THE ACTIVATION OF ATR IN RESPONSE TO DOUBLE-STRANDED DNA BREAKS

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ABSTRACT

Cells respond to the presence of damaged DNA by activating an interconnected web of signaling pathways that mediate the activities of proteins, which detect, repair, and restore DNA structure. In the presence of double-stranded DNA breaks (DSBs), these responses are primarily mediated by the ATM protein kinase. A related kinase, ATR, regulates the responses to dysfunctional DNA replication and is also activated, in an ATM-dependent manner, when DSBs occur during S-phase. The activation of ATR in response to DSBs is in part achieved by the ability of ATM to interact with TopBP1, an inducer of ATR activity. Additionally, in *Xenopus* egg extracts, the Mre11-Rad50-Nbs1 (MRN) complex is required to bridge ATM and TopBP1 together. With our current work, we show that CtIP, a known MRN-interacting protein, is recruited to DSB-containing chromatin and interacts with TopBP1 in a damage-dependent manner. A region containing the first two BRCT repeats of TopBP1 is essential for this interaction. Furthermore, two distinct regions of CtIP participate in mediating the association between CtIP and TopBP1. The first region includes two putative ATM/ATR phosphorylation sites that when mutated lead to a reduction in binding. Secondly, binding is diminished when an MRN-binding region in the N-terminal region of CtIP is deleted, indicative of the involvement of the MRN complex in mediating this interaction. In addition, the binding between CtIP and TopBP1 is diminished in Nbs1-depleted extracts and, reciprocally, the binding of Nbs1 to TopBP1 decreases in the absence of CtIP. This suggests the formation of a complex containing CtIP, TopBP1 and the MRN complex. When CtIP is removed from egg extracts, the levels of TopBP1 and Nbs1 in damaged nuclei are reduced, thereby compromising the activation of the damage response. Thus, CtIP interacts with TopBP1 in a damage-stimulated, MRN-dependent manner to mediate the activation of ATR in response to DSBs.

The chromatin environment in the vicinity of breaks influences the activation of the DNA Damage Response (DDR). In this report, we explore the involvement of the chromatin remodeling ATPase ISWI in the responses to DNA damage. We find that ISWI associates with ATR, ATRIP, and TopBP1 on DNA in the presence of damage. In addition, ISWI is a substrate of both ATM and ATR *in vitro*. Furthermore, the activities of

ATM and ATR stimulate an increase in the levels of ISWI on chromatin that contains DSBs. Finally, we assessed the role of ISWI in the activation of multiple damage responses in *Xenopus* egg extracts. Taken together, our work describes several previously uncharacterized features of ISWI with implications in the response to damaged and incompletely replicated DNA.

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INTRODUCTION

GENOMIC INSTABILITY AND CANCER

Cancer is a disease in which a group of cells loses the capacity to regulate growth and control of cell division. The tissue generated as a result of uncontrolled growth, referred to as neoplasia from the Greek word meaning "new growth," is likely to originate from a single cell, which by genetic misfortune or exposure to carcinogens suffers dynamic changes in its genome[1]. Initially, these lesions are confined to a few cells within a defined location. Via processes formally analogous to Darwinian evolution—shaped by interactions with neighboring cells and factors in the cellular microenvironment—the founder neoplastic population acquires a series of traits leading to the progressive conversion of the surrounding tissue into a highly malignant derivative, commonly known as a tumor, with deleterious effects to cellular and organismal homeostasis[2].

In humans, tumorgenesis is a multistep process that is characterized by the manifestation of several acquired traits that fundamentally alter cell physiology. At the turn of the millennium, Hanahan and Weinberg described six functional capabilities obtained by normal cells that drive tumor development[3]. Three of these hallmarks—self-sufficiency in growth signals, insensitivity to anti-growth signals, and the evasion of apoptosis—are reflective of the uncoupling of the cellular growth program from signals in

its environment. In order for the incipient neoplasias to maintain their large size as a result of uncontrolled growth, they develop the ability to sustain the process of growth of new blood vessels, or angiogenesis, to supply the growing cancerous tissue with oxygen and nutrients. Furthermore, to fulfill their "tumorgenic agenda," malignant cells overcome the cell-autonomous program to limit replication and develop a limitless replicative potential. Finally, as the tumor continues to grow it invades the adjacent tissue and colonizes non-adjacent tissues or organs in other parts of the body in a process referred to as metastasis.

The conceptual characterization of cancer has been further expanded by a decade of advancements in the understanding of the malignant transformation of cells or oncogenesis. The growing list of defining traits now includes the evasion of immune surveillance and a number of stress phenotypes including metabolic stress, mitotic stress and DNA damage and replication stress[4,5]. Additionally, the increased levels of instability at the nucleotide and chromosomal levels displayed by most human cancers, which, directly or indirectly, lead to the acquisition of these traits, are now regarded as a defined capability conducive to cancer development[3,6,7].

Two seminal papers by Bartkova, Gorgoulis and their colleagues[8,9] supplied evidence that the cellular mechanisms that ensure genomic stability serve as a barrier for tumorgenesis. By visualizing key phosphorylation events indicative of the activation of the DNA damage response (DDR), both groups could observe that in samples of precancerous lesions from early breast and colon tumors, melanomas, and lung and urinary bladder carcinomas, DDR markers accumulated relative to healthy specimens. Interestingly, in samples from more invasive tumors, the DDR indicators were absent. Furthermore,

numerous abnormalities and mutations in the proteins involved in DDR, including the tumor suppressor p53, were observed in lesions from more advanced stages of tumorgenesis[8]. These results are consistent with the observed high frequency of p53 mutations in most types of human cancers[10] and suggest that for the full-blown development of cancer, cells must overcome the blockage imposed by the pathways that monitor DNA damage and activate checkpoints that delay cell cycle progression. This barrier may be breached by mutation of the "genomic caretakers" involved in DDR[11]. Moreover, when healthy cells in culture are induced to overexpress growth factors or proteins with known oncogenic functions to recapitulate the excessive growth characteristic of human cancers, the cells display signs of replication stress including prematurely terminated DNA replication forks and double-stranded DNA breaks (DSBs), both of which lead to subsequent activation of DDR[12,13]. These key developments sparked the formulation of a model for cancer development where the activation of oncogenes stimulates aberrant proliferation accompanied by unscheduled initiation of DNA replication. The excessive amounts of DNA replication lead to replication stress and DNA damage, which provoke the activation of checkpoints to impede the progression of precancerous lesions into more invasive cancers[14]. In summary, these findings place the vigilance of genomic stability by DDR at a critical juncture in the transformation of healthy cells into malignancy.

In our estimation, gaining a better understanding of the activation and regulation of DDR will be fundamental in the development of novel therapies to contain cancer progression.

THE DNA DAMAGE RESPONSE (DDR)

DNA replication is a delicate, spatially and temporally coordinated process where the sequential assembly of factors onto DNA ensures that the genome of each cell is faithfully replicated once per cell cycle[15]. Even within this strict framework, subtle mistakes may occur when billions of bases are being copied. The incidence of errors is elevated in specific chromosomal loci that tend to acquire gaps and breaks. These regions, known as common fragile sites, are particularly sensitive to replication stress and are also frequently rearranged in tumor cells[16,17]. In addition, the highly reactive, oxygen-rich intracellular environment contains an abundance of by-products of cellular metabolism, such as reactive oxygen species (ROS) and free radicals, which pose a continuous threat to the stability of DNA molecules. Based on studies carried out in bacteria and in human cells, it has been estimated that DNA breaks occur anywhere from 0.2 to 50 per human genome replication in the absence of any exogenous insult[18,19]. Even if the conservative estimate is assumed, each one of these lesions may have catastrophic consequences, as they may give rise to gross chromosomal rearrangements such as translocations, duplications, inversions, and deletions that compromise the stability of the genome[20].

To cope with the consequences of normal cellular metabolism, which are continuously exacerbated by the exposure to external insults, cells have evolved surveillance mechanisms to monitor the genomic integrity and coordinate repair and cell cycle progression when damage is detected[21,22]. The response to damage is executed by a network of interacting pathways known as the DNA Damage Response (DDR)[23]. In the

presence of aberrant DNA structures, including nicks, gaps, single-stranded DNA (ssDNA), and double-stranded DNA breaks (DSBs), the activities of these pathways integrate the control of cell cycle transitions and the regulation of DNA repair mechanisms with elements of the ongoing cellular physiology, such as transcription and DNA replication[24]. The resulting circuitry orchestrates these different functionalities through the activity of sensors, transducers, mediators, and effectors that respond to specific types of DNA lesions and initiate the corresponding subroutines depending on the nature and severity of the damage.

In eukaryotes, two large phosphoinositide 3-kinase (PI3K)-related kinases (PIKKs) function as transducers to relay and amplify the damage signal when the integrity of the genome is compromised. In the presence of double-stranded DNA breaks (DSBs), the delay of cell cycle progression and the modulation of repair are mainly coordinated by the activity of the protein kinase ATM. ATM is the protein product of a gene that is defective in the autosomal disorder *ataxia telangiectasia* (A-T)[25]. A-T is a multisystem disease associated with a multitude of cancer-related abnormalities including clinical and cellular radiosensitivity, predisposition to lymphoid tumors, immunodeficiency, and chromosomal instability[26]. On the other hand, the cellular stress response pathways activated as a consequence of the presence of other DNA lesions—gaps, nicks, base adducts, prematurely terminated replication structures, and also DSBs—are mediated by the ATM-related protein kinase ATR[27]. ATR is essential for embryonic development and plays a crucial role in stabilizing the genome during DNA replication[28,29]. The only known link between ATR mutation and disease is a splice mutant of ATR found in a few patients of the rare

Seckel syndrome, characterized by intrauterine growth retardation, dwarfism, microcephaly, and mental retardation[30]. Large-scale proteomic analyses of proteins that become phosphorylated in response to DNA damage suggest that the ATM and ATR signaling networks are responsible for more than 900 regulated phosphorylation events in as many as 700 proteins[31,32]. The breadth of these responses confirms the central role of ATM and ATR at the apex of the signaling pathways of DDR.

A detailed description of the required sensors, interactions and events leading to the activation of these two important regulators of the stress response program will be discussed below. The downstream events carried out by mediator and effector proteins as a consequence of DDR activation are beyond the scope of our analysis. More information on the mediators and effectors of DDR can be found in[33–38].

THE ACTIVATION OF ATM: MORE THAN 1981

The serine/threonine (S/T) protein kinase ATM is a member of the phosphoinositide 3-kinase (PI3K)-related protein kinase (PIKK) family, which also includes ATM and Rad3-related protein (ATR), the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), mammalian target of rapamycin (mTOR), suppressor of morphogenesis in genitalia (SMG-1), and the transformation/transcription domain-associated protein (TRRAP)[39]. Four conserved domains—the FRAP-ATM-TRRAP (FAT) domain[40], a kinase domain (KD), the PIKK-regulatory domain (PRD)[41], and the FAT-C-terminal (FATC) domain[42]—are the defining architectural features of PIKKs. These

proteins display diverse biological activities, but specifically ATM, ATR, and DNA-PKcs play important roles in the response to DNA damage[43].

In unstressed cells, ATM exists as a catalitically inactive dimer[44]. Upon exposure to DNA damage, rapid autophosphorylation of ATM molecules occurs in *trans* allowing the inactive dimers to dissociate into active monomers capable of initiating the cellular response to damage. In humans, the phosphorylation event leading to monomerization occurs on the conserved FAT domain at serine 1981 (S1981) and likely permits the adjacent kinase domain to be more accessible to substrates[44]. Since the elucidation of the mechanism of activation by intermolecular autophosphorylation, phospho-S1981 has been equated to ATM activation. The simplistic view of a single event leading to ATM activation was swiftly challenged by the characterization of other damage-induced autophosphorylation sites on human ATM, S367 and S1893[45]. Mutation of any of these sites, or of the previously characterized S1981, to non-phosphorylatable residues, hindered the capacity of stably expressed ATM to rescue the radiosensitivity, genomic instability, and checkpoint-signaling characteristic of A-T cells[45]. In addition, ATM-deficient mice expressing ATM from a bacterial artificial chromosome (BAC) with the equivalent to human S1981 mutated to alanine (S1987A) as their sole ATM species, displayed no discernible defects in ATM-dependent responses and ATM-S1987A was recruited normally to laser-induced DSBs[46]. Contrary to the observation in mice, but in agreement with the initial model, data from A-T cells complemented with transgene-encoded ATM and expressing the eukaryotic endonuclease I-PpoI revealed that only the wild-type, but not a kinase dead or S1981A mutant ATM, could bind I-PpoI-induced breaks[47]. These

observations have been gathered from different experimental systems that have examined the localization of ATM at different times after the induction of damage (5 minutes after micro-irradiation[46] and 24 hours after IPpoI induction[47]). To clarify these discrepancies, So and colleagues monitored the real-time recruitment of fluorescently-tagged ATM proteins to micro-irradiated regions by live cell imaging[48]. Using this approach, they observe that, even though the initial recruitment is unaffected, the ablation of autophosphorylation at S1981 accelerates the dissociation of ATM from DSBs[48]. Their data suggest that S1981 autophosphorylation stabilizes ATM at DSBs and is therefore required for proper DDR activation.

Another facet of ATM activation, for which there is more consensus, is the requirement of the MRN complex for the recruitment of ATM to sites of damage, a pre-requisite for activation[48–54]. The MRN complex, composed of Mre11, Rad50, and Nbs1, is generally considered as the upstream sensor in the damage response pathways mediated by ATM[55]. Components of the MRN complex are among the first proteins detected at DSBs and their initial recruitment is independent of the activity of ATM[47,56,57]. The initial clues that hinted towards a functional interaction between ATM and the MRN complex came from the significant overlap in cancer susceptibility phenotypes associated with diseases that arise as a consequence of the deficiency of these proteins. *Mre11* mutations cause A-T-like disorder (ATLD) and hypomorphic mutations of *Nbs1* cause Nijmegen breakage syndrome (NBS). Patients suffering from these maladies exhibit immunodeficiency, microcephaly, chromosomal instability, and other symptoms that clinically resemble those of A-T[49,50]. The close relationship between these proteins was further evidenced by the

observation that cells isolated from NBS and ATLD patients are defective in the activation of ATM in response to radiomimetic chemicals[51]. Furthermore, experiments carried out *in vitro* with purified components showed that the activity of ATM towards its substrates, including p53 and Chk2, a principal effector kinase of DDR, was stimulated in the presence of the MRN complex[58].

Detailed analyses of the activities of the MRN complex and its components cemented the notion of MRN as a DNA damage sensor. Mre11 is a DNA binding protein with 3'–5' exonuclease and ssDNA endonuclease activities that are stimulated by the presence of Rad50[59]. The resulting Mre11-Rad50 (MR) complex preferentially binds to DNA ends[60]. This complex is stabilized on DNA by the presence of Nbs1, which is also responsible for the stimulation of several enzymatic activities of the complex, such as DNA duplex unwinding and the cleavage of hairpins. These ATP-dependent processing events require Rad50 to bind ATP in order to be executed[61]. The ATP binding of Rad50 is essential for the stimulation of ATM activity, suggesting that DNA end-processing by MRN plays a crucial role in the activation of ATM by the presence of DNA breaks[53,54]. The MRN complex can also support the tethering of DNA ends[50]. In *Xenopus* extracts, this DNA tethering function of MRN promotes the formation of a high molecular weight DNA damage-signaling complex that contains active ATM. The formation of this complex requires an intact Mre11 C-terminal domain that is deleted in some ATLD patients[62]. In addition to these processing events, the recruitment of ATM to DSBs is achieved by the direct interaction between ATM and Nbs1[52]. This interaction occurs through a defined carboxy-terminal motif that is required for the activity of ATM towards its downstream

targets. The C-terminal ATM binding motif of Nbs1 is conserved among the sensor proteins of other PIKKs and reflects a common mechanism for the recruitment and activation of PIKKs in response to damage[52].

In summary, the activation of ATM in response to DSBs can be viewed as a two-step process[54]. The first step entails the processing and tethering of loose DNA ends by the MRN complex and the subsequent recruitment of inactive ATM dimers to sites of damage. In a second step, the autophosphorylation of ATM is stimulated, leading to the generation of enzymatically active monomers that transduce the damage signal to a plethora of downstream substrates. Furthermore, the retention of these active ATM monomers on damage sites is critical for sustained DDR activity.

The specific circumstances leading to the autophosphorylation of ATM have yet to be clearly defined. A number of candidates that regulate dimer dissociation have been studied, among them, several protein phosphatases[63–65]. Stemming from the observation that treatment of cells with the protein phosphatase inhibitor okadaic acid (OA) induces autophosphorylation of ATM on serine 1981 in the absence of damage, Goodarzi and colleagues characterized an interaction between protein phosphatase 2A (PP2A) and ATM that is disrupted in the presence of damage[63]. Similar effects have been observed when the activity of the Wip1 phosphatase is compromised[65]. These observations suggest that ATM is continuously maintained in an inactive state by the activity of several phosphatases until stress is encountered. In addition to phosphorylation, the increase in the kinase activity of ATM in response to damage has been shown to be stimulated by the acetylation of the PIKK Regulatory Domain (PRD) of ATM by the Tip60 histone acetyltransferase[66].

Mutation of the lysine 3016 acetylation site inhibited ATM monomerization and prevented the phosphorylation of downstream targets[66].

Since the characterization of the 1981 autophosphorylation, changes in chromatin structure have been suspected to mediate dimer dissociation[44]. In the initial report by Bakkenist and Kastan, cells cultured in hypotonic media or treated with agents that relax chromatin structure lead to S1981 autophosphorylation in the absence of damage[44]. In a more recent study, the loss of irradiation-induced acetylation of histone H3 by the chromatin binding protein HMGN1 in *Hmgn1*$^{-/-}$ mice lead to defects in the activation of ATM[67]. Treatment of murine embryonic fibroblasts (MEFs) derived from *Hmgn1*$^{-/-}$ mice with histone deacetylase inhibitors reversed the decrease in S1987 autophosphorylation observed in the absence of HMGN1. These results add to a growing body of evidence that suggests that the loosening of chromatin structure stimulates the activation of ATM and its activity towards downstream substrates[44,47,67–73]. In light of the leading role played by ATM in DDR, we anticipate that much effort will be devoted in the immediate future to clearly illuminate the path leading to the activation of ATM.

THE ACTIVATION OF ATR: ATRIP, A TOP AND A TAIL

As detrimental as a single DSB may be, the lesions that trigger the activation of ATM are a relatively rare occurrence[18,19]. By contrast, ATR is activated during every S-phase to regulate the firing of replication origins and ensure the stability of replication forks[74–77]. The requirement of ATR in the maintenance of fork stability is indicated by the

increase in chromosomal fragmentation observed in embryos of ATR knockout mice, which additionally display severe defects in cellular proliferation, increased incidence of tumors, and lethality at early stages of embryogenesis[28,29]. Furthermore, ATR deficiency in mouse and human cells leads to an increase in the expression of common fragile sites, which are specific regions in the genome that have a tendency to exhibit gaps and breaks, and are particularly prone to genomic instability[75,78]. Likewise, elimination of Mec1 function, the budding yeast homolog of ATR, leads to fork stalling, accumulation of replication intermediates and chromosomal fragmentation, specifically in areas where replication fork progression is intrinsically slow, termed "replication slow zones."[79] The intimate relationship between ATR and the regulation of DNA synthesis is further evidenced by the requirement of replication for the activation of ATR-mediated damage response pathways that, in turn, inhibit the initiation of DNA replication in the presence of damage[80–82].

