

Chk2 Molecular Interaction Map and Rationale for Chk2 Inhibitors

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Abstract To organize the rapidly accumulating information on bioregulatory networks related to the histone γ -H2AX-ATM-Chk2-p53-Mdm2 pathways in concise and unambiguous diagrams, we used the molecular interaction map notation (<http://discover.nci.nih.gov/min>). Molecular interaction maps are particularly useful for networks that include protein-protein binding and posttranslational modifications (e.g., phosphorylation). Both are important for nearly all of the proteins involved in DNA double-strand break signaling. Visualizing the regulatory circuits underlying cellular signaling may help identify key regulatory reactions and defects that can serve as targets for anticancer drugs.

DNA double-strand breaks (DSB) are among the most severe genomic lesions. Their repair requires cells to arrest cell cycle progression to avoid further damage during replication or transcription (1–5). Cell cycle arrest may also allow the chromatin to switch from a metabolic (replicative) state to a repair state. DSB can be produced directly by ionizing radiation and certain anticancer drugs that bind to DNA (e.g., bleomycin, neocarzinostatin, topoisomerase II inhibitors, such as etoposide or doxorubicin). DSB can also result from conversion of single-strand breaks by DNA polymerase collisions or “replication fork collapse” at sites of damaged DNA. Replication DSB occur normally in cancer cells, but their frequency is markedly enhanced by topoisomerase I inhibitors such as the camptothecin derivatives (e.g., topotecan and irinotecan; ref. 6) and DNA alkylating agents.

The importance of Chk2, a protein involved in cell cycle arrest due to DSB, is indicated by its conservation in eukaryotes (7–10). However, yeast and vertebrate cells can survive without Chk2, although they are defective in cell cycle checkpoints and apoptosis after irradiation. Chk2 activation by camptothecin treatment has been reported to be defective in several colon carcinoma cell lines from the NCI 60 cell line screen (11). Chk2 inactivation in humans with normal p53 leads to Li-Fraumeni syndrome (12), supporting a genetic connection between Chk2 and p53 and the existence of a Chk2-p53 axis [see the molecular interaction map (MIM) Fig. 1]. Families with Chk2 deficiencies have been identified among patients with increased risk of breast cancer (10, 13, 14).

Chk2 functions as a kinase relay for ATM (refs. 9, 15, 16; see MIM), whose gene (*ataxia telangiectasia mutant*) is mutated in ataxia telangiectasia patients (2). It is generally accepted that

DNA damage triggers two main pathways (16). DSB activate the ATM-Chk2 axis, whereas replication lesions activate the ataxia telangiectasia and rad3-related kinase (ATR)–Chk1 axis. However, there is crosstalk between the two pathways and certain lesions activate both. In such cases, the ATM-Chk2 axis is activated first and transiently, whereas the ATR-Chk1 axis is activated secondarily and in a more sustained way (17). The association of Chk1 with replication damage is consistent with the cell cycle–dependent expression of Chk1, which increases during S-phase and peaks in G₂. In contrast, Chk2 is expressed throughout the cell cycle, consistent with its broader role in response to DSB produced both during S-phase and independently of replication (18).

Although Chk2 shares no sequence homology with Chk1, crosstalk between the Chk2 and Chk1 pathways is also indicated by the observation that several Chk2 substrates (e.g., Cdc25C and p53, highlighted in blue in Fig. 1) are also Chk1 substrates and are phosphorylated by both kinases on the same amino acid residues (9).

Chk2 MIM

Foreword. To organize the rapidly accumulating information on bioregulatory networks in concise and unambiguous diagrams, we crafted the MIM notation (ref. 19).¹ MIMs are particularly useful for networks that include protein-protein binding and posttranslational modifications (e.g., phosphorylation). Both are important for nearly all of the proteins involved in DNA damage signaling by the ATM-Chk2 axis. The MIM allows one to trace pathways and loops in the networks, thereby helping to interpret data and formulate testable hypotheses (Fig. 1).

