

## **SUPPLEMENTARY DATA**

### **SUPPLEMENTARY MATERIALS AND METHODS**

#### **Cell culture conditions and treatments**

hTERT-immortalized BS fibroblasts GM03509 (referred as BS) and chromosome 15 mini-chromosome-corrected BS fibroblasts (referred as A-15) (donated by Jerry Shay), GFP-BLM expressing BS fibroblasts GM08505 (referred as GFP-BLM) (donated by Nathan Ellis) were maintained with DMEM supplemented with 10% FCS, glutamine, sodium pyruvate and antibiotics. hTERT-immortalised RIDDLE patient cell line expressing the HA-tagged vector (referred as Vector) or HA tagged-RNF168 corrected Vector cell lines (referred as HA-RNF168) were maintained as described (Stewart et al, 2009). U2OS shRNF8 and U2OS shRNF168 (donated by Jiri Lukas) were maintained as described (Doil et al, 2009; Mailand et al, 2007). Treatment with hydroxyurea (HU) was for 16 hours. Cells were exposed to IR irradiation (5Gy) and then allowed to recover for 4 hrs. LLnL (10mM, Sigma) or MG132 (10mM, Calbiochem) treatments were carried out for the last 6 hr of the HU-treatment. Parallel plates were washed and allowed to grow for a further 6 hours (post-wash condition, +HU/PW). Dox regulated shutdown of RNF8 and RNF168 was done for 48 hours before the initiation of the experiments.

#### **siRNA mediated knockdown**

All the siRNAs used for the specific knockdowns were purchased from Dharmacon. The siRNA information for the specific genes are: RAP80 (On Target Plus smart pool L-006995-00-020); Ubc5a (Si Genome Smart pool M-009387-01-0005); Ubc13 (custom synthesis against the sequence CCA GAU GAU CCA UUA GCA A); RNF8 #1 (custom synthesis against the sequence GGA CAA UUA UGG ACA ACA A); RNF8 #2 (Si Genome Smart pool M-006900-01-0010); RNF168 #1 (custom synthesis against the sequence GAA GAG UCG UGC CUA CUG A); RNF168 #2 (Si Genome Smart pool M-007152-03-0010); PML (custom synthesis against the sequence CAC CCG CAA GAC CAA CAA CAU) and BLM (custom synthesis against the sequence AGC AGC GAU GUG AUU UGC A). The working concentrations for all the siRNAs were 100 picomoles. The siRNA knockdown was carried out using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. Cells were

transfected at 50-60% confluency and transfection was continued for 48 hrs, unless indicated otherwise. ON-TARGET plus Non-targeting siRNA #1 (D-001810-01-05) was used as the siRNA Control and was processed the same way as the experiment.

### **Expression, purification, interactions of proteins and helicase assays**

GST-tagged or His-tagged proteins were expressed in bacterial cells according to standard protocols at 18°C and subsequently purified by binding to either Glutathione S-Sepharose (GE Healthcare) or Nickel-NTA beads (Sigma). Purified proteins were obtained post-elution using a gradient of glutathione (for GST-tagged proteins) or imidazole (for His-tagged protein). For protein expression in eukaryotic cells, transfections were carried out in 293T cells with Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. Lysates were made 24-48 hours post-transfection. Whole cell lysates were made either in RIPA buffer or nuclear extracts prepared using a nuclear and cytoplasmic extraction kit (Pierce). For interactions, IPs were carried out with either whole cell or nuclear extracts using standard protocols (1 µg antibody/IP). The helicase assays were carried out according to published protocols (Brosh et al, 2000; Cheok et al, 2005) using 1nM of the forked duplex substrate and 1.5 nM of BLM (WT or 3K-R). The reaction was carried out at 37<sup>0</sup>C for 15 min and the reaction products resolved on 15% (w/v) polyacrylamide gel. The percent helicase substrate unwound was calculated by the following formula: % displacement = 100 X P/(S + P) where P is the product volume and S is the substrate volume. The values for P and S have been corrected after subtracting background values in the no enzyme and heat-denatured controls, respectively (Brosh et al, 2000).

