14-3-3 Proteins, FHA Domains and BRCT Domains in the DNA Damage Response

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Abstract

The DNA damage response depends on the concerted activity of protein serine/threonine kinases and modular phosphoserine/threonine binding domains to relay the damage signal and recruit repair proteins. The PIKK family of protein kinases, which includes ATM/ATR/DNA-PK, preferentially phosphorylate Ser-Gln sites, while their basophilic downstream effecter kinases, Chk1/Chk2/MK2 preferentially phosphorylate hydrophobic-X-Arg-X-X-Ser/Thr-hydrophobic sites. A subset of tandem BRCT domains act as phosphopeptide binding modules that bind to ATM/ATR/DNA-PK substrates after DNA damage. Conversely, 14-3-3 proteins interact with substrates of Chk1/Chk2/MK2. FHA domains have been shown to interact with substrates of ATM/ATR/DNA-PK and CK2. In this review we consider how substrate phsophorylation together with BRCT domains, FHA domains and 14-3-3 proteins function to regulate ionizing radiation-induced nuclear foci and help to establish the G_2/M checkpoint. We discuss the role of MDC1 a molecular scaffold that recruits early proteins to foci, such as NBS1 and RNF8, through distinct phosphodependent interactions. In addition, we consider the role of 14-3-3 proteins and the Chk2 FHA domain in initiating and maintaining cell cycle arrest.

Introduction

Protein kinases and phosphopeptide binding domains are critically important regulators of information flow in the DNA damage response. In this review, we discuss how 14-3-3 proteins, FHA domains, and BRCT domains cooperate to regulate the DNA damage induced cell cycle checkpoint as well as the assembly of multi-protein complexes at ionizing radiation induced DNA damage foci (IRIF). These proteins and domains specifically recognize short amino acid sequences surrounding a central phosphorylated serine or threonine residue, thereby coupling upstream protein kinase activation to downstream alterations in cell physiology through formation of phosphopeptide signaling complexes and networks. Phosphoserine/threonine-binding domain-containing proteins are involved in many levels of regulation of the DNA damage signaling network: setting up the DNA damage G_1, S and G_2 checkpoints, checkpoint maintenance, and coordinating the

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localization of signaling molecules at the DNA break. Here we discuss the molecular details of how phosphoserine/threonine recognition by three key families of phosphoserine/theronine binding domains function in the DNA damage response: 14-3-3 proteins, FHA-domains and BRCT-domains.

At the core of the DNA damage response are two distinct classes of protein kinases based upon their kinase phosphorylation motif. At the top of the DNA damage signaling cascade are the phosphatidylinositol-3-OH-kinase-like kinases (PI(3)KK) kinases ATM, ATR, and DNA-PK, which preferentially phosphorylate proteins at serines/threonines followed by glutamine (SQ sites) [1]. The second tier of DNA damage kinases that function downstream from the PI(3)KK kinases are the checkpoint effector kinases, Chk1, Chk2, and MK2 (Chk3) [2-5]. Functionally we can segregate the assembly of macromolecular complexes on chromatin from the cell cycle checkpoint by classifying foci formation as dependent on ATM substrates and ATM responsive phosphopeptide binding domains, whereas cell cycle checkpoint establishment essentially depends on Chk1/Chk2/MK2 substrates and Chk1/Chk2/MK2-responsive phosphopeptide-binding domains. As mentioned above, the kinase motifs of ATM/ATR/DNA-PK and the checkpoint kinases differ significantly: ATM/ATR/DNA-PK preferentially phosphorylate serine/threonine followed by glutamine, whereas Chk1/Chk2/MK2 are basophilic kinases that phosphorylate (Hydrophobic)-XRXXS motifs. The phospho-ligand binding specificity of domains that localize to foci generally overlap with the ATM kinase motif, such that these domains can bind to ATM substrates [6-9]. Conversely, the phosphopeptide binding specificity of domains that act in the cell cycle checkpoint response overlaps with checkpoint kinase motifs [10-14]. BRCT domains, for example, generally bind to phosphomotifs that overlap with the ATM kinase motif, and importantly, deselect for phosphomotifs that contain an arginine in the -3 position (relative to the phosphoserine), preventing BRCT domains from binding to motifs generated by checkpoint effector kinases [15] (Mohammad and Yaffe, unpublished data). In contrast, 14-3-3 proteins deselect phosphopeptides that contain a glutamine in the +1 position, making it unlikely that 14-3-3 proteins would bind ATM/ATR substrates [16] (Mohammad and Yaffe, unpublished data). Thus ATM/ATR are divided from the checkpoint kinases by the phosphorylation motif on a first level and further segregated by the phosphopeptide binding domains that these kinases recruit (see Figure 1).

The Players

14-3-3 proteins—The first phosphothreonine/phosphoserine binding proteins to be identified were 14-3-3 proteins [16, 17]. 14-3-3 proteins fold into cup-shaped homo and hetero-dimers; each 14-3-3 dimer is capable of binding to two phosentligands, with one ligand bound per monomer [16, 18]. Phosphopeptide binding affinity is mediated by a basophilic pocket that directly complexes to the phosphoserine/threonine phosphate within the ligand. There are seven 14-3-3 isotypes in mammalian cells: β, γ, e, η, σ, t, and ξ; all isotypes bind to small peptides containing either the motif RSX[pS/pT]XP (mode-1), RXXX[pS/pT]XP (mode-2), where X denotes any amino acid [16, 19]. A third motif, SWpTX (mode-3), located at the C-terminus of transmembrane proteins has also been identified [20]. While these motifs are useful in predicting the potential phosphopeptide binding sites of 14-3-3 ligands, there are a number of 14-3-3 ligands that deviate from this optimal motif, such as p53 which has a binding motif KGQSTpSRG [21] 14-3-3 proteins are highly abundant, expressed in all eukaryotic cells, and conserved in all eukaryotic organisms. These proteins have many important roles in a variety of cellular processes including the positive and negative regulation of kinases and phosphatases, membrane trafficking, cell cycle control and transcriptional regulation. A major role of 14-3-3 proteins, particularly the non-σ isoforms, lies in mediating the G_1/S and G_2/M cell cycle checkpoints after DNA damage. This occurs by suppressing Cyclin-Cdk activity through direct 14-3-3

