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An Unconventional Role of BMP–Smad1 Signaling in DNA Damage Response: A Mechanism for Tumor Suppression

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ABSTRACT

The genome is under constant attack by self-produced reactive oxygen species and genotoxic reagents in the environment. Cells have evolved a DNA damage response (DDR) system to sense DNA damage, to halt cell cycle progression and repair the lesions, or to induce apoptosis if encountering irreparable damage. The best studied DDR pathways are the PIKK-p53 and PIKK-Chk1/2. Mutations in these genes encoding DDR molecules usually lead to genome instability and tumorigenesis. It is worth noting that there exist unconventional pathways that facilitate the canonical pathways or take over in the absence of the canonical pathways in DDR. This review will summarize on several unconventional pathways that participate in DDR with an emphasis on the BMP-Smad1 pathway, a known regulator of mouse development and bone remodeling. J. Cell. Biochem. 115: 450–456, 2014.

THE CLASSICAL DNA DAMAGE RESPONSE

The size of the human genome is 3.3×10^9 base pairs and it encodes about 30,000 proteins. Mouse, the most commonly used animal model, has a genome of 2.8×10^9 base pairs [Pennacchio et al., 2003]. In non-dividing cells, genomic DNA is in general compacted into chromatin by histone proteins except the regions of active transcription, where the chromatin structure is loosened and DNA is exposed. In the S phase of dividing cells, chromatin is relaxed during DNA duplication and the newly synthesized DNA is quickly assembled into chromatin. In G2/M phase, the chromatin is further condensed and forms chromosomes, which are equally separated into two daughter cells at the end of cell division [Maldonado and Kapoor, 2011]. A human cell has 23 pairs of chromosomes with a length ranging from 0.2 to 20 μm [Redon et al., 2002]. The DNA will then revert to the chromatin structure in G1 phase after cell division.

Thus, the huge amount of DNA molecules in a cell (1.8-m long if not compacted) undergo dynamic change, including duplication, packing and unpacking, separation, and transcription. Errors can occur during these processes and DNA lesions are produced. There is increasing evidence that error prone replication is one of the major causes of spontaneously arising mutations in human, mouse, and yeast [Kunz et al., 1998; Stuart et al., 2000]. One study reported that DNA mutation rate in high eukaryotes is 0.1–100 per genome per division [Drake et al., 1998]. Another study estimated that the average human genome mutation rate is 7.26 per year [Kumar and Subramanian, 2002]. In addition, the genome is under constant attack by reactive oxygen species (ROS), which are generated in the mitochondria during energy production, and by various mutagens that exist in the environment, for example, ionizing radiation and chemical reagents [Lieber, 2010]. Due to the variety of the genotoxic insults, many types of DNA lesions are generated including double stranded DNA breaks (DSBs), single stranded DNA breaks, DNA crosslinks and intercalating, nucleotide and base modifications, insertions, deletions, and chromosome translocations. The most hazardous is double-stranded DNA breaks [Khanna and Jackson, 2001].

DNA damage can have detrimental effects on cells, tissue/organs, and the organisms. As the genetic material, genomic DNA encodes functional molecules, mainly proteins and a number of RNA species. DNA mutations may alter the expression and/or the sequence of encoded proteins. DNA duplication or deletions may affect the expression levels of affected proteins. Chromosomal translocation may generate new proteins, for example BCR-ABL that underlies the

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development of chronic myelogenous leukemia [Wong and Witte, 2004]. These may alter the functions of the affected proteins and eventually lead to problems in proliferation, differentiation, and/or survival of the cell. In the end, the mutations may cause a problem to the tissue and organ where the affected cells reside. It is well established that the accumulation of DNA damage underlies the etiology of tumor [Bartkova et al., 2005; Bartek et al., 2007]. In addition, there is increasing evidence to support the theory that aging is caused by accumulation of DNA damage and the activation of the DNA damage response (DDR) [Schmitt, 2003; Beausejour and Campisi, 2006; Ivanov and Adams, 2011; Jones and Rando, 2011].

