# Resolution of Recombination Intermediates: Mechanisms and Regulation

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DNA strand break repair by homologous recombination leads to the formation of intermediates in which sister chromatids are covalently linked. The efficient processing of these joint molecules, which often contain four-way structures known as Holliday junctions, is necessary for efficient chromosome segregation during mitotic division. Because persistent chromosome bridges pose a threat to genome stability, cells ensure the complete elimination of joint molecules through three independent pathways. These involve (1) BLM-Topoisomerase IIIα-RMI1-RMI2 (BTR complex), (2) SLX1-SLX4-MUS81-EME1 (SLX-MUS complex), and (3) GEN1. The BTR pathway promotes the dissolution of double Holliday junctions, which avoids the formation of crossover products, prevents sister chromatid exchanges, and limits the potential for loss of heterozygosity. In contrast to BTR, the other two pathways resolve Holliday junctions by nucleolytic cleavage to yield crossover and non-crossover products. To avoid competition with BTR, the resolution pathways are restrained until the late stages of the cell cycle. The temporal regulation of the dissolution/resolution pathways is therefore critical for crossover avoidance while also ensuring that all covalent links between chromosomes are resolved before chromosome segregation.

In response to replication stress, and as a consequence of endogenous DNA damage, human cells generate ~10–20 DNA double-strand breaks (DSBs) per day. These breaks are efficiently repaired by nonhomologous end joining (NHEJ) or homologous recombination (HR) and therefore do not pose a great threat to genome integrity. Some individuals, however, such as those mutated for *BRCA1* or *BRCA2*, carry genetic defects that affect the efficiency of these repair processes and are predisposed to breast and ovarian cancers (Venkitaraman 2014).

The recombination-mediated pathway for DSB repair is active in the S and $G_2$ phases of the cell cycle and generally involves interactions between sister chromatids. In some instances, however, recombination can occur between homologous chromosomes, rather than sister chromatids. Recombinational repair often leads to the formation of intermediates in which the recombining DNAs are covalently linked by the formation of four-way DNA junctions or Holliday junctions (HJs) (Holliday 1964; West 2003; Wyatt and West 2014). These intermediates need to be processed to allow proper chromosome segregation during mitotic division (Wechsler et al. 2011; Castor et al. 2013; Garner et al. 2013; Wyatt et al. 2013; Sarbajna et al. 2014).

## MECHANISMS FOR PROCESSING RECOMBINATION INTERMEDIATES

### Holliday Junction Dissolution

In human cells, there are three pathways for HJ processing. The first involves four proteins, the Bloom's syndrome helicase BLM, Topoisomerase IIIα, RMI1, and RMI2, which interact to form the BTR complex (Fig. 1A). This complex promotes the convergent migration of two HJs to produce a hemicatenane that can be processed by topoisomerase action (Wu and Hickson 2003). This Holliday junction "dissolution" pathway gives rise exclusively to non-crossovers. Dissolution therefore plays a key role in preventing excessive sister chromatid exchanges and limits the potential for loss of heterozygosity on those rare occasions when recombination occurs between homologous chromosomes. Individuals with mutations in BLM suffer from Bloom's syndrome (BS) and are predisposed to a broad spectrum of early onset cancers (Ray and German 1984; Hickson 2003). Cells derived from BS patients exhibit a high frequency of sister chromatid exchanges (Fig. 1C), defective chromosome segregation, and increased genome instability.

### Holliday Junction Resolution

The second and third pathways for the processing of recombination intermediates utilize structure-selective endonucleases (or "resolvases") that cut HJs to produce crossover (CO) and non-crossover (NCO) products (Fig. 1B). The elevated frequency of sister chromatid exchanges (i.e., COs between sister chromatids) observed in the BLM-deficient cells (Fig. 1C) is thought to be a consequence of resolution taking place in the absence of dissolution. The nucleases involved in these reactions are SLX1-SLX4 and MUS81-EME1, which interact to form the SLX-MUS complex (Andersen et al. 2009; Fekairi