### **Sister chromatid exchange (SCE) and host cell reactivation assays**

SCE was carried out according to standard protocols (Perry & Wolff, 1974). The whole experiment was carried out twice, and for each cell line SCE was scored blind. The p-values were obtained by Student's t-test. The conditions are two-tailed, unpaired data with unequal variance. The protocol for the host cell reactivation assay was adapted from (Slebos & Taylor, 2001). U2OS shRNF8 and U2OS shRNF168 cells were plated with and without doxycycline treatment. After the knockdown of the ligases by treatment with doxycycline, transfections were carried out in the absence of serum for 6 hr. Transfections were carried out with the substrate (pBHRF) alone or with different combinations of expression plasmids.

pBHRF encodes an intact, emission shifted. “blue” variant of GFP (BFP), with a 300-nucleotide stretch of homology to a nonfunctional copy of GFP. In the absence of homologous recombination, only BFP is present, whereas in the presence of active homologous recombination machinery, functional GFP is created. 24 hours after transfection, cells were treated with 1mM hydroxyurea for 16 hours. After that cells were trypsinised and analysed using a flow cytometer (BD FACS ARIA) for the rate of homologous recombination. Green and Blue fluorescence were simultaneously examined by exciting the cells using a 488nm Argon laser (EGFP) and a UV (350-360nm) laser (EBFP). Green and blue emissions were detected at 530nm and 424nm respectively. All comparisons between green/blue ratios were done on log-normal scale. The results presented were obtained from a minimum of four independent assay points.

### **In silico studies in determination of BLM ubiquitylation sites**

Two online softwares Ubpred (<http://www.ubpred.org/>) and Ubipred (<http://iclab.life.nctu.edu.tw/ubipred/>) were used for the prediction of putative ubiquitylation sites on BLM. The common predicted sites on BLM from the two ubiquitylation prediction softwares were hypothesized to be the sites of high probability on which RNF8 and/or RNF168 K63-linked ubiquitylation could happen. Initially the cut off limit for the sites was kept above 50%. Subsequently the stringency levels was enhanced and took only the sites which were common to both the programs and were predicted with a confidence level of above 70% were analysed for further studies. The analysis was carried out multiple times and it repeatedly gave eight probable sites. These lysines were mutated to arginines by site directed mutagenesis and the mutations were confirmed by automated sequencing. Each of the mutant proteins were expressed and purified along with the wild type and run on 10% SDS gels and stained by Coomassie to check for the proper molecular weight of the mutant proteins. Subsequently the mutants were experimentally verified for the loss of ubiquitylation by using in vitro ubiquitylation assays. Data for only three sites (lysines at 105, 225, 259) are presented in this manuscript as other predicted sites were determined to be not required for BLM ubiquitylation.

## SUPPLEMENTARY FIGURE LEGENDS

### Figure S1:

A. Levels of BLM are not altered due to siRNA mediated decrease in E2 conjugating enzymes and E3 ligases. GFP-BLM cells were transfected with either the control siRNA or siRNAs against Ubc5, Ubc13, RNF168 (#1 and #2) and RNF8 (#1 and #2). For the last 16 hrs the cells were treated with HU. The respective protein levels were determined by Western analysis using antibodies against BLM (A300-110A), Ubc5, Ubc13, RNF168, RNF8 and hsp90.

B. Lack of RNF168 affects the localization of BLM at the sites of stalled replication. RIDDLE syndrome cells complemented with an empty vector or HA-tagged RNF168 were grown in presence of HU. Immunofluorescence was carried out with antibodies against BLM (A300-110A), 53BP1 and the three anti-ubiquitin antibodies FK1, FK2 and K63. Scale 5 $\mu$ M.

C. E2 conjugating enzymes (Ubc5a/Ubc13) and E3 ligases (RNF8/RNF168) affected BLM recruitment to the sites of stalled replication. GFP-BLM cells were subjected to the following combinations of siRNA mediated ablations: RNF8 alone (#1 and #2); RNF8 and RNF168; RNF8 and Ubc5a; RNF8 and Ubc13; Ubc5a alone; Ubc13 alone; Ubc5a and Ubc13; RNF168 alone (#1 and #2); RNF168 and Ubc5a; RNF168 and Ubc13. Post-siRNA mediated knockdown, for the final 16 hours the cells were treated with HU. The fate of GFP-tagged BLM was correlated with 53BP1 recruitment at the sites of DNA damage. Nuclei have been stained using DAPI. Scale 5 $\mu$ M.

### Figure S2:

A. Coomassie gel of purified BLM, PML isoforms and E3 ligases. GST-BLM (1-1417), His-PML III (1-641), PML IV (1-633), His-RNF8 (1-485), His-RNF8 (1-485, C403S), GST-RNF168 (1-571) and GST-RNF168 (DRING) were purified, checked for Coomassie and used for ubiquitylation assays.

B. BLM and BLM (3K-R) have similar helicase activity. Helicase activity was carried out with BLM and BLM (3K-R) recombinant proteins. The experiment was repeated five times

and a typical result is represented (left). The quantitation of the extent of DNA displacement is on represented on the right.

