Launching a ubiquitination cascade at DNA breaks

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The *BRCA1* gene was cloned in 1994 as the first tumor suppressor of hereditary breast cancer, and it has been heavily studied ever since. The Brca1 protein is multifunctional and critical for the maintenance of genomic stability. Among its many roles, Brca1 is part of an E3 ubiquitin ligase important for homologous recombination (HR) and signaling of double-strand DNA breaks (DSBs). In response to DSBs, the checkpoint kinases ATM and ATR phosphorylate the histone variant H2AX adjacent to the breaks, which then recruits the mediator protein Mdc1 and other DNA repair and damage signaling proteins, including Brca1. Once recruited to DSBs, Brca1 colocalizes with these repair and signaling proteins in discrete nuclear foci. Both H2AX and Mdc1 are required for the formation of Brca1 foci, but how they contribute to Brca1 recruitment has not been clear. Part of this "missing link" was recently revealed. Rap80, a Brca1-associating protein, targets Brca1 to DSBs through its UIM domains, which recognize polyubiquitin chains (1–3). In this issue of PNAS, Wang and Elledge (4) present the discovery of Rnf8 as an E3 ubiquitin ligase that recognizes phosphorylated proteins at DSBs and generates the polyubiquitin chains recognized by Rap80, thereby connecting the phosphorylation- and ubiquitination-regulated steps during the recruitment of Brca1.

One important clue to the E3 ligase required for Brca1 recruitment is the specific binding of the UIM domains of Rap80 to Lys-63 (K63)-linked polyubiquitin chains (4). To generate polyubiquitin chains on substrates, an E3 ubiquitin ligase must work with an E2 ubiquitin-conjugating enzyme. Thus far, Ubc13 is the only known E2 conjugating enzyme that supports the formation of K63-linked polyubiquitin chains. In fact, Ubc13 was shown to be required for the formation of Brca1 foci (5). Ubc13 is known to interact with several E3 ligases (6). By screening these E3 ligases with siRNAs, Wang and Elledge (4) discovered that Rnf8 is the only E3 required for the formation of Brca1 foci.

Independently of Wang and Elledge (4), three other groups (7–9) also found that Rnf8 is required for Brca1 recruitment by using different approaches. From an RNAi screen, Kolas *et al.* (7) identified Rnf8 as a protein required for the formation of 53BP1 foci at DSBs. Huen *et al.* (8) and Mailand *et al.* (9), on the other hand, noticed that, in addition to a RING-finger domain characteristic for certain E3 ligases, Rnf8 possesses an FHA domain that can function as phosphopeptide-binding modules in some DNA repair and damage signaling proteins. Both Wang and Elledge (4) and Huen *et al.* (8) showed that a region of Rnf8 containing the FHA domain but not the RING finger is sufficient for foci formation at DSBs. The formation of Rnf8 foci requires H2AX and Mdc1 but not Brca1, Nbs1, and 53BP1 (8). A recent study by Elledge and colleagues (10) identified nine damage-induced phosphorylation sites (SQ/TQ) in Mdc1. Through a phosphopeptide library screen, Huen *et al.* (8) identified pTXXY/F as the optimal motif recognized by the FHA domain of Rnf8. Mdc1 contains four TQXF motifs that may be phosphorylated by ATM and recognized by Rnf8. The interaction of Rnf8 with Mdc1, as well as the recruitment of Rnf8 to DSBs, is disrupted by mutations in the four TQXF motifs of Mdc1 or the FHA domain of Rnf8 (7–9). Together, these results strongly suggest that Rnf8 is recruited to DSBs by phosphorylated Mdc1.

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How does Rnf8 generate polyubiquitin chains at DSBs? Rnf8 can function with multiple E2 ubiquitin-conjugating enzymes to form K63- or K48-linked polyubiquitin chains. Because Ubc13 is the only known E2 that generates K63-linked chains and because both Ubc13 and Rnf8 are required for the formation of Brca1 foci, Ubc13 and Rnf8 likely function as an E2–E3 complex at DSBs to catalyze the formation of K63-linked chains. Both histones H2A and H2AX are known to be ubiquitinated in response to DNA damage and are good candidates for the substrates of Rnf8–Ubc13. H2AX, in particular, was shown to be ubiquitinated in a Ubc13-dependent manner (5). Consistently, Mailand *et al.* (9) and Huen *et al.* (8) showed that the polyubiquitination of transiently expressed H2A and H2AX in cells requires Rnf8 and that purified Rnf8 promotes H2A polyubiquitination *in vitro*.

How is Brca1 recruited to polyubiquitin chains? Previous studies by three groups have identified Abra1, Rap80, and Brcc36 as the components of a Brca1-containing complex (the Brca1 A complex) (1–3). Among these proteins, Rap80 bears two UIM domains capable of recognizing polyubiquitin chains. Although these UIM domains alone are sufficient for foci formation, Wang and Elledge (4) found that the optimal localization of Rap80 to DSBs depends on both the UIM domains and the interaction between Rap80 and Abra1. Brcc36, which associates with Rap80 through Abra1, also is required for the formation of Rap80 foci (4). Thus, with the UIM domains of Rap80 directly recognizing polyubiquitin chains, the Rap80–Abra1–Brcc36 module of the Brca1 A complex targets Brca1 to DSBs.

