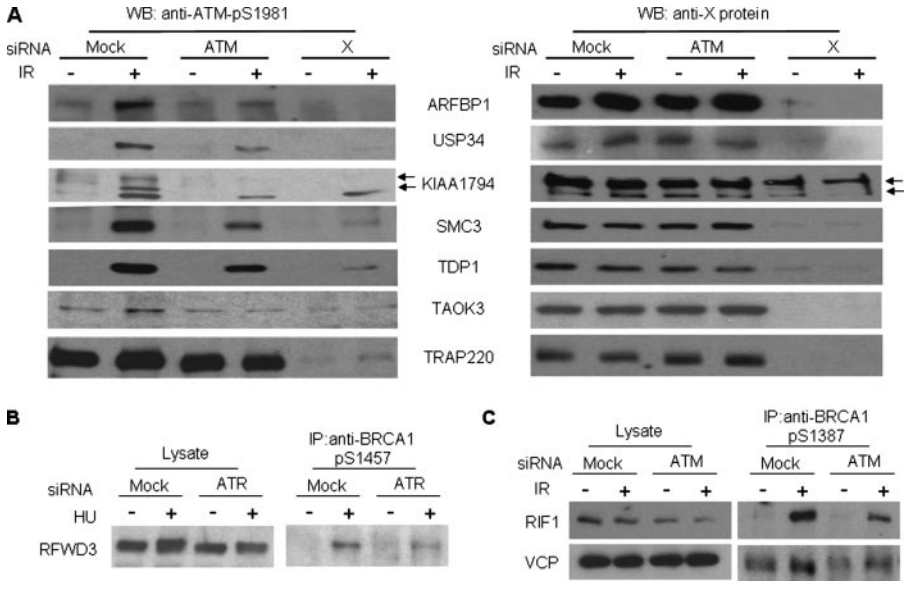

FIGURE 2. **Validation of a subset of candidate ATM/ATR substrates identified from phospho-specific antibody cross-reacting proteins.** *A*, Western blotting (*WB*) of HeLa whole cell lysates depleted of candidate proteins. HeLa cells transfected with siRNA to ATM or putative ATM substrates X (X = ARFBP1, USP34, KIAA1794, SMC3, TDP1, TAOK3, DRIP205) were treated with 10-Gy IR and harvested 2 h later. Duplicate membranes were blotted for anti-ATM-pS1981 or antibody to protein X. The *arrows* indicate two isoforms of KIAA1794 that are resolved on the 8% gel. *B* and *C*, immunoprecipitation (*IP*)/Western blotting of HeLa whole cell lysates depleted of ATR (*B*) or ATM (*C*). HeLa cells transfected with mock siRNA or siRNA to ATR (B) or ATM (C) were treated with HU (1 mM for 16 h) or IR (10 Gy, 2 h recovery). The immunoprecipitations were carried out with the indicated phospho-specific antibodies, and Western blotting was carried out with antibodies specific to RFWD3 (Fig. 2*B*), RIF1 and VCP (Fig. 2*C*).

extensively to disrupt the weak interactions. Thus, proteins identified by this method represent both potential ATM/ATR substrates and core components of the complex that associate with the substrates and have survived the stringent wash. For example, SMC1 was co-precipitated by its strong interaction with SMC3, the substrate recognized by ATM-pS1981 antibody (Fig. 1, *A* and *B*). Since interaction between two protein complexes is usually transient, the identification of protein complexes from different functional categories suggests that one or more proteins in each category are recognized by the phospho-antibodies as potential ATM/ATR substrates, and other core components in each protein complex come along with the substrate.