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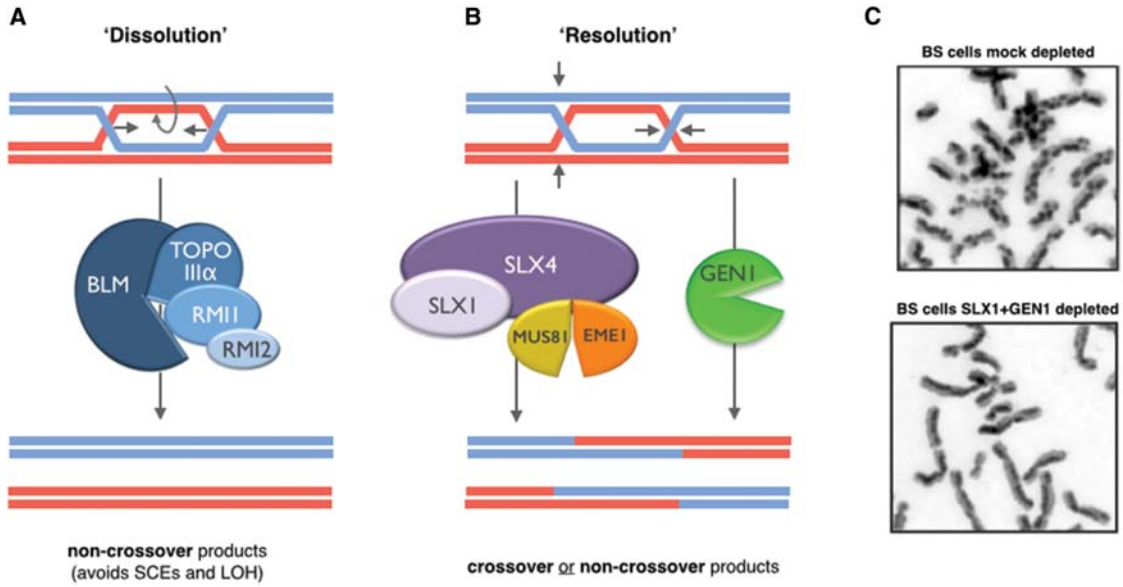


**Figure 1.** Mechanisms for the processing of recombination intermediates in mitotic human cells. The two mechanisms involve (*A*) "dissolution" or (*B*) "resolution." Dissolution is driven by the convergent migration of two Holliday junctions and topoisomerase-mediated dissolution of the resultant hemicatenane. The reaction involves BLM helicase, Topoisomerase IIIα, RMI1, and RMI2. Dissolution generates non-crossover products, thereby avoiding sister chromatid exchanges (SCEs) and the possibility for loss of heterozygosity (LOH) when recombination occurs between homologous chromosomes. Nucleolytic resolution is driven by two distinct pathways involving the SLX1-SLX4-MUS81-EME1 complex or GEN1 protein. Both generate crossovers and non-crossovers. (*C*, *top* panel) A high frequency of SCEs is observed in cells derived from individuals with Bloom's syndrome (BS). (*Lower* panel) The elevated SCE frequency observed in BS cells is largely dependent on the resolution pathways, as observed by depletion of SLX1 and GEN1.

et al. 2009; Muñoz et al. 2009; Svendsen et al. 2009; Castor et al. 2013; Garner et al. 2013; Wyatt et al. 2013), and GEN1 (Ip et al. 2008; Rass et al. 2010). Importantly, these three nucleases define two genetically distinct pathways for HJ resolution (Sarbajna and West 2014; Sarbajna et al. 2014). Because of the potential dangers involved in generating COs in mitotic cells, the resolution pathways must be tightly regulated in order to prioritize BTR-mediated HJ dissolution. The resolution pathways, however, play critically important roles in ensuring that any remaining HJs are resolved before chromosome segregation.

## REGULATION OF HOLLIDAY JUNCTION RESOLUTION

### Holliday Junction Processing in Yeast

Our first insights into how the dissolution/resolution pathways are temporally separated during the cell cycle came from studies carried out in *Saccharomyces cerevisiae*. During mitotic growth, most double HJs are processed at an early stage in the cell cycle by the yeast ortholog of the BTR complex, Sgs1-Top3-Rmi1 (STR), to generate NCOs. In *sgs1* mutants, however, the joint molecules persist until later in the cell cycle, at which time they are processed by Mus81-Mms4 (the ortholog of MUS81-EME1) and Yen1 (the ortholog of GEN1), generating both COs and NCOs (Ira et al. 2003; Dayani et al. 2011; Matos et al. 2011, 2013; Szakal and Branzei 2013). At first sight, therefore, HJ resolution appears to provide a backup pathway to STR-mediated HJ dissolution.