binding and inhibition of the Cdc25 family of phosphatases [10, 12-14, 22-24] and the 14-3-3 binding and activation of the Wee1 kinase, thereby maintaining Cdk1 in a phosphorylated and inactive state [25-28]. In contrast, the σ isoform of 14-3-3, which is upregulated after DNA damage, at least in part through p53 [29], may directly impair CDK2 activity [30], as well as control protein translation [31]. Conversely, in the presence of growth factors and the absence of DNA damage, activation of the protein kinase AKT downstream of PI(3)K facilitates transit through the cell cycle by promoting the phosphorylation and cytoplasmic sequestration of the CDK inhibitor p27[Kip1] [32, 33]. In addition, 14-3-3 binding to a major splicing isoform of the tumor suppressor protein IngI (Ing1b) may impair upregulation of the Cdk inhibtor p21[Waf1] [34]. We will discuss these events further at the end of the review. Finally, unlike FHA domains and BRCT domains, 14-3-3 proteins do not exist in modules within proteins, with the possible exception of the nonsense-mediated mRNA regulator SMG7 [35].

FHA domains—Forkhead Associated domains (FHA) domains are exclusively phosphothreonine-binding (and not phosphoserine-binding) proteins [36-38]. This domain family was originally discovered by bioinformatics studies of the Forkhead family of transcription factors [39]. FHA domains fold into a beta sandwich of two beta-sheets, with the greatest distinguishing feature in ligand selectivity being the identity of the amino acid 3 positions C-terminal to the phosphotheronine. FHA domains are found in hundreds of prokaryotic and eukaryotic proteins involved in transcription, DNA damage signaling and cell cycle regulation. Despite the presence of over half a dozen X-ray crystal structures of FHA domain:phosphopeptide complexes, the structural basis for exclusive phosphothreonine binding remains unclear. In this review we will discuss four FHA domain containing proteins: MDC1, RNF8, NBS1, and CHK2 (see Figure 2).

BRCT domains—BRCA1 C-terminal (BRCT) domains were originally discovered as a domain conserved in multiple DNA damage-response proteins [40, 41]. Named after one of the founding members, BRCA1, BRCT domains are often found as tandem repeats within a single protein. BRCA1, 53BP1, DNA LIgase IV, and MDC1, for example, contain 2 BRCT domains, while MCPH1 contains 3, Pax-2 interacting protein (PTIP) contains 6, and TopBP1 contains 8. A few BRCT domain-containing protein, including XRCC1, REV1, and a PARP family member contain only a single BRCT domain. There are approximately thirty BRCT domain-containing proteins in mammalian cells, and essentially all of these appear to have roles in DNA damage signaling and DNA repair. While not all BRCT domains function as phosphopeptide binding modules, a subset of tandem BRCT domains bind to phosphopeptides with a strong preference for aromatic residues in the position three amino acids C-terminal to the phosphoserine [6, 15]. In contrast to FHA domains which bind exclusively to phosphotheronine-containing peptides, BRCT domains generally bind stronger to peptides containing phosphoserines than phosphothreonine [15, 42]. The phosphopeptide binding activity of BRCT domains requires the presence of two BRCT domains in tandem. The structure of the tandem BRCT domains of BRCA1 bound to a phosphopepitde from BACH1 demonstrated how the individual BRCT domains fold into a phosphopeptide binding domain: the N-terminal BRCT primarily stabilizes the phophoserine, while the tandem BRCT domain fold forms a hydrophobic pocket which stabilizes the aromatic residue in the +3 position of the phospho-ligand [8, 43]. In addition to the clear roles of BRCT domain containing proteins in the DNA damage response, BRCA1 mutations have been identified in approximately fifty percent of hereditary breast cancers; cancer-associated mutations of BRCA1 often disrupt the BRCT domains of BRCA1 [44]. Cancer-associated point mutations in BRCA1 often fail to bind phosphopeptides [8], providing a physiological relevance to phosphopeptide binding activity in cancer

predisposition. In this review we will discuss the BRCT domain containing proteins MDC1, NBS1, BRCA1, 53BP1, as well as MCPH1 and PTIP (Figure 2).

The Play: Orchestrating the DNA Damage Response

The coordinated interaction of specific ATR/ATM substrates with BRCT- and FHA-domain containing molecules leads to the formation of multi-protein complexes within nuclear foci in DNA-damaged cells, amplifying the DNA damage signal, and leading to activation of the downstream effector kinases Chk1, Chk2, and MK2. The subsequent interaction of specific Chk1/Chk2/MK2 substrates with 14-3-3 proteins then establishes and maintains the G2/M cell cycle checkpoint. Below, we first discuss the role of phosphopeptide-binding domains in the formation of ionizing radiation-induced nuclear foci (IRIF), followed by a domain-centric view of DNA damage-induced G2/M arrest.

Ionizing Radiation Induced Nuclear Foci Formation (IRIF)

Ionizing Radiation Induced Nuclear Foci (IRIF) is an experimentally observed phenomena in which DNA damage signaling and repair proteins dynamically redistribute following treatment with agents that induce double strand breaks to regions around the putative break. Elegant studies using live-cell laser microirradiation have demonstrated that foci formation is regulated temporally: early events in IRIF formation include ATM acetylation [45, 46] followed by autophosphorylation at Ser-1981 [47], phosphorylation of the H2 histone variant H2AX at Ser-139 near the C-terminus to create γH2AX [48, 49], Mre11-Rad50-Nbs1 (MRN) complex relocalization, recruitment and phosphorylation of the modulator protein MDC1 into the γH2AXfoci [50-52], and the subsequent recruitment of the E3 ubiquitin ligaseRNF8 [53, 54]. Later events at foci involve the recruitment of BRCA1, and 53BP-1 [55]. The regulation of foci formation by BRCT and FHA domain containing proteins is an elegant example of a hierarchy of phosphopeptide binding interactions. MDC1 localization to IRIF seems to be a key event in this process, since MDC1 recruits the MRN complex as well RNF8 to IRIF [42, 56, 57]. The recruitment of the remaining proteins to IRIF can be classified as either Nbs1-dependent or RNF8-dependent events.

