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Eric J. Gapud
Washington University School of Medicine in St. Louis

Barry P. Sleckman
Washington University School of Medicine in St. Louis

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Unique and redundant functions of ATM and DNA-PKcs during V(D)J recombination

Eric J. Gapud and Barry P. Sleckman*

Department of Pathology and Immunology; Washington University School of Medicine; St. Louis, MO USA

Lymphocyte antigen receptor genes are assembled through the process of V(D)J recombination, during which pairwise DNA cleavage of gene segments results in the formation of four DNA ends that are resolved into a coding joint and a signal joint. The joining of these DNA ends occurs in G_1-phase lymphocytes and is mediated by the non-homologous end-joining (NHEJ) pathway of DNA double-strand break (DSB) repair. The ataxia telangiectasia mutated (ATM) and the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), two related kinases, both function in the repair of DNA breaks generated during antigen receptor gene assembly. Although these proteins have unique functions during coding joint formation, their activities in signal joint formation, if any, have been less clear. However, two recent studies demonstrated that ATM and DNA-PKcs have overlapping activities important for signal joint formation. Here, we discuss the unique and shared activities of the ATM and DNA-PKcs kinases during V(D)J recombination, a process that is essential for lymphocyte development and the diversification of antigen receptors.

Introduction

The V(D)J recombination reaction is initiated when the RAG-1 and RAG-2 proteins, which together form the RAG endonuclease, generate DNA DSBs at the border of two recombining gene segments.[1-3] This DNA cleavage event results in the formation of pairs of coding ends and signal ends that are then processed and subsequently joined by proteins of the non-homologous end joining (NHEJ) pathway, forming a coding joint and a signal joint, respectively.[4,5] Additionally, any rare unrepaired DNA ends must be prevented from being resolved aberrantly as potentially oncogenic chromosomal deletions or translocations.[6]

ATM and DNA-PKcs are members of the PI3-kinase-like serine/threonine kinase (PIKK) family that function during V(D)J recombination.[7] Each of these kinases have distinct activities during coding joint formation, with ATM promoting the stability of coding end complexes prior to joining and DNA-PKcs functioning in essential steps of coding end processing.[4,5,8-11] The function of these two kinases during signal joint formation has been less clear.[4,5,8,12-21] ATM and DNA-PKcs phosphorylate a host of downstream targets that function in DSB repair and DNA damage responses (DDRs).[22-26] Like all PIKKs, ATM and DNA-PKcs phosphorylate serines or threonines followed by a glutamine (SQ/TQ).[7] These two kinases can phosphorylate similar sets of target proteins in response to DSBs, and these two kinases can have overlapping activities in the repair of DSBs, such as those generated during immunoglobulin class switch recombination and V(D)J recombination.[20,25-27] Here, we discuss the functions of ATM and DNA-PKcs during V(D)J recombination and recent studies demonstrating their overlapping activities during signal joint formation.

ATM Function in DDR

ATM deficiency in humans leads to ataxia telangiectasia (A-T), a disease

*Correspondence to: Barry P. Sleckman; Email: Sleckman@immunology.wustl.edu

characterized by ocular telangiectasias, progressive cerebellar degeneration with ataxia, sensitivity to ionizing radiation, lymphopenia and a predisposition for lymphoid malignancies with translocations involving antigen receptor genes.[7,28,29] ATM deficiency in mice leads to many, but not all of the defects observed in ATM-deficient humans.[30] However, the lymphoid phenotypes observed in ATM-deficient mice are similar to those observed in humans with A-T.