Recent studies have shown how the Mus81-Mms4 and Yen1 nucleases are regulated throughout the cell cycle by stage-specific phosphorylation events that impose temporal control and lead to their sequential activation. In S phase, the Holliday junction resolving activity of Mus81-Mms4 is low, but it becomes elevated at the onset of mitosis by phosphorylation events mediated by the cell cycle kinases Cdc28/Cdk and Cdc5 (Fig. 2A; Matos et al. 2011, 2013; Gallo-Fernández et al. 2012; Szakal and Branzei 2013; Gritenaite et al. 2014). The primary target of phosphorylation is the Mms4 subunit of Mus81-Mms4. In contrast, Yen1's HJ resolvase activity is inhibited by phosphorylation events that occur at the $G_1$/S transition, and the nuclease remains inactive through S phase and $G_2$ (Fig. 2B). It is then activated by Cdc14-mediated dephosphorylation at the later stages of mitosis (Matos et al. 2011; Blanco et al. 2014; Eissler et al. 2014).

Remarkably, phosphorylation regulates the activity of Yen1 in two distinct ways. First, it was shown that Cdk-mediated phosphorylation of S679 inactivates Yen1's nuclear localization signal (NLS) and prevents nuclear import so that Yen1 accumulates in the cytoplasm (Kosugi et al. 2009; Blanco et al. 2014; Eissler et al. 2014). Second, high levels of S-phase Cdk-dependent phosphorylation directly reduce the DNA-binding affinity of Yen1 (Blanco et al. 2014). As cells enter anaphase, however, Cdc14 phosphatase dephosphorylates Yen1,