Early events: ATM activation, H2AX phosphorylation and MDC1 localization

One of the earliest events of foci formation is ATM activation, which leads to the phosphorylation of a histone variant H2AX [48, 58, 59]. H2AX compromises 5-10% of the total cellular histone H2A. Phosphorylation of H2AX by ATM occurs very near, but not at, the double strand break site and can extend outwards from the break for up to several megabases [60]. H2AX phosphorylation results in the generation of a C-terminal phosphomotif that is then recognized by the tandem BRCT domain containing protein MDC1 [42]. Recruitment of MDC1 to γH2AX is an early event in foci formation and is of fundamental importance to further signaling at the break [42] by the subsequent phospho-dependent recruitment of two complexes of proteins: RNF8-dependent proteins, and NBS1-dependent proteins [56, 57, 61-63].

MDC1- A critical scaffold

The DNA Damage modulator protein MDC1 contains an N-terminal FHA domain and tandem BRCT domains at the C-terminus, allowing it to function as a core scaffold that mediates events downstream from foci formation. There are three functional components to the MDC1 scaffold: the first is the tandem BRCT domains of MDC1 which bind to the phosphorylated C-terminus of H2AX [42], the second is a series of TQXF motifs on MDC1, which are phosphorylated by ATM and subsequently recruit the FHA domain-containing protein RNF8 [56, 57], and finally, a series of SDT repeats in MDC1 which are thought to be constitutively phosphorylated by Caesin Kinase 2 (CK2), and bind in a phospho-

dependent manner to a combination of the FHA domain and the tandem BRCT domains of NBS1 [61-64].

Although the tandem BRCT domains of PTIP and BRCA1 were the first BRCT domains to be described as phosphopeptide-binding modules [6, 15], later work demonstrated that the BRCT domains of MDC1 could also bind to phosphoserine-containing peptides. However, unlike the BRCT domains of PTIP and BRCA1, which can bind to internal phosphoserine-X-X-Aromatic motifs within proteins, oriented peptide library screening revealed that the MDC1 BRCT domains could only bind to phosphoserine-X-X-Aromatic motifs at the extreme C-terminus of a protein or peptide [42]. Intriguingly, the optimal recognition motif for the MDC1 BRCT domains closely matched the C-terminal sequence of γH2AX, while a mass spectrometry screen for proteins binding to the C-terminal peptide from γH2AX-binding proteins independently identified MDC1, and mapped the interaction to the tandem BRCT repeats. This unexpected discovery for MDC1 BRCT domain phospho-dependent C-terminal specificity was partially rationalized when the BRCT domain:γH2AX phosphopeptide crystal structure was solved, revealing that the peptide C-terminal carboxylate anion makes a pair of stabilizing salt bridge interactions with a conserved Arg residue that also salt bridges with a nearby Glu residue to help stabilize the BRCT fold [42, 65].

MDC1 recruitment to IRIF was shown to require the C-terminal phosphorylation of H2AX on Serine-139. MDC1 localization is γH2AX-dependent as MDC1 failed to form foci after IR in H2AX-/- MEFs [42]. Re-expression of wild-type H2AX in H2AX-/- MEFs restored MDC1 foci formation, however, re-expresssion of a non-phosphorylatable S139A, mutant of H2AX failed to restore MDC1 foci formation. In addition, overexpression of the BRCT domains of MDC1 resulted in a dominant negative phenotype, disrupting the localization of full-length MDC1 and abrogating NBS1 and 53BP-1 foci formation, thus placing MDC1 downstream of H2AX in the foci formation pathway, but upstream of NBS1 and 53BP-1 [42]. MDC1 knockdown cells also displayed a reduction in ATM autophosphorylation and a shortened duration of H2AX phosphorylation [42]. This implies that MDC1 both protects H2AX from dephosphorylation as well as functions to maintain the DNA damage checkpoint.

NBS1-dependent Foci

NBS1, a key component of the Mre11-Rad50-Nbs1 complex, contains an N-terminal FHA domain that closely abuts a tandem pair of BRCT domains [66, 67]. NBS1 was initially thought to directly interact with γH2AX, however, later experiments showed that the interaction of NBS1 with γH2AX required MDC1 [68]. Furthermore, NBS1 likely interacts with MDC1 constitutively [50], The molecular basis for recruitment of NBS1 to γH2AX foci through MDC1 was recently elucidated in a series of papers from 4 different research collaborations [61-64]. These studies demonstrated that NBS1 interacts with MDC1 through a unique phosphospecific interaction involving both the FHA domain and tandem BRCT domains of NBS1 and a repeated SDTD motif in MDC1. Mutation or deletion of either the FHA domain or the BRCT domains of NBS1 abrogated the interaction between NBS1 and MDC1 [61, 64]. Furhtermore, the repeated SDTD motifs in NBS1 are clustered in an acidic domain at the N-terminus of MDC1 with many of the motifs containing putative consensus CK2 phosphorylation sites. Curiously, the combined FHA domain and tandem BRCT domains of NBS1 were shown to bind to doubly phosphorylated SDTD phosphopeptides, while singly phosphorylated peptides failed to interact with NBS1 [61, 63]. The SDTD motifs in MDC1 were shown to be phosphorylated in vivo using orthophosphate labeling of U2OS cells [62], as well as by a phosphospecific antibody raised to that region [61], and phosphorylation at the SDTD sites was reduced after treatment of the cells with a CK2 inhibitor or following CK2 knockdown using RNAi [61, 63, 64]. Mutation of three of the six

SDTD motifs in MDC1 did not impair the ability of NBS1 to form foci, however, mutation of four or more SDTD motifs resulted in a reduction of NBS1 foci formation. However, despite all of these treatments MDC1 foci formation remained intact, clearly placing MDC1 upstream of NBS1. Finally, disruption of the NBS1 FHA domain resulted in a partial loss of the G2/M checkpoint [63].