ATM exists as an inactive homodimer that is recruited to DSBs by the Mre11-Rad50-NBS1 (MRN) complex early in the DSB response.[31-34] Association of the ATM homodimer with MRN at broken DNA ends leads to auto-phosphorylation at serine 1981 in humans (serine 1987 in mice), which converts the inactive dimer to an active monomer.[32,35-38] However, mice expressing a serine 1987 to alanine mutant of ATM do not exhibit defects in ATM-mediated DDR, suggesting that there are additional ways to promote the catalytic activity of ATM.[39] In this regard, there are several other SQ/TQ motifs in ATM that may compensate for the loss of; however, mutation of three of these motifs also had no effect on activation of DDR by ATM.[40,41]

ATM orchestrates many aspects of the DDR, including the initiation of DNA DSB repair, the enforcement of cell cycle checkpoints and, ultimately, the activation of cell death when DSBs persist unrepaired.[22,23] In addition, ATM can regulate cell type-specific processes that are not directly involved in canonical DDRs.[42,43] The regulation of these diverse processes occurs via the phosphorylation of many different proteins by ATM.[7,24] For example, ATM regulates transcription through the direct phosphorylation of transcription factors, such as p53, or indirectly by phosphorylating proteins that regulate transcription factor function, such as NEMO.[43-45] The G$_1$-S checkpoint is enforced by the Chk2 kinase after it has been phosphorylated by ATM.[46] Additionally, ATM phosphoryates each of the proteins in the MRN complex, possibly modulating their function in DNA repair and in further activation of ATM.[24,47-49] Finally, ATM can phosphorylate histone proteins such as the H2A variant H2AX to form γ-H2AX in chromatin

flanking DNA DSBs.[50,51] γ-H2AX promotes the retention of proteins at the DSB and may function in several aspects of the DDR, including enforcement of the G$_2$-M checkpoint and modulation of DNA end resection.[50,52-54]

DNA-PKcs Function in DDR

In mice, DNA-PKcs deficiency leads to severe combined immunodeficiency (Scid) due, at least in part, to a requirement for DNA-PKcs in coding joint formation (see below). DNA-PKcs mutations can also be found in humans with Scid.[55] Additionally, DNA-PKcs has broader functions in DDR, as evidenced by the sensitivity of DNA-PKcs-deficient cells to ionizing radiation.[4,5,56-58] DNA-PKcs is recruited to the site of a DNA DSBs by the Ku70/Ku80 proteins, and together these three proteins form the catalytically active DNA-PK complex.[4,5,59,60] DNA-PKcs is regulated by phosphorylation of a series of SQ/TQ motifs that are targets of ATM and/or DNA-PKcs itself.[61] Phosphorylation of DNA-PKcs may regulate several functions of this protein, including its kinase activity and its ability to promote DSB repair, possibly by driving dissociation of DNA-PKcs from broken DNA ends so that they can be joined.[61]

Like ATM, DNA-PKcs has many different substrates that function in several different aspects of DDR.[62,63] In addition to DNA-PKcs itself, these substrates include the NHEJ proteins Artemis, DNA Ligase IV, XRCC4-like factor (XLF)/ Cernunnos, XRCC4, Ku70 and Ku80, all of which have been shown to be phosphorylated by DNA-PKcs in vivo and/or in vitro.[64-68] DNA-PKcs can also phosphorylate H2AX in chromatin flanking DNA DSBs including RAG-mediated DSBs.[69-72] Moreover, in vitro DNA-PKcs can phosphorylate RAG-2, although the functional consequences of this phosphorylation, if any, are not clear.[73,74] Importantly, DNA-PKcs shares several phosphorylation targets in common with ATM (see below).[25,26]

The V(D)J Recombination Reaction

The second exon of all lymphocyte antigen receptor genes is generated by a

somatic rearrangement process known as V(D)J recombination.[75] V(D)J recombination is initiated when the RAG endonuclease introduces DNA DSBs at the border of two recombining gene segments and their flanking RAG recognition sequences (recombination signals, RSs).[1-3] RAG-mediated cleavage generates two hairpin-sealed coding ends and two signal ends, which are blunt and phosphoryalted. The two coding ends are processed and joined imprecisely as a coding joint, which remains within the chromosome and participates in completion of the second exon. The two signal ends are joined relatively precisely and on extrachromosomal excision circles when rearrangements occur by deletion of the region between the two gene segments. However, when rearrangement occurs by inversion of the region between the two gene segments, the signal joint remains chromosomal. Therefore, although not required to produce a complete antigen receptor gene, efficient signal joint formation is essential for maintaining chromosome integrity during rearrangements by inversion. Unrepaired signal ends could otherwise persist as chromosomal breaks or possibly be resolved as oncogenic chromosomal translocations or deletions.