ATR recognizes a wide array of aberrant DNA structures that trigger its kinase activity towards various downstream substrates, principally Chk1, the main effector kinase of ATR-mediated responses[27,83–85]. In order to undergo activation, ATR must be recruited onto sites of damage. It has long been reasoned that these different types of damage possess a common component that is recognized by ATR. Most data suggest that this structure contains single-stranded DNA, which is generally coated by the single-stranded DNA (ssDNA) binding protein RPA (Replication Protein A)[82,86–88]. Depletion of RPA from *Xenopus* egg extracts compromises the ability of ATR to associate with damaged chromatin, thereby jeopardizing its capacity to trigger a checkpoint response[82,87]. Similar

results have been observed in human and budding yeast systems, suggesting, in conjunction with what was observed in *Xenopus*, that RPA-coated ssDNA (RPA-ssDNA) is the common intermediate that serves as a signal to activate the ATR-mediated DDR[76,88]. Subsequently, using cell-free *Xenopus* nucleoplasmic extracts, Byun and colleagues demonstrated that the functional uncoupling of the MCM helicase and the replicative polymerase observed in the presence of damage accounts for the generation of unusually long stretches of ssDNA[89]. In addition to this mechanistic insight, findings from the same research group showed that ATR activity, as measured by the phosphorylation of Chk1, further increases in the presence of primed ssDNA. The level of Chk1 phosphorylation was also influenced by the amount of ssDNA adjacent to the primer[90]. These findings are consistent with the previously characterized requirement of RNA primer synthesis by primase for the phosphorylation of Chk1 in response to stalled replication forks[91]. Taken together, these results indicate that the consensus structure that maximally stimulates the activity of ATR contains long stretches of RPA-ssDNA adjacent to a double stranded- (ds-)/ssDNA juncture[92].

ATR is recruited onto the checkpoint-stimulating structure via its stable binding partner ATRIP (ATR-intercating protein), which directly binds to RPA[52,88,93,94]. ATRIP co-localizes with ATR on sites of DNA damage, and its knockdown in human cells using small interfering RNA (siRNA), reduces the levels of ATR expression, leading to a loss of DDR and cell death[93]. The interaction between ATR and ATRIP is mediated by a C-terminal ATR interaction motif on ATRIP, which physically and functionally resembles the ATM-binding motif of Nbs1 described above. Deletion of the last 32 residues of the

human ATRIP, which contain the PIKK-interacting module, abrogates the binding of ATR onto sites of damage and inhibits the phosphorylation of Chk1 in response to UV irradiation[52]. The N-terminus of ATRIP, on the other hand, contains a highly conserved acidic patch, the Checkpoint Recruitment Domain (CRD), which interacts with the basic cleft of the largest subunit of the RPA heterotrimer, RPA 70. Consistent with what can be observed with human proteins, mutating the CRD of Ddc2, the budding years homolog of ATRIP, prevents proper localization of Ddc2 to DNA breaks and sensitizes yeast cells to DNA-damaging agents[94]. Together, these findings define ATRIP as an obligate subunit of ATR, which is indispensable for the recruitment of ATR to RPA-ssDNA.

Once recruited onto sites of damage, ATRIP additionally stimulates the activity of ATR by mediating the interaction between ATR and TopBP1[41]. Vertebrate TopBP1, along with its fission and budding yeast homologs, Cut5 and Dpb11, have been characterized as having dual roles in the initiation of DNA replication and the regulation of checkpoint responses[95,96]. In *Xenopus* extracts, TopBP1 is required for the assembly of the checkpoint signaling machinery at sites of damage. The notion of TopBP1 as an essential regulator of ATR has been further substantiated by findings from our laboratory. The work of Akiko Kumagai describes a region of TopBP1 that specifically associates with ATR and strongly stimulates ATR kinase activity[97]. In a manner that is dependent on the presence of ATRIP, incubation of ATR with the ATR-activating domain (AAD) of TopBP1 leads to a dramatic increase in the intrinsic kinase activity of ATR. Furthermore, ectopic expression of an AAD-containing TopBP1 fragment in human cells stimulates the phosphorylation of physiological substrates of ATR in the absence of stress[97]. Likewise, subsequent work

from Toledo and colleagues showed precocious ATR activation by overexpression of the TopBP1 AAD[98]. In a heterologous experimental setup, a retrovirally expressed AAD of human TopBP1 could be selectively induced to localize to the nucleus of mouse cells. Induction of nuclear localization triggered ATR activity in the absence of damage[98]. In fact, continuous stimulation of ATR in this system drove cells into senescence, providing additional evidence of the tumor-suppressive potential of DDR[98]. Together, these findings have described a leading role for TopBP1 to establish its position as a prominent actor in the ATR activation stage.

Within the framework of DDR, ATR is a transducer of the DNA damage signal to a host of downstream mediators and effectors. As is the case with ATM, a sensing complex is required to detect damage and enhance the recruitment and retention of ATR on sites of damage. In response to replication stress, this sensor is the Rad9-Hus1-Rad1 (9-1-1) complex[99,100]. The 9-1-1 complex forms a heterotrimeric ring-shaped molecule that structurally resembles the proliferating cell nuclear antigen (PCNA), a sliding clamp that is loaded around DNA at sites of ongoing replication to enhance polymerase processivity[100]. During normal DNA replication, loading of PCNA is achieved by the activity of a clamp loader, the pentameric Replication Factor C (RFC)[15]. In an analogous manner, a damage specific clamp loader, Rad17, together with the four smaller RFC subunits, loads the 9-1-1 complex onto sites of damage independently of ATR[101,102]. Both, Rad17 and 9-1-1 display increased affinity towards DNA ends adjacent to RPA-ssDNA, underlying the importance of this unique structure in damage recognition by sensors[103]. The recognition of similar structures by ATRIP and Rad17/9-1-1 suggests that, the proximity of these factors, which

are independently recruited to lesions, is what stimulates their activity. Evidence to support this model comes from work done in budding yeast, where the induced co-localization of Mec1-Ddc2 and 9-1-1 onto DNA is sufficient to trigger the activation of the Chk1 homolog Rad53 in the absence of damage[104]. In agreement with this proximity model, the budding yeast 9-1-1 complex has been reported to directly activate Mec1 *in vitro*[103].

The proximity-induced direct activation of ATR by 9-1-1 has not been observed in any other organism. Instead, what has been clearly demonstrated in eukaryotes is that, in response to stalled replication forks, the 9-1-1 complex activates checkpoint signaling by regulating the interaction between ATR and TopBP1[105,106]. In *Xenopus*, the interaction between 9-1-1 and TopBP1 is mediated by the first two Brca1 C-terminus (BRCT) repeats of TopBP1. BRCT repeats are phosphopeptide interacting modules common in proteins involved in DNA damage and cell cycle repair[107]. Other than mediating the interaction with 9-1-1, the BRCT I-II region of *Xenopus* TopBP1 is required to establish the interaction between ATR-ATRIP and TopBP1, a prerequisite for Chk1 activation[106]. The binding of 9-1-1 to the BRCT I-II of TopBP1 occurs via the extensively modified C-terminal tail of Rad9, which has been previously shown to interact with TopBP1[105,108]. Specifically, the constitutive phosphorylation of the Rad9 C-terminal tail on serine 373 (S373) in critical for the interaction with TopBP1. In addition, the activity of Rad17 has proven to be instrumental in the establishment of the TopBP1/9-1-1 interaction. Immunodepletion of Rad17 from egg extracts compromises the recruitment of 9-1-1 and reduced the accumulation TopBP1 on stalled forks thereby affecting Chk1 activation. Whereas addition of wild-type Rad17 restored these defects, an ATP-binding deficient

mutant of Rad17 could not[109]. These recent finding by our laboratory have lead to the formulation of a model that summarizes our current understanding of the 9-1-1-mediated activation of ATR in response to stalled replication forks[109]. Initially, Rad17 loads 9-1-1 onto DNA, which already contains a basal level of TopBP1 required for the initiation of replication. Subsequently, an increase in the level of TopBP1 on chromatin is stimulated by the presence of both Rad17 and 9-1-1. Finally, the accumulated TopBP1 interacts with ATR-ATRIP leading to an increase in the activity of ATR to its downstream substrates.

THE ATM-TO-ATR SWITCH

Many different aberrant DNA structure induce the activity of ATR. Among them, double-stranded DNA breaks (DSBs) are capable of triggering an ATR-mediated response. Until recently, how this was achieved remained unknown. To address this issue, Jazayeri and colleagues took advantage of the fact that ionizing radiation (IR) induces the activation of both ATM- and ATR-mediated damage responses[110]. By monitoring the response to IR in A-T and ATR-Seckel cells, they observed that, although the phosphorylation of most ATM-specific substrates is compromised in ATM deficient A-T cells, activation of these substrates occurs normally in Seckel cells, which display compromised ATR responses. In a similar fashion, ATR-specific substrates are still phosphorylated in A-T cells. Conversely, the phosphorylation of Chk1 is defective in both cell types. In addition to ATM, the IR-induced phosphorylation of Chk1 requires the nuclease activity of Mre11 in order to process DSBs and generate the ssDNA-RPA needed to activate ATR. Moreover,

the activation of ATR by ATM in response to DSBs is restricted to S and G2 phases of the cell cycle and requires the activity of a cyclin-dependent kinase (CDK)[110]. This requirement of CDK in this process is consistent with the observed requirement of CDK1 in budding yeast to promote RPA-ssDNA formation and Mec1 activation during S and G2[111].

Subsequently, CtIP, which physically and functionally interacts with Mre11, was shown to promote ATR activation in response to DSBs by stimulating end resection and generation of RPA-ssDNA. CtIP, which is specifically recruited to DSBs at S and G2 phases, is also required for efficient repair of breaks by homologous recombination (HR)[112]. CtIP is the eukaryotic homolog of the budding yeast Sae2 and fission yeast Ctp1 proteins[113]. The abnormal phenotypes of isolated sae2 null mutants—unresected DSBs, defects in repair by HR, and damage sensitivity—are almost identical to the ones conferred by some rad50 and mre11 mutant alleles. These observations have prompted Sae2 to be considered as the "fourth member" of MRX, the budding yeast MRN complex[114]. The ability of CtIP to stimulate the resection of DSBs is partly modulated by the phosphorylation of two conserved residues by CDK, serine 327 (S327) and threonine 847 (T847)[115]. Mutation of S327 compromises the capacity of CtIP to generate RPA-ssDNA, leading to defects in HR, specifically in G2 and M phases. Conversely, repair by microhomology-mediated end joining (MMEJ), a more error-prone homology-independent pathway, is not affected in the absence of S327 phosphorylation[115]. The phosphorylation on S327 is also critically important to mediate the interaction between CtIIP with the tumor suppressor protein BRCA1[116]. Together, these results lead to the proposal of a model

where the CDK-stimulated resection of ends modulated by CtIP promotes a switch from repair by the error-prone MMEJ pathway to error-free HR as cells progress through S-phase. This transition allows increased fidelity of repair and promotes genomic stability in the face of DNA damage[112]. In addition, we speculate that the expansion of RPA-ssDNA achieved by the activity of CtIP, which is conducive to ATR activation, might represent an amplification step in the response to DSBs, which is exclusive to S-phase when DNA is more sensitive to insults.

The activation of ATR in response to stalled forks not only requires the RPA-ssDNA template for activation, but also the TopBP1 stimulatory activity and the localization to sites of damage by a sensor complex. Interestingly, ATM stimulates the interaction between ATR and TopBP1 in the presence of DSBs[117]. ATM phosphorylates TopBP1 within the AAD on serine 1131 of the *Xenopus* protein, to enhance the association between TopBP1 and ATR-ATRIP. The DSB-induced activation of ATR is additionally enhanced by the interaction between TopBP1 and the MRN complex[118]. TopBP1 specifically interacts with Nbs1 and abrogation of this interaction leads to a decrease in Chk1 phosphorylation in egg extracts supplemented with DSB-containing DNA templates. The interaction between TopBP1 and Nbs1 requires the same BRCT I-II region required for the association of TopBP1 with the 9-1-1 complex[118].

Taken together, these findings suggest that the ATR response to DSBs is mediated in a manner very similar to the response to stalled replication forks, but contains an additional level of processing that is suggested to be exclusive to stages of the cell cycle where homologous sequences are available for repair by HR. In this view, the MRN

complex senses the initial signal, leading to the recruitment of ATM and triggering a "normal" ATM-mediated response. As these ends become processed for repair, RPA-ssDNA, which is required for repair by HR, is generated [119]. The presence of RPA-ssDNA is sensed by proteins involved in ATR activation, namely ATR-ATRIP and TopBP1, leading to the stimulation of ATR activity towards its downstream substrates. It remains to be seen if any additional sensors are required to recruit ATR-ATRIP to resected DSBs, or if the MRN complex fulfills that role.

A biphasic DSB response involving ATM and ATR has been human cell extracts containing defined DNA structures[120]. In this experimental setup, ATM becomes activated by strctures composed of a ds/ssDNA junction with either a blunt end or a short single-stranded overhang (SSO). As the length of the SSO increases, ATM activation is attenuated but ATR activation is potentiated. *In vivo* results were consistent with this observation, as ATM-mediated phosphorylation events are observed earlier than ATR-mediated events in cells treated with IR. In addition, the appearance of indicators of ATR activation, in this case Chk1 phosphorylation, can be accelerated by the overexpression of CtIP. The overexpression of CtIP along with Exo1, a nuclease with known roles in DSB resection, also lead to a decrease in S1981 phosphorylation of ATM[120–123]. These results underscore the importance of resection in mediating the ATM to ATR switch.

In the following chapter we will discuss our most recent findings, which advance our understanding of the involvement of CtIP, TopBP1, and the MRN complex in the activation of ATR in response to DSBs.

CHAPTER II

CTIP INTERACTS WITH TOPBP1 TO MEDIATE THE RESPONSE TO DNA DOUBLE-STRANDED BREAKS (DSBS) IN *XENOPUS* EGG EXTRACTS

ABSTRACT

In the presence of double-stranded DNA breaks (DSBs), TopBP1-dependent activation of ATR is achieved by the ability of TopBP1 to interact with ATM. ATM phosphorylates TopBP1 on serine 1131 to enhance the interaction between ATR and TopBP1 thereby increasing the kinase activity of ATR towards its downstream substrates. In *Xenopus* egg extracts, the Mre11-Rad50-Nbs1 (MRN) complex is also required to bridge ATM and TopBP1 together. In this report we show that CtIP, which is recruited to DSB-containing chromatin, interacts with TopBP1 in a damage-dependant manner. An N-terminal region containing the first two BRCT repeats of TopBP1 is essential for this interaction. Furthermore, two distinct regions in the N-terminus of CtIP participate in mediating the association between CtIP and TopBP1. The first is a region that includes a potential ATM/ATR phosphorylation site on serine 275 (S275). Secondly, binding is also diminished when an MRN-binding region spanning residues 25–48 is deleted, indicative of a role for the MRN complex in mediating this interaction. This was further evidenced by a decrease in binding between CtIP and TopBP1 in Nbs1-depleted extracts and a reciprocal decrease in the binding of Nbs1 to TopBP1 in the absence of CtIP, suggestive of the formation of a complex containing CtIP, TopBP1 and the MRN complex. When CtIP is immunodepleted from egg extracts, the activation of the response to DSBs is compromised and the levels of TopBP1 and Nbs1 on damaged nuclei are reduced. Thus, CtIP interacts with TopBP1 in a damage-stimulated, MRN-dependent manner to mediate the activation of ATR in response to DSBs.

INTRODUCTION

Eukaryotic cells have evolved a series of intricately choreographed processes to ensure that their DNA is swiftly repaired if harmed by the continuous assault of metabolic byproducts and exogenous insults[22]. A failure in repair is usually associated with multiple pathological disorders, including cancer[7]. Genomic safeguarding is achieved by the activation of checkpoints that delay cell cycle progression while damage is being repaired[35,124]. Members of phosphoinositide 3-kinase (PI3K)-related protein kinase (PIKK) family of protein kinases, ATM and ATR, function as the principal regulators of the response to damaged DNA[25,27,74]. ATM specifically responds to double-stranded DNA breaks (DSBs), the most deleterious form of damage[14]. Conversely, ATR is activated in response to different types of DNA lesions, including DSBs, and has been largely characterized as the main apical kinase in the response to stalled replication forks[27].

The activation of ATR is a highly regulated process that is partly modulated by its interaction with other proteins. Among these factors is the ATR-Interacting Protein (ATRIP) that is crucial for the checkpoint regulatory functions of ATR[88,93]. ATRIP is required to establish a critical association between ATR and the BRCA1 C-terminus (BRCT) repeat-containing protein, TopBP1, which is essential for the activation of the ATR-ATRIP kinase complex[41]. In *Xenopus* egg extracts and in human cells, the exogenous introduction of a TopBP1 fragment that interacts with the ATR-ATRIP complex, the ATR-Activating Domain (AAD), triggers an increase in the kinase activity of ATR towards a number of substrates including Chk1, the main downstream effector kinase

of the DNA Replication Checkpoint[97]. The activation of ATR-ATRIP by TopBP1 is further regulated by differentially specified interactions determined by the nature of the initial source of damage[106,109,117,118]. In response to DSBs, the TopBP1-mediated activation of ATR is achieved through the phosphorylation of TopBP1 by ATM[117]. ATM phosphorylates TopBP1 on serine 1131 (S1131), a modification that leads to the strengthening of the interaction between ATR and TopBP1 thereby enhances the activity of ATR. The activation of ATR in response to DSBs is additionally regulated by the capacity of TopBP1 to interact with the Mre11-Rad50-Nbs1 (MRN) complex, specifically with Nbs1[118]. The MRN complex is one of the first factors to be localized to DSBs and, together with ATM, is responsible for the generation of stretches of single-stranded DNA (ssDNA) that are subsequently coated by Replication Protein A (RPA)[51,110]. The production of ssDNA (RPA-ssDNA) is crucial for proper localization of the ATR-ATRIP complex to sites of damage and therefore another important determinant of ATR activity[88,89,110,120].

Among the agents that along with ATM and the MRN complex are required for the formation of the checkpoint-stimulating RPA-ssDNA is CtIP[57,112–114,120,125]. CtIP was initially identified in yeast two-hybrid screens as an interacting protein of the transcriptional co-repressor CtBP (CtBP-Interacting Protein)[126,127]. Subsequently, it has been described as an ATM substrate that is recruited to DSBs to mediate the activation of repair by homologous recombination (HR)[112,115,116,128–132]. In fission yeast and in higher eukaryotes the recruitment of Ctp1 and CtIP, respectively, to DSBs is partly mediated by the activity of the MRN complex[57,113,133]. CtIP has been shown to physically interact with

all the components of the MRN complex through multiple points of contact and the absence of either CtIP or Mre11 decreases the frequency of HR repair to levels similar to those achieved by the depletion of Rad51, a critical regulator of the HR pathway[112,133,134]. Likewise, fission yeast cells lacking Ctp1 displayed defects in HR similar to those observed in *nbs1Δ* and *mre11Δ* mutant strains. In addition, *ctp1Δ* strains were more sensitive to DNA-damaging agents because of inefficient formation of RPA-ssDNA adjacent to breaks [113]. Similar results were observed in human cells, where knockdown of CtIP by siRNA resulted in a reduction of RPA accumulation in irradiated regions thereby compromising the recruitment of ATR to DSBs, abolishing ATR activity[112].