Cells have evolved a DDR pathway to sense DNA lesions and repair them, and if the lesions are too extensive, the cell will commit suicide [McGowan and Russell, 2004; Lavin et al., 2005] (Fig. 1). The function of the highly conserved canonical DDR is to maintain genome integrity. At the core of the DDR are the phosphatidylinositol 3-kinase-related kinases (PIKKs), especially Atm, Atr, and DNA-PKcs [Lempiainen and Halazonetis, 2009]. They are a family of protein kinases that can phosphorylate many protein substrates on Ser/Thr residues [Kastan and Lim, 2000; Khanna and Jackson, 2001; Sun et al., 2012; Zannini et al., 2012]. DNA-PKcs is activated by DSBs and is involved in non-homologous end joining (NHEJ) repair. Atm is also activated by DSBs whereas Atr is activated by ssDNA as well as other DNA lesions [Wang et al., 2011]. Once activated, Atm, Atr, and DNA-PKcs are relocalized to the DNA damage sites, which are usually described as DNA damage-induced foci. It is estimated that more than a hundred proteins are assembled at the foci, which act as a center for DNA repair and signal transduction [Lukas et al., 2011; Thorpe and Rothstein, 2012]. The effector proteins such as Chk1, Chk2, and p53 goes on and off these foci and are phosphorylated by PIKKs in response to DNA damage [Wu, 2012].

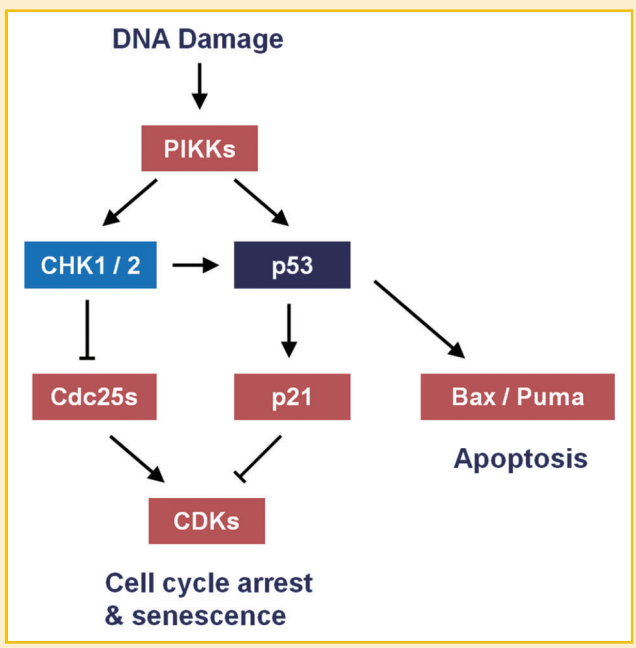

Fig. 1. The conventional DNA damage response pathways.

Phosphorylation by PIKKs can activate Chk1, Chk2, and p53. Chk1 and Chk2 can further phosphorylate and activate p53 [Lempiainen and Halazonetis, 2009; Xiao et al., 2006]. Activated Chk1 and Chk2 also phosphorylate Cdc25 proteins, a family of dual specificity protein phosphatase [Boutros et al., 2007; Rudolph, 2007] (Fig. 1). The phosphorylation inactivates Cdc25 to halt cell cycle progression under genotoxic stress. Cdc25A, Cdc25B, and Cdc25C are required for the activation of cyclin-dependent kinases (CDKs) by removing two phosphate groups, for G1 to the S phase progression and for M phase entry [Rudolph, 2007]. Therefore, in response to DNA damage, Chk1 and Chk2 activate the cell checkpoints via Cdc25-Cdc2 and cause cell cycle arrest at G1 or G2 phase.

p53 is a transcription regulator that can activate or repress gene transcription [Riley et al., 2008]. In response to DNA damage, p53 is phosphorylated at multiple sites and its protein level is highly elevated, mainly via protein stabilization. p53 can turn on genes such as p21, Bax, Puma, and Mdm2 [Vousden and Lane, 2007]. p21 is a CDK inhibitor and can induce cell cycle arrest at G1 phase (Fig. 1). p53-p21 also induces cell senescence, an aging process of the cell. Bax and Puma can induce programmed cell death. There is also evidence that p53 itself might be translocated into mitochondria and induce cell apoptosis [Mihara et al., 2003]. The consequences of p53 activation in response to genotoxic stress are cell cycle arrest, apoptosis, and/or senescence. Therefore, cells with damaged genome are prohibited to give rise to offspring as long as the DNA lesions are not fixed. Based on this, p53 is designated as "the guardian of the genome."

