Discussion
Mutations in Bloom helicase (BLM), a member of the highly conserved RecQ helicase family, leads to an autosomal recessive cancer predisposition syndrome called Bloom Syndrome (BS). Though rare, BS patients are unique as they are predisposed to multiple forms of cancer ranging from solid tumors to lymphoma (German, 1993). It has also been reported that BLM heterozygotes also have a higher rate of developing cancer (Gruber et al., 2002). This unique feature of BLM helicase has given it a status of a “caretaker tumor suppressor” – potentially controlling vital regulatory processes that suppress the neoplastic transformation process and thereby maintain the genomic stability (Hickson, 2003). Hence the loss of BLM in BS patients leads to an approximately 10-fold increase in the rates of sister chromatid exchange (SCE) [which are thought to arise due to homologous recombination (HR)] (Chaganti et al., 1974), interhomologous recombination and loss of heterozygosity (LaRocque et al., 2011). To study BLM functions, multiple mouse models (both complete knockouts and hypomorph systems) have been developed (Chester et al., 1998; Goss et al., 2002; LaRocque et al., 2011; Luo et al., 2000b; McDaniel et al., 2003). Though none of the three mice models recapitulate all aspects of BS, each of them recapitulate the certain cellular and tumorigenic features of the syndrome. The role of BLM is possibly at a global level as mutation of murine BLM gene causes global genome destabilization (Chester et al., 2006).

The functions of BLM have been extensively worked out from a biochemical perspective. The centrepiece of BLM biochemistry is its function as a 3'-5' helicase capable of acting on a variety of conventional and less common substrate (reviewed in (Bachrati and Hickson, 2003)). Curiously BLM also possesses a single strand annealing activity (Cheok et al., 2005), the physiological function of which is yet to be elucidated. Much of the functions of BLM during HR are due to its interactions with different protein complexes. BLM is a part of the BLM-topoisomerase III-RMI1/2 (BTR) complex (Chang et al., 2005; Singh et al., 2008; Xu et al., 2008; Yin et al., 2005). Components of the BTR complex are required in the later stages of the HR subpathways to “dissolve” i.e. remove double Holliday Junction Structures (DHJs) into non-crossover products (Plank et al.,
BLM can also directly interact with pro-recombinogenic core HR proteins like RAD51 (Bischof et al., 2001; Sengupta et al., 2003; Wu et al., 2001a) and RAD54 (Srivastava et al., 2009). The lab and others have demonstrated that BLM can disrupt RAD51 nuceloprotein filaments and thereby control an early stage of HR (Bugreev et al., 2007; Tripathi et al., 2007). The functional interaction of BLM with RAD54 occurs during multiple stages of HR (Chu et al., 2010). BLM also interacts with accessory anti-recombinogenic proteins like p53 (Sengupta et al., 2003) and 53BP1 (Tripathi et al., 2008; Tripathi et al., 2007) and control their functions during HR. In contrast to the above “anti-recombinogenic” roles that are consistent with the hyper-recombinogenic phenotypes of the BS cells, BLM also has a “pro-recombinogenic” function. As part of this helicase-independent function BLM can promote resection and stimulate the nucleolytic activity of human Exonuclease 1 (hExo1) and DNA2 helicase/nuclease (Nimonkar et al., 2011; Nimonkar et al., 2008). A satisfactory explanation for BLM to possess two opposite enzymatic activities under physiological conditions is yet to be obtained. BLM is also known to have a role during DNA replication. BLM having the helicase activity and phosphorylated at threonine99 (Thr99) is required for two aspects of the cellular response to replicative stress namely efficient replication-fork restart and suppression of new origin firing (Davies et al., 2007). BLM can promote the regression of stalled forks in vitro (Machwe et al., 2006; Ralf et al., 2006), which in theory allows the bypass of a DNA lesion that blocks the forks and thereby causes the restart of the DNA replication. Once regressed, a Holliday Junction (HJ) is formed which can be migrated back by BLM to restore a functional replication fork (Heller and Marians, 2006).