The parallels exhibited by CtIP and TopBP1 in mediating the activation of ATR by regulating the formation of RPA-ssDNA in an ATM- and MRN-dependant fashion prompted us to explore the possibility of an interaction between these two checkpoint proteins. Using the *Xenopus* egg extracts we were successful in characterizing a damage-stimulated interaction between CtIP and TopBP1 that requires the first two BRCT repeats of TopBP1. The adjacent ATM/ATR consensus phosphorylation sites S273/S275 are also required for this interaction and it is further stimulated by the presence of Nbs1. The absence of CtIP in egg extracts compromises the activation of ATR in response to DSBs by reducing the level of TopBP1 and Nbs1 on damaged chromatin suggesting that CtIP in conjunction with TopBP1 and the MRN complex work together to stimulate the activity of ATR in response to DSBs.

EXPERIMENTAL PROCEDURES

PREPARATION OF EGG EXTRACTS**:** *Xenopus* egg extracts were prepared as described in [135]. To elicit a checkpoint response extracts were treated with 50 μg/ml of annealed oligonucleotides poly(dA)$_{70}$-poly (dT)$_{70}$ (pA-pT) in the presence of 3 μM tautomycin as described in [33]. To prepare extracts containing chromatin, extracts were supplemented with 3000 demembranated *Xenopus* sperm nuclei/μl. Replication blocks were induced by the addition of aphidicolin at 50 μg/ml. Double-stranded DNA breaks were created by the addition of either the restriction endonuclease PflMI (final concentration of 1 U/μl) or EcoRI (0.5 U/μl). When noted, caffeine was added at a final concentration of 5 mM. Nuclear [136] and chromatin fractions [137] were isolated according to previously described methods. For isolation of fractions from extracts containing caffeine, all buffers were supplemented with 5 mM caffeine.

ANTIBODIES: A DNA fragment encoding amino acids 628–856 of *Xenopus* CtIP was generated by PCR and cloned into a pET-His6 expression vector. The His6-CtIP (628–856) protein was expressed in *Escherichia coli*, isolated with nickel agarose, and used for production of rabbit antibodies at a commercial facility. Affinity-purified antibodies against *Xenopus* versions of ATM, ATR, TopBP1, Nbs1, RPA70, Cdc45 and Orc2 were described previously [87,97,118,138]. Anti-human Mcm2 (BM28), anti-FLAG and control rabbit antibodies (IgG fraction) were purchased from Cell Signaling Technology, Zymed Laboratories and Sigma, respectively.

PRODUCTION OF ^{35}S-LABELED PROTEINS: ^{35}S-labeled proteins were synthesized *in vitro* using the TnT System (Promega). For production of ^{35}S-labeled full-length (amino acids 1-856) and truncated forms of CtIP (amino acids 1–430, 1–359, 1–298 1–247 and 415–856), PCR-generated DNA fragments encoding these regions were cloned into pBluescript and the resulting plasmids were used as templates in the TnT system. Mutant versions of the proteins were produced using QuikChange Kit (Stratagene). ^{35}S-Chk1 was generated as described in [139]

PRODUCTION OF RECOMBINANT PROTEINS: Recombinant, full-length HF-TopBP1 with both hemagglutinin and His6 tags at the N-terminal end and a FLAG tag at the C-terminal end was produced in baculovirus-infected Sf9 cells. Full-length wild-type (WT), DI-II and BRCT I-II versions of TopBP1 were generated as described in [118]. Point mutations were produced using the QuikChange Kit (Stratagene).

PULLDOWNS OF RECOMBINANT TOPBP1 FROM *XENOPUS* EGG EXTRACTS: Recombinant TopBP1 (WT, KKAM, DI-II, BRCT I-II and BRCT I-II-KKAM) (0.5 μg) bound to anti-FLAG antibody beads was incubated in egg extracts (100 μl) containing 100 μg/ml cycloheximide and 3 μM tautomycin in the absence or presence of 50 μg/ml pA-pT. When noted, caffeine was added at a final concentration of 5 mM. The beads were isolated by centrifugation and washed three times in CHAPS buffer (10 mM HEPES-KOH, pH 7.5, 0.1% CHAPS, 150mM NaCl, 2.5 mM EGTA, 20 mM β-glycerolphosphate and 0.5% NP-40) and twice with HEPES-buffered saline (HBS) (10 mM HEPES-KOH, pH 7.5, 150mM NaCl). Bound proteins were subjected to SDS-PAGE and immunoblotting.

IMMUNOPRECIPITATIONS: For interphase immunoprecipitations, interphase extracts (100 μl) were incubated under constant agitation for 45 min at 4ºC with Affiprep-protein A beads (Bio-Rad) coated with anti-TopBP1 (3 μg) or control IgG antibodies (3 μg). The beads were isolated by centrifugation and washed three times in CHAPS buffer and twice with HBS. Bound proteins were subjected to SDS-PAGE and immunoblotting. For nuclear immunoprecipitates, nuclei isolated from extracts (250 μl) were subsequently lysed by the addition of half the volume of the original extract in CHAPS buffer and incubation at 4ºC with rotation for 30 min. Lysates were spun down in fixed rotor centrifuge for 10 min at 14,000 x g. An equal volume of 20 mM HEPES-KOH (pH 7.5) was added to supernatant. Diluted lysates were incubated with protein A beads coated with anti-TopBP1 (3 μg), anti-CtIP (3 μg) or control antibodies (3 μg) under constant agitation for 45 min. at 4ºC. Beads were washed 3 times in CHAPS buffer and twice in HBS before addition of 2X-SDS sample buffer for SDS-PAGE and immunoblotting analysis.

IMMUNODEPLETIONS: For immunodepletion of CtIP, interphase extracts (100 μl) were incubated at 4ºC for 60 minutes with 25 μg of CtIP antibodies bound to 20 μl of protein A beads. Equal amounts of rabbit IgG antibodies were used as a control. After incubation, beads were recovered by gentle centrifugation and the supernatants were treated for a second round of depletion. Resulting supernatants were then used in the specified experiments. Nbs1 was immunodepleted as described [138].

RESULTS

CTIP ASSOCIATES WITH DSB-CONTAINING CHROMATIN IN *XENOPUS* EGG EXTRACTS

The *Xenopus* egg extract system has been a valuable resource for the molecular characterization of the pathways that mediate the response to stalled replication forks. The tools generated to study the activation of these checkpoint pathways have recently been utilized in the dissection of the response to double-stranded DNA breaks (DSBs)[57,117,118]. In these studies, DSBs were generated by supplementing egg extracts with the restriction endonuclease EcoRI. Treatment of extracts with EcoRI only yielded a modest activation of the DNA damage response as gauged by the phosphorylation of Chk1 in comparison to the stronger activation caused by the DNA polymerase inhibitor aphidicolin (Figure 1A, compare lanes 2 and 3). To generate a more robust response to DSBs we used the endonuclease PflMI. This enzyme, which produces non-complementary DNA ends and provokes a very robust Chk1 response (Figure 1A, lane 4). Treatment with PflMI also caused a dramatic increase in the nuclear accumulation of RPA, while only causing a modest increase in the phosphorylation of Chk2 (Figure 1A, lane 8).

With this system, we set out to characterize the involvement of CtIP in the DSB response. Upon treatment with PflMI a considerable decrease in the mobility of CtIP was observed (Figure 1B), similar to the slower-migrating, hyper-phosphorylated form of CtIP that arises in response to ionizing radiation (IR) and topoisomerase inhibitors in human cells[112,128]. Nbs1, a member of the MRN complex involved in the early stages of DNA damage sensing and TopBP1, a crucial regulator of the activation of ATR, both displayed

similar changes in mobility in response to PflMI. In extracts treated with the aphidicolin we could observe a modest increase in the accumulation of CtIP in nuclear fractions but no discernible change in mobility. The replication initiation factor Cdc45, on the other hand, displayed significant nuclear accumulation in response to replication blocks triggered by aphidicolin but no changes upon treatment with PflMI when compared to untreated controls.

To further characterize the behavior of CtIP in the response to DSBs, we examined its recruitment to chromatin in response to damage (Figure 1C). In the absence of damage or in the presence of replication blocks, CtIP only marginally loaded onto chromatin. Conversely, we could observe a substantial accumulation of CtIP on chromatin treated with PflMI. The chromatin-bound CtIP also displayed the decreased mobility shift seen in nuclear fractions. PflMI treatment also triggered an increase in the recruitment of TopBP1, Nbs1 and the ssDNA-binding protein RPA onto damaged chromatin. Increased binding of a plethora of replication and checkpoint factors in response to damage is regulated by ATM and ATR. Inhibition of these kinases with caffeine disrupts checkpoint signaling and thus leads to a failure to activate the downstream effector kinase Chk1[33]. Interestingly, however, certain proteins involved in the regulation of Chk1 activation and other checkpoint functions are seen to increase dramatically on damaged chromatin in the presence of caffeine[87]. To assess if CtIP falls within this class of proteins, we looked at the DSB-induced chromatin binding of CtIP in the presence of caffeine (Figure 1D). This was indeed the case, as the levels of CtIP on PflMI-treated chromatin are further increased in extracts supplemented with caffeine. In fact, treatment with caffeine alone or along with

aphidicolin leads to a subtle increase of CtIP binding onto chromatin, implying a strong correlation between the checkpoint functions of ATM and ATR and the chromatin binding of CtIP. Taken together, our results indicate that a modified form of CtIP is loaded onto chromatin containing DSBs and that this recruitment is sensitive to the presence of a functional checkpoint apparatus.

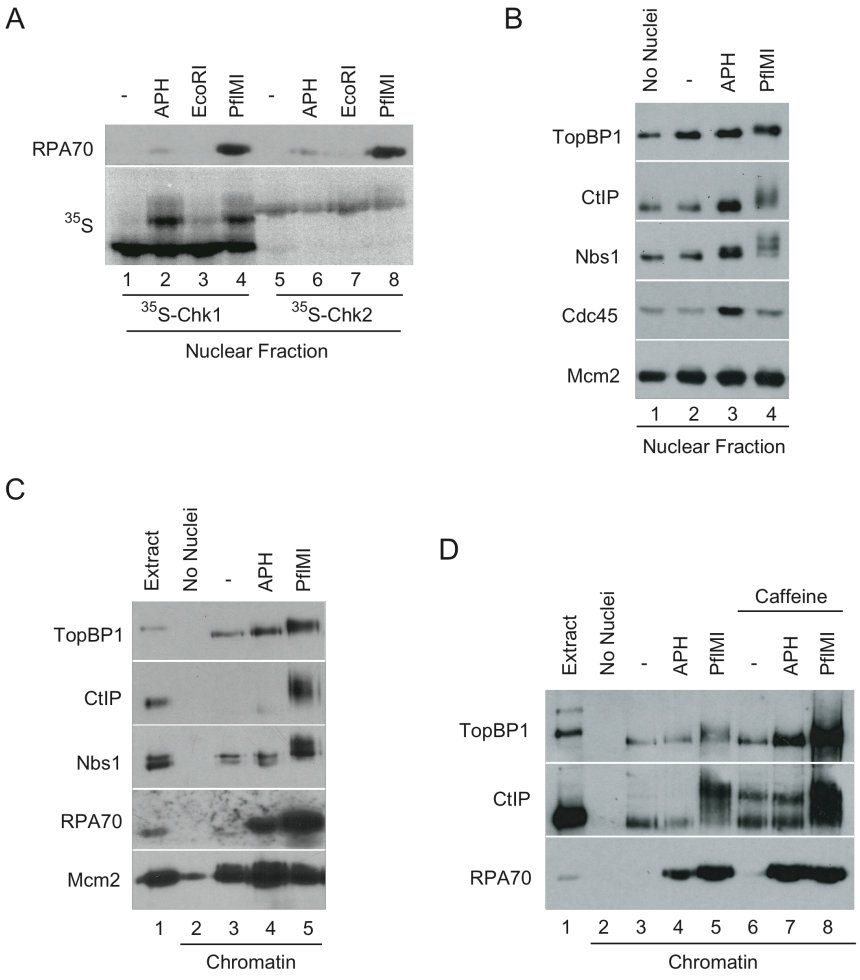


Figure 1: CtIP associates with DSB-containing chromatin

(A) Egg extracts containing ^{35}S-Chk1 (lanes 1–4) or ^{35}S-Chk2 (lanes 5–8) and demembranated sperm nuclei were incubated with no checkpoint inducer (lanes 1 and 5) or sperm nuclei treated with 50 μg/ml aphidicolin

(lanes 2 and 6), 0.5 U/μl of EcoRI (lanes 3 and 7) or 1 U/μl of PflMI (lanes 4 and 8). Nuclear fractions were isolated and analyzed by phosphorimaging and immunoblotting with anti-RPA antibodies.
(B) Egg extracts lacking (lane 1) or containing sperm nuclei (lanes 2–4) were incubated without checkpoint inducer (lane 2), 50 μg/ml aphidicolin (lane 3), or 1 U/μl PflMI (lane 4). Nuclear fractions were subjected to SDS-PAGE and immunoblotted with anti-TopBP1, anti-CtIP, anti-Nbs1, anti-Cdc45, and anti-Mcm2 antibodies.
(C) Egg extracts lacking (lane 2) or containing sperm nuclei (lanes 3–5) were incubated without checkpoint inducer (lane 3), 50 μg/ml aphidicolin (lane 4), or 1 U/μl PflMI (lane 5). Chromatin fractions were subjected to SDS-PAGE and immunoblotted with anti-TopBP1, anti-CtIP, anti-Nbs1, anti-RPA70, and anti-Mcm2 antibodies. One microliter of extract was loaded in lane 1 as a loading control.
(D) Chromatin fractions were isolated from egg extracts lacking (lane 2) or containing sperm nuclei treated as above in (C) (lanes 3–5) or in the presence of 5 mM caffeine (lanes 6–8). One microliter of extract was loaded in lane 1 as a loading control.

CtIP INTERACTS WITH TopBP1 IN *XENOPUS* EGG EXTRACTS

Activation of checkpoint signaling in response to DSBs is mainly regulated by the activity of ATM. The initial activation of ATM in turn leads to an increase in ATR activity [120]. In egg extracts, the transition from the initiating function of ATM to the subsequent activation of ATR is in part achieved by the ATM-dependent phosphorylation of TopBP1 [117]. TopBP1 molecules that have been phosphorylated on S1131 interact more strongly with ATR. This interaction is a crucial step in the initiation of ATR signaling in response to damage[97,117]. In U2OS cells, CtIP has also been shown to be involved in the recruitment to sites of irradiation as well as in its activation[112]. The involvement of both TopBP1 and CtIP in the activation of ATR in response to DSBs prompted us to investigate if these proteins displayed any functional interactions in *Xenopus* egg extracts. This was indeed the case, as TopBP1 immunoprecipitates (IPs) from interphase extracts supplemented with the checkpoint-stimulating synthetic oligonucleotides dA_{70}-dT_{70} (hereafter termed pA-pT) contained CtIP (Figure 2A). This damage-stimulated interaction was also noticeable in IPs from nuclear lysates of extracts treated with PflMI (Figure 2B). We could also observe the

reciprocal interaction, as TopBP1 was present in CtIP nuclear IPs, albeit with a reduced degree of damage-dependence.

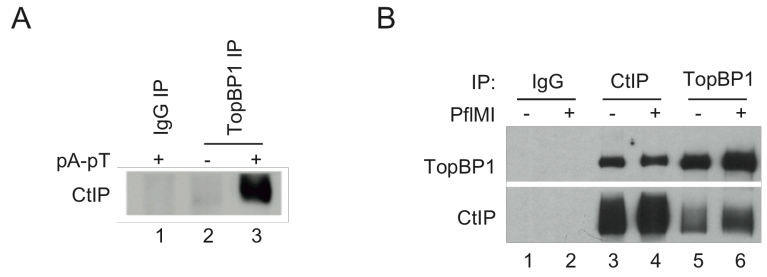


Figure 2: CtIP interacts in a regulated manner with TopBP1 in *Xenopus* egg extracts

(A) Control IgG (lane 1) and anti-TopBP1 immunoprecipitates (IP) from interphase extracts incubated in the absence (lane 2) or presence of 50 μg/ml pA-pT (lane 3) were immunoblotted for CtIP.
(B) Control IgG (lanes 1–2), anti-CtIP (lanes 3–4) and anti-TopBP1 (lanes 5–6) immunoprecipitates (IP) from nuclear fractions isolated from interphase extracts supplemented with demembranated sperm nuclei in the absence or presence of 1 U/μl of PflMI. Isolated nuclear fractions were subjected to SDS-PAGE and immunoblotted with anti-TopBP1 and anti-CtIP antibodies.

THE BRCT I-II REGION OF TOPBP1 IS NECESSARY AND SUFFICIENT FOR ASSOCIATION WITH CTIP

Having determined that CtIP and TopBP1 associate in a checkpoint-induced manner, we investigated which region of TopBP1 is responsible for this interaction. CtIP interacts with the breast cancer tumor suppressor BRCA1 through its BRCT domains in a highly regulated manner to modulate the response to DSBs and the activation of repair pathways throughout the cell cycle[115,116,128–131,140,141]. TopBP1 contains eight BRCT domains distributed throughout the length of the protein (Figure 3A). To assess the necessity of specific BRCT domains of TopBP1 in mediating the interaction with CtIP we

used a series of deletion mutants of recombinant full-length TopBP1 (HF-TopBP1) lacking one or more BRCT repeats[106,117]. These FLAG-tagged mutants were added to extracts in the absence and presence of pA-pT oligomers and subsequently retrieved for analysis (data not shown). Using this approach, we found that the mutant version of TopBP1 lacking its first two BRCT repeats (ΔI-II) fails to interact with CtIP (Figure 3B). The region comprising BRCT repeats I-II is also responsible for the association of TopBP1 with the Nbs1 subunit of the MRN complex (Mre11-Rad50-Nbs1), also a key regulator of the response to DSBs[118]. The interaction between TopBP1 and Nbs1 is compromised when the phosphate-binding pocket within the first BRCT repeat of TopBP1 is disrupted by mutating two adjacent lysines, K154 and K155 to alanine and methionine respectively (designated as the KKAM mutant)[118]. To determine if the mode of CtIP binding to TopBP1 is similar to or mediated by the presence of Nbs1, we incubated HF-TopBP1-KKAM in egg extracts and carried out the pull-downs described above. The KKAM version of TopBP1 displayed a decrease in binding to CtIP, as opposed to a full abrogation of the interaction as seen with Nbs1 (Figure 3B).

To characterize further the requirement of the BRCT I-II region for the CtIP-TopBP1 interaction we utilized a FLAG-tagged fragment of TopBP1 that only contains the first two BRCT repeats (HF-BRCT I-II). This fragment strongly interacted with CtIP in a checkpoint-regulated manner (Figure 3C). As was the case with the full-length protein, the binding of CtIP was decreased when the KKAM mutation was introduced into HF-BRCT I-II.