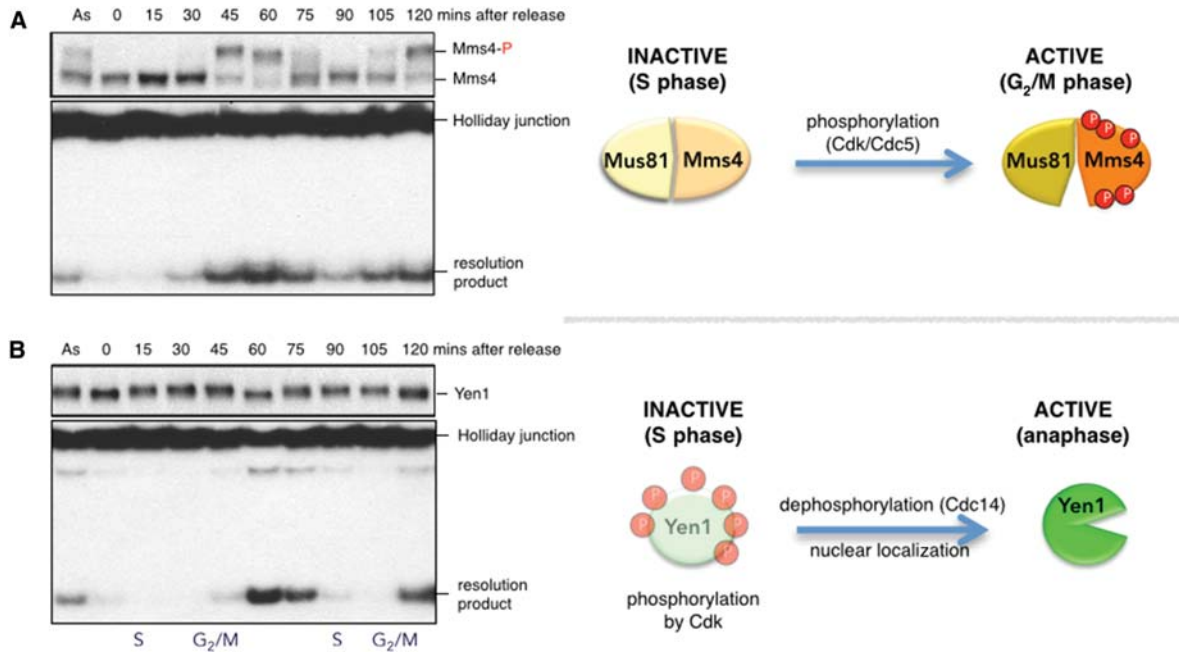


**Figure 2.** Cell cycle regulation of Holliday junction resolution activities in yeast. (*A*, *left*) *S. cerevisiae* cells, carrying Mms4-Myc9, were synchronized using α-factor and, following release, samples were taken at the indicated times. Extracts were prepared and analyzed for Mms4 (*upper* panels) by Western blotting, and immunoprecipitates were assayed for their ability to cleave $^{32}$P-labeled Holliday junctions in vitro. Approximate stages of the cell cycle are indicated below. (Adapted from Matos et al. 2011.) (*Right*) Schematic diagram indicating how Mus81-Mms4 is activated at $G_2$/M by cell cycle–dependent phosphorylation events mediated by Cdk and Cdc5. (*B*, *left*) As *A*, except that the cells carried Yen1-Myc9. Extracts were analyzed for Yen1 by Western blotting and immunoprecipitates were assayed for Holliday junction cleavage. (*Right*) Schematic indicating the activation of Yen1 by Cdc14 phosphatase-mediated removal of S phase–specific inhibitory phosphorylation events. Dephosphorylation activates Yen1 for DNA binding and mediates its nuclear relocalization at mitosis.

triggering its activation and entry to the nucleus (Blanco et al. 2014; Eissler et al. 2014; Garcia-Luís et al. 2014). These studies therefore revealed that Mus81-Mms4 and Yen1 promote consecutive and temporally separable waves of HJ processing, at metaphase and anaphase, respectively.

### Holliday Junction Processing in Human Cells

***The SLX-MUS Complex.*** The clues gained from studies with yeast provided an opportunity to determine how the resolvases are regulated in human cells. The mechanisms of regulation observed were similar in principle, but quite different in detail. Like Mus81-Mms4, the MUS81-EME1 protein becomes phosphorylated at the onset of mitosis, in reactions driven by the cyclin-dependent kinase CDK1, which promotes phosphorylation of the regulatory subunit EME1 (Fig. 3A,B; Matos et al. 2011). In contrast to yeast, however, phosphorylation does not activate MUS81-EME1 directly, but it instead promotes the association of MUS81-EME1 with the 5′-flap endonuclease SLX1-SLX4, to form the SLX1-SLX4-MUS81-EME1 (SLX-MUS) complex (Fig. 3C; Wyatt et al. 2013). Given that MUS81-EME1 is a very poor HJ resolvase and yet is very active on nicked HJs, the combination of MUS81-EME1 with SLX1-SLX4, a potent nickase on a variety of DNA secondary structures, provides the cell with a novel mechanism to combine two existing nucleases to form a highly active HJ resolvase (Wyatt et al. 2013).

The mechanisms by which CDK1-mediated phosphorylation events enhance the interaction between MUS81-EME1 and SLX1-SLX4 are presently unknown, but it is likely that the SLX-MUS complex might be directly stabilized by multiple phosphorylation events. The Polo-Like Kinase PLK1 was also found to interact with SLX4 (Svendsen et al. 2009), but in contrast to CDK1, its activity appears to be dispensable for the bulk of SLX-MUS complex formation at the $G_2$/M transition (Fig. 3B; Wyatt et al. 2013). Interestingly, in response to phosphorylation, XPF-ERCC1 also associates with SLX1-SLX4 and MUS81-EME1, but the precise role of this additional nuclease within the complex is unknown.

The SLX-MUS complex, in which a 3′-flap endonuclease (MUS81-EME1) is combined with a 5′-flap endonuclease (SLX1-SLX4), is unlike any other HJ resolvase (Wyatt et al. 2013). The paradigm for HJ resolution is provided by *Escherichia coli* RuvC, a homodimeric protein that uses its two active sites to introduce a pair of coordinated and symmetrical nicks into strands that are diametrically opposed across the junction (Dunderdale et al. 1991; Iwasaki et al. 1991; Bennett et al. 1993). The two nicks are introduced within the lifetime of the protein–DNA complex, and the reaction products are a