While the role of BLM during the late-functions after DNA damage (replication and HR) have been mechanistically dissected, comparatively little is known at the mechanistic level about the early functions of BLM, immediately after exposure to the different types of deleterious lesions. BLM seems to play a role at both the sensing of the DNA damage and transmission of damage signal to the downstream effector proteins (reviewed in (Tikoo and Sengupta, 2010). BLM
stimulates the ATPase and chromatin remodelling activity of RAD54 (Srivastava et al., 2009), which may enhance the capability of other sensor proteins to mount a robust DNA damage response. Absence of BLM impairs the ability of the Chromatin Accessibility Factor-1 (CAF-1) to be recruited to the sites of DNA damage (Jiao et al., 2004). The accumulation of BLM to the sites of damage is transitory (having a residence time of 7.2 seconds) (Srivastava et al., 2009) and has been reported to be independent of the known sensor proteins like ATM and NBS1 (Karmakar et al., 2006). Infact during replication stress BS cells have compromised accumulation of BRCA1 and NBS1 complexes (Davalos and Campisi, 2003).

Various post-translational modifications on BLM have been reported which possibly influences its diverse functions during DNA damage response. ATR-mediated phosphorylation of BLM at Thr99 and Thr122 were not required for its recruitment to the site of stalled replication (Davies et al., 2004). However phosphorylation of BLM at Thr99 was required for its interaction and colocalization with 53BP1 (Tripathi et al., 2008) and subsequent recovery from replication stress induced S-phase arrest (Davies et al., 2004). Dephosphorylation of the Chk1-mediated constitutive phosphorylation of BLM at Ser646 was essential for BLM to be recruited to the sites of damage (Kaur et al., 2010). Sumo modification of BLM at Lys317 and Lys331 was essential for BLM and RAD51 interaction at the damaged replication forks (Eladad et al., 2005; Ouyang et al., 2009), thereby subsequently affecting the role of BLM during HR. It is to be noted that while these modifications on BLM are essential for it’s (i.e BLM’s) ability to carry out its subsequent interaction and functions, none of them are a definite prerequisite for the recruitment of the helicase to the sites of damage.

Recruitment of proteins to the site of DNA damage is a highly ordered, hierarchical process controlled by certain key elements [reviewed in (Stewart, 2009; Tikoo and Sengupta, 2010)]. In the last few years it has become increasingly obvious that two E3 ligases, RNF8 and RNF168, play very important roles in the regulation of the DNA damage response by catalyzing the recruitment of two important downstream factors – RAP80/Abraxas/BRCA1 complex and 53BP1 to the sites of DNA damage (Kim et al., 2007; Liu et al., 2007; Wang and Elledge,
RNF8 itself is recruited to the chromatin via a ATM-mediated phosphorylation on the conserved (TQXF)4 motif on MDC1 when the latter accumulates at the site of the lesion (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007). Once present on the damaged chromatin RNF8 (in conjunction with its specific E2 conjugating enzyme Ubc13) catalyzes K63-linked ubiquitylation on its preferred substrates, H2A and H2AX. The second E3 ligase, RNF168, functions downstream of RNF8 and is in fact recruited to the site of damage via binding to ubiquitylated histones catalyzed by RNF8/Ubc13 (Doil et al., 2009; Stewart et al., 2009). Once recruited RNF168 amplifies the ubiquitin signal on the histones, which are recognized by a zinc finger, ubiquitin binding protein called RAP80 via its ubiquitin binding motif (UIM). RAP80 recruits BRCA1/BARD1 complex to the site of DNA damage via an adaptor protein called Abraxas (ABRA1) (Kim et al., 2007; Liu et al., 2007; Wang and Elledge, 2007; Wang et al., 2007; Yan et al., 2007b).

Though the recruitment of 53BP1 was found to be a convenient readout for the presence of functional RNF8 and RNF168, the exact mechanistic details which governed the binding of 53BP1 to specific histone mark (H4K20me) were till recently unknown. It has been recently discovered that two TUDOR domain containing lysine demethylase, JMJD2A and JMJD2B having a high affinity binding specificity for H4K20me are polyubiquitylated by RNF8 and RNF168 and subsequently degraded after induction of DNA damage (Mallette et al., 2012). Hence demasking of this histone mark after DNA damage leads to be recognized by 53BP1, thereby allowing the latter's recruitment to the sites of the damage. A complementary mechanism regulating the recruitment of 53BP1 involve AAA-ATPase p97/VCP mediated extraction of JMJD2A/B from the H4K20me histone mark (Meerang et al., 2011). Interestingly AAA-ATPase p97/VCP may also be involved in RNF8 and RNF168-dependent removal of another H4K20me binding protein L3MBTL1, thereby adding another mechanistic detail regulating the recruitment of 53BP1 to the damage sites (Acs et al., 2011).