C. Expression of BLM ubiquitylation mutants. EGFP tagged wildtype BLM, BLM (K105R), BLM (K225R), BLM (K259R), BLM (3K-R) were transiently transfected into 293T cells. Western analysis of the transfected lysates was carried out with antibodies against BLM and hsp90.

D. Lack of BLM ubiquitylation affects its recruitment at the sites of damage after IR. 293T cells were transfected with EGFP-tagged wildtype BLM or BLM (3K-R) mutant. Post-transfection the cells were irradiated with IR and allowed to recover for 4 hrs. GFP-BLM cells were tracked. The nuclei have been stained using DAPI. Scale 5 $\mu$ M.

E. N-terminal region of BLM interacts with RNF8. (Left) Coomassie gel indicating the purification of GST-tagged BLM, its four fragments and His-tagged RNF8. (Right) Soluble His-tagged RNF8 was interacted with either Glutathione-Sepharose bound GST or GST-tagged wildtype BLM or its fragments. Sepharose bound His tagged RNF8 was detected by western analysis with antibodies against His.

### **Figure S3:**

A. Ablation of RNF8 and RNF168 in U2OS shRNF8 and U2OS shRNF168 cells leads to increase in HR. U2OS shRNF8 and U2OS shRNF168 cells were grown in the presence and absence of doxycycline (Dox), and also in the presence or absence of HU. SCE assays were carried out in the above four conditions. (\*) indicates  $p < 0.05$ .

B. Cells from RIDDLE patients have elevated SCE levels. SCE assays were carried out in RIDDLE syndrome cells complemented with the Vector or HA-tagged RNF168. The cells were grown in absence or presence of HU treatment. (\*) indicates  $p < 0.05$ .

**Figure S4:**

Lack of RNF168 affects the localization of BLM at the laser induced DSBs. U2OS shRNF168 cells were grown in absence or presence of Dox and subjected to laser induced double strand breaks. Immunofluorescence was carried out with antibodies against BLM (A300-110A) and  $\gamma$ H2AX. Nuclei were stained with DAPI. Scale 5 $\mu$ M.