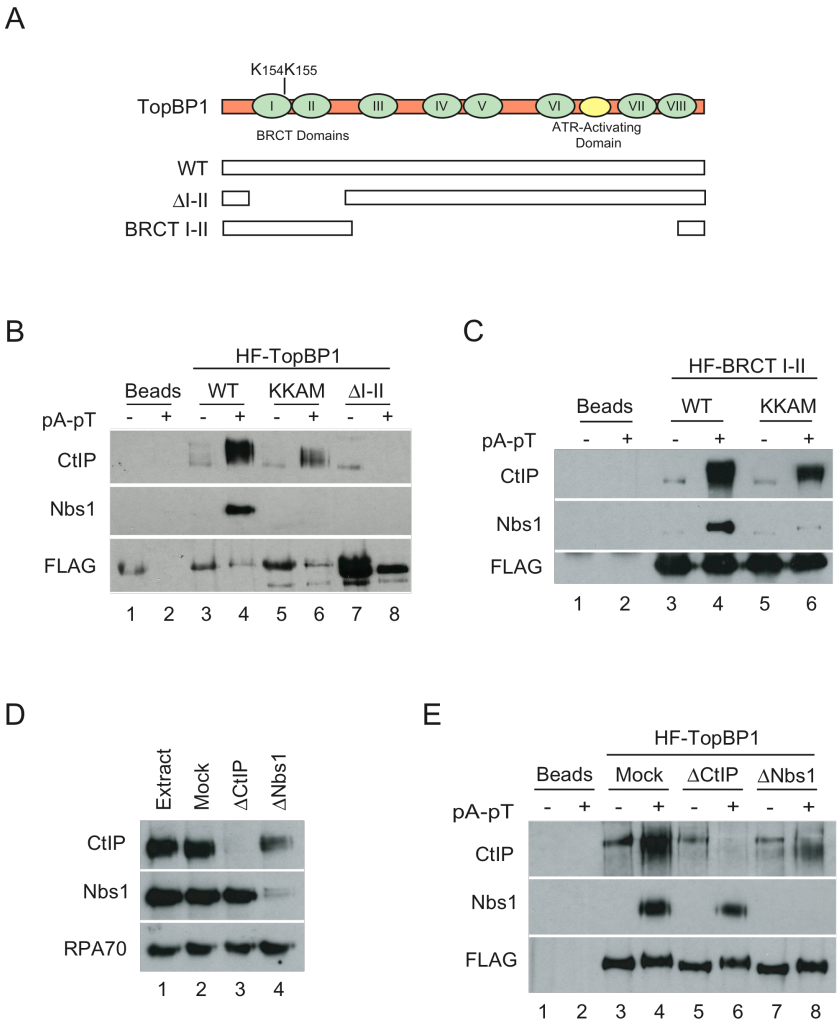


Figure 3: The BRCT I-II region of TopBP1 is necessary and sufficient for association with CtIP

(A) TopBP1 domain architecture. Roman numerals denote BRCT domains of TopBP1. Full-length HF-TopBP1 (WT) and versions of TopBP1 lacking residues 96–282 (ΔI-II) or 360–1485 (BRCT I-II) produced in Sf9 cells. Location of residues mutated in KKAM mutants is specified in schematic.
(B) Anti-FLAG antibody beads containing no recombinant protein (lanes 1 and 2) or the indicated versions of HF-TopBP1 (lanes 3–8) were incubated in egg extracts in the absence or presence of pA-pT. Beads were retrieved and immunoblotted with anti-CtIP, anti-Nbs1, and anti-FLAG antibodies.
(C) Anti-FLAG antibody beads containing no recombinant protein (lanes 1 and 2) or wild-type (lanes 3 and 4) and KKAM (lanes 5 and 6) versions of HF-TopBP1-BRCT I-II were incubated in egg extracts in the absence or presence of pA-pT. Beads were retrieved and immunoblotted with anti-CtIP, anti-Nbs1, and anti-FLAG antibodies.

(D) Representative samples of extracts that were left undepleted (lane 1), mock-depleted with control IgG antibodies (lane 2), depleted with anti-CtIP antibodies (lane 3), or depleted with anti-Nbs1 antibodies (lane 4). Extracts were immunoblotted with anti-CtIP, anti-Nbs1, and anti-RPA antibodies.
(E) Anti-FLAG antibody beads containing no recombinant protein (lanes 1 and 2) or wild-type HF-TopBP1 (lanes 3-8) were incubated in the absence or presence of pA-pT in egg extracts that were either mock-depleted with control IgG antibodies (lanes 3 and 4), depleted with anti-CtIP antibodies (lane 5 and 6), or depleted with anti-Nbs1 antibodies (lanes 7 and 8). Beads were retrieved and immunoblotted with anti-CtIP, anti-Nbs1, and anti-FLAG antibodies. In the CtIP immunoblot, the band in lanes 3, 5 and 7 represents cross-reacting band, not CtIP signal, as it is present even in CtIP-depleted extracts.

In order to define more precisely the contribution of Nbs1 in mediating the interaction between CtIP and TopBP1 we carried out HF-TopBP1 pull-downs in extracts from which we had immunodepleted Nbs1 (Figure 3D). In the absence of Nbs1, the amount of CtIP that interacts with TopBP1 in response to pA-pT is reduced akin to the decreased binding observed with KKAM mutants (Figure 3E). Interestingly, in extracts where CtIP had been removed by immunodepletion, the degree to which Nbs1 interacts with TopBP1 in response to damage is also reduced. Taken together, these results indicate that the first two BRCT repeats of TopBP1 are required for association with CtIP. Additionally, Nbs1 and CtIP reciprocally mediate their association with TopBP1 in response to damage, which suggests the existence of a larger complex that would minimally be composed of CtIP, TopBP1 and presumably the whole MRN complex.

TWO DISTINCT REGIONS IN THE N-TERMINUS OF CTIP MEDIATE ASSOCIATION WITH TOPBP1

We next performed a structure-function analysis of CtIP to determine the region within CtIP necessary for interacting with TopBP1. We initiated these studies by generating ^{35}S-labeled fragments of the N-terminal (residues 1–430) and C-terminal (residues 415–856) halves of CtIP. The radiolabeled proteins were incubated in egg

extracts containing HF-TopBP1. Upon retrieval of HF-TopBP1 with anti-FLAG antibody beads, we could readily observe that in the presence of pA-pT, the N-terminal, but not the C-terminal, fragment of CtIP could bind efficiently to beads containing HF-TopBP1 (Figure 4A). While this manuscript was in preparation, You and colleagues[57] characterized a region of *Xenopus* CtIP spanning residues 479–584, termed the damage-recruitment (DR) motif, which is sufficient for recruitment to EcoRI-treated chromatin in egg extracts. Based on our studies, this region appears not to be required for the association of CtIP with TopBP1. This is consistent with the fact that we have observed the CtIP-TopBP1 interaction in interphase extracts that lack chromatin.

Next, to more precisely define the region required for this interaction, we tested the capacity of smaller N-terminal fragments to bind TopBP1 (Supplementary Figure 1A). Among the generated fragments, the one containing residues 1–359 maintains the capacity to robustly bind TopBP1, whereas the 1–298 fragment is not as efficient in binding while still displaying the large decrease in mobility characteristic of the other binding competent peptides. Moreover, binding is further compromised but not completely abolished in a smaller 1–247 fragment that also fails to shift in response to pA-pT. These studies loosely define a region between residues 247–359 that is modified in response to pA-pT and is involved in the damage-dependent interaction between CtIP and TopBP1 with an additional contribution from another region that further strengthens the interaction.

The first two BRCT repeats of TopBP1 are required for the interaction with CtIP (Figure 3). Previous work has demonstrated that tandem BRCT repeats form a phosphopeptide-binding pocket to mediate their interactions[107,142]. Therefore, we

endeavored to look for potential phosphorylation sites within amino acids 247–359 of CtIP that might be involved in the establishment of the association with TopBP1. This region contains a sequence that is homologous to the known CDK phosphorylation site (SPVF) at S327 in human CtIP (Supplementary Figure 1B). Phosphorylation of this residue by CDK mediates the functionally important interaction with BRCA1[115,116,130]. However, mutation of this critical residue to alanine did not affect the capacity of CtIP to bind TopBP1 in egg extracts (Figure 4B). Another candidate residue within this region is the well-conserved ATM/ATR phosphorylation consensus sequence (S/T-Q) on serine 275 of the Xenopus protein (Supplementary Figure S1C). Because in the *Xenopus* protein this site lies directly upstream of another SQ site (containing S273), we mutated both serine 273 and 275 to alanine within the context of full-length CtIP (to create the CtIP-2AQ mutant) and then performed binding assays to measure the contribution of these sites in mediating the interaction with TopBP1 (Figure 4C). The binding of the 2AQ mutant to TopBP1 was reduced in comparison with wild-type CtIP (Figure 4C, lane 10).

The N-terminal region of human CtIP has been shown to be involved in regulating the response to DSBs by interacting with components of the MRN complex[133]. Specifically, residues 22–45 of human CtIP mediate the interaction with the MRN complex and are required for proper localization of CtIP to sites of damage and activation of the G2/M checkpoint in response to γ-irradiation in HeLa cells. The corresponding residues (25–48 in *Xenopus*), which are highly conserved among higher eukaryotes (Supplementary Figure S1D), were deleted from *Xenopus* CtIP to assess the extent to which this MRN binding region is involved in mediating the interaction with TopBP1. Consistent with the

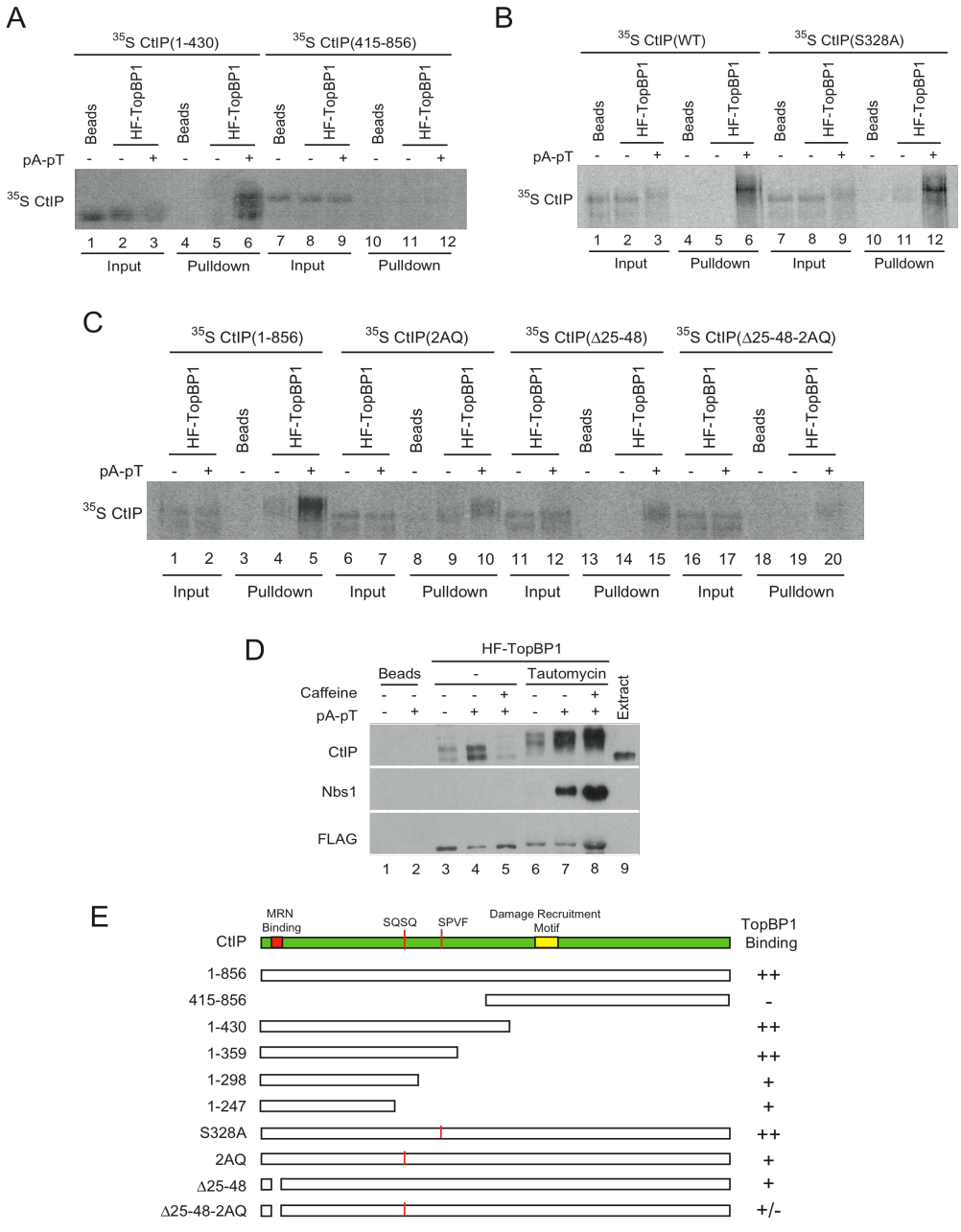
decrease in the strength of the CtIP-TopBP1 interaction in Nbs1-depleted extracts (Figure 3E), the degree of binding of CtIP(Δ25–48) to TopBP1 was significantly lower that that of full-length CtIP (Figure 4C, lane 15). Moreover, in a mutant version of CtIP lacking the MRN-binding domain in combination with the mutated SQ sites, CtIP(Δ25–48-2AQ), we could observe an additive effect, with the interaction being compromised further than for the Δ25–48 and 2AQ mutations alone (Figure 4C, lane 20). It is worth noting that the interaction was not fully abolished in the combined mutant, raising the possibility of a contribution by an additional site or motif in mediating this interaction.

In all of the pull-down experiments described above, extracts were supplemented with the potent phosphatase inhibitor tautomycin to stabilize the checkpoint-stimulated phosphorylation events triggered by pA-pT[33]. Omission of tautomycin from these incubations essentially abolished the interaction between TopBP1 and Nbs1. On the other hand, we could still observe significant, albeit reduced, binding of CtIP to TopBP1 under these conditions (Figure 4D, lane 4). Utilizing this system, we examined the effect of caffeine on the Nbs1-independent interaction between TopBP1 and CtIP. We could readily observe that in the absence of tautomycin, the interaction between CtIP and TopBP1 was abolished when the activities of ATM and ATR were inhibited by caffeine (Figure 4D, lane 5).

Figure 4: Two distinct regions in the N-terminus of CtIP mediate association with TopBP1

(A) Anti-FLAG antibody beads containing no recombinant protein (lanes 1, 4, 7 and 10) or wild-type HF-TopBP1 (lanes 2–3, 5–6, 8–9 and 11–12) were incubated in egg extracts containing ^{35}S-CtIP N-terminal (1–430) (lanes 1–6) and C-terminal fragments (415–856) (lanes 7–12) in the absence or presence of pA-pT. The beads were retrieved and bound ^{35}S-labeled proteins were detected by SDS-PAGE and phosphorimaging (lanes 4–6 and 10–12. Lanes 1–3 and 7–9 are representative samples of extracts used in pull-downs.

(B) Anti-FLAG antibody beads containing no recombinant protein (lanes 1, 4, 7 and 10) or wild-type HF-TopBP1 (lanes 2–3, 5–6, 8–9, and 11–12) were incubated in egg extracts containing full-length ^{35}S-CtIP wild-type (WT) (lanes 1–6) and S328A (lanes 7–12) in the absence or presence of pA-pT. The beads were retrieved and bound ^{35}S-labeled proteins were detected by SDS-PAGE and phosphorimaging (lanes 4–6 and 10–12. Lanes 1–3 and 7–9 are representative samples of extracts used in pull-downs.



(C) Anti-FLAG antibody beads containing no recombinant protein (lanes 3,8,13 and 18) or wild-type HF-TopBP1 (lanes 1–2, 4–7, 9–12, 14–17 and 19–20) were incubated in egg extracts containing full-length ^{35}S-CtIP wild-type (WT) (lanes 1–5), 2AQ (lanes 6–10), Δ25-48 (lanes 11–15) and Δ25-48-2AQ (lanes 16–20) in the absence or presence of pA-pT. The beads were retrieved and analyzed for binding as described in (B).
(D) Anti-FLAG antibody beads containing no recombinant protein (lanes 1 and 2) or wild-type HF-TopBP1 (lanes 3–8) were incubated in egg extracts in the absence (lanes 3–5) or presence (lanes 6–8) of tautomycin. Checkpoint activation was induced by the addition of pA-pT (lanes 4–5, 7–8) and PIKK activity was inhibited by supplementing extracts with 5 mM caffeine (lanes 5 and 8). Beads were retrieved and immunoblotted with anti-CtIP, anti-Nbs1, and anti-FLAG antibodies. One microliter of extract was loaded in lane 9 as a loading control.
(E) Summary of the capacity of indicated forms of CtIP to interact with TopBP1.

In summary, as represented in Figure 4E, two distinct regions within the N-terminus of CtIP are required for the interaction with TopBP1. The first being the region that mediates the binding to the MRN complex at residues 25–48 and a second larger region from amino acids 247 to 359. This second area harbors a conserved putative ATM/ATR phosphorylation at S275, which when mutated, along with another adjacent SQ site, to a non-phosphorylatable residue (2AQ), partially compromises the interaction. Combining the 2AQ mutant with a mutant missing the MRN binding region (Δ25–48) displayed an additive effect, suggesting that these two events, the association with the MRN complex and the phosphorylation by a PIKK, separately mediate the interaction between CtIP and TopBP1.

CtIP mediates damage-dependant nuclear accumulation of TopBP1 in response to DSBs in *Xenopus* egg extracts

CtIP is required for the recruitment of ATR to laser-induced DSBs in human cells [112] and to EcoRI-treated chromatin in *Xenopus* egg extracts[57]. As the recruitment of ATR to DSBs is a function of TopBP1 activity and in light of the interaction described above we

examined how the depletion of CtIP affected loading of TopBP1 onto chromatin in response to DSBs. To assess the effect of the absence of CtIP we generated an antibody directed against a C-terminal region of CtIP (amino acids 628–856) and used it to successfully immunodeplete CtIP from egg extracts (Figure 5A).

We proceeded to determine the effect of depleting CtIP on the activation of the checkpoint response to DSBs. First, we examined the effect of PflMI treatment on the accumulation of checkpoint factors together with the activation of Chk1 in nuclear fractions of CtIP-depleted extracts. There is ample evidence to support the role of CtIP in DNA end resection, which ultimately leads to the accumulation of RPA[57,112,113]. Consistent with these observations, we could note a decrease in the PflMI-induced nuclear accumulation of RPA in CtIP depleted extracts in comparison with mock-depleted extracts (Figure 5B). The loss of resection and ssDNA generation likely to be occurring in the absence of CtIP also leads to a compromise in the activation of Chk1, along with a mild decrease in the levels of ATM and ATR. Moreover, the levels of TopBP1 were dramatically reduced in the absence of CtIP. Depletion of CtIP removed a small amount of TopBP1 from interphase extracts, possibly due to their association (Figure 5A). However, the decrease in nuclear accumulation of TopBP1 in the absence of CtIP is much more pronounced. Moreover, in the absence of damage, the nuclear accumulation of TopBP1 in CtIP-depleted extracts is only slightly affected.