In addition to revealing the role of Rnf8 in Brca1 recruitment, Wang and Elledge (4) also present a comprehensive analysis of the anatomy of the Brca1 A complex. They found that Abra1 functions as a scaffold in this complex. The N and C termini of Abrca1 interact with Brca1 and Rap80, respectively. Abra1 and Brcc36 associate with each other through the coiled-coil domain on each protein. Rap80 uses an internal region to associate with Abra1 and depends on Abra1 to associate with Brca1 and Brcc36. Previous studies also have suggested that the Brca1 A complex contains additional components, including Bard1 and Brcc45 (2). Because Abra1, Bach1, and CtIP interact with the BRCT domains of Brca1 in a mutually exclusive manner (1), the Abra1-containing Brca1 A complex is distinct from the Bach1-containing B and CtIP-containing C complexes.

The studies by Wang and Elledge (4) and the other three groups (7–9) suggest an attractive model for the recruitment of Brca1 A complex to DSBs. In the presence of DSBs, H2AX is phosphorylated by ATM in the vicinity of the breaks, allowing Mdc1 to be recruited through a phosphorylation-directed in-

Author contributions: X.H.Y. and L.Z. wrote the paper.

The authors declare no conflict of interest.

See companion article on page 20759.

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teraction. The localization of Mdc1 to DSBs enables ATM to phosphorylate Mdc1, thus presenting binding sites for the FHA domain of Rnf8. The recruited Rnf8–Ubc13 then generates polyubiquitin chains at DSBs, which are subsequently recognized by Rap80 in the Brca1 A complex. This model not only provides a mechanism by which Brca1 is recruited to DSBs but also connects the initial ATM-mediated phosphorylation events to the subsequent ubiquitination cascade catalyzed by Rnf8 and Brca1.

It is important to note that Rnf8 is required for the foci formation of both Brca1 and 53BP1 (7, 9). Unlike the Brca1 A complex, 53BP1 is not known to directly recognize polyubiquitin chains. Instead, 53BP1 uses its tandem Tudor domains to bind to histone H4 dimethylated at K20 (11). Because the methylation of H4 K20 is not induced by DNA damage, it was proposed that the recruitment of 53BP1 to methylated H4 reflects a change of chromatin structure in the vicinity of DSBs. The requirement of Rnf8 for 53BP1 recruitment led Mailand *et al.* (9) to propose a second attractive model. In this model, the ubiquitination mediated by Rnf8 promotes restructuring of chromatin at DSBs and indirectly facilitates the recruitment of DNA repair and DNA damage signaling proteins such as 53BP1.

The models emerging from the studies described above also raise several new questions. The foremost question is why and how Rnf8 and Brca1, two distinct ubiquitin ligases, function together at DSBs. The Brca1-containing E3 ligase can interact with different E2s, such as Ubc13 and UbcH5, to assemble either K63- or K6-linked polyubiquitin chains (12–14). The Brca1–UbcH5 interaction and the *in vitro* E3 ligase activity of Brca1 are stimulated by DNA damage in a Ubc13-dependent manner (5), suggesting that both the activity and specificity of Brca1 are regulated by DSBs. Interestingly, the UIM domains of Rap80 recognize not only K63-linked but also K6-linked polyubiquitin chains (2), suggesting a possible role for Brca1 in propagating itself at DSBs. Yet another layer of regulation may come from the deubiquitinase Brcc36, a component of the Brca1 A complex. Biochemical reconstitution of the Rnf8–Ubc13 and Brca1 A complexes would help to address how Rnf8, Brca1, and Brcc36 function in concert.

Finding the substrates of Rnf8 and Brca1 critical for DNA repair and damage signaling is another main challenge. Although Rnf8 can promote the formation of polyubiquitin chains on H2A and H2AX, it is still unclear whether these histones are the key substrates of Rnf8 that recruit Brca1 and other proteins. A recent study showed that the polyubiquitination of H2AX promotes the release of H2AX from damaged chromatin (15), implying that ubiquitination down-regulates the function of H2AX at DSBs. Furthermore, conflicting results were reported on whether the phosphorylation of H2AX is a prerequisite for its polyubiquitination and whether Brca1 is important for the ubiquitination of H2AX (5, 8, 15). How the Brca1 A complex functions at DSBs also is unclear. Despite the clear requirement of Rnf8 for the recruitment of the Brca1 A complex, depletion of Rnf8 did not affect the phosphorylation of a number of ATM/ATR substrates (9). Furthermore, although CtIP is ubiquitinated by Brca1 in response to DSBs (16), it is unlikely a substrate of the Brca1 A complex because of the mutually exclusive binding of CtIP and Abra1 to Brca1.

It is important to note that Brca1 exists in multiple complexes in cells (1). Bach1, a unique component of the B complex, is implicated in HR (17). CtIP, a unique component of the C complex, is required for the G_2/M checkpoint in response to DSBs (16). It is conceivable that the Brca1 B and C complexes also are localized to sites of DNA damage, but how they are recruited and how they coordinate with the A complex remain to be determined.

In summary, the study by Wang and Elledge (4), together with the other three studies (7–9), has revealed an exciting link between the phosphorylation and the ubuiqutination events at DSBs. These studies will undoubtedly impact future investigations on Brca1 regulation and the roles of ubiquitination in safeguarding the genome.

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