DNA mutations are one major driving force for cancer development especially if they occur in tissue stem cells and progenitor cells. Mutations may activate an oncogene and/or inactivate a tumor suppressor gene, thus rendering the cell growth advantage [Hanahan and Weinberg, 2011; Wu, 2013]. Therefore, DDR, by eliminating cells with DNA damage, is believed to constitute a barrier for tumorigenesis. Disruption of the DDR is required for tumorigenesis [Bartkova et al., 2005]. That is why DDR molecules are frequently mutated in human cancer samples. In particular, p53 is mutated in more than 50% of the primary human tumors [Vogelstein et al., 2000]. In addition to acting as a tumor suppression mechanism, DDR is also activated in tumor chemotherapy as well as radiotherapy. Reactivation of p53 has been clearly shown to be effective in cancer therapy [Lane et al., 2011].

THE ALTERNATIVE DDR PATHWAY–THE P38MAPK PATHWAY AND THE NF-κB PATHWAY

While the PIKK-p53 and the PIKK-Chk1/2 pathways are deemed the predominant DDR mechanisms, a number of studies have shown that the p38-MK2 pathway also plays an important role in response to DNA damage, especially in the absence of p53 [Reinhardt and Yaffe, 2009]. Activation of p38 is downstream of Atm/Atr. Activated p38MAPK has been shown to phosphorylate p53 under various stress conditions, which helps the optimal activation and induction of p53 [Harris and Levine, 2005]. In p53 deficient cells but not p53 proficient cells, p38-MK2 is required for Cdc25a-mediated S phase checkpoint and Cdc25b-mediated G2/M checkpoint [Xiao et al., 2006; Reinhardt et al., 2007]. In response to DNA damage, p38 and MK2 are

translocated into the cytoplasm, where it phosphorylates hnRNPA to stabilize Gadd45a, and phosphorylates PARN to block Gadd4a degradation. Gadd45a helps MK2 to sequester Cdc25b/c and block cell entry into the G2/M phases [Reinhardt et al., 2010]. p38/MK2 also upregulates miR-34c, which targets c-Myc expression and prevents DNA replication [Cannell et al., 2010]. On the other hand, a recent study shows that p38/MK2/AATF pathway can repress p53-driven apoptosis in DDR [Hopker et al., 2012].

The NF-κB pathway is activated by various stimuli, including mitogen, cytokines, and ROS (inflammatory and immune signals) [McCool and Miyamoto, 2012]. NF-κB activation plays a role in the lymphocyte development and cancer progression [Hadian and Krappmann, 2011; Yang et al., 2011]. Early studies have shown that Atm could phosphorylate IκBα and this mediates TNFα-induced degradation of IκBα [Jung et al., 1997]. In addition, Atm has been shown to phosphorylate NEMO on Ser85, which promotes NEMO nuclear export. Along with NEMO, Atm is also translocated into the cytoplasm, where it leads to the activation of IKK and thereafter NF-κB [Huang et al., 2003]. Genotoxic stress-induced NF-κB activation requires PARP-1 and Atm, and the Atm/Traf6/CIAP pathway, which goes through Tab1/2-Tak1 [Hinz et al., 2010]. One function of NF-κB activation under genotoxic stress is to promote cell survival. In acute myeloid leukemia and myelodysplastic syndrome patients, activated Atm accounts for the activation of NF-κB, which causes radio-resistance [Grosjean-Raillard et al., 2009]. On the other hand, cells derived from patients who carry ATM mutations showed high radiosensitivity due to the deficiency of NF-κB activation [Ahmed and Li, 2007]. Activation of NF-κB is also involved in the accelerated aging via production of proinflammatory cytokines [Osorio et al., 2012], as well as in DNA double strand break repair [Sakamoto et al., 2012; Volcic et al., 2012]. Thus, NF-κB pathway seems to have cell context dependent functions in DDR.

THE ALTERNATIVE DNA DAMAGE RESPONSE PATHWAY–THE BMP–SMAD1 PATHWAY

THE BMP–SMAD1 PATHWAY

More than a dozen proteins constitute the BMP family, which belongs to the TGFβ superfamily [Canalis et al., 2003]. The BMPs were initially purified from the bone powder and they showed powerful osteogenic ability. Injection of BMPs into the muscle could induce ectopic bone formation. Now BMP2 and BMP7 are approved by FDA of the US to treat bone fractures and non-unions [Li, 2008]. In osteoblast lineage, BMPs stimulate osteogenic differentiation of the mesenchymal stem cells and are also involved in the aging process of MSC and osteoblasts [Kua et al., 2012; Ma et al., 2012]. In addition, BMPs have been found in the serum and is a niche molecule for intestinal, follicle, neuronal, and other tissue stem cells, where they are involved in the maintenance of tissue stem cells [Zhang and Li, 2005]. Moreover, BMPs are essential for early mouse development [Varga and Wrana, 2005; Li, 2008]. Deletion of some BMPs, BMP receptors, and downstream signaling molecules such as Smad1 and Smad5, has been reported to lead to early embryonic lethality.