In the last few years BRCA1 complex and 53BP1 have both been implicated in various aspects of HR. It was demonstrated that RAP80-dependent
recruitment of BRCA1 to the sites of DNA damage suppressed the otherwise exaggerated BRCA1-driven HR (Hu et al., 2011). 53BP1 also inhibits HR in BRCA1-deficient cells by blocking resection of DNA breaks (Bunting et al., 2010). Interestingly the lab had shown that 53BP1 itself acts as anti-recombinogenic protein in a BLM-dependent and independent manner (Tripathi et al., 2007). Based on the facts about the role of BLM in DNA damage response (as described earlier), it was hypothesized that like BRCA1 complex and 53BP1, BLM will also be recruited to the sites of DNA in an ubiquitylation-dependent manner. This thesis provides compelling evidence in favour of the hypothesis.

Though broadly BLM is recruited to the sites of DNA damage in a RAP80/RNF8/RNF168-mediated ubiquitylation dependent process, the finer mechanistic details are different when compared to either BRCA1 or 53BP1. For example, BLM binds constitutively to the extreme N- and C-terminal regions of RAP80 (Figure 3.13) within the PML NBs even in absence of any DNA damage (Figure 3.3). In presence of DNA damage, BLM is ubiquitylated by both RNF8 and RNF168 via K63-linkage at lysine 105, 225, 259 (Figure 3.38, 3.39). The binding of BLM with RAP80 infact enhances the RNF8/RNF168-mediated ubiquitylation (Figure 3.24), allowing RAP80 to regulate the stability of BLM (Figure 3.15, 3.16). The ubiquitylated BLM led to its enhanced binding to RAP80 (Figure 3.2, 3.4, 3.5) in a UIM-dependent manner (Figure 3.14). It is hypothesized that RAP80, known to interact with ubiquitylated histones at the sites of DNA damage, in turn recruits BLM to the damaged chromatin.

Even though for long time histones H2A and H2AX were identified as the substrates for RNF8 and RNF168, an increasing repertoire of RNF8/RNF168 substrates have been identified in recent months. Apart from BLM (this study), RNF8 and RNF168 have been shown to ubiquitylate substrates like NPM1 (Koike et al., 2010), JMJD2A, JMJD2B (Mallette et al., 2012) and L3MBTL1 (Acs et al., 2011). However among these novel substrates, BLM is probably the only one where the specific residues at which the K63-linked ubiquitylation occur have been biochemically validated (Figure 3.38) and subsequently verified in vivo (Figure 3.40). Using ubiquitylation mutants it has been demonstrated that the loss of this
post-translational modification completely eliminates the recruitment and subsequent retention of BLM at the sites of DNA damage. Instead the BLM, which is mutated at Lys105, 225, 259 remains accumulated within the nucleolus (Figure 3.40).

The question can be asked regarding the reason for nucleolar accumulation of BLM not only in ubiquitylation mutants but also in absence of RNF8. By an unknown mechanism it was observed that depletion of RNF8 also led to the destabilization of PML NBs (Figure 3.36). Hence the loss of its primary storage site (i.e. the PML NBs) probably forces the non-ubiquitylated BLM to instead accumulate in the nucleolus. These events also indicate that RNF8 is the primary E3 ligase required for the ubiquitylation of BLM at the PML NBs (Figure 3.25). Like for the other RNF8 substrates (for example histone H2A and H2AX), RNF168 probably amplifies the RNF8-dependent ubiquitylation signal (Figure 3.21-3.25). However this amplification of the K63-linked ubiquitylation is necessary, as without RNF168, BLM cannot be retained at the sites of DNA damage (as observed in siRNA mediated ablation or in cells from RIDDLE syndrome patients) (Figure 3.28A, 3.30, 3.32). Interestingly BLM can utilize two E3 ligases (RNF8 and RNF168) and two E2 conjugating enzymes (Ubc5a and Ubc13) during K63-linked ubiquitylation (Figure 3.22, 3.25 and data not shown). Though Ubc13 is the more well known E2 conjugating enzyme for RNF8 and RNF168, Ubc5a is equally effective as a E2 conjugating enzyme with both RNF8 and RNF168 (Figure 3.22 and data not shown).