We used the following criteria to validate the identified proteins as ATM/ATR substrates. First, they must be phospho-proteins; second, they are phosphorylated in an IR/HU-inducible and ATM/ATR-dependent manner; and third, they must contain sequences similar to the SQ peptides used to raise the phospho-antibodies. As shown in Fig. 1*B*, when the lysate was treated with calf intestine phosphatase (*CIP*), no band was recognized by ATM-pS1981 antibody by Western blotting, demonstrating that all bands correspond to phospho-proteins. Furthermore, most of the proteins were phosphorylated in an IR- and HU-inducible manner (Fig. 1, *B* and *C*). To determine the identity of the protein band and to test whether the phospho-Western blot signal is ATM-dependent, we transfected siRNAs against ATM and the candidate protein that has a similar molecular weight to the protein band identified by immunoprecipitation/MS (supplemental Table 1). The cell lysate was analyzed by Western blotting of duplicate blots with either the phospho-antibody or a specific antibody against the candidate protein. As shown in Fig. 2*A*, the knockdown of either ATM or ARFBP1 reduced the p480 phospho-signal, suggesting that p480 is ARFBP1 and that this protein is phosphorylated in an ATM-dependent manner; this is confirmed by the reduction of the Western blotting signal of ARFBP1 on the duplicate membrane. Using this approach, we found that ARFBP1, USP34,

TABLE 1

Comparison of phospho-specific antibody epitopes with sequences within the identified cross-reacting proteins (S/T, D/E, and I/L are treated as the same residue in the analysis)

The identical residues to antibody epitopes are underlined.

Protein	ATM, pS1981, EGSQST	BRCA1, pS1387, LSSQSD	BRCA1, pS1457, LTSQKS
Rad18	HFSQSK		
RNF20	LQSQSS, LSSQSS	LQSQSS, LSSQSS	
RNF40		LKSQVD, LRSQAL	
RFWD3			VSSQGV, ISSQAT
ARFBP1/ HUWE	WGSQLG, CSSQSS, ESSQSE	LSSQEM, CSSQSS, ESSQSE	LSSQEM, ATSQAG, VASQKR
EDD1		LGSQPQ, LISQAQ, SSSQSQ, SSSQSS, SSSQSD, LASQSS	
ZBTB2		LASQGA, TSSQQE	
BTBD12		CSSQTQ, LASQTY, LSSQSS	SRSQKS, LASQTY, LSSQSS
USP15	ENSQSE		
USP28		SSSQDV, SSSQDY, STSQEP	SSSQDV, SSSQDY, STSQEP
USP34	DASQTT, QGSQES		
VCP	SLSQSN	IVSQLL, SLSQSN	IVSQLL
BAT3	LSSQTS, LDSQTR	LDSQTR, LSSQTS	LDSQTR, LSSQTS
KIAA1794		LSSQEE, SSSQCS, ASSQAT, QCSQSL, DFSQST,	
SMC3	GGSQSS,	GGSQSS	GGSQSS
TAOK3	TGSQSS,	TGSQSS, LESQKK,	
TDP1	SGSQED, QGSQKD, AGSQEP		
RIF1		LESQES	
TRAP220	GHSQST, LNSQSQ, SQSQSG, ESSQSG, NSSQSG, SGSQGP, GSSQSK	ESSQSG, NSSQSG YSSQGS, GSSQSK	

KIAA1794, SMC3, TAOK3, and TDP1 are phosphorylated in an IR-and HU-inducible and ATM/ATR-dependent manner. Analyses of amino acid sequences of these proteins reveal that they often contain a stretch of at least 4 amino acid residues or multiple stretches of 3 amino acid resides surrounding the SQ site that are identical to the phosphopeptide antigen, suggesting that these sequences are recognized by the phospho-antibodies employed in this study. Thus, these proteins are most likely to be ATM/ATR substrates (Table 1). The protein band p250 is identified as TRAP220, but its phosphorylation is neither dependent on ATM/ATR nor IR/HU-inducible. Thus, some proteins cross-reacting to phospho-antibodies are not ATM/ATR substrates.

Some of the candidate proteins identified from the immunoprecipitation/MS analysis are not recognized by the same phospho-antibody in Western blots. To demonstrate that they are potential ATM/ATR substrates, we immunoprecipitated these proteins with the phospho-antibody and Western blotted with antibodies specific to these proteins. Using this method, we found that RFWD3 is phosphorylated in an ATR-dependent manner in response to HU (Fig. 2*B*), and RIF1 and VCP are phosphorylated in an ATM-dependant manner in response to IR (Fig. 2*C*). Substrates validated by this method could be associated proteins, but since they have survived the stringent wash and all contain sequences similar to the peptide antigen, thus it is equally possible that they are kinase substrates. Using both approaches demonstrated in Fig. 2, we validated nine ATM/ATR substrates among our collection of candidates. We conclude that the above approach is capable of finding ATM/ATR substrates.