Like TGFβ, BMPs bind to cell surface-localized BMP receptor (BMPR) I and II, leads to BMPRI phosphorylation and activation by the constitutively active BMPRII. BMPRI then phosphorylate Smad1, 5, 8 (B-Smads) at the C′ terminal SXS motif. The SXS phosphorylated B-Smad1 form a dimmer with co-Smad, Smad4 and are translocated into the nucleus, where they bind to the Smad-binding element (SBE) to turn on or off targeted genes. Once fulfilled its functions, Smad1 is dephosphorylated by protein phosphatases such as PPM1A and is transported back into the cytoplasm [Lin et al., 2006; Kokabu et al., 2010]. There is increasing evidence that B-Smads continuously shuttle between the nucleus and cytoplasm.

Studies have suggested that BMP-Smad signaling has a tumor suppression function [Li, 2008]. BMPRIA mutations have been reported to be associated with the development of juvenile polyposis [Howe et al., 2001]. Deletion of Smad1 and Smad5 in somatic cells of ovaries and testes leads to development of metastatic granulosa cell tumors and metastatic testicular tumors respectively [Pangas et al., 2008]. Study on how BMP-Smad1 signaling prevents tumor formation is just getting underway.

A LINK BETWEEN THE ATM–P53 PATHWAY AND THE BMP–SMAD1 PATHWAY

Studies of mouse lines deficient for Atm, p53, Mdm2, or c-Abl, a Tyr kinase involved in DDR [Li, 2005; Pendergast, 2005; Wang et al., 2006; Zhang et al., 2013], demonstrated that these proteins have a regulatory role in bone homeostasis, especially osteoblast differentiation and bone formation [Li et al., 2000; Hishiya et al., 2005; Lengner et al., 2006; Rasheed et al., 2006; Wang et al., 2006; Zambetti et al., 2006; Kua et al., 2012]. Further studies revealed that these proteins affect osteogenic differentiation through the BMP-Smad-Osx/Runx2 pathway [Lengner et al., 2006; Ma et al., 2012]. These genetic evidence established a link between the Am-p53 DDR pathway and the developmentally essential BMP-Smad1 pathway. The implication from these findings can be two fold: the Atm-p53 pathway might make use of the BMP-Smad1 pathway to modulate development and tissue homeostasis; and that BMP-Smad1 pathway is involved in DDR. The latter could provide a molecular basis whereby the BMP-Smad1 pathway executes its tumor suppression activity [Li, 2008].

DNA DAMAGE ACTIVATES THE BMP–SMAD1 PATHWAY

In various cell types including HeLa, mouse embryonic fibroblast, mouse osteoblasts, mouse embryonic stem cells, DNA damage generated by ionizing radiation, doxorubicin, or hydroxyurea, can activate BMP-Smad1 signaling, indicating that this is a rather common cellular event. Activation is manifested by an increase in the SXS-phosphorylation of Smad1, nuclear entry of Smad1, an increase of Smad1 protein, and turn-on of BMP target genes [Chau et al., 2012]. In contrast, TGFβ responsive Smad2 and Smad3 are not activated, suggesting a specific function of BMP-Smad1 signaling in DDR. Compared to BMP2-induced activation, DNA damage-induced Smad activation has a few unique features. First, DNA damage induces upregulation of Smad1 at the protein level, but not Smad5 or Smad8, whereas BMP2 stimulation does not markedly affect the protein levels of Smad1, Smad5, or Smad8. Second, DNA damage-induced Smad1 activation is long-lasting compared to BMP2-indced Smad1 activation. In MEFs, BMP2-induced Smad1/5/8 SXS phosphorylation peaks within one hour and then declines quickly. However, Smad1 activation under DDR is maintained at high levels up to 12 h. Third, under DNA damage, Smad1 appears to be localized

at some speckles that do not overlap with the DNA damage-induced H2AX positive foci [Chau et al., 2012].