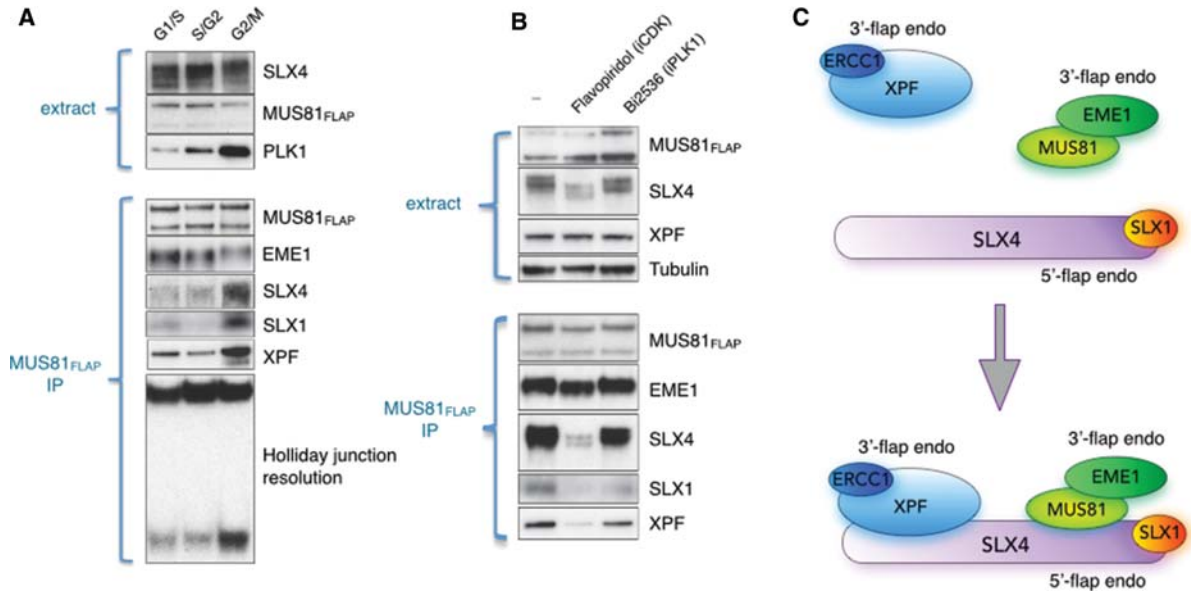


**Figure 3.** Cell cycle stage–specific formation of the SLX1-SLX4-MUS81-EME1 complex in human cells. (*A*) HeLa cells expressing FLAP-tagged MUS81 were blocked at various stages of the cell cycle by treatment with thymidine ($G_1$/S), camptothecin (S/$G_2$), or nocodazole ($G_2$/M). The FLAP tag comprises GFP (green fluorescent protein) and Flag affinity tags. Cell extracts and GFP immunoprecipitates (IPs) were probed for the indicated proteins by Western blotting. The IPs were also assayed for the ability to cleave $^{32}$P-labeled Holliday junctions. The $G_2$/M fractions showed: increased Holliday junction resolvase activity, EME1 phosphorylation, and the presence of SLX1-SLX4 and XPF-ERCC1, compared with the other cell cycle stages. (*B*) Formation of the SLX1-SLX4-MUS81-EME1-XPF-ERCC1 complex, and EME1 phosphorylation, are blocked by CDK inhibition using Flavopiridol, but are relatively insensitive to the PLK1 inhibitor Bi2536. (Adapted from Wyatt et al. 2013.) (*C*) Schematic diagram indicating the $G_2$/M-specific formation of the SLX1-SLX4-MUS81-EME1-XPF-ERCC1 complex.

pair of nicked duplexes in which the nicks can be ligated. In contrast to RuvC, the SLX-MUS complex contains a pair of heterodimeric flap endonucleases, with each contributing one active site to the cleavage reaction. Recent studies with SLX-MUS complexes in which one nuclease was inactivated revealed that SLX1 catalyzes the initial, rate-limiting incision, and that MUS81 introduces the second cut on the opposing strand of like polarity (Wyatt et al. 2013). Although this reaction is reminiscent of those catalyzed by canonical HJ resolvases, only a fraction of the products made by SLX-MUS can be ligated. The majority of the cleavage products contain gaps and flaps that require further cellular processing before ligation.

***GEN1 Holliday Junction Resolvase.*** GEN1 is a structure-selective endonuclease that promotes the cleavage of 5′-flap structures, replication forks, and HJs (Ip et al. 2008; Rass et al. 2010). The mechanism of action of GEN1 with HJs is similar to that exhibited by RuvC, indicating that, unlike SLX-MUS, GEN1 is a canonical HJ resolvase. In contrast to Yen1, however, which is regulated by changes to its phosphorylation status that influence its activity and subcellular localization, GEN1 is regulated primarily by nuclear exclusion (Fig. 4; Matos et al. 2011; Chan and West 2014). Consequently GEN1 can only access and cleave recombination intermediates when the nuclear membrane breaks down at mitosis. Nuclear exclusion is facilitated by the presence of a nuclear export sequence (NES) located within the carboxy-terminal region in GEN1.