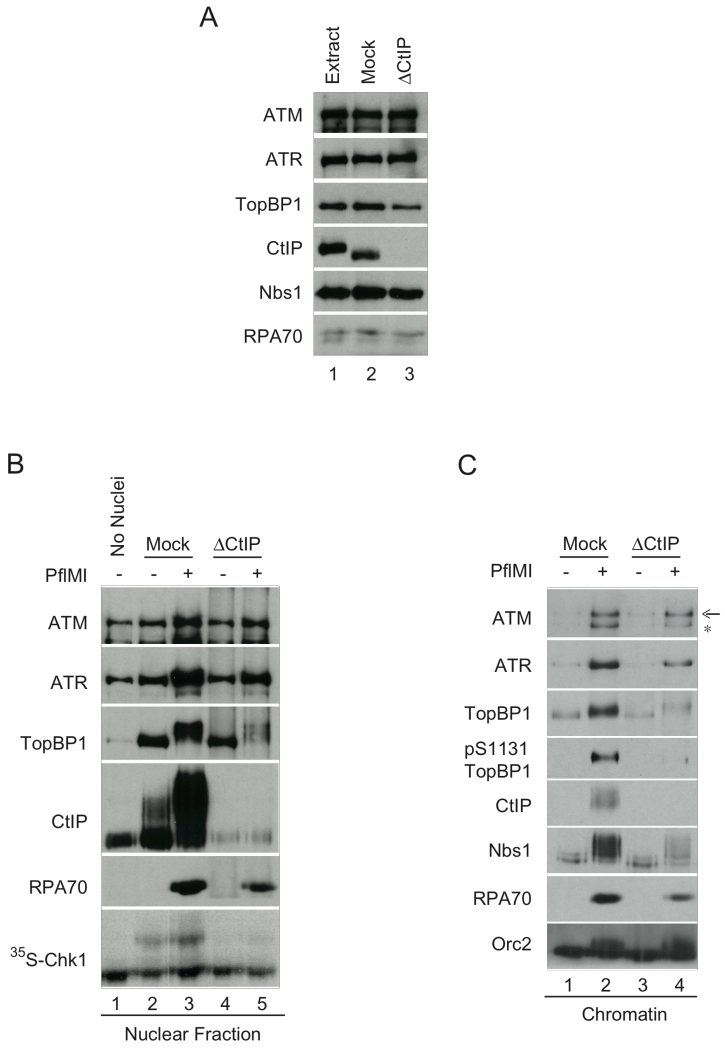
To probe further the effects of the absence of CtIP on the accumulation of TopBP1, we isolated PflMI-treated chromatin from depleted extracts and examined the chromatin binding of TopBP1 and other checkpoint factors. Consistent with the observations in

nuclear fractions, TopBP1 levels were markedly diminished, with only a minimal amount of protein associating with DSB-containing chromatin in the absence of CtIP (Figure 5C). Consequently, the critical ATM phosphorylation of the ATR-Activating Domain (AAD) of TopBP1 on S1131 that is required for proper activation of ATR in response to DSBs was severely compromised. Furthermore, the levels of ATR on damaged chromatin were noticeably reduced and a decrease in the chromatin accumulation of RPA was observed. Conversely, the levels of ATM, even though reduced in nuclear fractions, did not decrease substantially on chromatin in the absence of CtIP. In contrast to a previous report[57], we observed that the levels of Nbs1 on chromatin were significantly reduced in CtIP-depleted extracts. The levels of Nbs1 in egg extracts, on the other hand were not reduced, discounting the possibility of co-depletion (Figure 5A). Taken together, our data strongly suggest that CtIP is required for the increase in nuclear accumulation and chromatin association of TopBP1 and Nbs1 in response to damage, important steps in the activation of ATR in response to DSBs.

Figure 5: CtIP mediates damage-dependent nuclear accumulation of TopBP1 in response to DSBs in *Xenopus* egg extracts

(A) Representative samples of extracts that were either left undepleted (lane 1), mock-depleted with control IgG antibodies (lane 2), or depleted with anti-CtIP antibodies (lane 3). Extracts were immunoblotted with anti-ATM, anti-ATR, anti-TopBP1, anti-CtIP, anti-Nbs1, and anti-RPA antibodies.
(B) Egg extracts containing ^{35}S-Chk1 in the absence (lane 1) or presence of sperm nuclei that were either mock depleted with control IgG antibodies (lane 2–3) or depleted with anti-CtIP antibodies (lane 4–5) were incubated with no checkpoint inducer (lanes 2 and 4) or in the presence of 1 U/μl of PflMI (lanes 3 and 5). Nuclear fractions were isolated and subjected to SDS-PAGE and phosphorimaging to detect radiolabeled Chk1 and blotted with anti-ATM, anti-ATR, anti-TopBP1, anti-CtIP, and anti-RPA70 antibodies.
(C) Egg extracts that were either mock-depleted with control IgG antibodies (lane 1–2) or depleted with anti-CtIP antibodies (lane 3–4) were supplemented with sperm nuclei in the absence or presence of 1 U/μl of PflMI. Chromatin fractions were isolated and subjected to SDS-PAGE and immunoblotted with anti-ATM,

anti-ATR, anti-TopBP1, anti-phospho[S-1131]TopBP1, anti-CtIP, anti-Nbs1, anti-RPA70, and anti-Orc2 antibodies. ATM is denoted with an arrow, whereas residual ATR signal from previous blotting is marked with an asterisk.



DISCUSSION

ATR is activated in response to a wide range of DNA structures ranging from DSBs and base adducts to the intermediates that arise upon stalling of replication forks. These observations led to the model that these different structures generate a common intermediate that triggers the activation of ATR[27]. Various lines of evidence have suggested that this structure contains RPA-coated single-stranded DNA (RPA-ssDNA)[88, 89,110,120]. A protein that is critical in the generation of RPA-ssDNA, particularly in response to DSBs, is CtIP[57,112,113]. Another integral component of the activation of ATR is TopBP1 whose presence, even in the absence of damage, is sufficient to stimulate the kinase activity of ATR towards many of its substrates[97,98]. In this report, we have explored the possibility of an interaction between CtIP and TopBP1, two critical components for ATR activation, in *Xenopus* egg extracts. We have found that CtIP and TopBP1 interact in a regulated manner in response to checkpoint activation by synthetic oligonucleotides (pA-pT) and DSBs generated by restriction endonuclease.

To understand the nature of this interaction, we generated a series of TopBP1 deletion mutants lacking one or more of its BRCT domains, which have been shown to be involved in mediating damage-stimulated interactions with other checkpoint proteins[106,118]. Utilizing this approach, we determined that the region of TopBP1 containing BRCT domains I and II is necessary and sufficient to mediate the interaction with CtIP. These are the same domains required for the interaction between TopBP1 and Nbs1. Further analysis revealed that a version of TopBP1 with a mutation in the BRCT I-II region that abolishes

the interaction with Nbs1, TopBP1-KKAM, partially compromises the capacity of CtIP to bind TopBP1. Moreover, depletion of Nbs1 from egg extracts also reduced the interaction to a similar extent. It has previously been demonstrated by work from our laboratory that the presence of Nbs1 is critical for another key aspect of the function of TopBP1, namely its ability to bridge the interaction between ATM and TopBP1. In the absence of Nbs1, TopBP1 cannot interact with ATM thereby hindering the ability of ATR to be activated by ATM in response to DSBs[118]. With our current work, we have further shown that CtIP participates in the establishment of this interaction as indicated by a decrease in the levels of Nbs1 in TopBP1 pull downs from CtIP-depleted extracts.

The MRN complex mediates the recruitment of CtIP to sites of damage to resect DSBs and give rise to RPA-ssDNA[57,112,113,133]. Recently, Chen and colleagues[133] have characterized a region in the N-terminus of human CtIP (residues 22–45) that is critical for targeting CtIP to DNA breaks. This region is capable of interacting with the MRN complex, although it is not unique since the C-terminus also has the capacity to bind MRN components. Our structure/function analysis of the *Xenopus* CtIP reveals that the N-terminal and not the C-terminal region of CtIP is required for the interaction with TopBP1. Furthermore, TopBP1 can only pull down roughly half the amount of a full-length CtIP mutant lacking the homologous N-terminal region required for MRN binding, CtIP(Δ25–48), than it would of wild-type CtIP. This outcome is not surprising in light of the levels of CtIP binding to TopBP1 observed in Nbs1 depletions and KKAM pulldowns. Overall, these results indicate that the regulated interaction between TopBP1 and CtIP in response to damage is partially but not fully mediated by the presence of the MRN complex,

suggesting the formation of a larger damage-stimulated complex that would at least be composed of the MRN complex, CtIP, and TopBP1.

BRCT repeats mainly interact with phosphorylated substrates[107,116,142]. Phosphorylation of CtIP on serine 327 (S327) of the human protein mediates the formation of a CtIP-BRCA1 complex that is involved in regulating the delay in cell cycle progression and Chk1 activation in irradiated HeLa cells[116]. Subsequent work in avian DT40 cells further highlights the importance of this phosphorylation event, where complementation of CtIP null cells with CtIP-S327A leads to a decrease in the generation of ssDNA and defects in homologous recombination (HR)[115]. We explored the possibility of the corresponding *Xenopus* site might be required for the interaction with TopBP1. Mutation of serine 328 to a non-phosphorylatable alanine (S328A) had no effect on the interaction. This result is consistent with the notion that even though the phosphorylation of S327 in the human and chicken CtIP is required for checkpoint activation, this phosphorylation event is not induced by DNA damage[116] and the TopBP1-CtIP interaction only occurs in the presence of damage.

To further explore the events that are required for the interaction between TopBP1 and CtIP we looked at other potential phosphorylation sites within the region that was determined to be involved in the interaction (residues 247–359). Since the interaction occurs in response to damage, we turned our attention to potential ATM/ATR sites. A couple of S/T-Q sites in CtIP have been demonstrated to be phosphorylation sites for ATM[128]. Both of these sites lie in the C-terminal region of CtIP, which is not required for the TopBP1 interaction. Sequence alignments with other vertebrate homologs pointed

towards two directly adjacent ATM/ATR consensus SQ sites within the 247–359 region, the second of which, S275, is highly conserved. When both of these serines were mutated to alanine within full-length CtIP, the binding of the resulting 2AQ mutant to TopBP1 was lower relative to the wild-type protein. Binding was further reduced when these mutations were combined with a deletion (Δ25–48) that affects binding to MRN. In addition, under conditions where TopBP1 does not interact with Nbs1, the interaction between TopBP1 and CtIP is sensitive to caffeine. When viewed together, the additive effect of the combination mutant (CtIP[Δ25–48-2AQ]) and the caffeine sensitivity in the absence of the TopBP1-Nbs1 interaction, present a picture where the presence of the MRN complex and the checkpoint-stimulated activity of PIKKs comprise two separate modes in which the interaction between CtIP and TopBP1 is regulated.

As mentioned above, CtIP also interacts with MRN through its C-terminal domain[115]. It is likely that our combination mutants, made in the context of the full-length protein, still interact with MRN through the C-terminal region. These issues will likely be clarified by performing pull-down experiments with a recombinant CtIP containing the different mutant combinations. We devoted a lot of time and resources to obtain a functional CtIP protein that could be used for this purpose and were unsuccessful in doing so. Current efforts are underway to resolve this issue.

While this manuscript was in preparation, You and colleagues[57] published an article characterizing *Xenopus* CtIP. Consistent with their results, we observed that a highly modified form of CtIP binds to chromatin containing DSBs in egg extracts. In addition, in the presence of replication blocks even though nuclear accumulation of CtIP is evident,

mobility is not as severely affected and it did not bind to chromatin. Interestingly, we observed that when caffeine was added to inhibit the activity of ATM and ATR the levels of CtIP on chromatin increased even in the absence of damage or when replication is blocked. Furthermore, a massive accumulation was observed when caffeine was added in combination with PflMI, mirroring the behavior displayed by other checkpoint factors, including TopBP1 and RPA. These data strongly suggest that CtIP plays a key role in the response to DSBs in egg extracts.

Consistent with what has been observed in mammalian cells[112], the activation of Chk1 in response to DSBs is defective in the absence of CtIP. Also, as You et al.[57] demonstrated with EcoRI treatment, the nuclear and chromatin levels of RPA substantially decreased in response to PflMI in ΔCtIP extracts. Likewise, the levels of ATR are reduced but not those of ATM. These observations are concordant with the model presented by Shiotani and Zou[120] of the involvement of CtIP in the biphasic response to DSBs. Using human cell extracts containing defined DNA structures, they could observe ATM activation in the presence of structures with double-stranded ends and short single-stranded overhangs (SSOs). As the length of the overhangs increases or when linearized plasmids incubated with nucleases are used as templates, the activity of ATM is reduced whereas the activity of ATR increases. In HeLa cells treated with ionizing radiation (IR) this ATM to ATR switch, gauged by Chk1 and Chk2 phosphorylation, was enhanced by the overexpression of CtIP and Exo1, an alternative nuclease with known roles in DSB resection[121–123]. When CtIP and Exo1 were co-expressed the activity of ATM is reduced and the level of Chk1 phosphorylation increases at earlier time points suggesting that the

switch from ATM to ATR signaling is stimulated by the progressive resection of ends by nucleases, which include CtIP.

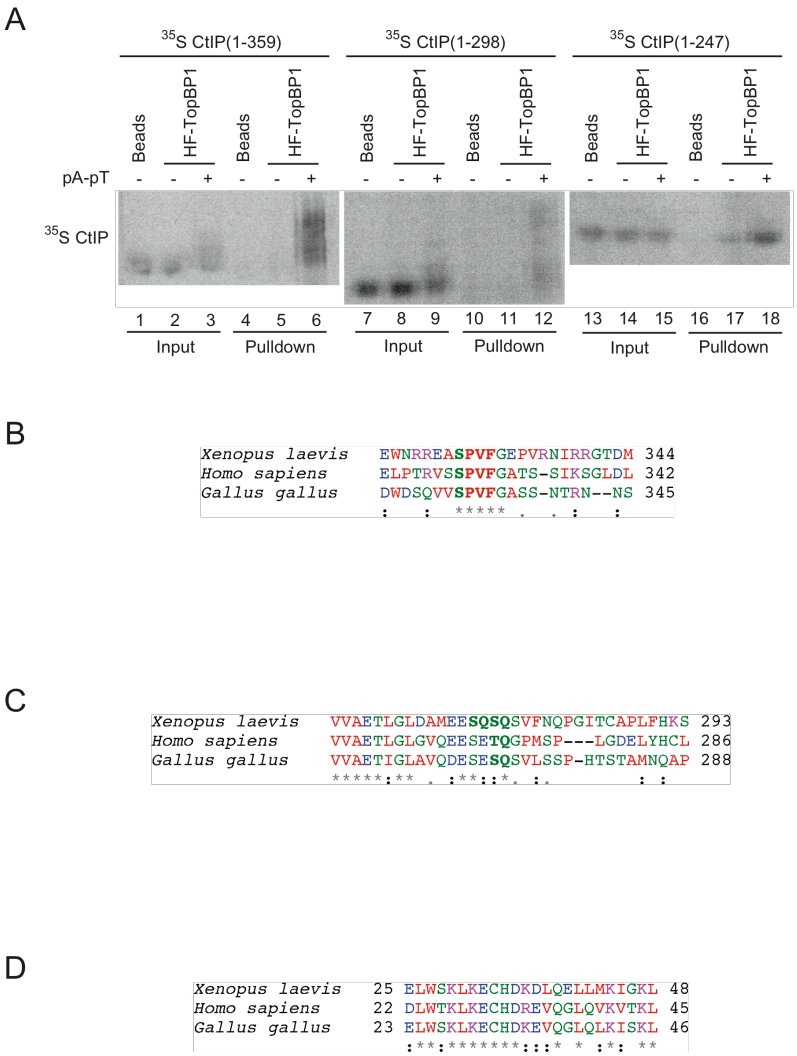
Our work adds a key mechanistic insight into the impact of the lack of resection by CtIP in the transition from ATM to ATR signaling. In the absence of CtIP, the amount of TopBP1 in the nucleus and on chromatin is drastically reduced. Concomitantly, phosphorylation of S1131 of TopBP1 by ATM is abrogated. Furthermore, the nuclear (data not shown) and chromatin levels of Nbs1 were significantly reduced, in contrast to what was reported by You et al.[57]. The levels of Nbs1 in egg extracts on the other hand were not significantly reduced by the depletion of CtIP (Figure 5A). It is worth noting that PflMI triggers a stronger checkpoint response than EcoRI and the nuclear and chromatin accumulation of checkpoint proteins is greater with PflMI (Figure 1A and our unpublished data). The robust accumulation of proteins makes variations in amount more noticeable by amplifying even subtle differences.

In summary, we have described a damage-stimulated interaction between CtIP and TopBP1. Phosphorylation of CtIP on S273/S275, by what is likely to be ATM, partly mediates this interaction and it is further enhanced by presence of the MRN complex. In the absence of CtIP the recruitment of both TopBP1 and Nbs1 to damaged chromatin is reduced thereby compromising the checkpoint response to DSBs. These observations strongly suggest that CtIP, TopBP1 and the MRN complex collaborate to incite the transition from the initial ATM response to the subsequent ATR activity in response to DSBs.

CONTRIBUTIONS AND ACKNOWLEDGEMENTS

The work presented in this chapter was done in collaboration with Hae Yong Yoo. Dr. Yoo provided Figure 2A and a wealth of preliminary data that was subsequently confirmed and expanded by our work. The interaction between CtIP and TopBP1 was further corroborated by analysis of TopBP1 pull downs by mass spectrometry. The pull-down experiments were carried out in our laboratory by Akiko Kumagai. Anna Shevchenko and Andrej Shevchenko at the Max Planck Institute of Molecular Cell Biology and Genetics in Dresden, Germany, carried out the mass spectrometry analysis.

We would like to thank all the members of our laboratory for insightful discussions and comments on the data that went into this chapter, which is currently in the process of being finalized for journal submission. In particular, we would like to thank Sanjay Kumar for detailed comments on the text.



Supplementary Figure 1: Sequence conservation of CtIP regions required for binding TopBP1

(A) Anti-FLAG antibody beads containing no recombinant protein (lanes 1, 4, 7, 10, 13 and 16) or wild-type HF-TopBP1 (lanes 2–3, 5–6, 8–9, 11–12, 14–15 and 17–18) were incubated in egg extracts containing ^{35}S-CtIP N-terminal fragments 1–359 (lanes 1–6), 1–298 (lanes 7–12) and 1–247 (lanes 13–18) in the absence or presence of pA-pT. The beads were retrieved and bound ^{35}S-labeled proteins were detected by SDS-PAGE and phosphorimaging (lanes 4–6, 10–12 and 16–18). Lanes 1–3, 7–9 and 13–15 are representative samples of extracts used in pull downs. Results for each fragment were obtained from separate experiments.
(B) Sequence alignment of *Xenopus*, human, and chicken CDK phosphorylation site.
(C) Sequence alignment of *Xenopus*, human, and chicken CtIP region around the putative ATM/ATR site necessary for binding to TopBP1.
(D) Sequence alignment of *Xenopus*, human and chicken Nbs1 binding domain of CtIP (as described in [133]) necessary for binding to TopBP1.