RAG DSBs are processed and joined by the NHEJ pathway of DNA DSB repair.[4,5] Elements of this pathway that function during V(D)J recombination include DNA Ligase IV, XRCC4, Artemis, XLF, DNA-PKcs, Ku70 and Ku80.[4,5,76,77] XRCC4 associates with and stabilizes DNA ligase IV, promoting its essential activity in ligating both signal and coding ends.[4,5] The Ku70 and Ku80 members of the DNA-PK complex are also required for the repair of both signal and coding ends.[4,5] DNA-PKcs is required for coding joint formation, and this function is discussed below. The Artemis nuclease is required primarily to open hairpin-sealed coding ends for subsequent joining.[4,5] Although not required for signal joint formation, Artemis contributes to signal joint diversification.[78]

Patients have been described that have lymphopenia and IR-sensitive fibroblasts due to XLF mutations, suggesting that XLF functions in the repair of RAG-mediated DSBs.[76,77] However, XLF-deficient mice

have largely normal lymphocyte development and XLF-deficient lymphocytes undergo efficient chromosomal and extra-chromosomal V(D)J recombination.[79] In contrast, recombination of extrachromosomal substrates is significantly compromised in XLF-deficient mouse embryonic fibroblasts and stem cells.[79,80] Thus, there is a differential requirement for XLF in the repair of RAG-mediated DSBs in lymphoid and non-lymphoid cells. Interestingly, a recent study demonstrated that combining a deficiency of XLF with either ATM or H2AX deficiency leads to a severe block in coding joint formation similar in magnitude to what is observed in NHEJ-deficient cells.[81] Signal joint formation was also inhibited in cells deficient for both XLF and ATM but was not examined in cells deficient for both XLF and H2AX.[81] As lymphocytes deficient in H2AX have no measurable defects in the repair of RAG-mediated DSBs in G_1-phase cells and the repair defects in ATM-deficient lymphocytes are mild (see below), these findings demonstrate that XLF has important activities in the repair of RAG-mediated DSBs that overlap with ATM and H2AX.[8-11,30,81-84] However, the nature of these shared activities is currently unknown.

Experimental Approaches to Study V(D)J Recombination

Over 20 years ago, Gellert and colleagues developed the first experimental approach for studying the V(D)J recombination reaction in cultured cells.[85] This approach involved the introduction of plasmid substrates containing appropriate RS pairs into transformed pre-B cells that express RAG. Rearrangement of substrates recovered from cells could then be assayed by differential antibiotic selection of bacteria transformed with rescued plasmid substrates.[85] In subsequent years, modifications of this approach have allowed recombination to be assayed in non-lymphoid cells co-transfected with plasmids encoding RAG-1 and RAG-2.[86] Moreover, plasmid recombination can be analyzed by PCR rather than antibiotic drug selection.[87] Many important mechanistic features of both the cleavage and joining steps during V(D)J recombination were elucidated using this

approach. However, mechanisms that rely on chromatin or chromosomal context cannot be studied using this approach, because normal chromatin configurations would not necessarily be recapitulated on extrachromsomal substrates.

To study chromosomal V(D)J recombination, Rosenberg and colleagues developed an approach using Abelson murine leukemia virus-transformed pre-B cell lines, hereafter referred to as abl pre-B cells.[88] These cells were transformed with a temperature-sensitive Abelson murine leukemia virus and underwent G_1 cell cycle arrest, inducing RAG expression and rearranging the endogenous immunoglobulin (Ig) light (L) chain κ locus when cultured at the non-permissive temperature.[88] Recently, a similar approach has been developed that involves treating abl pre-B cells with the abl kinase inhibitor Gleevac, which causes G_1 cell cycle arrest, induction of RAG expression and robust rearrangement of the endogenous *IgL*κ locus as well as chromosomally integrated retroviral substrates.[8,89] These different approaches have been used effectively to study chromosomal V(D)J recombination.