It can be hypothesized that the regulation of BLM during HR can partially depend on its upstream DNA damage sensor and transmission function. Indeed it was observed that the high level of HR observed in absence of BLM is due to the lack of the accumulation of BLM at the sites of damage due to absence of ubiquitylation at Lys residues 105, 225, 259 (Figure 3.40, 3.42). Specifically wildtype BLM but not the BLM mutated at the ubiquitylation sites could rescue the high rate of HR in BS cells. lnfact lack of either RNF8 or RNF168 also led to an elevated recombinogenic phenotype (Figure 3.47), indicating that BLM and the two E3 ligases maybe in the same epistatic pathway. Hence targeting BLM directly
to the chromatin during DNA damage (by tagging BLM with either FHA domain of MDC1 or with full-length H2AX) can rescue the hyper-recombination caused due to the absence of either RNF8 or RNF168 (Figure 3.49) and also lead to the recruitment of BLM at the sites of DNA damage (Figure 3.44).

Hence based on the results the mechanism of BLM recruitment to the site of DNA damage has been elucidated. Interestingly the mechanism has elements that are both common and also different when compared to the classic DNA damage response pathway. It will interesting to know whether the ubiquitylation effects on BLM, post-generation of stalled replication forks, remain conserved when cells are exposed to different types of DNA damage. In recent past it has been demonstrated that the RNF8-mediated recruitment of the CHD4 (the catalytic subunit of the NuRD chromatin remodelling complex), leads to the unfolding of the higher-order chromatin structure and thereby regulating the signalling and repair of DNA damage (Larsen et al., 2010; Luijsterburg et al., 2009; Smeenk et al., 2010). Based on the effect of BLM on the chromatin remodelling activity of RAD54 (Srivastava et al., 2009) and its (i.e. BLM's) effect on CAF1 recruitment (Jiao et al., 2004), it will be perhaps important to know whether ubiquitylated BLM recruited to the sites of DNA damage can regulate the remodelling activities of the NuRD complex. It is well known that response to any DNA damage has to be controlled in a temporal manner. Hence identification of the deubiquitylating enzymes (DUBs) that specifically target BLM or ubiquitin chain editing mechanisms (Newton et al., 2008) that can regulate the spectrum of BLM ubiquitylation at the site of damage need to be identified so that the role of BLM during the entire DNA damage response can be elucidated in full detail. During this processes integration of the various BLM post-translational modifications (like ubiquitylation with phosphorylation and sumoylation) with the varied functions of BLM will be achieved – thereby allowing a better appreciation of the in vivo functions of this important caretaker tumor suppressor.
Supplementary Data
Experiment #1: To determine whether p53 and RAP80 interact

Wildtype p53, apart from functioning as a transcriptional regulator of its downstream targets, is also known to play a direct role in varied but important functions such as DNA repair and recombination (Sengupta and Harris, 2005). p53 is known to accumulate at the sites of DNA damage including stalled replication (Sengupta et al., 2003).

![Figure S1: RAP80 interacts with p53](image)

Figure S1: RAP80 interacts with p53. Interaction between GST RAP80 wild type and *in vitro* transcribed and translated S\(^{35}\) labelled p53. Interaction was seen between full length RAP80 and *in vitro* transcribed and translated S\(^{35}\) labelled p53.

RAP80 has also been shown to play a role in homologous recombination and repair (Wang et al., 2007; Yan et al., 2007a) and in recruiting proteins like 53BP1 (Doil et al., 2009; Stewart et al., 2009), BRCA1 (Kim et al., 2007; Sobhian et al., 2007; Wang et al., 2007; Yan et al., 2007b) and BLM (this thesis) to the sites of DNA damage. For this purpose, it was decided to investigate whether RAP80 and p53 interact which can be the basis of p53 recruitment to the site of DNA
Supplementary Data

damage. To determine the direct interaction between RAP80 and p53, GST tagged construct of RAP80 was used in GST pull down assays with in vitro transcribed and translated S\textsuperscript{35} labelled wild-type p53 construct. An interaction with p53 was observed for RAP80; thereby validating that p53 is a novel interacting partner of RAP80 (Figure S1).