Molecular species and events in the MIM fall into three categories indicated to the right of the MIM: (a) chromatin, (b) sensors and kinases, and (c) effectors. Symbol definitions are shown below the MIM. Arabic numbers in the map refer to text and literature references listed in the Supplementary Data and

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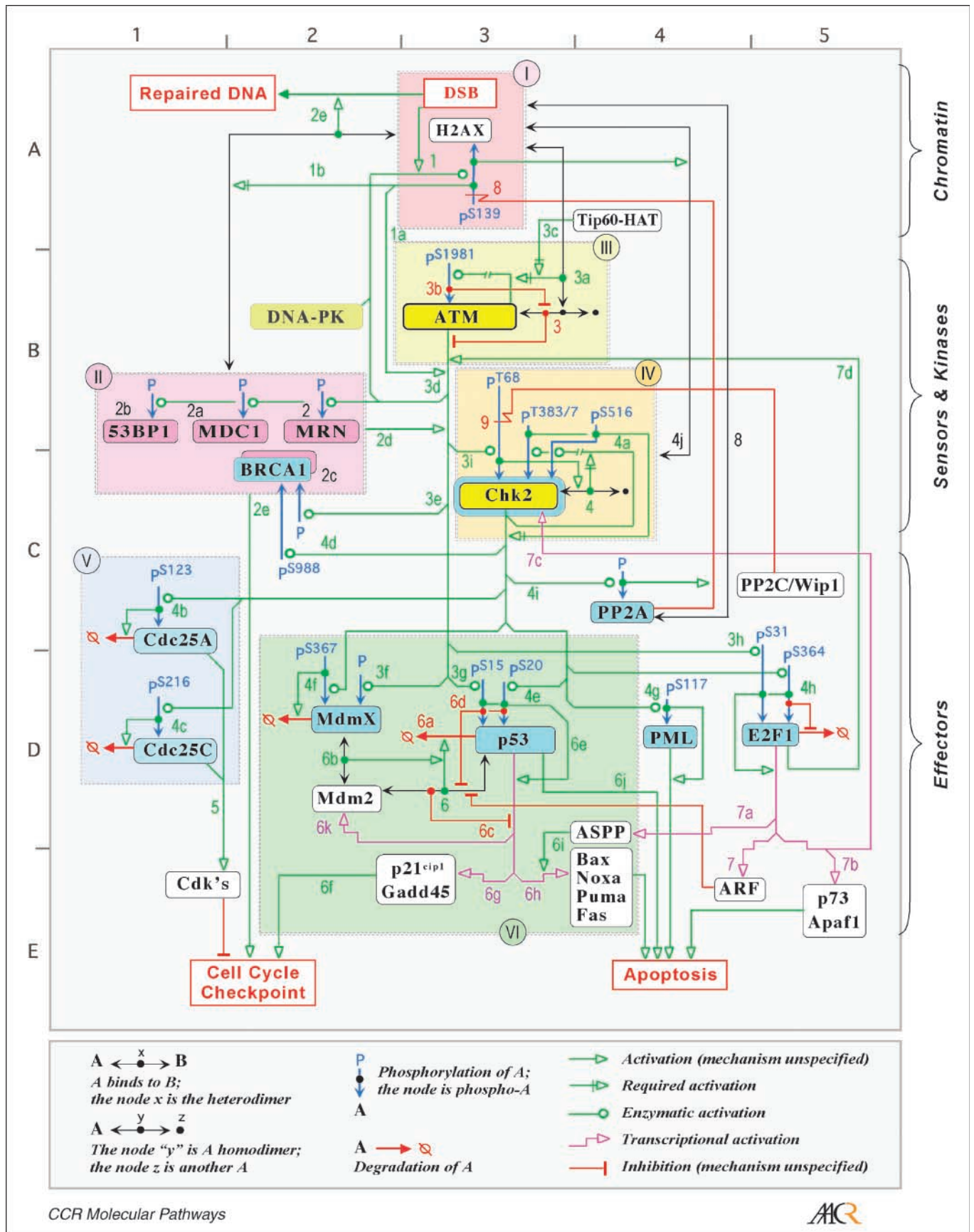
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Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>) and at the Discover Website.¹

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¹ <http://discover.nci.nih.gov/mim>.