ATM Function during V(D)J Recombination

The lymphoid phenotypes of ATM-deficient mice and humans suggest that ATM is an important component of responses to RAG-mediated DNA DSBs.[29,30] Analysis of extrachromosomal plasmid recombination substrates in ATM-deficient fibroblasts failed to reveal any defects in V(D)J recombination.[90] In sharp contrast, however, analysis of chromosomal V(D)J recombination in ATM-deficient abl pre-B cells revealed a partial block in coding joint formation leading to an accumulation of unrepaired coding ends.[8,81] Similar defects were observed in primary ATM-deficient lymphocytes in vivo.[9-11] This defect in coding joint formation is due to the function of ATM in promoting the stability of unrepaired coding ends in post-cleavage complexes until they are joined by NHEJ. In the absence of ATM, coding ends are lost from post-cleavage complexes and are frequently resolved aberrantly as chromosomal deletions or translocations.[8,83,91] In contrast to

coding joint formation, ATM appears to be dispensable for signal joint formation.[8,20,81]

The kinase activity of ATM is required to promote coding end stability, suggesting that ATM phosphorylates downstream target proteins that perform a stabilizing function.[8] In this regard, each of the components of the MRN complex are phosphorylated by ATM in response to DNA DSBs.[24,47-49] Rad50 has direct DNA binding activity that tethers sister chromatids during homology-mediated repair.[33,34] Nbs1, presumably with Rad50 and Mre11, localizes to RAG DSBs generated at the endogenous T-cell receptor α locus in developing thymocytes.[92] Moreover, cells expressing hypomorphic versions of Mre11 or Nbs1 have defects in the repair of RAG-mediated DSBs, which, although less severe, are similar in nature to those observed in ATM-deficient cells.[93,94] From these observations, it is tempting to speculate that MRN stabilizes coding ends in post-cleavage complexes, and that phosphorylation of Mre11, Nbs1 or Rad50 by ATM augments this activity. However, MRN is also required for the activation of ATM in response to DNA DSBs, and thus, the V(D)J recombination defects in MRN-deficient cells could be due, in part, to impaired activation of ATM.[33-38] The ATM-mediated formation of γ-H2AX at the DSB site may also contribute to stability by, for example, increasing MRN retention.[52,83]

DNA-PKcs Functions during V(D)J Recombination

DNA-PKcs is required for coding joint formation due to its function in promoting the hairpin-opening activity of Artemis.[4,5,68,95] Indeed, DNA-PKcs activates the Artemis endonuclease via direct phosphorylation of Artemis and/or via the association of Artemis with phosphorylated DNA-PKcs.[68,95] DNA-PKcs also participates in the repair of blunt-ended DNA breaks, suggesting that it may play a role in the resolution of both open coding ends and blunt phosphorylated signal ends.[96-99] However, the function of DNA-PKcs in signal joining has been unclear. Lymphocytes with a naturally occurring DNA-PKcs mutation that causes murine Scid have low levels of DNA-PKcs protein

and exhibit severe defects in coding joint formation but have only mild defects in signal joining.[18-21,100-103] Moreover, signal joining is minimally impaired in lymphocytes from mice with gene-targeted null DNA-PKcs mutations.[17,18] However, other studies have demonstrated a more severe signal joining defect in cells with compromised DNA-PKcs function.[12-16] Moreover, several of these studies have demonstrated defects in signal joint fidelity in DNA-PKcs-deficient cells. The variability in these different studies may be due, in part, to the analysis of different cell types (lymphoid vs. non-lymphoid), different DNA-PKcs mutations and signal joining at endogenous antigen receptor versus extrachromosomal plasmid recombination substrates. Together, these studies suggest that DNA-PKcs has independent functions during signal joining; however, the relative contributions of unique DNA-PKcs activities to efficient signal joining remain to be resolved.