CHAPTER III

A ROLE FOR THE CHROMATIN REMODELING ATPASE ISWI IN THE DNA DAMAGE RESPONSE (DDR) OF *XENOPUS* EGG EXTRACTS

ABSTRACT

The study of the structural requirements for the activation of the DNA Damage Response (DDR) has largely focused on the topology directly adjacent to sites of damage. Recently, more attention has been placed upon the role of the global chromatin environment in mediating these processes. In this chaper, we explore the involvement of the chromatin remodeling ATPase ISWI in the response to damaged DNA in *Xenopus* egg extracts. We find that ISWI associates with ATR, ATRIP, and TopBP1 on DNA in response to DNA damage. Furthermore, ISWI is phosphorylayted by ATR and ATM on a conserved residue, which is adjacent to a domain that is critical for the binding of ISWI onto chromatin. Additionally, the activities of ATM and ATR stimulate an increase in the levels of ISWI on chromatin that contains double-stranded DNA breaks (DSBs). Furthermore, we assess the role of ISWI in the activation of the responses to DSBs and replication blocks in *Xenopus* egg extracts. Taken together, our work describes several previously uncharacterized features of ISWI with implications in the response to damaged and incompletely replicated DNA.

INTRODUCTION

The maintenance of the integrity DNA molecules is of paramount importance to achieve cellular homeostasis. Eukaryotic cells handle the continuous onslaught of intra- and extracellular insults by multiple means. One of the first layers of defense that a cell employs to shield its genetic material is by packaging DNA around globular protein complexes called histones. The resulting array of DNA molecules spooled around histones is further compacted to generate a larger insulating structure called chromatin. Even in this compact conformation DNA molecules are still at risk of being broken or mutated. In cases where damage does occur, cells respond through the activation of a network of signal transduction cascades called checkpoints that modulate the control of cell cycle transitions, DNA replication and DNA repair[21].

Two large phosphoinositide 3-kinase (PI3K)-related protein kinase (PIKKs) sit at the top of these damage-signaling pathways to sense the presence of damage and transduce the signal to a plethora of downstream targets[35,124]. ATM, the protein product of a gene that is defective in the rare autosomal disorder *ataxia telangiectasia* (A-T) (reviewed in [25]) mediates the response to double-stranded DNA breaks (DSBs). DSBs occur naturally as V(D)J and meiotic recombination intermediates but increase in frequency by exposure to mutagens, at eroded telomeres or in conditions that challenge replication and lead to fork collapse. Failure to repair DSBs in a timely manner may contribute to the genomic instability that characterizes the majority of human cancers[7,14].

A second PIKK and close relative of the ATM kinase, ATR responds to multiple types of damage. These lesions, including DSBs, base adducts and structures that arise when replication forks stall, are processed to form a common intermediate that leads to ATR activation[27]. A growing body of evidence defines this ATR-activating structure as long stretches of single-stranded DNA (ssDNA) coated by Replication Protein A (RPA) (RPA-ssDNA) adjacent to a juncture between single-stranded and double-stranded DNA[88,89,110,120]. The initial damage structures are sensed and subsequently processed by the activity of multiple nucleases to generate the template for activation[112–114,121,123]. RPA binds ssDNA and the resulting ssDNA-RPA is recognized by the ATR-Interacting Protein (ATRIP)[88,93]. Furthermore, ATRIP mediates the association between ATR and TopBP1[41]. TopBP1 is a BRCA1 C-terminus (BRCT) repeat containing protein that interacts with ATR and leads to an increase in its kinase activity[97]. *In vitro* studies in *Xenopus* egg extracts and human and mice tissue culture cells showed that even in the absence of damage overexpression of the key regulatory domain of TopBP1, which mediates the association to ATR, the ATR Activating Domain (AAD), leads of an increase in ATR activity[97,98]. Once activated by TopBP1 ATR can then phosphorylate its downstream substrates that include replication and repair proteins as well as the effector serine/threonine kinase Chk1 (reviewed in [27]).

The activation of ATR illustrates that a defined DNA template is required for the full manifestation of the checkpoint response. In the case of ATM, as observed by supplementing *Xenopus* egg extracts with linear DNA fragments, a minimum of 200 base pairs (bp) flanking a DSB is required for proper activation[143]. This length roughly

coincides with the amount of DNA wrapped around a single nucleosome, suggesting that the displacement of histones from the vicinity of damage sites may play a role in regulating ATM activity. Consistent with these findings, human cells that are cultured in hypotonic conditions or treated with drugs that lead to chromatin relaxation display an increase in the critical autophosphorylation of ATM on serine 1981 even in the absence of damage[44]. Furthermore, murine embryonic stem cells with reduced amounts of linker histone H1, which is one of the main factors involved in high-order chromatin compaction, display enhanced checkpoint signaling in response to ionizing radiation[71]. This effect could also be reproduced by treating wild-type cells with histone deacetylase inhibitors implying that an open chromatin conformation amplifies the response to DNA damage. From these and other reports[67,70,144–150], a picture is beginning to emerge in which chromatin acts as a selective barrier for the activation and amplification of the checkpoint, which highlights the importance of chromatin structure in the DNA Damage Response (DDR).

In this study, we have characterized a damage-dependent interaction between ATR and the ATP-dependent nucleosome remodeling factor ISWI. This highly conserved protein, found in organisms ranging from yeast to mammals, is the primary "motor" of a number of chromatin remodeling complexes involved in mobilizing nucleosomes to make the chromatin template fluid and allow the appropriate regulation of transcription, DNA replication, and chromosome segregation[151,152]. The initial characterization of this protein in *Xenopus* revealed that it very tightly associates with chromatin in a cell cycle-regulated manner and is required to maintain periodic nucleosomal arrays[153,154]. We have accumulated some evidence to suggest that the binding of ISWI onto chromatin is

enhanced in the presence of DNA breaks in a manner that depends on the activity of ATM and ATR. The absence of ISWI from egg extracts compromises the response to different types of DNA damage in conjunction to delaying cell cycle progression, DNA replication and displaying the characteristic disruption of nucleosomal spacing. Taken together our results suggest that ISWI actively plays a role in the response to DNA damage in *Xenopus* egg extracts.

EXPERIMENTAL PROCEDURES

PREPARATION OF EGG EXTRACTS: *Xenopus* egg extracts were prepared as described in [135]. Interphase extracts were supplemented with 3,000 demembranated *Xenopus* sperm nuclei/μl. Aphidicolin was used at 50 μg/ml. EcoRI used at a final concentration of 0.5U/μl and PflMI used at 1U/μl. Sperm chromatin was UV irradiated with 300 J/m^2 unless otherwise noted. Caffeine was added at a final concentration of 5 mM and wortmannin used at 100 μM. Nuclear fractions [138] and chromatin fractions [137] were isolated according to previously described protocols. One-step chromatin isolations were carried out according to [87].

PRODUCTION OF RECOMBINANT ISWI IN INSECT CELLS: Full-length ISWI clone (MGC:79877 IMAGE:6317687) was obtained from Open Biosystems. A PCR-generated version with NcoI and SpeI sites was cloned into pFastBac with downstream FLAG and polyhistidine tags (HF-ISWI). Recombinant baculoviruses were produced using the Bac-to-Bac system (Invitrogen). Sf9 insect cells were infected with recombinant baculovirus for protein expression following the manufacturer's specifications. Proteins used in pull-down experiments were purified with nickel agarose. Proteins used to assess chromatin binding and as substrates in kinase assays were purified with Anti-FLAG M2 beads (Sigma). Mutant versions of the protein were produced using QuikChange Kit (Stratagene).

PRODUCTION OF RECOMBINANT ISWI FRAGMENTS IN BACTERIA: PCR-generated ISWI fragments spanning regions corresponding to amino acids 473–533, 692–752 and 803–863

were amplified and cloned into pGEX-4T3 vector using BamHI and XhoI sites. Constructs were expressed in *Escherichia coli* and purified using glutathione beads. Eluted proteins were used as substrates in ATR kinase assays described below. Mutant versions of the protein were produced using QuikChange Kit (Stratagene).

ATR KINASE ASSAY: FLAG-ATRIP-Δ222 was incubated in egg extracts following the specifications detailed in [97]. ATR-ATRIP complexes were recovered and subsequently used for kinase assays following the protocol detailed in the publication mentioned above.

ANTIBODIES: ISWI cDNA encoding amino acids 671–1019 of ISWI clone reported by [154] (corresponding to 649–1046 of clone used [155]) in a pRSetB expression vector with a downstream polyhistidine coding sequence was expressed in *Escherichia coli*, isolated with nickel agarose beads and used for production of rabbit antibodies at a commercial facility. The pRsetB-ISWI (671–1019) construct was a kind gift from Dr. Marcel Méchali. Purified antigen was also used as a substrate in ATR kinase assays described above. Affinity-purified antibodies against *Xenopus* versions of ATM, ATR, TopBP1, Nbs1, RPA70, Chk2, and Orc2 were described previously [84, 87, 97, 118, 138].

IMMUNODEPLETION OF ISWI: For immunodepletion of ISWI, interphase extracts (100 μl) were incubated at 4ºC for 60 min with 30 μg of ISWI antibodies bound to 20 μl of Affiprep-protein A beads (Bio-Rad). Equal amounts of rabbit IgG antibodies were used as a control. After incubation, beads were recovered by gentle centrifugation and the supernatants were treated for a second round of depletion. Resulting supernatants were then used in the specified experiments.

NUCLEOSOME SPACING ASSAY: The nucleosome spacing assay using micrococcal nuclease was carried out according to [153] with some modifications. Briefly, demembranated sperm nuclei were incubated in egg extracts at 3,000 nuclei/μl. After 90 min incubation in the presence of α^{32}P-dATP, nuclei were isolated from extracts to obtain a total of 500 μg of labeled DNA. Isolated nuclei were resuspended in 60 μl micrococcal nuclease (MNase) digestion buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM $CaCl_2$). MNase was added to a final concentation of 20 U/mg DNA and incubated at room temperature. At the indicated times, reactions were stopped by the addition of 30 mM EDTA, 0.5% SDS and 500 μg/ml proteinase K and incubation at 37ºC. Samples were subsequently RNase treated and DNA extracted by phenol:chloroform and ethanol precipitation. Samples were run on 1.8% agarose gel in TBE.

DNA REPLICATION ASSAY: DNA Replication Assays were performed by measuring the incorporation of α^{32}P-dATP as described previously [137].

PRODUCTION OF ^{35}S-CHK1- ^{35}S-labeled-Chk1 proteins were synthesized as previously described [139].

OLIGONUCLEOTIDES AND LINEARIZED PLASMIDS: Annealed oligonucleotides $poly(dG)_{70}$-$poly(dC)_{70}$ (termed pG-pC) were added to extracts at a final concentration of 50 μg/ml to induce checkpoint response as previously described [33]. Circular and linearized plasimds were prepared according to specification in [84] and added to extracts to a final concentration of 10 ng/μl.

ISWI FLAG NUCLEAR PULL-DOWN: 25ng/μl of recombinant full-length wild type or mutant ISWI proteins (described above) were incubated in egg extracts for 90 min. Nuclei

were isolated from these extracts and subsequently lysed by the addition of half the volume of the original extract in CHAPS buffer (10 mM HEPES-KOH, pH 7.5, 0.1% CHAPS, 150mM NaCl, 2.5 mM EGTA, 20 mM β-glycerolphosphate, and 0.5% NP-40) and incubation at 4ºC with rotation. Lysates were spun down in fixed rotor centrifuge for 10 min at 14,000 rpm. An equal volume of 20 mM HEPES-KOH (pH 7.5) was added to supernatant. Four percent (4%) of total volume was collected and used as loading control (Input). Diluted lysates were incubated with Flag-coupled Protein G magnetic beads and incubated for 60 min. Beads were washed 4 times in CHAPS buffer and twice in HBS (10 mM HEPES-KOH, 150mM NaCl) before addition of 2X-SDS sample buffer for SDS-Page and immunoblotting analysis.

PROTEIN BINDING TO LINEARIZED PLASMIDS: Streptavidin-coated beads bound to linearized biotinylated plasmids were generated as described preiously [156]. Only one end of the single biotinylated plasmids (1X) will bind to the streptavidin beads and the unbound end will mimic DNA ends to which proteins involved in the response to DNA damage or repair will bind. Double biotinylated plasmids (2X) mimic a closed circular DNA plasmid that does not elicit a DNA Damage Response (data not shown). Beads were added to egg extracts at 50 ng/μl and recovered through centrifugation. Recovered beads were washed twice in CHAPS buffer and twice in HBS before addition of 2X-SDS sample buffer for SDS-Page and immunoblotting analysis.

H1 KINASE ASSAY: Egg extracts (1 μl) were diluted into 100 μl EB (80 mM β-glycerolphosphate, 20 mM EGTA, 15 mM $MgCl_2$, and 1 mM DTT added fresh). Diluted extracts (5.5 μl) were mixed with H1 kinase assay mix (14.5 μl) (2x H1 buffer: 40 mM

HEPES-NaOH, pH 7.3, 10 mM EGTA and 20 mM $MgCl_2$, 5 mg/ml histone H1, 1 mM ATP, 1 mCi/100 μl γ^{32}P-ATP) on ice. The mixtures were incubated at room temperature for 15 min. Reactions were stopped by adding 20 ml 2X SDS sample buffer and 5 min of boiling before being loaded onto SDS-Page gel.

RESULTS

ISWI ASSOCIATES WITH ATR IN *XENOPUS* EGG EXTRACTS IN RESPONSE TO DNA DAMAGE

In an effort to identify proteins that collaborate with ATR in the DNA Damage Response (DDR), an ATR pull-down was carried out in egg extracts containing damaged chromatin (Akiko Kumagai, Anna Shevchenko, and Andrej Shevchenko, unpublished data). One of the isolated proteins was the *Xenopus* ortholog of the chromatin remodelling ATPase ISWI. To investigate the regulation of this interaction within the context of the checkpoint activity of ATR, recombinant ISWI (HF-ISWI) purified from baculovirus infected Sf9 cells was added to egg extracts containing chromatin treated with different DNA damaging agents and subsequently recovered to determine the types of damage that stimulate this interaction (Figure 1A). HF-ISWI isolated from nuclear lysates of extracts supplemented with undamaged sperm chromatin did not display any appreciable interaction with ATR. Conversely, in the presence of chromatin that contains double-stranded DNA breaks (DSBs) generated by the restriction endonuclease EcoRI or in extracts where replication has been blocked by the DNA polymerase inhibitor aphidicolin (APH), the recovered HF-ISWI is found to associate with ATR. Additionally, ATRIP and TopBP1, both of which bind to and are required for the activation of ATR in response to damage[88,97] also interact with HF-ISWI in the presence of damage. The activation of ATR by TopBP1 and ATRIP depends upon the ability of ATRIP to bind to Replication Protein A (RPA), which coats stretches of single-stranded DNA (ssDNA) that are exposed adjacent to breaks or when replication is blocked[41,89,110]. Interestingly, binding of RPA to HF-ISWI was not

observed implying that the interactions with ATR, ATRIP and TopBP1 are not occurring on the RPA-ssDNA directly adjacent to sites of damage.

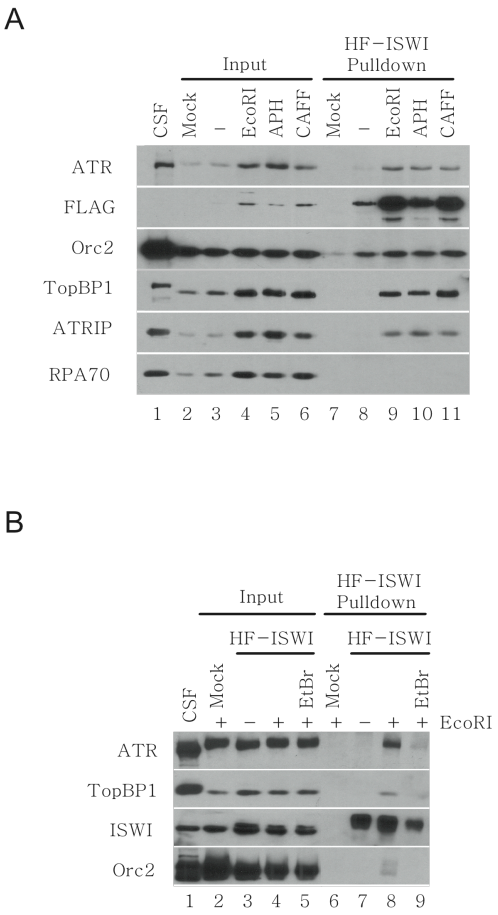


Figure1: ISWI Interacts with ATR in a Manner that Depends on Damaged DNA and Phosphorylation

(A) Nickel-purified recombinant FLAG and polyhistidine-tagged ISWI (HF-ISWI) was incubated in egg extracts (lanes 3–6 and 8–11) containing untreated sperm chromatin (lanes 3 and 8) or chromatin that was subjected to treatment with EcoRI (lanes 4 and 9), aphidicolin (APH) (lanes 5 and 10) or caffeine (CAFF) (lanes 6 and 11). FLAG beads were incubated with nuclear lysates from these extracts (lanes 2–6 are initial extract aliquots) and beads were reisolated and immunoblotted as indicated. Lanes 2 and 7 contain no recombinant protein. One microliter of CSF extract loaded onto lane 1 as a loading control.
(B) ISWI recovered from nuclear lysates treated with EcoRI as described in (A). Nuclear lysates used for samples in lanes 5 and 10 were supplemented with 50 μg/ml Ethidium Bromide (EtBr).

HF-ISWI also pulls down the origin specifying protein Orc2 even in the absence of damage, indicative of the strong association of ISWI with replicating chromatin[153,154]. Furthermore, supplementing egg extracts with caffeine to increase origin firing[77,157] similarly leads to the association between HF-ISWI and DDR factors in the absence of damage. To assess the importance of chromatin in mediating the interactions between ISWI and components of the DDR pathway, nuclear lysates were supplemented with ethidium bromide (EtBr) to disrupt DNA structure (Figure 1B). The addition of EtBr substantially diminished the capacity of HF-ISWI to interact with DDR proteins, as well as Orc2. Taken together, these results indicate that ISWI interacts with ATR, ATRIP, and TopBP1 on DNA in response to DSBs and replication blocks.

ISWI IS AN ATR SUBSTRATE

In light of the damage-stimulated interaction between HF-ISWI and ATR, we sought to determine if ISWI is an ATR substrate. In *Xenopus* egg extracts the exogenous addition of a TopBP1 fragment that interacts with the ATR-ATRIP complex, the ATR-Activating Domain (AAD), triggers an increase in the kinase activity of ATR as evidenced by the increase in phosphorylation of the model substrate PHAS-I[97] (Figure 2B, lane 7). Initially, a C-terminal fragment of ISWI corresponding to amino acids 694–1046 was used as a substrate. As shown in Figure 2B, the ATR-ATRIP complex stimulated by the AAD of TopBP1 displayed good kinase activity towards ISWI(694–1046) (Figure 2B, lane 3). This phosphorylation event was not observed when the kinase complex was supplemented

with a mutant version of the TopBP1-AAD (W1138R) that fails to activate ATR (Figure 2B, lanes 4 and 8).