The effects of artificially expressing GEN1 in the nucleus were investigated by constructing a GEN1 variant with an inactivated NES and three nuclear localization signals fused to the carboxyl terminus of the protein (Fig. 5A,B; Chan and West 2014). Cells expressing this nuclear localized version of GEN1 (GEN1$^{nuc}$) exhibited an increased frequency of sister chromatid exchanges, indicating its ability to compete with the BTR pathway of HJ dissolution and increase CO formation (Fig. 5C).

The need for additional regulatory control in yeast may be due to the fact that this organism undergoes a closed mitosis in which the nuclear envelope remains intact. As a consequence, active import machineries have to be used to shuttle Yen1 into the nucleus. In contrast, in human cells, the physical barrier is automatically disassembled at the $G_2$/M transition, so that cells only need to maintain GEN1 export to prevent its accumulation in the nucleus. Once the nuclear membrane is dissolved, GEN1 will gain access to, and resolve, any persistent covalent bridges that link sister chromatids and so enable their segregation.

## DEFECTS IN HJ PROCESSING LEAD TO MITOTIC CATASTROPHE

The cellular importance of HJ processing in mammalian cells is clear from the synthetic lethality observed in cells depleted for BLM (dissolution pathway) and SLX4 (a resolution pathway) or SLX4 and GEN1 (both resolution pathways) (Garner et al. 2013; Wyatt et al.

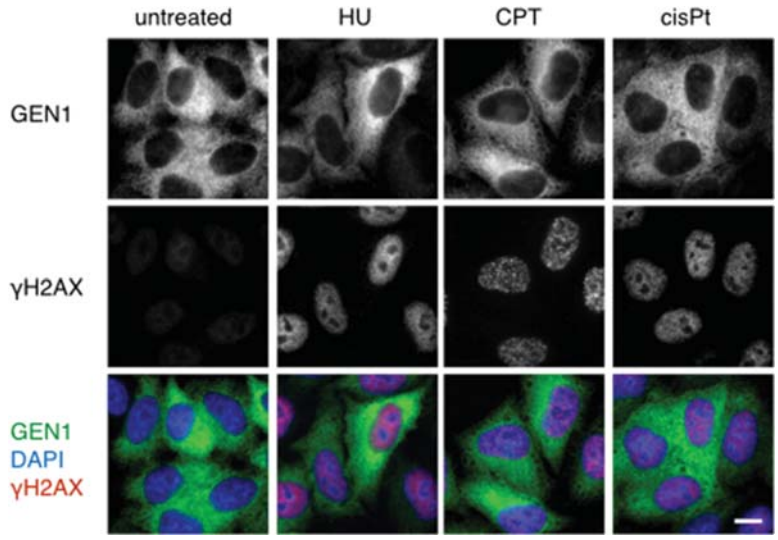


**Figure 4.** Cytoplasmic localization of GEN1. HeLa cells expressing GEN1-GFP were treated with or without the replication inhibitor hydroxyurea, the topoisomerase inhibitor camptothecin or the DNA damaging agent cisplatin. The cellular localization of GEN1 was then determined by immunofluorescence, using DAPI, which stains DNA, and γH2AX, a signal for DNA damage, as markers. (Adapted from Chan and West 2014.)