Overlapping Activities of ATM and DNA-PKcs

During V(D)J recombination, ATM and DNA-PKcs both phosphorylate SQ/TQ motifs and have overlapping protein targets in response to DNA DSBs.[25,26] ATM and DNA-PKcs deficiency combined in B cells leads to a more severe DSB repair defect during immunoglobulin class switch recombination than when cells lack only one of these proteins.[25] Moreover, mice deficient in ATM and DNA-PKcs exhibit early embryonic lethality, whereas those deficient in either ATM or DNA-PKcs are viable.[104,105] Thus, ATM and DNA-PKcs have overlapping functions in DDR that could also be relevant to the repair of RAG-mediated DSBs.

Indeed, using different approaches, Gapud et al. and Zha et al. demonstrated that efficient signal joint formation relies on redundant functions of ATM and DNA-PKcs.[20,27] Gapud et al. examined chromosomal signal joint formation in abl pre-B cells generated from *ATM*^{-/-} or *Scid* mice.[20] An isolated deficiency of either ATM or DNA-PKcs in these cells had no significant effect on the kinetics or levels of chromosomal signal joint formation.[20] However, treatment of *ATM*^{-/-} cells with

DNA-PKcs kinase inhibitors or *Scid* cells with an ATM kinase inhibitor led to a significant block in chromosomal signal joint formation.[20] Moreover, knockdown of ATM in *Scid* pre-B cells also led to a similar block in signal joint formation.[20] In agreement with these findings, Zha et al. showed that ATM and DNA-PKcs doubly deficient cells have a profound defect in chromosomal and extrachromosomal signal joint formation, which is linked mechanistically to a requirement for the kinase activity of at least one of these proteins.[27] Finally, Gapud et al. demonstrated that ATM and DNA-PKcs have overlapping activities in preventing aberrant resolution of unrepaired signal ends as potentially oncogenic chromosomal translocations and deletions.[20] Thus, signal joint formation requires a kinase-dependent function shared by ATM and DNA-PKcs, implying that these proteins have common downstream targets participating in the resolution of RAG-mediated breaks.

We envision three non-mutually exclusive general mechanisms by which shared downstream targets of ATM and DNA-PKcs function in the signal joining process (**Fig. 1**). In the first model, ATM and DNA-PKcs could phosphorylate proteins that promote signal joining (**Fig. 1A**). In this regard, several NHEJ factors that function during signal joining are phosphorylated by DNA-PKcs and/or ATM either in vitro or in vivo, including DNA Ligase IV, XRCC4, Ku70 and Ku80.[64,66,67] However, analyses of cells with selected SQ/TQ motif mutations of XRCC4, Ku70 and Ku80 failed to reveal any defects in coding joint formation.[64,66,67] Signal joint formation was analyzed only in cells with XRCC4 mutations, but, again, no defects were observed.[66]

In the second model, ATM or DNA-PKcs may be required to evict proteins from signal ends that would inhibit ligation (**Fig. 1B**). In this regard, phosphorylation of DNA-PKcs by itself or by ATM may be important for promoting dissociation of DNA-PKcs from DNA ends.[61] However, DNA-PKcs-deficient cells require ATM kinase activity for efficient signal joint formation, suggesting that targets other than DNA-PKcs itself must be phosphorylated by ATM or DNA-PKcs for efficient signal joint formation.

Similarly, the Artemis nuclease can also be phosphorylated by ATM or DNA-PKcs.[68,106-109] Artemis is not required for signal joining, but signal ends can be exonucleolytically processed by Artemis prior to joining, demonstrating that Artemis has access to signal ends during V(D)J recombination.[78] Unrepaired signal ends in cells deficient in both ATM and DNA-PKcs exhibit increased nucleolytic resection when compared with unrepaired signal ends in DNA Ligase IV-deficient cells that express ATM and DNA-PKcs.[20] Thus, ATM or DNA-PKcs may promote signal joining by removing Artemis from signal ends.