Xenopus ISWI contains three serine/threonine-glutamine (S/T-Q) consensus ATR sites (Figure 2A). To determine the specific ATR phosphorylation site on ISWI, small GST fragments of the regions containing the putative phosphorylation sites were expressed and purified from bacteria to be used as substrates in ATR kinase assays (Figure 2C). The GST-ISWI(803–863) fragment was robustly phosphorylated in the presence of an active ATR complex whereas the GST-ISWI(692–752) was only marginally phosphorylated and GST-ISWI(473–533) was not phosphorylated at all. To confirm the specificity of this phosphorylation, threonine 832 was mutated to a non-phosphorylatable alanine (T832A) and the resulting mutant fragment was incubated with the active ATR complex. As shown in Figure 2D, ATR does not phosphorylate GST-ISWI(803–863-T832A). The minimal phosphorylation of the GST-ISWI(692–752) is also compromised in the T721A mutant. ATM, which shares the same consensus phosphorylation sites as ATR, can also phosphorylate ISWI at T832 *in vitro* (data not shown). In addition, full-length ISWI was only minimally phosphorylated in these kinase assays likely due to diminished accessibility of the kinase complex to the phosphorylation site in *in vitro* conditions (data not shown).

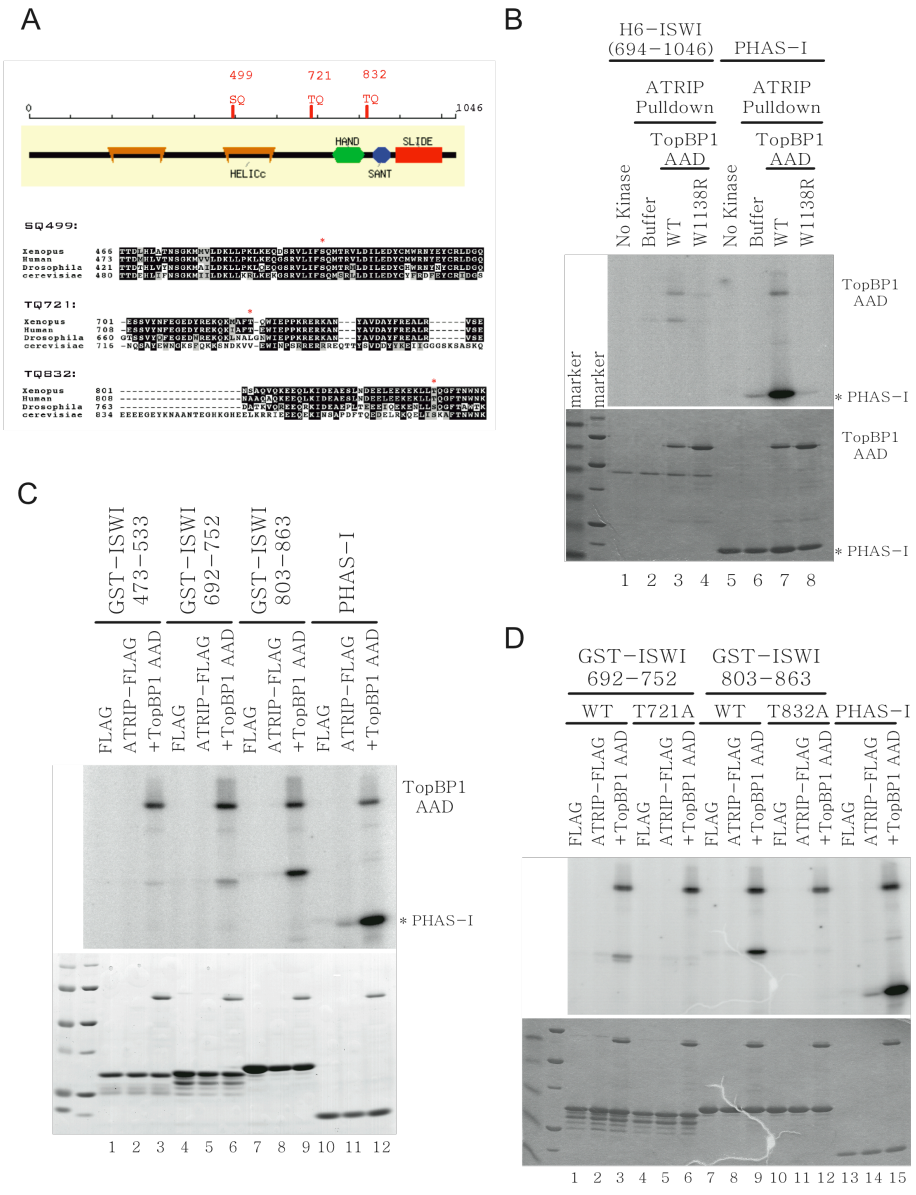
Figure 2: ISWI is an ATR Substrate

(A) Sequence alignment of human, fly and budding yeast regions around potential ATR phosphorylation sites in *Xenopus* ISWI along with their locations relative to conserved domains in the protein.
(B) Recombinant ISWI C-terminus (H6-ISWI[694–1046]) was incubated with γ^{32}P-ATP in the absence (lane 1) or presence of protein eluted from a FLAG-ATRIP-Δ222 pull-down supplemented with either buffer (lane 2), wild-type TopBP1 AAD (ATR Activation Domain GST-TopBP1[972–1279]) (WT) (lane 3), or W1138R mutant of the AAD that fails to activate ATR (lane 4). Same conditions of lanes 1–4 used in lanes 5–8 with

PHAS-I as a substrate to confirm ATR activity. Top panel is phosphorimage. Lower panel is Coomassie Brilliant Blue stain to assess protein loading.

(C) ISWI GST fragments containing potential phosphorylation sites were incubated with eluates from FLAG bead only pull-down (lanes 1,4,7 and 10), FLAG-ATRIP-Δ222 pull-down (lanes 2,5,8 and 11) or FLAG-ATRIP-Δ222 pull-down supplemented with GST-TopBP1-AAD (lanes 3,6,9 and 12) and analyzed as described in (A).

(D) ISWI GST fragments (lanes 1–3 and 7–9) and their corresponding non-phosphorylatable AQ mutants (lanes 4–6 and 10–12) were subjected to ATR kinase assays as described above. PHAS-I used as a control for ATR activity (lanes 13–15).



PIKK-Dependent Increase in the Binding of ISWI onto DSB-Containing Chromatin

ISWI binds to chromatin at the exit of mitosis and remains bound throughout DNA synthesis even in conditions when DNA replication is blocked[154]. In agreement with these observations, we could consistently see a gradual increase in the chromatin binding of ISWI onto chromatin after CSF release to coincide with the timing of replication (Figure 3A, see Figure 4D for typical replication kinetics). Furthermore, inhibition of replication did not affect the chromatin binding of ISWI as evidenced by no detectable changes in chromatin accumulation in response to aphidicolin treatment (Figure 3B, lanes 4 and 5). On the other hand, the binding of ISWI onto chromatin slightly increased in the presence of double-stranded DNA breaks (DSBs) created by EcoRI (Figure 3, lane 6). Because of the robust association of ISWI onto replicating chromatin this subtle increase in binding to EcoRI-treated sperm chromatin displayed a high degree of variability between experiments. Therefore, as an alternative method, we examined the binding of ISWI to bead-bound linearized plasmids incubated in egg extracts (Figure 3C). In this experimental setup we could appreciate a rapid increase in the binding of ISWI onto single-biotinylated linearized plasmids (1x Biotin) that mimic DSBs. The kinetics of binding to loose DNA ends was similar to those exhibited by other checkpoint response factors, mainly ATM. Binding of ISWI onto plasmids with no loose ends (2x Biotin) progressively increased at later time points clearly illustrating the affinity of ISWI towards DNA in general and potentially explaining the observed variability of binding in experiments with sperm chromatin in egg extracts.

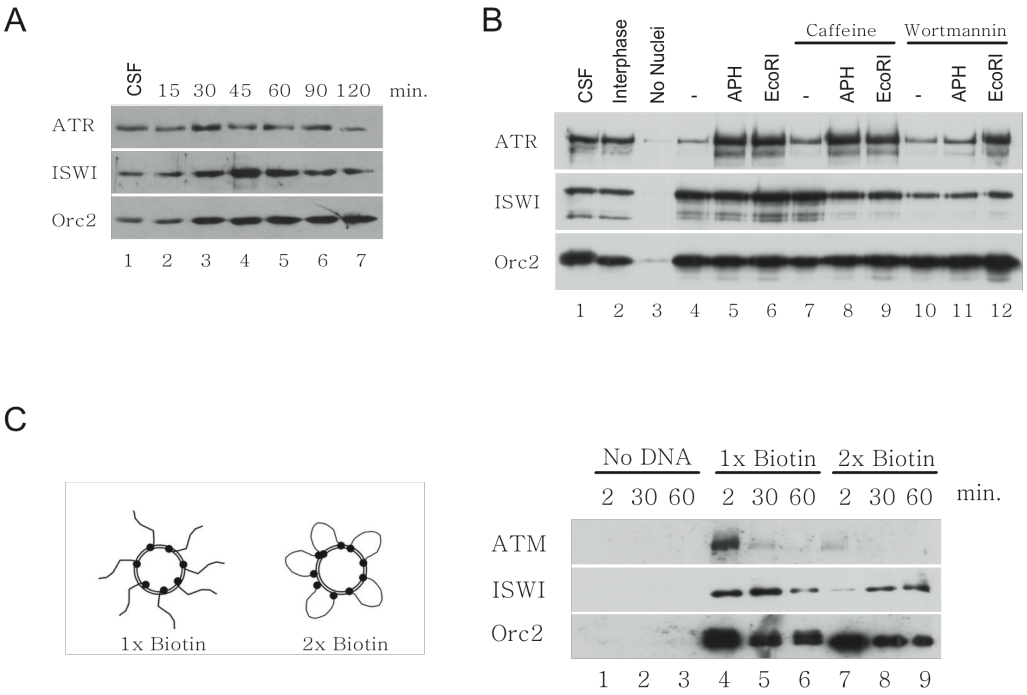
ATR phosphorylates ISWI on threonine 832, which lies at the boundary of a SANT domain, a conserved domain among chromatin remodeling proteins, which confers DNA and nucleosome binding properties (Figure 2A) (reviewed in [158]). We proceeded to gauge the importance of T832 in mediating the increased binding of ISWI onto chromatin with DSBs. Initially, chemical inhibitors of ATM and ATR were used to determine if the activity of these kinases is responsible for the increased binding of ISWI onto DSB-containing chromatin. Inhibition of the checkpoint-stimulating activity of both ATM and ATR in the presence of caffeine abolished the DSB-stimulated increase of ISWI on chromatin (Figure 3B). Interestingly, treatment of caffeine alone in the absence of damage, which leads to an increase in origin density and origin firing[77,157], resulted in an increase of chromatin-bound ISWI akin to the levels observed in the presence of EcoRI. The increase in the amount of replicating chromatin in the presence of caffeine could concomitantly lead to an increased recruitment of ISWI onto chromatin. The damage-independent increase caused by the addition of caffeine was not a consequence of PIKK inhibition, as treatment of extracts with wortmannin, which more strongly inhibits ATM, did not display this behavior. In fact, treatment with wortmannin produced a decrease in the binding of ISWI onto chromatin independent of damage.

To specifically determine the importance of T832 in mediating the binding of ISWI onto DSB-containing chromatin, we evaluated the chromatin binding of a mutant of HF-ISWI with T832 mutated to alanine (HF-ISWI-AQ). Endogenous ISWI strongly associates with chromatin and the binding of the recombinant HF-ISWI proteins onto chromatin was minimal. To circumvent this limitation, we immunodepleted the endogenous ISWI from

egg extracts and looked at the binding of the recombinant proteins onto undamaged and EcoRI-treated chromatin in depleted extracts (Figure 3D). With the use of a polyclonal antibody generated towards the C-terminus of ISWI we could remove the vast majority of ISWI from egg extracts (Figure 3D, lane 3 and Figure 4A). Wild-type HF-ISWI (HF-ISWI-WT) bound effectively to undamaged chromatin and its association greatly increased in the presence of damage. In this particular experiment, HF-ISWI-AQ bound normally onto undamaged chromatin whereas the increased binding onto EcoRI treated chromatin was not observed. Taken together, these results indicate that the increase of ISWI binding onto DSB-containing chromatin is mediated by the activity of ATM and/or ATR. We are hesitant to draw any definitive conclusions about the role of the phosphorylation of ISWI on threonine 832 because we observed some variability in the binding of HF-ISWI-AQ onto damaged chromatin.

Figure 3: PIKK-Dependent Increase in the Binding of ISWI onto DSB-Containing Chromatin

(A) Chromatin fractions were isolated at indicated timepoints after release from CSF arrest. A one-step chromatin isolation protocol was used in these experiments (see Experimental Procedures for details). Isolated chromatin fractions were subjected to SDS-PAGE and immunoblotted as indicated.
(B) Chromatin fractions were isolated from extracts in the absence (lane 3) or presence of untreated sperm chromatin (lanes 4, 7 and 10) or chromatin that was subjected to treatment with aphidicolin (APH) (lanes 5, 8 and 11) or EcoRI (lanes 6,9 and 12). Lanes 7–9 were treated with 5 mM caffeine (CAFF). Lanes 10–12 were treated with 100 μM wortmanin (WORT). One microliter of M-phase extract (CSF) (lane 1) or Interphase (lane 2) as loading controls.
(C) Right panel: Cartoon depicting single (1x biotin) or double (2x biotin) biotinylated linearized plasmids bound to streptavidin-coated beads. See Experimental Procedures for bead preparation protocol. Left panel: Bead-bound plasmids were incubated with interphase extracts. At the indicated times, beads were reisolated and immunoblotted as indicated.
(D) Extracts were treated with anti-ISWI antibodies (lanes 3 and 5–7) or control IgG (lanes 2 and 4) were subjected to two successive rounds of depletion. Wild-type (WT-HF-ISWI) (lane 6) or T832A mutant (HF-ISWI-AQ) (lane 7) were added back to depleted extracts. Extracts were supplemented with undamaged (lanes 8, 10, 12 and 14) and EcoRI treated (lanes 9, 11, 13 and 15) sperm chromatin. After incubation, chromatin was reisolated and immunobloted as indicated.



DEPLETION OF ISWI COMPROMISES PROGRESSION THROUGH INTERPHASE

Having established that ISWI associates with and is a substrate of ATR we proceeded to characterize the effects of the lack of ISWI on cell cycle progression in

Xenopus egg extracts. As previously reported[153,154], we could observe that the depletion of ISWI from egg extracts disrupted the spacing of nucleosomal arrays as evidenced by the loss of the periodic distribution of nucleosomes on chromatin incubated in ΔISWI extracts (Figure 4B). Subsequently, we assayed the entry of these depleted extracts into mitosis by monitoring the phosphorylation of histone H1 (Figure 4C). Undepleted extracts enter mitosis 150 minutes after release from MPF arrest whereas depleted extracts are slower, entering mitosis 60 minutes later, at 210 minutes post-release. The same lag was observed when assaying the entry into mitosis by looking at the phosphorylation state of cyclin B2 (data not shown).

Work done in human cells by Varga-Weisz and colleagues has shown that a delay in cell cycle progression in the absence SNF2H, the human homolog of ISWI, was caused by reduced rates of nucleotide incorporation throughout S-phase[159]. These data suggest that the delay of entry into mitosis seen in ISWI-depleted extracts might be a consequence of a slowing of DNA replication. Conversely, in the initial characterizations of *Xenopus* ISWI [153, 154] noted that the absence of ISWI did not compromise the timely completion of DNA replication. In our experiments we could reproducibly observe a reduction in the total amount of α^{32}P-dATP incorporation in the absence of ISWI relative to mock depleted extracts (Figure 4D, n=3). The nature of the discrepancy with previous work done in *Xenopus* remains unclear as other effects of the depletion are consistent with the previously published work.

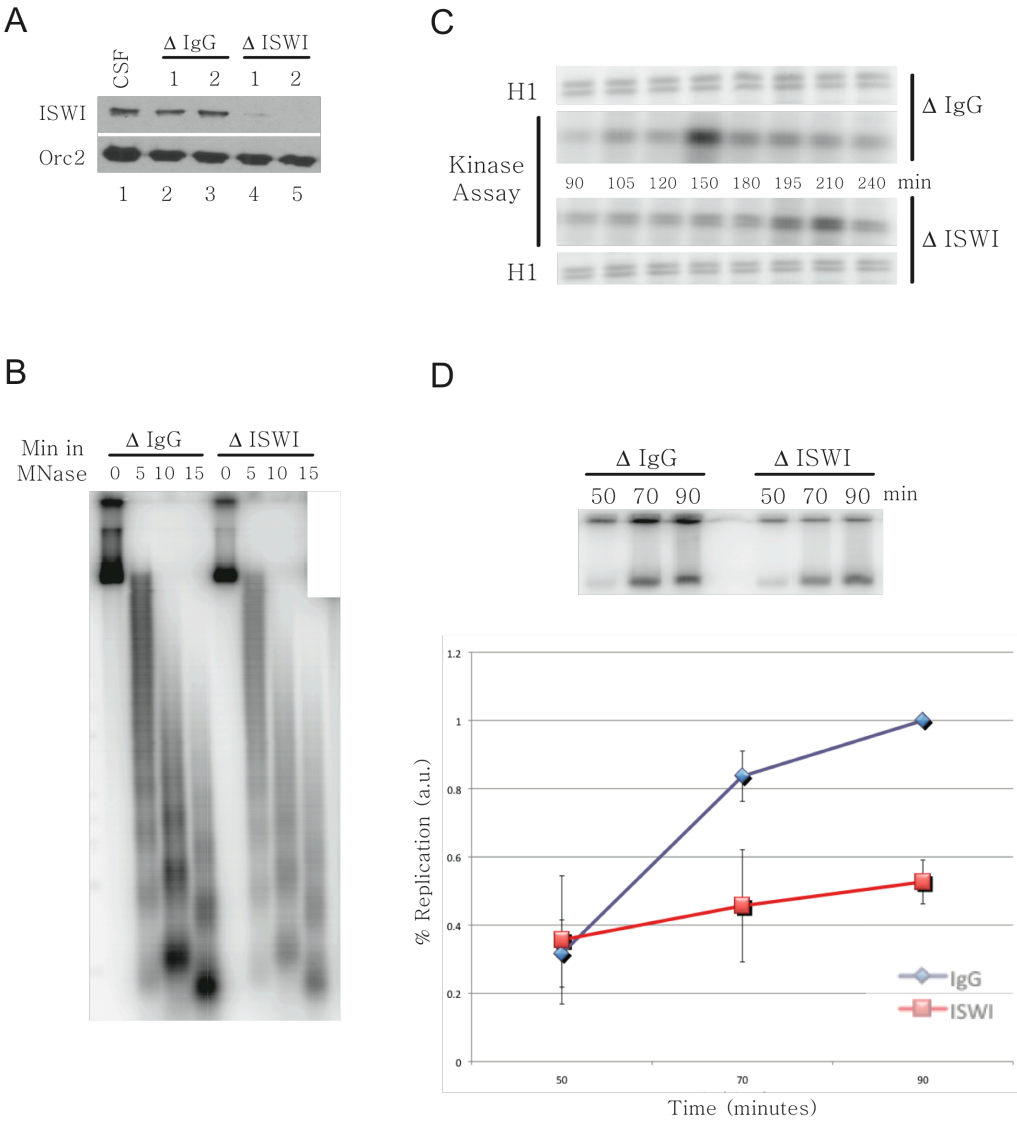


Figure 4: Multiple Effects of ISWI Depletion from Interphase Extracts
(A) One microliter of extracts treated with control-IgG or anti-ISWI antibodies from two successive rounds of depletion.
(B) Phosphorimage of nucleosome spacing assays from control-IgG- or ISWI-depleted extracts.
(C) H1 kinase assays from control-IgG- or ISWI-depleted extracts. Top and bottom panels labeled "Histone H1" are a Coomassie Brilliant Blue-stained gel to assess loading. Middle panels labeled "Kinase Assay" reflect α^{32}P-dATP incorporation.
(D) DNA Replication from control-IgG- or ISWI-depleted extracts. Bottom panel (graph) is a compilation of three separate experiments (n=3) of which the top panel is a representative example.