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at the Discover Web site.¹ Maps at the Discover Web site are interactive (electronic). The user can navigate MIM and link to electronic molecular databases. Clicking on a molecular species activates links to Genbank, Entrez Nucleotide, Entrez Genome, PubMed, GeneCards, MatchMiner, CGAP, MedMiner, and Glossary. Clicking on the annotation numbers opens a text window describing the molecular interaction and listing references, with links to PubMed. The symbology and complete reference list can be found in Supplementary Data and at the Discover Web site¹ (Chk2 Map). Furthermore, we recently published a detailed MIM for the p53-Mdm2 module (20).

Brief description of the Chk2-ATM-p53 MIM. The input signal "DSB" is shown in a box outlined in red at the top of the map. The outputs, "Cell Cycle Checkpoints" and "Apoptosis" are also shown in red boxes at the bottom. "Repaired DNA," another output, is shown to the left of the DSB. The signaling pathways are labeled with number-letter combinations followed by the map coordinates.

The pink box (Ⓐ, A3) indicates a group of chromatin events at the DSB site. DSB stimulate phosphorylation of histone H2AX on serine 139 by DNA-PK and ATM [1,A3]. Phosphorylated H2AX (known as γ -H2AX) then further stimulates (or amplifies) the DSB-induced stimulation of ATM [1a,A2]. γ -H2AX is also required for binding of sensor proteins to altered chromatin [1b,A2] and for keeping the ends the DSB in close proximity (21). Protein complexes containing BRCT domains are grouped in the purple box at left (Ⓐ). They include 53BP1, MDC1, Nbs1 (which is part of the trimeric MRN complex [2,B2] with Mre11 and Rad50), and BRCA1 [C2]. Those proteins contribute to the full activation of ATM, at least in part by providing scaffolds that enable ATM to recruit and process its various substrates [2d,B2] (22, 23). Mre11-Rad50 can directly act as a repair nuclease [2e,A2].

Box Ⓑ shows events in ATM activation. ATM normally exists as an inactive dimer [3,B3]. Binding of the ATM dimer to altered chromatin changes its configuration, allowing autophosphorylation (in *trans*) at serine 1981. The histone acetyl transferase TIP60 is also required for ATM activation [3c,A3]. Phosphorylation dissociates the ATM dimer, fully activating ATM. ATM phosphorylates the BRCT domain sensor proteins [3d-e,B1-C3], perhaps providing positive feedback between ATM and the BRCT-containing proteins. ATM directly phosphorylates many other molecules (only some of which are represented on the map): MdmX [3f,D2], p53 [3g,D3], E2F1 [3h,D5], and Chk2 [3i,C3]. It is striking that four of the nine ATM substrates shown are also Chk2 substrates (discussed below).

The Chk2 activation loop is shown in box Ⓒ. Phosphorylation of Chk2 at threonine 68 by ATM [3i,C3] induces Chk2 dimerization, which is required for Chk2 activity [4,C4]. Once dimerized, Chk2 is further activated by autophosphorylation in *trans* at residues 383, 387, and 516 [4a,B4]. Thus, Chk2 is active as a dimer, whereas ATM is active as a monomer. Chk2

phosphorylates a range of proteins involved in cell cycle control and apoptosis. The Chk2 substrates besides itself are shown in blue: cdc25A [C1], cdc25C [D1], MdmX [D2], p53 [D3], PML [D4], E2F1 [D5], and phosphatase 2A (PP2A [C4]).

Checkpoint activation causes cell cycle arrest. Under normal conditions, Cdc25 phosphatases (box Ⓓ) stimulate the activation of cyclin-dependent kinases [5,D1]. Two Cdc25s are Chk2 substrates: Cdc25A and Cdc25C, which are required to promote cell cycle progression through G₁-S and G₂, respectively. Cdc25A and C are both inactivated following phosphorylation by Chk2 [5a and 5b,D1]. The BRCT domain proteins (box Ⓔ) also contribute to cell cycle checkpoint control [2e,C2].