The large number of potential ATM/ATR substrates identified in the proteomic screen poses a significant challenge for functional characterization. When we categorized the proteins into functional modules based on the known function or functional motifs in these proteins, we found many proteins involved in DDR, DNA repair, chromosomal cohesion, and transcriptional regulation, which are expected from ATM/ATR substrates (supplemental Table I). We also found that proteins in the ubiquitin-proteasome system (UPS) are overrepresented (Table 1). This suggests the involvement of protein ubiquitylation in DNA damage response, a notion that is well known in yeast and begins to be appreciated in mammalian cells.

The UPS proteins include E3 ubiquitin ligases (Rad18, RNF20, RNF40, RFWD3, ARFBP1, EDD1), BTB domain proteins (ZBTB2 and BTBD12) that may recruit protein degradation targets to E3 ubiquitin ligase complexes (22–26), ubiquitin hydrolases (USP15, USP28, and USP34) (27, 28), a polyubiquitin-binding protein (VCP), and a ubiquitin-like domain containing protein (BAT3) (29, 30). Importantly, these proteins also contain stretches of amino acid sequences flanking the SQ sites that are similar to the phospho-antigens (Table 1), and the number of peptides detected in MS increases when cells are treated with IR or HU, suggesting that they are potential ATM/ATR substrates or at least tightly associated with the substrates. We thus focused our subproteome functional characterization on 10 UPS proteins for their roles in DNA damage response (Fig. 3).

If these candidates are *bode fide* ATM/ATR substrates, they may participate in regulation of DNA damage checkpoint, DNA repair, or apoptosis. Ideally, we would like to map the precise phosphorylation sites of these candidates and test the loss-of-function mutants for their functions. Unfortunately, it is too labor intensive for a single lab to carry out such analysis on 10 proteins. We decided to examine whether the UPS proteins are required to regulate DNA damage checkpoints.

We carried out intra-S and G_2/M checkpoint assays in HeLa cells depleted of these genes using siRNA. In response to IR, the intra-S checkpoint is activated to mainly suppress late origin firing and is measured by a temporary decrease of nucleotide incorporation following IR. Three days after siRNA knockdown, we irradiated the cells with 10-Gy IR and measured nucleotide incorporation 1 h post-irradiation. Of the 10 genes