RNF8-dependent Foci –linking phosphorylation and ubiquitinylation

RNF8 is an E3 ubiquitin ligase that contains a RING finger domain and an FHA domain. RNF8 forms foci after DNA damage in an MDC1-dependent manner [53, 56, 57, 69]. Interestingly 53BP-1 and BRCA1 but not NBS1 foci formation is abrogated in the RNF8 knockdown cells. The RNF8 FHA domain is necessary for recruitment of RNF8 to foci; a version of RNF8 missing the FHA domain failed to form foci, whereas deletion of the RING finger domain had no effect on RNF8-containing IRIF. However, expression of the RNF8 FHA domain alone in RNF8 siRNA treated cells failed to restore 53BP-1 and BRCA1 foci formation, indicating that the RING finger domain RNF8 is required for the localization of 53BP-1 and BRCA1 to IRIF. The FHA domain of RNF8 interacts with MDC1 in a phospho-specfic manner through a repeated pTQXF motif in MDC1[53, 56, 57]. An X-ray crystal structure of the RNF8 FHA domain:phosphopeptide complex revealed a structural arginine that was important for phosphopeptide binding [56], as well as the molecular basis for the unusual pT+3 aromatic selection, a motif selectivity feature that is more common in tandem BRCT domains than in FHA domains. A mutant version of RNF8, R61Q, abrogated foci formation as well as the interaction between RNF8 and MDC1 [56, 57]. These data explain the requirement of the RNF8 FHA domain for recruitment of RNF8 into foci. The requirement for the RNF8 RING finger domain for BRCA1 and 53BP1 foci formation became clear when the enzymology of RNF8-mediated ubiquitnation was examined, and the data then tied to prior studies on BRCA1-interacting proteins. RNF8 promotes the localization of poly-K63 ubiquitin conjugates to foci, is required for H2A and H2AX ubiquitinylation after DNA damage, and the RING finger domain is essential for this function.

How does this connect RBF8 to BRCA1? It had been previously demonstrated that BRCA1 BRCT domains are necessary and sufficient for IRIF formation. In addition, a subset of cancer associated mutations of BRCA1 fail to form foci after DNA damage. The tandem BRCT domains of BRCA1 interact with phosphopeptides, with a preference for peptides that contain an aromatic in the +3 position [6, 15]. Several known BRCA1 ligands including BACH1 and CtIP contain these phosphomotifs, however none of these ligands were found to be necessary for BRCA1 foci formation. Several independent studies were performed to identify the BRCA1 interacting proteins that are responsible for BRCA1 IRIF. These screens identified several proteins including RAP80, a protein that contains tandem Ubiquitin Interacting Motifs (UIM) and Abraxas, a coiled coiled protein [70-72]. BRCA1 was shown to interact directly with Abraxas, with Rap80 binding to BRCA1 through Abraxas. siRNA depletion of Rap80 resulted in the loss of BRCA1 foci formation. Interestingly, the UIM domains of Rap80 which are sufficient for Rap80 foci formation through binding to poly-K63 and poly-K6 ubiquitinylated lysines; however, a version of Rap80 missing the UIM domains was still able to form foci, albeit inefficiently[70]. Collectively, the model that has emerged is that the FHA domain of RNF8 recruits RNF8 to MDC1, the RING finger of RNF8 results in the polyK63-ubiquitination of histone H2A and histone H2AX and this in turn recruits BRCA1 through its interaction with Rap80 (Figure 3). This model does not explain all the data, however. Knockdown of RNF8 prevents Rap80 accumulation at sites of DNA damage, and greatly attenuates BRCA1 IRIF. Nevertheless, Rap80 cannot be solely responsible for BRCA1 recruitment, since knockdown of Rap80 results in a reduction of BRCA1 foci formation that is not as severe as the reduction in BRCA1 foci formation that

occurs after MDC1 knockdown. Therefore Rap80 independent mechanisms of BRCA1 recruitment likely exist. In addition, knockdown of Rap80 only reduced the intensity of BRCA1 recruitment when cells were damaged by laser microirradiation rather than ionizing radiation. Furthermore, BRCA1 may not interact with Rap80 during S-phase delay, as Rap80 was reported not to interact with BRCA1 after thymidine treatment [73]. There may also be histone ubiquitinylation-independent mechanisms for BRCA1 localization to IRIF, since a large number of proteins are phosphorylated on SQ motifs at sites of DNA damage [74], providing potential binding sites for the BRCT domains, and a component of BRCA1 function does not depend entirely on Rap80. Finally, despite the role of Rap80 in BRCA1 recruitment to IRIF, Rap80 does not appear to be critical for 53BP-1 foci formation [70].

Interestingly, like BRCA1 and MDC1, RNF8 selects aromatic residues three positions C-terminal from the phosphoresidue. Notably, BRCT domains prefer to bind to phosphoserine motifs over phosphotheronine motifs, whereas FHA domains bind to phosphotheronine motifs exclusively. This constitutes an elegant division of labor between FHA domains and BRCT domains; BRCT domains binding to ATM/ATR/DNAPK substrates phosphorylated on serines, while the FHA domain of RNF8 binds to ATM/ATR/DNAPK phosphorylated on theronines.