After DNA cleavage, the RAG proteins avidly bind signal ends and inhibit their ligation in vitro.[110-112] ATM and DNA-PKcs could function to displace RAG-1 and RAG-2 from signal ends, allowing them to be joined. In this regard, both RAG-1 and RAG-2 have several SQ/TQ motifs that could be targets for ATM or DNA-PKcs, and RAG-2 can be phosphorylated by DNA-PKcs in vitro.[73] However, analyses of RAG-1 and RAG-2 with mutations of some of these SQ/TQ motifs have led to conflicting conclusions regarding their potential function during V(D)J recombination.[73,74]

In the third model, ATM and DNA-PKcs could have overlapping functions that promote the accessibility of signal ends to repair machinery (**Fig. 1C**). In what may be a related finding, the H1 linker histone inhibits ligation of chromatinized DSB substrates in vitro, and DNA-PKcs-mediated phosphorylation of H1 diminishes its ability to associate with chromatinized DNA breaks.[113] Moreover, recent studies have shown that ATM-dependent ubiquitylation of the histone H2B is important for efficient DSB repair, possibly due to a role in promoting chromatin accessibility at broken DNA ends.[114] Importantly, however, Zha et al. have demonstrated that efficient signal joining on extrachromosomal plasmid substrates also requires the overlapping activity of ATM and DNA-PKcs.[27] Although these substrates may associate with histone proteins, they are unlikely to adopt a higher-order chromatin structure. Thus, ATM and DNA-PKcs may modulate chromatin structure to allow for chromosomal signal

joining but likely also have additional relevant activities.

As discussed above, the histone variant H2AX is also phosphorylated by ATM or DNA-PKcs to form γ-H2AX in chromatin flanking DNA DSBs, including RAG-mediated DSBs.[50,51,69,92] However, H2AX-deficient cells have no demonstrable defect in chromosomal coding joint formation; thus, neither H2AX nor γ-H2AX is absolutely required for the repair of coding ends.[82-84] Emerging studies demonstrate that XLF and H2AX have essential overlapping activities in chromosomal coding joint formation.[81] Whether H2AX has any independent functions, or functions overlapping with XLF during chromosomal signal joint formation is unknown. However, because cells deficient in ATM and DNA-PKcs should express XLF, the loss of γ-H2AX in these cells cannot explain the observed defect in chromosomal signal joint formation unless ATM and DNA-PKcs also function redundantly upstream of XLF.

Finally, ATM and DNA-PKcs may also have overlapping activities during coding joint formation. DNA-PKcs is required to promote the hairpin-opening activity of Artemis, and, in this regard, ATM cannot significantly compensate for loss of DNA-PKcs. Whether DNA-PKcs can provide some measure of coding end stability in ATM-deficient cells has not been examined. Moreover, ATM and DNA-PKcs could conceivably have overlapping activities in coding joint formation downstream of hairpin opening or DNA end stabilization. Such activities may be similar or identical to those required for the joining of signal ends.

Concluding Remarks

The ATM and DNA-PKcs kinases are both activated by DNA DSBs. Although these proteins have unique phosphorylation targets and activities in DDR, mounting evidence demonstrates that they also have shared targets and activities. These overlapping activites can be partial, as is the case with γ-H2AX formation; in the absence of ATM, γ-H2AX is generated by DNA-PKcs in chromatin flanking RAG DSBs, but at levels lower than observed when ATM is present.[69] In