SMAD1 IS A SUBSTRATE OF ATM

Since Atm and other PIKKs are sensors of DNA damage and critical signaling molecules, Smad1 activation, upregulation and nuclear entry in response to DNA damage are tested in Atm−/− cells or in the presence of PIKK inhibitor caffeine. It was found that Atm activation is required for DNA damage-induced Smad1 activation. Further studies show that Atm is necessary and sufficient to phosphorylate Smad1 on Ser239, a residue located at the linker region, in response to DNA damage (Fig. 2). Cell-based studies and in vitro kinase assay demonstrated that Smad1 is a bone fide substrate of Atm kinase. Mutation of Ser239 to Ala diminished DNA damage-induced Smad1 activation and up-regulation as well as nuclear entry. These data suggest that DNA damage-induced Smad1 activation is downstream of Atm. S239 is only present in Smad1 and Smad5, but not in Smad8, Smad2, Smad3, or Smad4 (Fig. 2). However, Smad5 is not a good substrate for Atm, at least compared to Smad1 [Chau et al., 2012]. This may explain why only Smad1 is activated and up-regulated in response to DNA damage.

A SEQUENTIAL SMAD1 PHOSPHORYLATION

Atm is mainly localized in the nucleus and once activated by DNA damage, it is relocalized to the chromatin, including the DNA damage-induced foci. However, only activated (SXS phosphorylated) Smad1 is localized in the nucleus. In theory, only SXS phosphorylated Smad1 molecules are accessible to active Atm. Indeed, it was found that SXS-deleted Smad1 cannot be phosphorylated by Atm anymore in vivo. In the presence of BMPR inhibitor or deficiency, DNA damage could not phosphorylate Smad1 on S239, nor could it activate or up-regulate Smad1 [Chau et al., 2012]. Thus, DNA damage-induced Smad1 activation/upregulation requires cooperation of the BMP-BMPR and Atm (Fig. 3). In another word, DNA damage could only extend or enhance the pre-existing BMP-Smad1 signaling. If Smad1 is not activated in cell, DNA damage-induced p53 activation is compromised, which will promote tumorigenesis. In sum, DNA damage-induced Smad1 activation/upregulation requires sequential phosphorylation events: BMPRI-mediated SXS phosphorylation in the cytoplasm and Atm-mediated S239 phosphorylation in the nucleus.

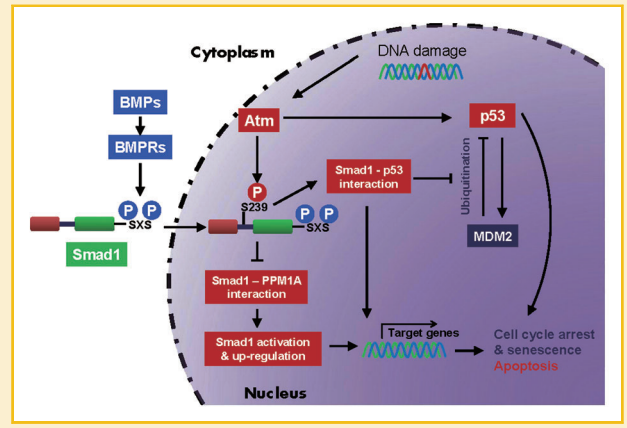

Fig. 3. A working model showing activation of Smad1 under genotoxic stress and possible mechanisms by which Smad1 executes its function in DDR.

PPM1A IN SMAD1 ACTIVATION/UPREGULATION IN DDR

The next question is how Ser239 phosphorylation facilitates Smad1 activation and up-regulation? DNA damage-induced Smad1 activation and upregulation mainly occur in the nucleus. One mechanism by which S239 phosphorylation promotes Smad1 activation is by interfering with the interaction between Smad1 and PPM1A (Fig. 3). PPM1A is a Smads phosphatase that antagonizes the function of Smads. Although PPM1A is present in both the cytoplasm and nucleus, only Smad1-PPM1A complex in the nucleus is disrupted in response to DNA damage. These results support the theory that Atm-mediated Smad1 Ser239 phosphorylation disrupts Smad1-PPM1A interaction in the nucleus and therefore extend Smad1 SXS phosphorylation and Smad1 nuclear localization.