**Experiment #2: Determine whether Fbw7γ and RAP80 interact**

RAP80 is known to interact via its UIM’s to the K63-linked ubiquitin chains amplified by RNF8 and RNF168 at the damaged chromatin (Doil et al., 2009; Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007; Stewart et al., 2009). As an exploratory project, we wanted to investigate whether RAP80 also interacts with the degradative E3 ligase Fbw7γ. To determine the direct interaction between RAP80 and Fbw7γ, GST tagged construct of RAP80 wildtype was used in GST pull down assays with in vitro transcribed and translated S\textsuperscript{35} labelled wild-type Fbw7γ construct. An interaction with Fbw7γ was observed for RAP80, thereby validating that Fbw7γ is a novel interacting partner of RAP80 (Figure S2A). Subsequently, deletion fragments of RAP80 namely pGEX4T-1-RAP80 (1-719) i.e pGEX4T-1 RAP80 (1-79), pGEX4T-1 RAP80 (1-124), pGEX4T-1 RAP80 (1-428), pGEX4T-1 RAP80 (1-460) and pGEX4T-1 RAP80 (1-583) (Figure S2B) were expressed and the purified recombinant proteins were used for the interaction with in vitro transcribed and translated S\textsuperscript{35} labelled Fbw7γ. In contrast to the wildtype RAP80, all RAP80 deletion fragments exhibited a weak interaction with Fbw7γ (Figure S2C). This indicated that the conformation of RAP80 was possibly an essential ingredient for its interaction with Fbw7γ.
Figure S2: RAP80 interacts with Fbw7γ. A. Interaction between GST RAP80 wild type and *in vitro* transcribed and translated S\(^{35}\) labelled Fbw7γ. Interaction was seen between full length RAP80 and *in vitro* transcribed and translated S\(^{35}\) labelled Fbw7γ. B. Schematic representation of various C-terminal deletions of RAP80. C. Interaction between GST RAP80 wild type, C-terminal deletions and *in vitro* transcribed and translated S\(^{35}\) labelled Fbw7γ. The gels were dried and signals obtained by autoradiography.
Experiment #3: Determine whether RAD51 and RAP80 interact

RAP80 has also been shown to play a role in homologous recombination and repair (Wang et al., 2007; Yan et al., 2007a). RAP80 also plays a role in recruiting proteins like 53BP1 (Doil et al., 2009; Stewart et al., 2009), BRCA1 (Kim et al., 2007; Sobhian et al., 2007; Wang et al., 2007; Yan et al., 2007b) and BLM (this thesis). RAD51 known to be a core homologous recombination (HR) protein, which stimulates the synaptic phase of the recombination process by acting as the recombinase (Holthausen et al., 2010). For this purpose, it was decided to investigate whether RAP80 and RAD51 interact which can be the basis of RAD51 recruitment to the site of DNA damage. To determine the direct interaction between RAP80 and RAD51, GST tagged construct of RAP80 was used in GST pull down assays with in vitro transcribed and translated S\textsuperscript{35} labelled wild-type RAD51 construct. RAD51 and RAP80 interact; thereby validating that RAD51 is a novel interacting partner of RAP80 (Figure S3A). Subsequently, deletion fragments of RAP80 namely pGEX4T-1-RAP80 (1-719) i.e pGEX4T-1 RAP80 (1-79), pGEX4T-1 RAP80 (1-124), pGEX4T-1 RAP80 (1-428), pGEX4T-1 RAP80 (1-460) and pGEX4T-1 RAP80 (1-583) (Figure S3B) were expressed and the purified proteins used for the interaction with in vitro transcribed and translated S\textsuperscript{35} labelled RAD51. In contrast to the wildtype RAP80, all RAP80 deletional fragments exhibited a weak interaction with RAD51 (Figure S3C). This indicated that the conformation of RAP80 was possibly an essential ingredient for its interaction with RAD51.
Figure A3: RAP80 interacts with RAD51. A. Interaction between GST RAP80 wild type and *in vitro* transcribed and translated S\(^{35}\) labelled RAD51. Interaction was seen between full length RAP80 and *in vitro* transcribed and translated S\(^{35}\) labelled RAD51. B. Schematic representation of various C-terminal deletions of RAP80. C. Interaction between GST RAP80 wild type, C-terminal deletions and *in vitro* transcribed and translated S\(^{35}\) labelled RAD51. Interaction was seen between full length RAP80 and *in vitro* transcribed and translated S\(^{35}\) labelled RAD51 as well as all of the RAP80 fragments.

**Experiment #4: Generation of a stable cell line expressing EGFP tagged wildtype BLM in BS3509-hTERT cells**

To study the *in vivo* dynamics of BLM helicase, a lentiviral mediated GFP-BLM stable cell was generated line in BS3509-hTERT cells. Hence to generate the
stable cell line the full length EGFP- BLM (1-1417) was cloned in pIRES vector using its SpeI and NotI sites (Figure S4A).