MCPH1 – A parallel pathway to MDC1

MCPH1 (also called BRIT1) contains three BRCT domains; a single N-terminal one, and a C-terminal tandem pair. The MCPH1 C-terminal BRCT domains have phosphopeptide binding activity similar to the MDC1 tandem BRCT domains, requiring a C-terminal carboxylic acid for phosphopeptide binding (Mohammad, Jackson and Yaffe, unpublished). The most likely MCPH1 tandem BRCT domain ligand is γH2AX, since ectopically expressed MCPH1 forms foci in MDC1-/- MEFs, but not in H2AX-/- MEFs. This suggests that MCPH1 binding to the C-terminus of H2AX is likely to act through a pathway parallel to MDC1 [75]. The functional relevance of this interaction is unclear, since knockdown of MCPH11 does not impair foci formation of any other known DNA damage molecules. MCPH1 deficient cells do, however, show impaired intra-S and G2/M checkpoint responses [76]. The levels of both BRCA1 and Chk1 are reduced in MCPH1 deficient cells, likely as a consequence of MCPH1 modulation of BRCA1 and Chk1 gene transcription through binding of MCPH1 to E2F1 [77, 78]. In addition, an MCPH1-E2F1 complex may regulate the expression of many other DNA damage signaling genes [77]. Therefore it is unclear if the loss of checkpoint function in MCPH1 deficient cells is due to the loss of some direct MCPH1 function in DNA damage responses, or is a secondary effect due to impaired expression of BRCA1 and Chk1. Interestingly TopBP1 has also been shown to interact with E2F1 as well, and this interaction has been demonstrated to require the last three BRCT domains of TOPBP1. Whether the interaction between the TopBP1 BRCT domains and E2F1 and the MCPH1 BRCT domains and E2F1 occurs through the same site, or is phospho-dependent is unclear. However, it is distinctly possible that E2F1 is phosphorylated at one of the SQ/TQ sites after DNA damage and that this interaction recruits MCPH1 or TOPBP1 DNA damage and regulates the expression of many key DNA repair and signaling proteins.

MCPH1 deficient cells have a variety of interesting pleiotropic phenotypes. Both cells derived from MCPH1-defective patients and MCPH1-/- MEFs exhibit premature chromosome condensation, the rescue of which requires the N-terminal BRCT domain [79, 80]. While there are some insights into MCPH1 function, it is still unclear what the role of MCPH1 in the mammalian DNA damage is per se. A recent report showed that the Condensin II complex bound tightly to MCPH1, and noted that both MCPH1-/- MEFs and

Condensin II-depleted cells were defective in DBA repair by homologous recombination [80].

PTIP: More BRCT domains than molecular functions

Pax2 Transactivation domain interaction protein (PTIP) is a six-BRCT domain containing protein that has roles in transcription and the DNA damage response. The PTIP knockout mouse provided the first hint that PTIP might be involved in the DNA damage response [81]. PTIP-/- embryos died at E9.5, and MEFs derived from PTIP-/- embryos failed to proliferate, recapitulating a phenotype similar to that seen for other DNA damage response proteins involved in DNA synthesis. Interestingly, PTIP-/- embryos exhibited increased TUNEL staining, however rather than the pyknotic morphology typically observed in apoptotic cells, TUNEL positive PTIP-/- cells had a diffuse morphology, possibly indicating the presence of fragmented chromosomes [81]. The C-terminal tandem BRCT domains of PTIP were found to be a phosphopeptide binding module, with a preference for hydrophobic amino acids in the +3 position [15]. PTIP was found to form IRIF, although the BRCT domains required for foci formation remains controversial. Two studies found that the phosphopeptide binding C-terminal BRCT domains are necessary for foci formation [15, 82], however, a recent study found the central BRCT domains are necessary for foci formation, and not the phosphopeptide binding BRCT domains [83]. PTIP was also found to interact with 53BP1 in a phospho-dependent manner [15], and this interaction likely requires the last four BRCT domains of PTIP [84]. The requirement for four BRCT domains for phosphopeptide binding is a novel, however, it may be that the middle two BRCT domains stabilize the interaction with 53BP1, as it is a non-optimal phospholigand for the last two PTIP BRCT domains.

Exactly what PTIP does to control the DNA damage response and coordinate DNA repair remains elusive. Much of the work performed on PTIP has focused on the interaction of PTIP with MLL2, MLL3, and MLL4 complexes [85] and the putative role of PTIP in transcription regulation. A recent study identified two PTIP containing complexes, a high molecular weight complex that co-purifies with MLL proteins, and a low molecular complex that purifies with PTIP-associated protein 1 (PA1). PA1 is recruited to IRIF by PTIP [83]. In addition, Gong and colleagues demonstrated that PTIP did not form foci in the absence of H2AX, MDC1 or RNF8. Interestingly, despite the in vitro and in vivo interaction between PTIP and 53BP1, PTIP and 53BP1 appear to be recruited to foci independently [83].

A number of important questions in the field remain unanswered, including the interplay between MCPH1 and MDC1, both of which seem to be interacting with γH2AX with similar affinities. It has been demonstrated that MCPH1 recruitment to foci does not depend on γ-H2AX; however, as MDC1 has been demonstrated to be a protein that coordinates and recruits several proteins to foci, the question remains, what is the role of MCPH1 in foci formation? Could MCPH1 be specifically involved in checkpoint exit? It has been demonstrated that MDC1 is degraded after DNA damage, suggesting that MCPH1 could bind to and facilitate the dephosphorylation of γH2AX after MDC1 destruction. It is also possible that MCPH1 regulates repair processes like HR, while MDC1 might functions primarily in the early stages of the DNA damage signaling response. Another facet of phosphopeptide binding in protein recruitment to foci after DNA damage is the question of uniqueness in ligand binding. It is not intuitive that recruitment of phosphopeptide binding proteins into nuclear foci should depend exclusively on a single ligand. Given the large number of ATM substrates, why should proteins like BRCA1 bind to such a limited number of ligands after DNA damage, especially when 14-3-3 proteins typically bind hundreds of distinct ligands. It seems likely that there are other factors involved in specifically localizing proteins to foci.