The p53 module (Ⓜ; ref. 20) is a major target of Chk2. Under normal conditions, p53 [D3] levels are kept low because p53 is degraded by Mdm2 [D2]. Binding of p53 to Mdm2 [6,D3] results in p53 polyubiquitination, which then induces p53 degradation [6a,D3]. Binding of MdmX to Mdm2 may further activate Mdm2 [6b,D2]. Binding of Mdm2 to p53 inhibits p53 by two additional mechanisms: transcriptional inhibition of p53 target genes [6c,D3] and export of p53 from the nucleus (not shown). Chk2 activates p53 by at least three mechanisms: two resulting from direct phosphorylation of p53 [4e,D3] and one indirectly by phosphorylation of MdmX [4f,D3]. Phosphorylation of p53 at serine 20 [4e,D3] inhibits its binding to Mdm2 [6d,D3] and therefore increases p53 levels. p53 phosphorylation at serine 20 also enhances p53 transcriptional activity [6e,D3]. Phosphorylation of MdmX [4f,D3] leads to MdmX degradation and thereby reduces p53 inhibition by Mdm2. This convergence of several mechanisms for p53 activation by Chk2 is remarkable. The apparent robustness of the molecular network underscores the importance of p53 activation by Chk2.

Two outcomes of p53 activation are represented in the MIM: cell cycle arrest and apoptosis. Cell cycle arrest [6f,E2] is mediated by the transcriptional activation of genes exemplified by CDKN1 (p21^{waf1/cip1}) and Gadd45 [6g,E3]. In contrast, transcriptional activation of genes such as *Bax*, *Noxa*, *Puma*, and *Fas* [6h,E4] favors apoptosis. ASPP1 and ASPP2 associate with p53 on the promoters of proapoptotic genes [6i,E4] and switch the transcriptional activity of p53 toward apoptosis. p53 can also stimulate apoptosis by actions at mitochondria (for instance, by targeting Bax and Bak) [6j,D4] (20). Finally, p53 can inactivate itself by transactivating Mdm2 gene expression (negative feedback) [6k,D2]. Thus, a coherent picture of the control of p53 emerges. First, p53 is rapidly stabilized/activated by a convergent set of phosphorylations of both p53 and MdmX by Chk2. Later, p53 activates cell cycle arrest and apoptosis by transcriptional activation. Finally, p53 can turn itself off by transactivating Mdm2.

Two other Chk2 substrates further reinforce the proapoptotic function of Chk2: PML and E2F1. Phosphorylation of PML at serine 117 [4g,D4] enhances PML-dependent apoptosis. Phosphorylation of E2F1 at serine 364 [4h,D5] stabilizes E2F1

Fig. 1. MIM centered on the ATM-Chk2-p53 axis. A fully annotated version, including references for each molecular species and interaction, can be found in the Supplementary Data, and as an electronic interactive version at the Discover Web site.¹ Bottom, symbols and convention keys. Top, MIM describing the interactions between a DSB (*input box at the top*) and three output boxes ("Repaired DNA," "Cell Cycle Checkpoint," and "Apoptosis"). The six shaded boxes (modules/nodes) numbered with roman numerals are as follows: Ⓐ, chromatin; Ⓑ, sensor proteins with BRCT domains (shaded in purple); Ⓒ, ATM activation node; Ⓓ, Chk2 activation node; Ⓔ, cell cycle checkpoint; and Ⓜ, p53 node. Blue boxes, Chk2 substrates. Arabic numbers, annotations used in the text. These annotations are fully explained individually in Supplementary Data and in the electronic MIM at the Discover Web site¹ with complete sets of references directly linked to PubMed.

and induces apoptosis in both p53-dependent and p53-independent ways. As a transcription factor, E2F1 transactivates p14 (ARF) [7,E4], which activates p53 by inducing its dissociation from Ndm2. E2F1 also transactivates ASPPs [7a,E5] and Chk2 [7c,C3] and activates ATM [7d,B5], which further activates p53 and both p53-dependent and p53-independent apoptosis (see above). Finally, E2F1 can induce apoptosis independently of p53 by transactivating p73 and Apaf1 [7b,E5].