![Supplementary Data](image)

Figure S4: Generation and characterization of stable cell line pIRES BLM WT (1-1417) in BS3509-hTERT cells. (A) Mini prep DNA of the pIRES-GFP-BLM (1-1417) was prepared, digested with SpeI and NotI and checked for the release of the respective insert. B. Analysis for BLM expression in six clones. Western analysis was carried out with anti-BLM and hsp 90 antibodies. Cells were grown asynchronously and the lysates were prepared. Western analysis was carried out using anti-BLM antibody.

The analysis for BLM expression in six clones was carried out using western blotting against anti BLM antibody. EGFP-tagged BLM was expressed to varied levels in all the analysed clones. Hsp90 was used as a loading control (Figure S4B).

**Experiment #5: Generation of the constructs pIRES EGFP BLM-2K and pIRES EGFP BLM-3K**

As part of a strategy to generate stable lines, which would express EGFP-tagged mutant BLM which are incapable of undergoing ubiquitylation, two mutant BLM constructs were generated. These constructs were: pIRES-GFP-BLM (1-1417) (2K) i.e (BLM mutated in K105R and K225R) and pIRES-GFP-BLM (1-1417) (3K) i.e (BLM mutated in K105R, K225R and K259R). The mutant EGFP-tagged BLM 2K and 3K were cloned in pIRES vector using SpeI and NotI sites (Figure S5A, S5B).
Figure S5: Cloning of piRES EGFP BLM-2K and piRES EGFP BLM-3K. A. Mini prep DNA of the piRES-GFP-BLM (1-1417) (2K) i.e (BLM mutated in K105R and K225R) was prepared, digested with SpeI and NotI and checked for the release of the respective insert. B. Mini prep DNA of the piRES-GFP-BLM (1-1417) (3K) i.e (BLM mutated in K105R, K225R and K259R) was prepared, digested with SpeI and NotI and checked for the release of the respective insert.

Experiment #6: Determine whether BLM and RNF8/RNF168 act in the same pathway of homologous recombination

It is known that BS cells have a high rate of homologous recombination (Karow et al., 2000a; Chaganti et al., 1990). To determine whether the high rate of HR observed in absence of BLM had an effect on the elevated rates of HR seen in absence of RNF8 or RNF168, a host cell reactivation assay was carried out in presence or absence of the respective ligases and in absence of BLM.
Figure S6: Shutdown of RNF8/RNF168 expression in absence of BLM increases homologous recombination in an additive manner. U2OS shRNF8 (A) and U2OS shRNF168 (B) cells (grown with or without Dox treatment) were transfected with pBHRF construct along with either control siRNA or siRNA against BLM. Before harvesting the cells were grown in presence of HU for 16 hrs. Under each experimental condition the GFP/BFP ratio (as measured via FACS analysis) was determined as readout for homologous recombination. The results were obtained from a minimum of four assay points.

An additive increase in the level of recombination (i.e. the GFP/BFP ratio) was observed when both BLM and RNF8 or RNF168 were absent (Figure S6A, S6B). These results indicate that apart from BLM additional factors involved in negatively regulating homologous recombination also get recruited via RNF8/RNF168 mediated ubiquitylation. The additive recombination rate in absence of the ligase and BLM is probably a reflection of the combinatorial affect of the lack of recruitment all these factors at the site of the DNA damage.

Experiment #7: Determine the extent of RAD51 ubiquitylation in absence or presence of RNF168

Extracts from Vector (from the Riddle Syndrome patients) and the complemented HA-RNF168 cells (with without HU-treatment) were immunoprecipitated with K63-linkage specific ubiquitin antibody. Probing the immunoprecipitate with anti-RAD51 antibody revealed non-compromised RAD51 mono- and poly-ubiquitylation, in both absence and presence of replication stress (Figure S7A, S7B). This indicated that RAD51 ubiquitylation in vivo does not depend on RNF168.
Figure S7: K63-linked ubiquitylation of RAD51 is not compromised in Vector cells from RIDDLE cells. A. Lysates were prepared from untreated (-HU) or treated (+HU) Vector and complemented HA-RNF168 cells. Lysates were immnoprecipitated with either K63-linked ubiquitin antibody or the corresponding IgG. The immunoprecipitates were probed with antibodies against RAD51 (Calbiochem, rabbit polyclonal).