Checkpoint Response

In addition to the specific localization of proteins to sites of DNA damage, the DNA damage response coordinates a checkpoint response which prevents mitotic entry in the presence of large amounts of unrepaired double strand breaks. In contrast to foci formation, where the emphasis is on localizing molecules to a concentrated area, the checkpoint response must communicate the presence of DNA breaks to the rest of the cell. While Chk1, Chk2 and MK2 cooperate to regulate this response, the discussion below focuses on Chk2 as it is the only checkpoint kinase that contains a phosphopeptide binding domain. Reviews of Chk1 and MK2 signaling are available elsewhere [86, 87].

The CHK2 FHA Domain

Chk2 is a key downstream effector kinase of ATM which contains an N-terminal SQ/TQ rich region (SCD), a phosphopeptide-binding FHA a domain, and a C-terminal kinase domain. The FHA domain of Chk2 preferentially binds to phosphothreonine with Isoleucine, Leucine or Valine in the +3 position [38]. Upon DNA damage, activated ATM phosphorylates Chk2 on Thr68, creating a weak Chk2 FHA domain binding site, and some studies have suggested that this phosphorylation of Chk2 results in its homo-oligomerization through the binding of the FHA domain of one Chk2 molecule to the phospho-T68 site of another Chk2 molecule [88, 89]. This interaction facilitates kinase activation by the trans autophosphorylation of Thr383 and Thr387 on the activation-loop of Chk2. Studies using phosphoantibodies have demonstrated that Thr68 phosphorlyation proceeds Thr383 and Thr387 phosphorylation after DNA damage, consistent with this model [90]. However, the affinity of the Chk2 FHA domain for the phospho-T68 site is weak, with a K_D of ~25-30uM [38], suggesting that this interaction is unlikely to be solely responsible for stable homo-oligomerization. These data can be partially resolved with the recent kinase domain structure of Chk2, which provided evidence for T-loop interactions between molecules resulting in Chk2 kinase domain dimerization [91]. It may be that there is synergy between the kinase domain interaction and the FHA domain interaction that, when coupled, results in a productive Chk2 autophosphorylation and activation. It is unlikely that the Chk2 kinase domain on its own is capable of a productive interaction that induces autophosphorylation, as expression of the Chk2 kinase domain alone in bacteria does not result in Chk2 autophosphorylation [91]. Full-length Chk2, which contains the FHA domain, however, does autophosphorylate; indicating a role for the FHA domain in Chk2 activation.

There are two mutations that were thought to be associated with the Li-Fraumani syndrome [92] and were used to study the role of the Chk2 FHA domain in Chk2 kinase activation: the Chk2 FHA domain mutant Ile157 to Thr (I157T) which does not disrupt the phosphopeptide binding activity of the FHA domain, and the mutation Arg145 to Trp (R145W) which is structurally disrupted, and does not bind phosphopeptides [38, 88-90, 93]. Finally, Arg117A is a mutant based on the crystal structure that fails to bind phosphopeptides [38]. Interestingly all three mutants when overexpressed in 293T cells failed to interact with BRCA1 after HU treatment [38]. Therefore, there may be another binding surface on the FHA domain that stabilizes protein-protein interactions, distinct from those mediated by phosphopeptide binding [38]. One issue that is obscuring the mechanism of Chk2 activation is the use of the FHA mutant R145W, which not only inactivate the FHA domain, but clearly disrupt the structure of the protein. Several studies used this phosphopeptide binding-defective mutant as a negative control, and the data from these experiments is therefore difficult to interpret due to a lack of structural integrity of Chk2 proteins carrying these mutations. Data obtained using this mutant has led to significant confusion in the field as to whether the Chk2 FHA domain is, or is not, required for Chk2 activation. Interestingly, Chk2 phosphorylation on T68 is still observed in the FHA phospho-binding mutant Arg117A, indicating that the phosphopeptide binding function of the FHA domain is not

required for Chk2 phosphorylation [38]. However, the Chk2 hypermobility shift seen after IR was not observed in the Arg117A mutant [38], implying that the FHA phosphopeptide binding activity was required for of the CHK2 kinase activation.

Finally, the role of the Chk2 FHA domain in Chk2 activation was recently evaluated using an artificial construct in which the Chk2 FHA domain was ligated to the T68 phosphopeptide through a synthetic thioester peptide linkage [5]. The authors found that despite the low affinity of the free FHA domain for the T68 phosphospeptide, dimer formation was observed with Chk2-FHA: pT68 ligated molecules. Interestingly, this study also followed up on an observation made by another group, which had found that Chk2 phosphorylates the FHA domain in trans [89], and that the phosphorylated FHA domain had a reduced affinity for the T68 peptide. Li *et al* also observed this phenomena and identified an autophosphorylation site on human Chk2 at Ser140 that is properly positioned to disrupt the phosphopeptide-binding activity. In the unphosphorylated Chk2 FHA domain, the side chain of Ser140 makes a hydrogen bonding contact with the phosphate oxygen of the ligand; this interaction cannot occur when this residue is phosphorylated [5]. Thus, a model for Chk2 activation after DNA damage that embraces all of this data would suggest that Chk2 phosphorylation at T68 by ATM, causes Chk2 dimerization, which results in T-loop phosphorylation and activation of the kinase. Finally, dimer formation is disrupted by autophosphorylation of the Chk2 FHA domain, releasing active Chk2 monomers.