Figure 1. Models for overlapping activities of ATM and DNA-PKcs during V(D)J recombination: (A) ATM and DNA-PKcs may phoshorylate factors that are directly involved in signal joint formation or may phosphorylate proteins that indirectly promote the activity of joining factors. (B) ATM and DNA-PKcs may phoshorylate proteins that directly inhibit ligation, leading to their eviction from the DNA ends, or may phosphorylate proteins that actively evict these inhibitory proteins. (C) ATM and DNA-PKcs may directly phosphorylate histones in a way that makes signal end-associated chromatin accessible or may phosphorylate proteins that actively modify chromatin structure at RAG DSBs.

contrast, ATM and DNA-PKcs are completely redundant during chromosomal signal joint formation.[20,27] The full spectrum of DDR that can be mediated by DNA-PKcs and/or ATM and the extent of functional overlap between these two kinases in these different responses remain to be determined. Importantly, there may

be undefined DDR functions that will not be revealed by analyzing cells with isolated deficiencies in ATM or DNA-PKcs, as highlighted by recent studies. Thus, identifying the full spectrum of DDR may require the analysis of cells with both isolated and combined deficiencies of ATM and DNA-PKcs.
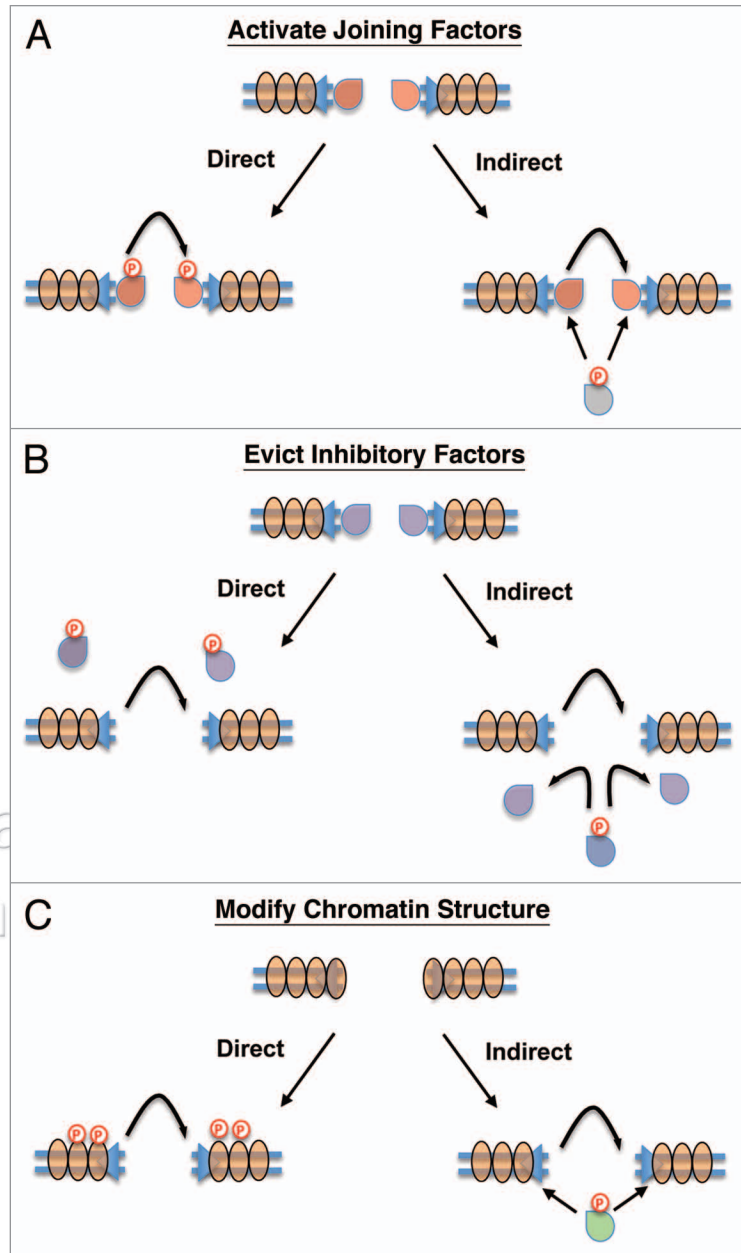
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