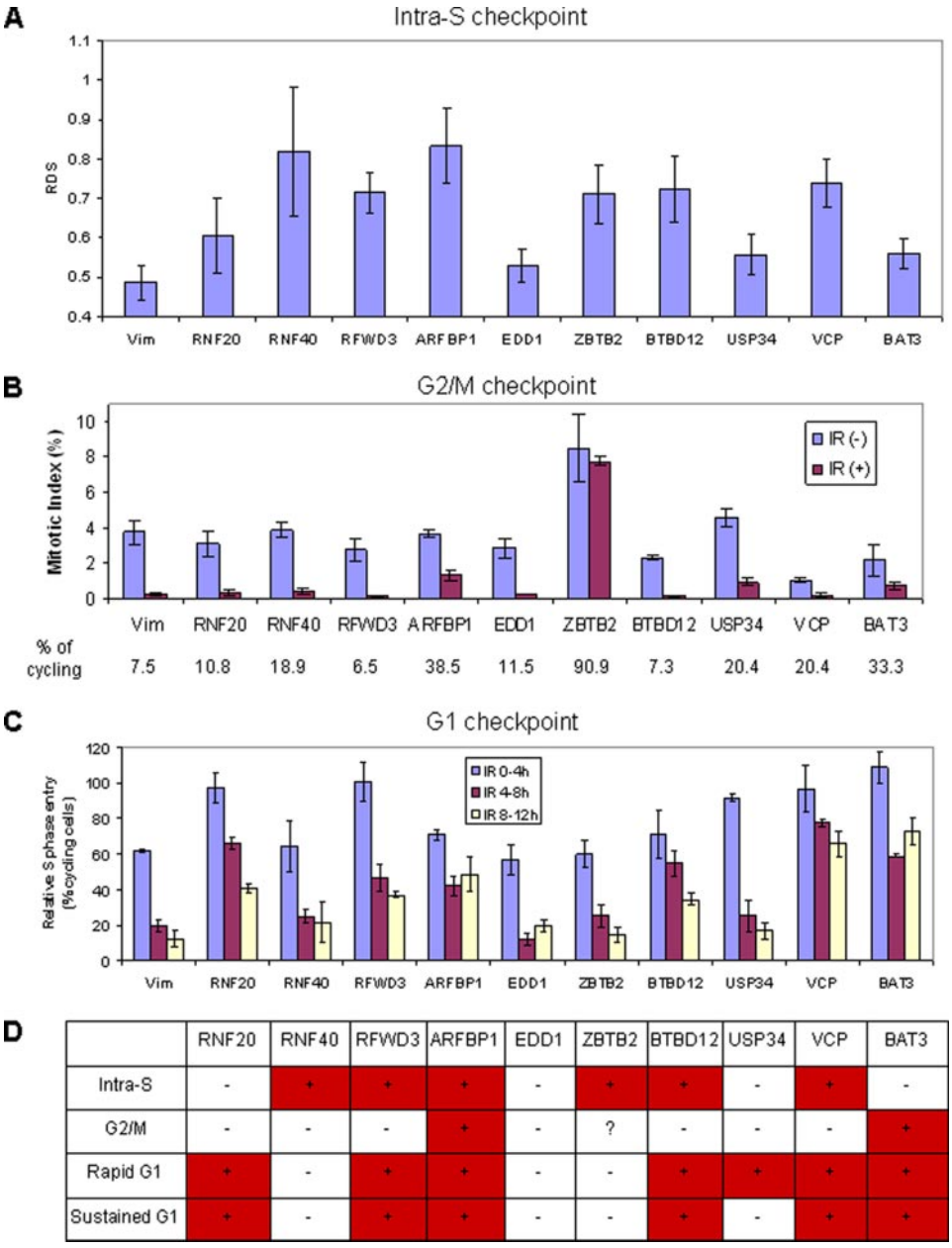

FIGURE 3. **Requirements of 10 UPS genes for the regulation of intra-S, G_2/M and G_1 checkpoints.** *A*, measurements of intra-S checkpoints by radio-resistant DNA synthesis (*RDS*) of HeLa cells transfected with siRNA against the UPS genes listed. The *error bars* represent standard deviation of at least three repeated siRNA transfection experiments. Quintuplet samples were measured for each experiment. *B*, measurements of G_2/M checkpoint by reduction of mitotic index of HeLa cells transfected with siRNA. SiRNA transfected cells were irradiated with 10-Gy IR and mitotic index measured after 1-h recovery. Cells grown on coverslips were immunostained with an antibody against histone H3-pS10 to identify mitotic cells. The *error bars* represent standard deviation of two repeated siRNA transfection experiments. *C*, measurements of G_1 checkpoints by relative S phase entry in U2OS cells transfected with siRNA. Cells were irradiated with 10-Gy IR, and S phase entries between 0–4 h, 4–8 h, and 8–12 h were measured by pulse labeling the cells with CldU at the beginning and IdU at the end of each time intervals. Relative S phase entry was calculated as the ratio of S phase entry after IR to that of cycling cells in 4-h interval. The *error bars* represent standard deviation of at least two repeated siRNA transfection experiments. *D*, summary of checkpoint assay results in *A–C*. Genes whose knockdown caused checkpoint defects are marked in *red*.

tested, six exhibited radio-resistant DNA synthesis significantly above a control siRNA (siVim) transfected cells (Fig. 3*A*), indicating that these UPS proteins are required to regulate the intra-S phase checkpoint. The G_2/M checkpoint is activated to prevent cells with damaged DNA from entering mitosis. We irradiated siRNA-transfected cells with 10-Gy IR and measured the mitotic index 1 h post irradiation. The population of histone H3-pS10 positive cells, which correspond to mitotic cells, was visualized using a microscope. Knocking down 2 out of the 10 genes leads to a profound G_2/M checkpoint failure that cells accumulate more than 4 times as many mitotic cells as in control transfected cells (compare >30% with 7.5% relative mitotic index for control transfected cells). Interestingly, siRNA transfection of ZBTB2 causes a marked increase in mitotic index before IR and virtually no further decrease after IR, indicating that ZBTB2 may play an essential role in mitotic progression, which complicates the interpretation of its role in G_2/M checkpoint control.