14-3-3 proteins – Coordinators of Checkpoint Effector Kinase Function

Progression from the G_2 phase of the cell cycle into mitosis depends on the activity of the CyclinB/Cdk1 complexes. Cdk1 activity is primarily regulated through inhibitory phosphorylation on Thr-14 and Tyr-15 in the ATP-binding loop. The phosphorylation of these two sites is positively regulated by the kinases Myt1, a dual-specificty kinase which preferentially phosphorylates T14 [94], and Wee1, a tyrosine kinase which targets Y15 [95], and negatively regulated by members of the Cdc25 family of phosphatases, primarily Cdc25B and Cdc25C. In response to DNA damage, the checkpoint kinases Chk1, Chk2, and MK2 indirectly regulate CyclinB/Cdk1 activity by activating Wee1 and inhibiting Cdc25B and Cdc25C, all in a 14-3-3 dependent manner [12, 13, 26, 28, 96]. 14-3-3 binding to Cdc25B and Cdc25C results in both sequestering of these phosphatases in the cytoplasm and modest suppression of catalytic activity [22, 97, 98], Conversely, 14-3-3 binding to Wee1 increases its catalytic activity of [27]. In addition to Cdc25 and Wee1, 14-3-3 proteins may regulate p53 transactivation activity [21, 99], potentially enhancing the expression of additional mitotic kinase inhibitors such as p21^{Waf1}.

Concluding Remarks

Both foci formation and the DNA damage checkpoints depend on the integrated activity of distinct kinase pathways and phosphopeptide binding domains. To prevent molecular signals from getting crossed, there appear to be multiple levels of regulation. After DNA damage, BRCT domains appear to bind to substrates of ATM, ATR, and DNA-PK to facilitate foci formation, while 14-3-3 proteins preferentially bind to substrates of Chk1, Chk2 and MK2 to block Cdk activation. Both positive and negative selection appear to be involved; BRCT domains discriminate modestly against motifs generated by the basophilic checkpoint kinases while 14-3-3 proteins have reduced affinity for ATM and ATR substrates by moderately discriminating phospho-motifs with glutamine in the +1 position. Thus, these phosphopeptide-binding domains have evolved to bind the ligands of the appropriate kinase class, while rejecting substrates of the incorrect kinase class.

It is likely that these types of phospho-dependent protein-protein interactions are just one step in a complex network of post-translational modifications that control DNA damage

responses. The discovery that RNF8 acts as a bridge between protein kinase signaling and histone ubiquitinylation suggests that additional phospho-dependent coupling to histone sumolation, neddylation, methylation and acetylation will turn out to be equally important in the chromatin reorganization steps that are critical for DNA damage signaling and repair.

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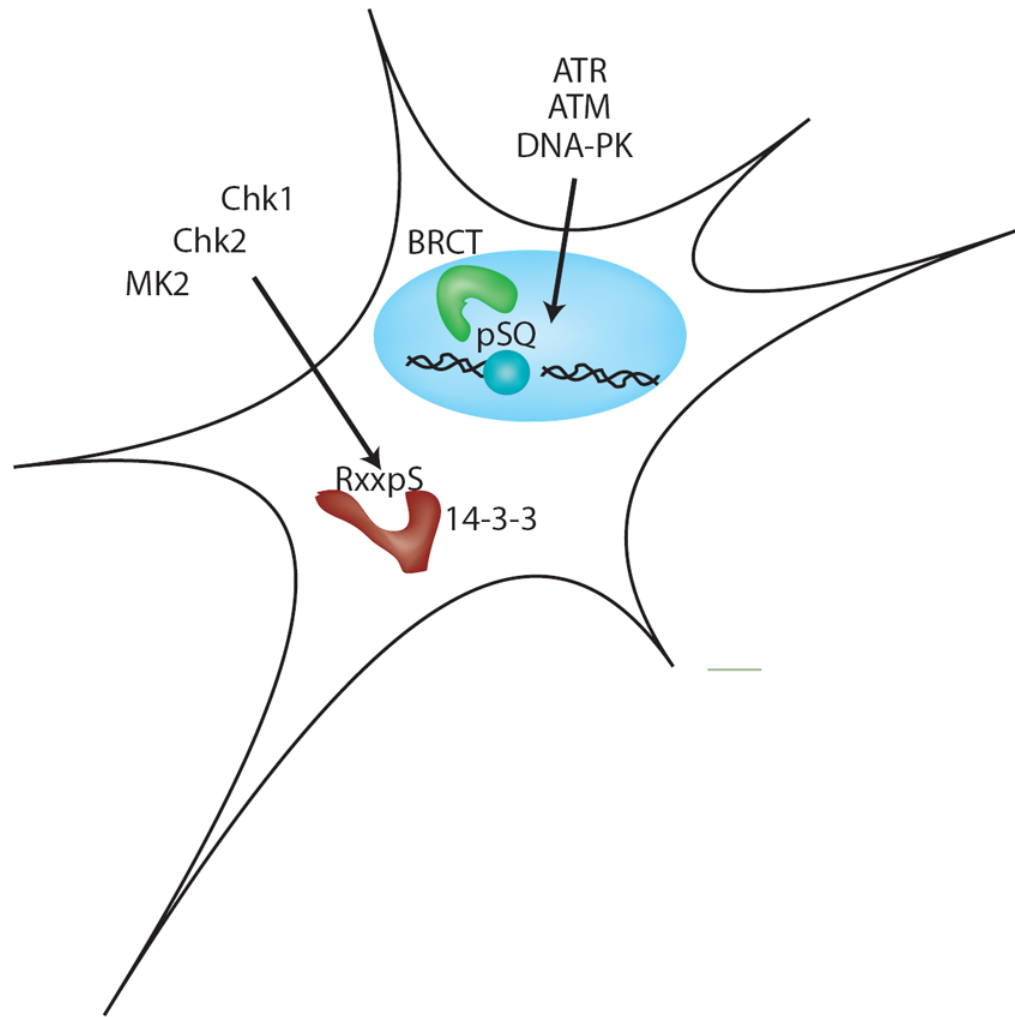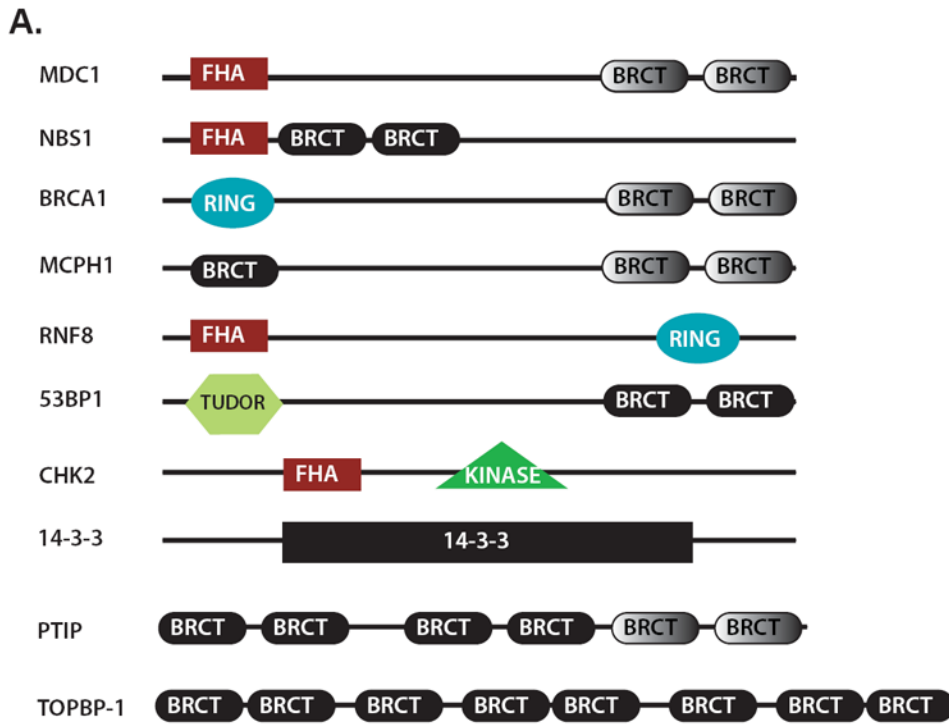
Figure 1. Kinase class-specific phosphopeptide-binding domains
The DNA damage-responsive phosphopeptide binding domains can be segregated by kinase motif. ATM/ATR/DNA-PK are gluamine-directed kinases that recruit a subset BRCT domains and FHA domains. Significant accumulation of ATM/ATR/DNA-PK substrates is observed at Ionizing Radiation Induced Foci (IRIF). Conversely, checkpoint kinases preferentially phosphorylate $\Phi x R x x S \Phi$ sites, where Φ denotes hydrophobic amino acids sites, and a subset of their substrates result in the generation of 14-3-3 motifs. Selectivity in interactions are a result of the positive selection for binding to the substrates of the proper kinase family and negative selection to prevent binding to substrates of the incorrect kinase family.