The last Chk2 substrate in the MIM is phosphatase 2A (PP2A [C4]) whose activation by Chk2 [4i,C3] induces its recruitment to γ -H2AX foci [8,C4]. γ -H2AX dephosphorylation [8,A3] is critical for cell recovery following DSB and checkpoint activation. Failure to dephosphorylate γ -H2AX increases cell sensitivity to DNA damage. The phosphatase 2C (PP2C/Wip1) has recently been implicated in turning off Chk2 activation by ATM by dephosphorylating threonine 68 of Chk2 [9,B3]. Because *Wip1* is a p53-responsive gene, both PP2A and PP2C probably act as negative feedback loops within the Chk2 molecular network that terminates checkpoint control once repair has taken place. Overall, these linked phosphorylation-dephosphorylation couples suggest intricate control possibilities, the elucidation of which will be an interesting challenge for theory and experiments (24, 25).

Overview of the Chk2-ATM-p53 MIM. The MIM underlines some remarkable features of the ATM-Chk2-p53 axis/network with its several positive and negative feedback loops, convergence of signals to achieve key molecular outputs, and coupled ATM- and Chk2-mediated phosphorylation of p53 and other dual ATM-Chk2 substrates. The positive feedback loops include: (a) ATM activation (module III); (b) Chk2 activation (module IV); (c) the H2AX-ATM activation loop (between modules I and III) with the BRCT-containing proteins (module II) acting as amplifier; and (d) E2F1-induced activation of both ATM and Chk2. These positive feedback loops suggest a regulated (and perhaps temporally sustained and/or synergistic) activation of each step of the pathway.

Convergence of cycle arrest/checkpoint signals cell from both the Cdc25 module and the p53-p21^{cip1/waf1} network underlines their importance for DSB response. Convergence is also illustrated within the p53 module (VI), where p53 is activated both directly by Chk2 and indirectly by MdmX-Mdm2. Such coherences can contribute to robustness of the system.

The significance of the dual phosphorylation by ATM and Chk2 of most of the substrates (p53, MdmX, E2F1, and Chk2 itself) remains to be understood. Dual phosphorylations may provide fine tuning for conditional activation. Finally, negative feedback loops, such as the Mdm2-p53 interaction and the PP2A-mediated inactivation of γ -H2AX and the Wip1-mediated inactivation of Chk2, suggest that the ATM-Chk2-p53 network may be autoregulated by switching itself off to allow transient cell cycle inhibition coupled with DNA repair.

Clinical Translational Advances

Two main observations justify the development of Chk2 inhibitors. First, a significant fraction of tumors and precancerous lesions overexpress activated Chk2 (26, 27). Hence, such tumors may be “dependent upon” (“addicted to”) activated Chk2. Chk2 may be required to respond to DNA lesions resulting from the genomic instability and aberrant replication that characterize a large fraction of tumors, thereby allowing the transformed and malignant cells to survive.

Second, Chk2 is activated in tumor cells by a wide range of commonly used chemotherapeutic agents, including ionizing radiation, topoisomerase inhibitors, and DNA-targeted agents. Because of the dual function of Chk2 (induction of p53-dependent apoptosis and cell cycle checkpoint activation coupled with DNA repair), it is plausible that a Chk2 inhibitor would increase the therapeutic indices of DNA-targeted agents (9). In tumors defective for apoptosis (and p53), the Chk2 inhibitor would act primarily as a checkpoint inhibitor and prevent DNA repair, whereas in normal tissues (with functional p53) the Chk2 inhibitor would primarily block apoptosis and subsequent side effects.

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