## SUPPLEMENTARY REFERENCES

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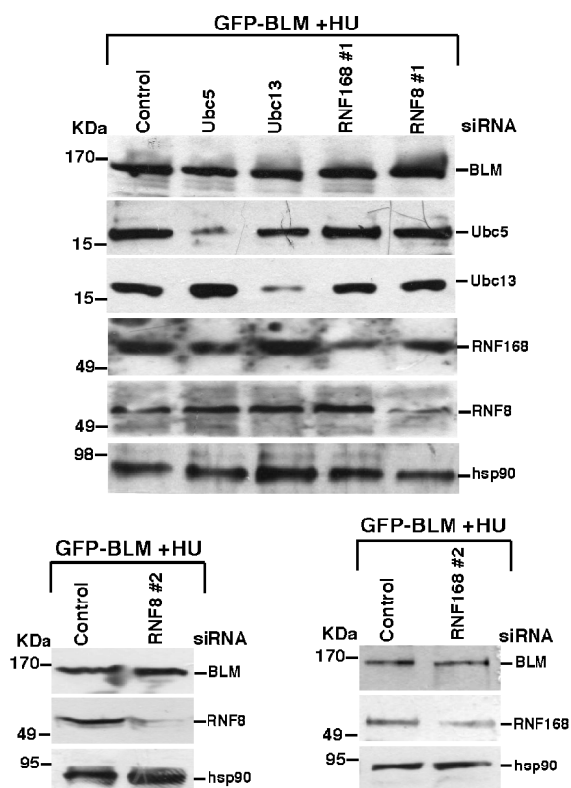
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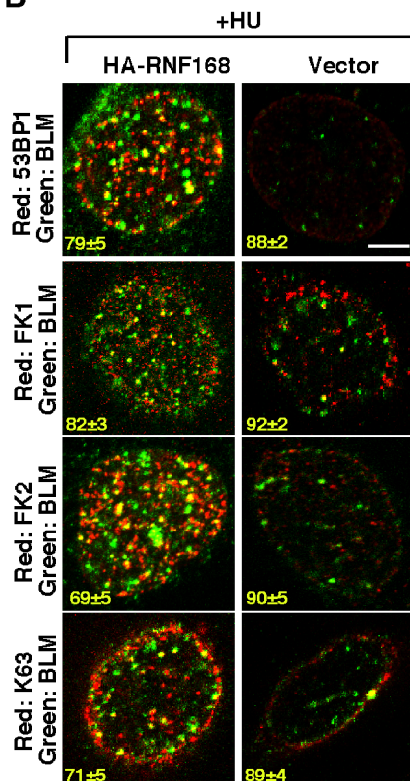
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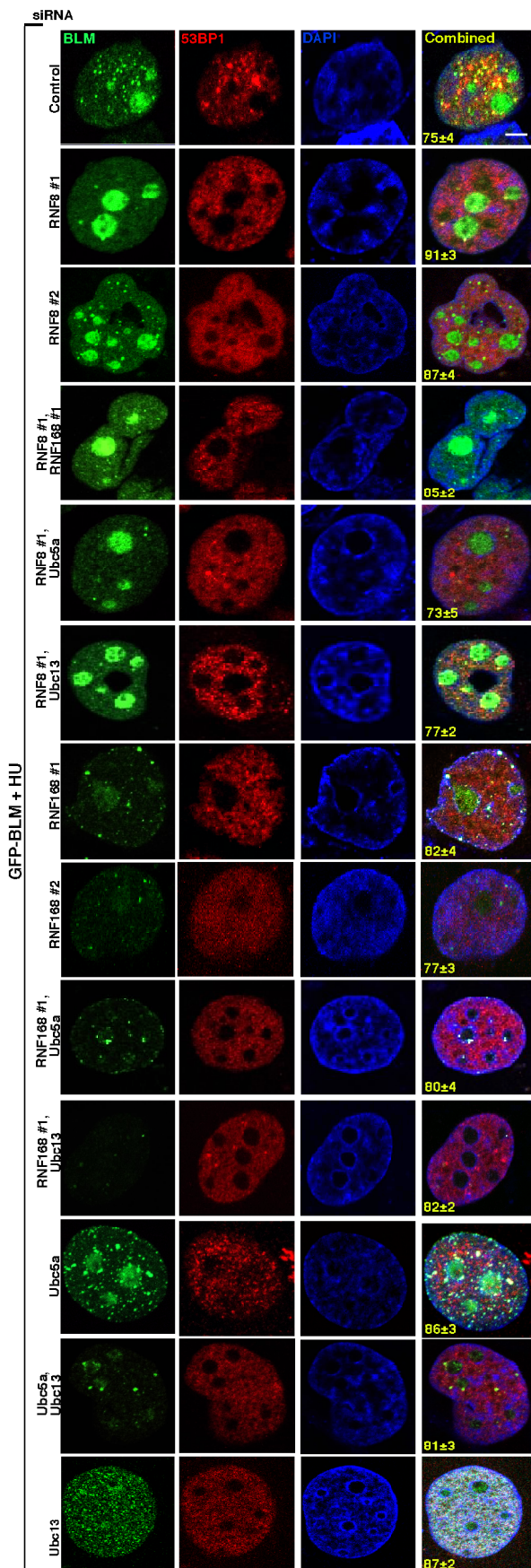
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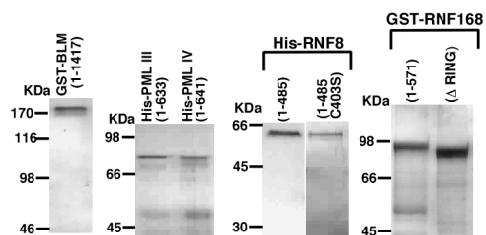


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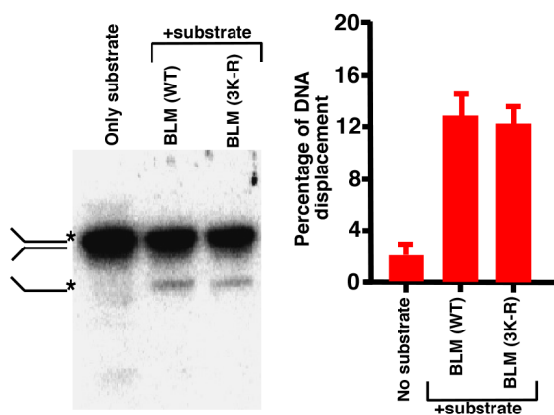