Figure 2. Protein domain architecture and phospho-motif selectivity for DNA damage-response proteins

A. The domain structure of the proteins that will be discussed in this review. BRCT domains in gray have empirically determined phosphopeptide binding activity.

B. Table summarizing the domains, selected binding motifs and example ligands. "X" stands for any amino acid, "pS" stands for phosphoserine, "pT" for phosphothreonine and ψ, for aromatic residues.
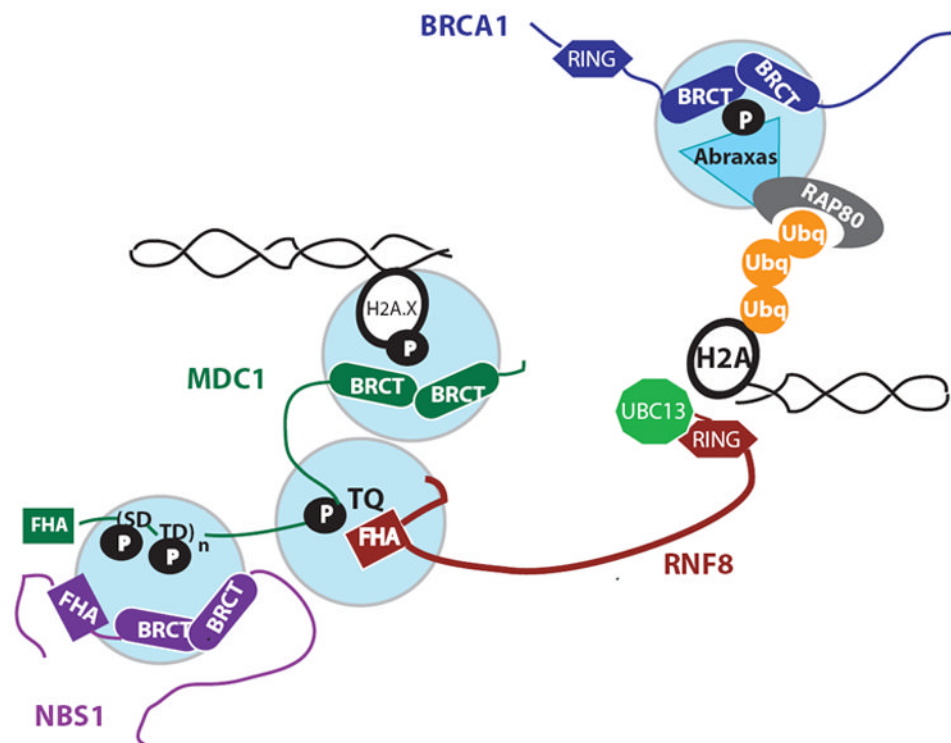
Figure 3. Overview of protein-protein interactions in IRIF formation
ATM is activated rapidly after IR treatment, resulting in the phosphorylation of H2AX at Ser-139. Phosphorylation of H2AX generates an optimal phosphobinding epitope that is recognized by the BRCT domains of MDC1 [42]. MDC1 recruits RNF8 and NBS1 through phospho-dependent interactions. NBS1 regulates the localization of the MRN complex and amplifies ATM activation [61-64], while RNF8 recruitment is required for BRCA1 and 53BP1 localization to IRIF [56, 57].