THE P53-SMAD COMPLEX

Previous studies have established a physical and functional interaction between p53 and the TGFβ responsive Smads, Smad2/

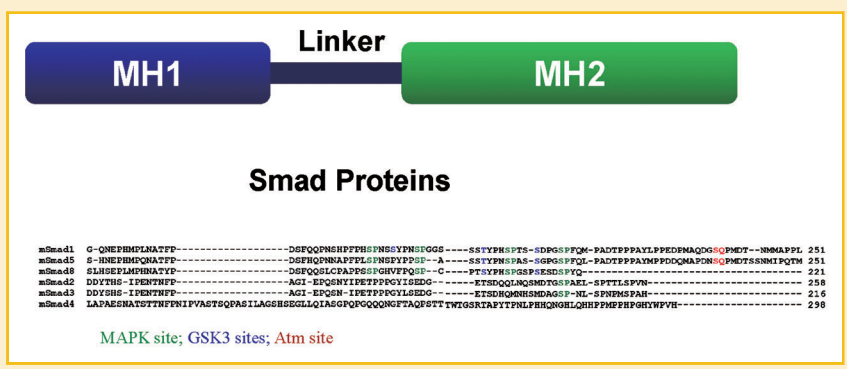

Fig. 2. The domain structure of Smad1 (uper) and alignment of the linker regions of Smad1, 5, 8, 2, 3, 4 (bottom). The known phosphorylation sites in the linker region are shown in different colors.

3. Wild-type p53 forms a complex with Smad2/3 and coordinately induces the transcription of a number of tumor suppressor genes including p21 [Cordenonsi et al., 2003]. In addition, p53-Smad2/3 has been reported to repress alpha-fetoprotein transcription [Wilkinson et al., 2005]. p53 has also been reported to represses TGFβ-induced collage I expression [Ghosh et al., 2004]. On the other hand, mutant p53 interferes with the transcriptional activation of TGFβ target genes [Kalo et al., 2007]. In addition, Mutant p53 can form a complex with Smads and the p53 family member p63, which inhibits p63-mediated activation of genes to promote metastasis [Melino, 2011].

Our studies showed that in DDR, the increased amounts of Smad1, which is also phosphorylated on both C-terminal SXS and Ser239, form a complex with p53 and inhibits Mdm2-mediated p53 ubiquitination and degradation. As such, Smad1 deactivation leads to a compromised induction of p53 in response to DDR. Thus, one of the functions of Smad1 activation in DDR is to facilitate p53 induction, indicating a transcription-independent function of Smad1. At the transcriptional level, activated Smad1 might execute its function in the following ways. First, activated and up-regulated Smad1 might turn on or off BMP target genes. Second, p53-Smad1 complex, just like p53-Smad2/3 complex, might positively or negatively regulate p53 target genes. Third, p53-Smad1 complex might positively or negatively regulate Smad1 target genes (Fig. 3). Some of the affected genes might be involved in modulating DDR.

OTHER SMADS AND DNA DAMAGE

Although TGF-Smad2/3 are not activated by genotoxic stress, recent studies show that TGFβ1 inhibitor could reduce DNA damage-induced foci formation (H2AX) and cell death [Bouquet et al., 2011], and that TGFβ signaling also represses the transcription of p53 via E2F4-Smad2/3 interaction, which enhances drug resistance in precancerous cells [Lopez-Diaz et al., 2013]. In addition, recent studies also imply that other members of the Smad family are involved in DDR. For example, both Smad7 and phospho-Smad2 were found to be colocalized on the nuclear foci generated by ionizing radiation. Foci assembly of p-Smad2 but not Smad7 requires Atm activation. On the other hand, foci assembly of Smad7, but not p-Smad2 requires TGFβ-TGFBR1 signaling. The function of foci assembly of Smad7 and p-Smad2 remains unclear [Wang et al., 2013]. Furthermore, Smurf2, a E3 lipase for Smad proteins, has also been found to localized to the foci and regulates DDR and genome stability [Blank et al., 2012]. In addition, Smurf1/2 promotes the dimmer formation between Mdm2 and MdmX, thus promotes p53 degradation [Nie et al., 2010].

THE PERSPECTIVES

While the PIKK-Chk1/2 and PIKK-p53 pathways in DDR have been well studied, the unconventional pathways activated by DNA damage are emerging as important players in DDR as well as in tumorigenesis. Here we summarized the new development on such pathways including NF-κB, p38MAPK-MK2, and Smad1. Under genotoxic stress, activation of these pathways seems to be downstream of the PIKKs. So they are in theory parallel to the PIKK-Chk1/2 and the PIKK-p53 pathways. In addition, these pathways may interact with and crosstalk with the conventional PIKK-Chk1/2/p53 pathways. They cooperate with the PIKK-Chk1/2/p53 pathways to decide the cell fate in response to genotoxic stress. Moreover, these pathways can also be activated by environmental cues in PIKKs-independent manners. Therefore, these unconventional pathways may integrate signals from the microenvironment to modulate cell fate under genotoxic stress. Future work might identify more unconventional DNA damage responsive pathways that play important roles in DDR and tumorigenesis.

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