ASSESSMENT OF RESPONSES TO REPLICATION BLOCKS AND DSBS IN THE ABSENCE OF ISWI

Depletion of ISWI causes defects that compromise the timely progression of extracts through interphase. To further explore the defects that arise in ISWI-depleted extracts, we examined the activation of the checkpoint response to different types of DNA damage in the absence of ISWI. Initially, the response to incompletely replicated DNA was analyzed by treating sperm chromatin with UV or by supplementing the extract with the polymerase inhibitor aphidicolin. Both of these types of damage lead to the nuclear accumulation of the critical checkpoint response factor TopBP1 and DNA polymerase α, as well as the activation of the downstream effector kinase Chk1 (Figure 5B, lanes 2–4). In the absence of ISWI, we could sometimes observe an amelioration of these responses in extracts treated with aphidicolin. Conversely, no effect on checkpoint responses was observed when ISWI was depleted from extracts containing UV-treated chromatin. We further examined the impact that the loss of ISWI has on checkpoint activation by looking at the chromatin binding of two key checkpoint response factors TopBP1 and Nbs1 in response to damage (Figure 5C). Nbs1 is a component of the Mre11-Rad50-Nbs1 (MRN) complex, which is among the first responders to DSBs[50,51,110]. Increasing doses of UV irradiation led to an increase in the binding of TopBP1 and Nbs1 onto chromatin with a maximal accumulation of these factors at 300 J/m^2 (Figure 5C lanes 1-4). Addition of aphidicolin to extracts led to an increase similar to what was optimally seen with UV irradiation (Figure 5C lane 5). Similar to the defects seen in nuclear fractions, the absence of ISWI somewhat compromised the accumulation of TopBP1 and Nbs1 on chromatin in

response to aphidicolin (compare lanes 5 and 10), whereas both still accumulated normally on UV-treated chromatin.

Next, we assesed the impact of the depletion of ISWI in the response to DSBs. For this purpose we incubated annealed poly-dG poly dC oligos (pG-pC) in the extract, which selectively activate Chk2 and not Chk1[84]. Also, extracts containing linearized plasmids with different ends were added to extracts to activate the DSB response (Figure 5D). In all cases, the absence of ISWI did not affect the phosphorylation of Chk2 in response to breaks. Finally, we looked at the effect that the lack of ISWI might have on the response to PflMI-treated chromatin. This restriction endonuclease triggers a more robust checkpoint response than EcoRI because the ends produced as a result of cleavage are not complementary. Similar to the defects displayed above in response to aphidicolin, the lack of ISWI leads to a decrease of the nuclear accumulation of a wide range of checkpoint response factors, including TopBP1 and Nbs1, as well as a substantial reduction in the activation of Chk1 (Figure 5E). These experiments were repeated multiple times in various iterations and the results displayed a high degree of variability. This may be due to the efficiency of the depletion as a residual amount of the protein remained in nuclear fractions. The differential depletion of other ISWI binding partners, which have been shown to be co-depleted with ISWI antibodies ([153] and data not shown) could also contribute to the inconsistency. Regardless of the variability, we venture to conclude that the absence of ISWI and ISWI-containing complexes partially compromises the ability of egg extracts to respond to specific types of DNA lesions.

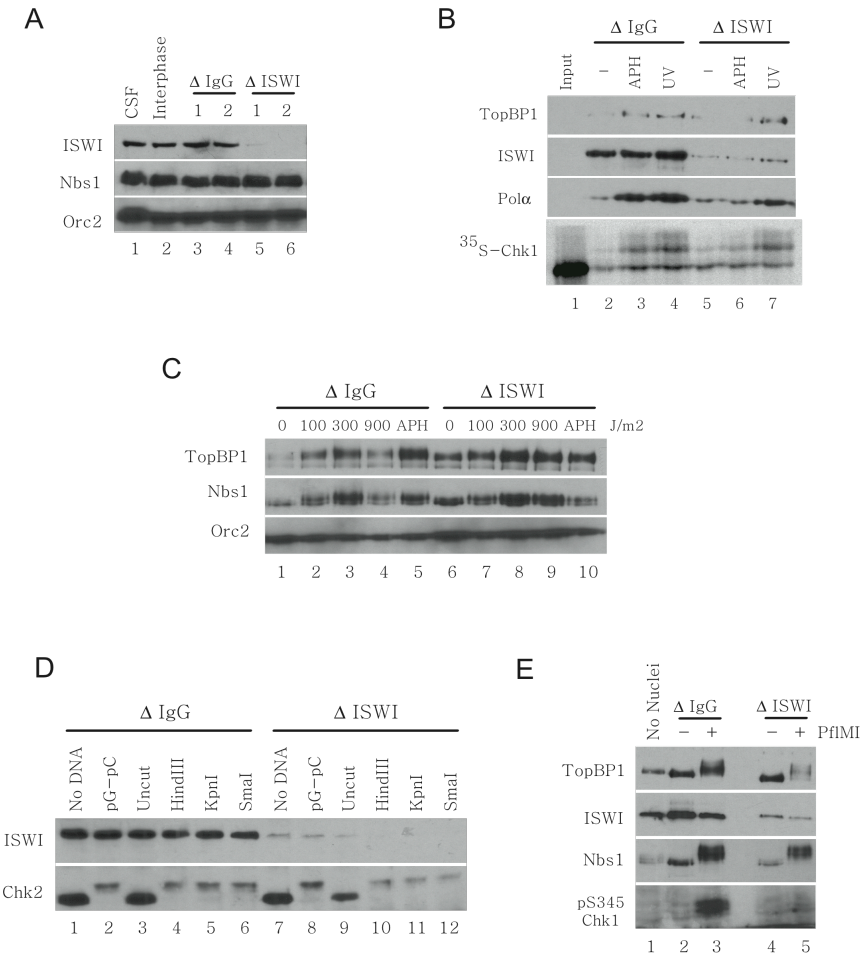


Figure 5: Effects of ISWI Depletion on the Response to Damaged DNA

(A) One microliter of extracts treated with control IgG or anti-ISWI antibodies from two successive rounds of depletion.
(B) Nuclear fractions from extracts treated with control IgG (lanes 2–4) or anti-ISWI antibodies (lanes 5–7) containing ^{35}S-Chk1 and incubated with aphidicolin (APH) (lanes 3 and 6) or UV-damaged (lanes 4 and 7) sperm chromatin. Lane 1 only contains one microliter ^{35}S-Chk1 as a loading control.
(C) Chromatin fractions from extracts treated with control-IgG (lanes 1–5) or anti-ISWI antibodies (lanes 6–10) containing sperm chromatin subjected to increasing amounts UV-damage (lanes 1–4 and 6–9) or supplemented with aphidicolin (APH) (lanes 5 and 10) were immunobloted as indicated.
(D) Extracts treated with control-IgG (lanes 1–6) or anti-ISWI antibodies (lanes 7–12) incubated in the absence of DNA (lanes 1 and 7) or supplemented with poly(dG)-poly(dC) annealed oligonucleotides (pG-pC) (lanes 2 and 8) or pBluescript (pBS). pBS was either added uncut (lanes 3 and 9) or linearized with HindIII (lanes 4 and 10) to produce 5' overhangs, KpnI (lanes 5 and 11) to produce 3' overhangs or SmaI (lanes 6 and 12) to produce blunt ends. After incubation, one microliter of extracts was subjected to SDS-Page analysis and immunobloted as indicated.
(E) Nuclear fractions from extracts treated with control IgG (lanes 2–3) or anti-ISWI antibodies (lanes 4–5) in the absence (lane 1) or presence of undamaged (lanes 2 and 4) or PflMI-treated (lanes 3 and 5) sperm chromatin were immunoblotted as indicated.

DISCUSSION

The studies aimed at uncovering the structural determinants required to initiate the DNA Damage Response (DDR) have largely focused on establishing the length and type of DNA ends that are necessary to trigger the activation of ATM and ATR[90,92,110,120,143,160]. This work has demonstrated, mostly based on experiments done *in vitro* using synthetic oligonucleotides and linearized plasmids, that double-stranded ends, primed single-standed DNA (ssDNA), RPA-coated ssDNA and junctions between single- and double-stranded DNA are involved in mediating these responses. Interestingly, in all cases the length of DNA adjacent to these structures is critical for optimal checkpoint function. These results raise the possibility that in the physiological context of chromatin the area surrounding breaks and stalled forks must be cleared of obstructions, namely histones, to allow DDR components to recognize the checkpoint-inducing signatures and launch a response. To examine this hypothesis, we have investigated the potential involvement of the chromatin remodeling ATPase ISWI in the DDR. With this work, we have characterized novel interactions between ISWI and the critical DDR components ATR, ATRIP, and TopBP1 on DNA in a damage-dependent manner.

In a similar DNA-dependent manner, we can observe an interaction between ISWI and the origin specifying protein Orc2. The interaction with Orc2 is consistent with the affinity of ISWI with replicating chromatin. The involvement of ISWI in replication has also been observed in studies of the human ISWI isoform SNF2H (sucrose non-fermenting 2 homolog). The absence of a complex including SNF2H and Acf1 (ATP-utilizing

chromatin assembly and remodeling factor 1), which localizes to pericentromeric heterochromatin, interferes with late S-phase replication, an effect that can be reversed by "loosening" chromatin with DNA methylation inhibitors[159]. Consistent with these observations, we see a decrease in the rate replication when ISWI is immunodepleted from egg extracts (Figure 4D). Furthermore, the Williams syndrome transcription factor (WSTF), which together with SNF2H forms the WICH complex, is targeted to replicating foci by its interaction with DNA polymerase processivity clamp PCNA[111]. The association of ISWI with active replication forks might also explain why in the presence of caffeine we notice an increase in the interaction of ISWI and DDR components in the absence of damage. Caffeine inhibits a damage-independent function of ATM and ATR, which is required for regulating origin density and origin firing[76,77]. Caffeine treatment also leads to an increase in the levels of ISWI on chromatin relative to untreated controls (Figure 3B, lane 7). The abnormally high level of firing origins may lead to the eventual collapse of some forks leading to the generation of checkpoint-inducing structures. This will in turn stimulate the recruitment of ATM and ATR to failed forks with no downstream consequences in checkpoint activation since the checkpoint-activating function of ATM and ATR is inhibited by caffeine. The increase in abundance of both of these proteins in the vicinity of damage might explain the observed caffeine-stimulated interactions in the absence of induced damage.

ATR preferentially binds to DNA that contains damage and this binding stimulates its kinase activity[85]. The observed interaction between ISWI and ATR is occurring on damaged DNA, which should be enriched for active ATR molecules. This prompted us to

explore the possibility of ISWI being a substrate of ATR. *Xenopus* ISWI contains three potential ATR phosphorylation sites distributed throughout the protein. The first potential site lies within a highly conserved nucleotide-binding region found in a wide variety of helicases and helicase-related proteins. This site S499, conserved in all the observed ISWI homologs from yeast to humans, was not phosphorylated by ATR. A second site, T721, lying in an otherwise uncharacterized region of the protein that displays a lower degree of conservation was marginally phosphorylated by ATR and ATM. Finally, T832, which is highly conserved in eukaryotic ISWI homologs, was robustly phosphorylated by both ATM and ATR. This site lies directly upstream of the SANT DNA-binding domain, which in conjunction with the adjacent SLIDE domain is required to mediate the substrate recognition and chromatin remodeling activities of ISWI[161,162]. Mutation of this residue to a non-phosphorylatable alanine did not compromise the ability of ISWI to interact with ATR (data not shown). Conversely, albeit with some level of uncertainty, it diminishes the binding of ISWI onto chromatin that contains DSBs. The regulation of ISWI activity by post-translational modifications was recently observed by work done in *Drosophila*. Corona and colleagues observed the previously uncharacterized poly-ADP-ribosylation of ISWI by PARP. Poly-ADP-ribosylation inhibited the DNA and nucleosome binding abilities of ISWI as well as its ATPase activity *in vitro*[163]. It is tempting to speculate that phosphorylation of T832 by ATR and/or ATM increases the affinity of the SANT/SLIDE region to broken DNA ends thereby retaining the already chromatin-bound ISWI onto sites of damage to mobilize nucleosomes in the vicinity of breaks.

To further investigate the potential for a functional involvement of ISWI in the DDR, we immunodepleted ISWI from egg extracts and monitored the activation of the checkpoint response. The high affinity of ISWI to chromatin made it troublesome to fully immunodeplete it from egg extracts. Even in these non-optimal depletions we could observe the effect of the absence of ISWI on the periodic spacing of nucleosomes. Additionally, we noticed a delay in the progression of ISWI-depleted extracts through interphase and into mitosis, accompanied by a reduction in the rate of replication as discussed above.

The delay in mitotic entry in the absence of ISWI suggested an involvement in the regulation of cell cycle transitions and a likely role in checkpoint function. Upon looking at the activation of the checkpoint response in the absence of ISWI, we noticed a reduction in the phosphorylation of Chk1 in response to aphidicolin. The response to UV damage on the other hand was unaffected in the absence of ISWI. Accordingly, the chromatin binding of TopBP1 and Nbs1 was reduced on ISWI-depleted chromatin exclusively in response to aphidicolin and not UV irradiation. In fact, a slight increase in the levels of these factors on UV-treated chromatin was observed in the absence of ISWI. The repair of UV adducts is largely resolved by the process of nucleotide excision repair (NER). Upon recognition of damage, 24–32 nucleotides surrounding the damaged site are excised. The incisions generated for this purpose have been shown to be stimulated in the presence of the ISWI containing ACF complex[164]. It is conceivable to envision that the absence of ISWI would hinder the repair process of UV damage by NER and lead to a sustained checkpoint response as evidenced by the retention checkpoint factors on chromatin.

In the presence of DSB-containing chromatin, checkpoint activation is mildly compromised in the absence of ISWI. As with aphidicolin treatment, Chk1 activation is reduced, as is the nuclear accumulation of TopBP1. In response to DSBs, the activation of ATR in *Xenopus* egg extracts is mediated by the concerted activities of TopBP1 and Nbs1. Both of these, which are reduced on DSB-containing chromatin in the absence of ISWI, mediate the transition from the initial damage sensing by ATM to a subsequent ATR activation[117,118]. Within this transition the activity of several partially redundant nucleases is required to resect the double-stranded end to generate the required ssDNA-RPA template for activation[165]. In our view, ISWI might be instrumental in reconfiguring chromatin in the vicinity of the break by sliding histones away from the damage to allow the generation of the desired template with the appropriate length of vacant DNA for full checkpoint activation. Moreover, the lack of an interaction between ISWI and RPA further suggests that ISWI is not functioning directly on the checkpoint-activating template but more likely in close proximity. This might explain why the activation of the checkpoint was not fully abrogated in the absence of ISWI, hinting at the more likely involvement of ISWI in the amplification and persistence of the response. The observed effects were somewhat variable, likely due to inefficient depletion of ISWI. Also, other ISWI related factors might be involved in this process and to appreciate a full effect those might also have to be depleted.

Taken together, these observations underlie the importance of chromatin structure in mediating the activation of the cellular response to DNA damage. Our current work has been an effort to lay a foundation for future work, which will hopefully elucidate a better-

defined mechanistic framework to understand the interplay between the activation of the checkpoint response and the changes in local and global chromatin configuration in response to damage.

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We are greatly indebted to Akiko Kumagai for sharing unpublished data of proteins that interact with ATR in response to damage. Briefly, ATR was recovered from egg extracts containing damaged chromatin and the recovered proteins were analyzed by mass spectrometry. The pull-down experiments were carried out in our laboratory by Akiko Kumagai. Anna Shevchenko and Andrej Shevchenko at the Max Planck Institute of Molecular Cell Biology and Genetics in Dresden, Germany, carried out the mass spectrometry analysis. Their contributions are also greatly appreciated. Additionally, we would like to extend our gratitude to Dr. Marcel Méchali at the CNRS Institute of Human Genetics in Montpellier, France, for kindly providing the pRsetB-ISWI (671–1019) construct used as an antigen for antibody generation and as a substrate in ATR kinase assays (Figure 2B). We would also like to thank Karen Wawrousek and Barbara Fortini (Campbell Lab, Caltech) for sharing reagents and providing technical support to carry out the experiments detailed in Figure 3C. Finally, we would like to thank all the members of our laboratory for insightful discussions and comments on the data that went into this chapter.

CHAPTER IV

CONCLUDING REMARKS

The link between the cellular machinery that monitors the integrity of DNA and human pathologies associated with losing these critical functions has provoked a rapid expansion of knowledge on how cells respond to DNA damage. Among the most critical advances, which have allowed a better conceptual comprehension of these responses, is having gained a better understanding of the interconnectedness that exists within the pathways that ensure genomic stability. By now, it has been well established that the signal transduction cascades that operate in response to different types of lesions share a lot of the same components and can stimulate the activity of one another. In addition, piercing observations as to how these responses are activated in the physiological context of chromatin are beginning to emerge.

The traditional DNA Damage Response (DDR) paradigm suggested that the protein kinase ATR is activated by replication stress, and ATM, a related kinase, is activated in response to double-stranded DNA breaks (DSBs) in a parallel pathway[23]. With our current work, detailed in Chapter II, we have gained a better mechanistic understanding of how ATR is activated in response to DSBs. The activation of ATR in response to DSBs is achieved by the concerted activities of various proteins. Initially, the MRN complex recruits and activates ATM on sites of damage[48–54]. ATM then activates ATR by enhancing its interaction with a critical activator TopBP1[117]. In addition, the activity of ATR is further stimulated by the interaction between TopBP1 and the MRN complex[118].

Here we describe the role of an additional protein in this response, CtIP. In our experiments, CtIP, which is recruited to DSBs to stimulate the processing of breaks to generate a proper template for repair by homologous recombination (HR), interacts with TopBP1 in a damage-stimulated manner[57,112–114,120,125]. The interaction between CtIP and TopBP1 is mediated by the sensor MRN complex and by the phosphorylation of CtIP by either ATM or ATR, which share the same consensus phosphorylation site. Furthermore, the stimulatory MRN/TopBP1 interaction, previously described by members of our laboratory, is partly compromised in the absence of CtIP. Removal of CtIP from egg extracts also severely affects the activation of ATR in the presence of DSBs. Taken together, our results more strongly implicate CtIP in the activation of ATR in response to DSBs by forming a large ATR-stimulating complex, minimally composed of CtIP, TopBP1, and the MRN complex.

In Chapter III we have explored the role of the chromatin remodeling ATPase ISWI in DDR. Herein we describe an interaction between ISWI, ATR, and two principal ATR activators ATRIP and TopBP1. This interaction, which occurs on DNA, is dependent on the presence of damage. In addition we characterize an ATR (and ATM) phosphorylation site on ISWI that is conserved among other metazoan ISWI homologs. Furthermore, we gathered a number of observations to suggest that the chromatin remodeling activity of ISWI is, in some manner yet to be determined, involved in mediating the cellular response to DNA damage.

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