The G_1 checkpoint arrests cells with damaged DNA at the G_1/S boundary. G_1 checkpoint is the least understood checkpoint among the DNA damage checkpoints, at least in part due to the lack of an accurate and sensitive assay. Current G_1 checkpoint assay, which measures G_1 accumulation by flow cytometry, is unable to accurately measure cell cycle progression immediately after DNA damage induction. We developed an imaging-based G_1 checkpoint assay by pulse-labeling cells with two bromodeoxyuridine analogs at various times before and after DNA damage induction. By distinguishing cells that have entered S phase after DNA damage induction from the replicating cells at the time of damage induction, this assay allows for accurate measurement of G_1-S transition with a better time resolution. We have validated this assay by measuring the G_1 arrest of known checkpoint proteins and confirmed the existence of p53-independent rapid response (within 4 h post-IR) and p53-dependent sustained arrest (after 4 h).[4] We used this assay to examine in details whether the UPS genes are involved in both rapid and sustained G_1 arrest in U2OS cells. We first pulse-labeled the cells with CldU to mark the starting point and then labeled

[4] Y. Wang and J. Qin, submitted for publication.

them with IdU 4 h later to mark the end point. Using CldU- and IdU-specific antibodies, we scored cells that were CldU-negative and IdU-positive as having entered S phase during the 4 h period after IR. We calculated the S phase entry as the percentage of CldU-negative and IdU-positive cells among DAPI-stained cells. By comparing relative S phase entry (ratio of S phase entry after and before IR in the 4 h period) between 0–4 h, 4–8 h, and 8–12 h following 10-Gy IR, we evaluated the G_1 checkpoint in U2OS cells depleted of USP genes by siRNA transfection. In control siRNA-transfected cells, relative S phase entry is decreased to 60% during the first 4 h after IR as a result of rapid G_1 checkpoint activation and is further decreased to and maintained below 20% starting from 4 h after IR. Of the 10 genes tested, 7 genes are required to regulate the rapid G_1 checkpoint 4 h after IR, and 6 genes are required to regulate the sustained G_1 arrest (Fig. 3*C*). Up to now, only Cyclin D1 degradation has been shown to regulate the rapid G_1 response (4), and Cdc25A is implicated in this process by biochemical evidence (31, 32). Our screen therefore has significantly increased the number of potential G_1 checkpoint regulators and reinforced the notion that protein degradation is an important mechanism for the rapid G_1 arrest. Moreover, the finding that UPS proteins are also required for sustained G_1 arrest thus expands its regulators beyond p53-$p21^{CIP}$. It is conceivable that the UPS may regulate the sustained G_1 arrest through p53-dependent transcriptional program as transcription co-regulators or through other novel pathways yet to be discovered. Detailed mechanistic insights await the identification of substrates of the UPS proteins.

As summarized in Fig. 3*D*, 9 of 10 UPS genes tested are required for at least one cell cycle checkpoint, and 1 gene, ARFBP1, regulates three checkpoints. Our checkpoint assays utilize siRNA pools that are composed of four sequences to maximize knockdown, the possibility of off-target effect cannot be excluded. Nevertheless, the high success rate for identifying checkpoint genes demonstrates that a proteomic screen for ATM/ATR substrates combined with a functional screen is highly effective in selecting protein candidates for further elucidation of the signaling pathways mediated by ATM/ATR. The use of large number of phospho-SQ antibodies to screen for ATM/ATR substrates has revealed an extensive network of proteins in DNA damage response.[5] When combined with functional categorization and application of functional screens for checkpoint, DNA repair or apoptosis, potential substrates as listed in supplemental Table I can be similarly screened and placed into different pathways in the ATM/ATR-regulated DNA damage response network, expending the conceptual framework of DNA damage response. Our report is an example of applying this strategy to reveal the involvement of UPS among the ATM/ATR substrates in mammalian checkpoint control.

[5] S. J. Elledge, personal communication.

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