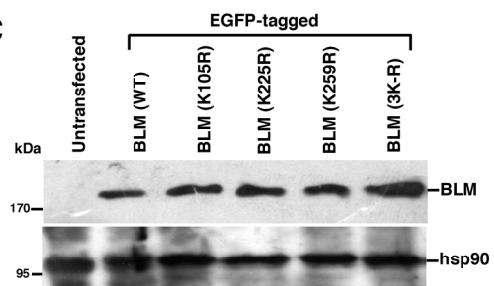
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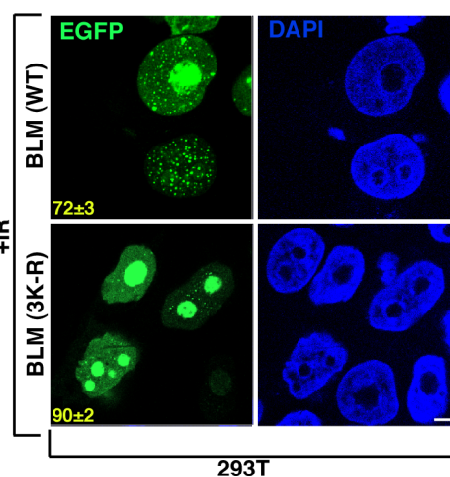
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**C**



**D**



**E**

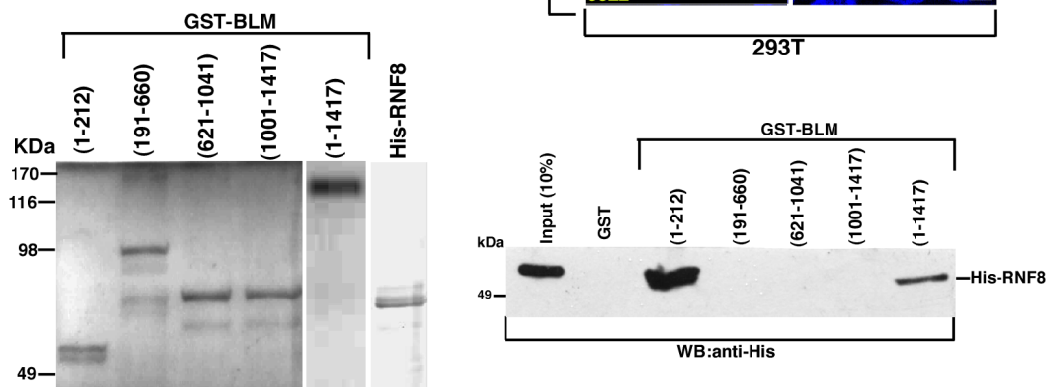


Figure S3  
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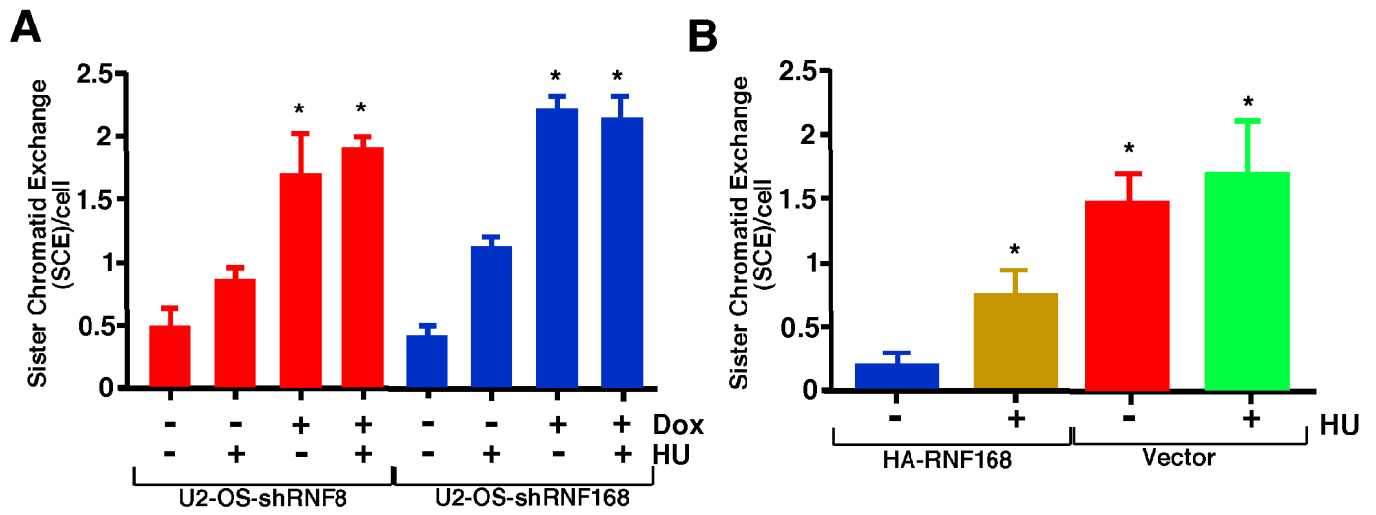


Figure S4  
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