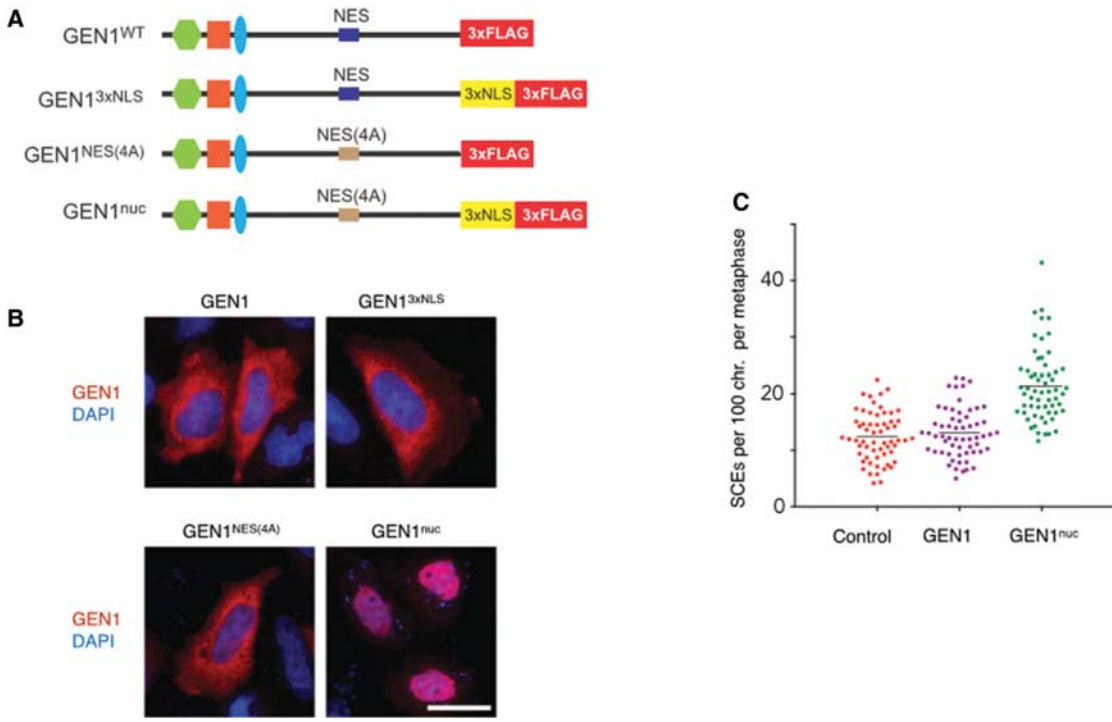


**Figure 5.** A nuclear export sequence is important for the cytoplasmic localization of GEN1. (*A*) Schematic diagram of GEN1 constructs showing the location of the putative nuclear export sequence (NES). The constructs include wild-type GEN1 ($GEN1^{WT}$), a GEN1 mutant carrying alanine substitutions of four leucine/isoleucine residues within the NES ($GEN1^{NES(4A)}$), and two derivatives carrying three synthetic nuclear localization signals (NLSs), namely $GEN1^{3NLS}$ and $GEN1^{nuc}$. (*B*) $GEN1^{nuc}$, which has a mutated NES together with three NLSs, localizes to the nucleus as determined by immunofluorescence as described in Fig. 4 legend. (*C*) The nuclear localization of $GEN1^{nuc}$ is associated with an increased frequency of sister chromatid exchanges, most presumably because the constitutively nuclear GEN1 competes with the BTR-mediated pathway of Holliday junction dissolution. (Adapted from Chan and West 2014.)

2013). Recent studies have shown that mortality is likely to stem from gross chromosomal abnormalities and mitotic defects (Wechsler et al. 2011; Garner et al. 2013; Wyatt et al. 2013; Sarbajna et al. 2014). Loss of SLX4 and GEN1 leads to severe chromosomal instability, exemplified by the appearance of elongated and highly segmented chromosomes (Wechsler et al. 2011). Similar defects have been observed in BLM-depleted SLX4 null cells and in cisplatin-treated cells depleted of SLX4 and GEN1 or MUS81 and GEN1 (Garner et al. 2013; Sarbajna et al. 2014). Segmentation occurs at equivalent positions on the two sister chromatids and can be rescued by expression of a bacterial HJ resolvase such as RusA (Wechsler et al. 2011; Garner et al. 2013). Because the observed chromosome indentations are effectively free of the SMC2 condensin, it has been suggested that persistent chromatid bridges cause defects in chromosome condensation rather than chromosome breakage (Wechsler et al. 2011).

In addition to driving chromosome instability, persistent sister chromatid entanglements also impede chromosome segregation, as exemplified by the high frequency of anaphase bridges and lagging chromosomes (Garner et al. 2013; Wyatt et al. 2013; Sarbajna et al. 2014). Defective segregation also leads to the formation of micronuclei and to the appearance of irregular and catastrophic nuclei (Garner et al. 2013; Sarbajna et al. 2014). In addition, these cells exhibit prolonged mitoses and often fail to divide, which leads to the formation of multinucleate cells. Finally, depletion of SLX-MUS and GEN1 leads to elevated levels of 53BP1-positive nuclear bodies in $G_1$, indicating transmission of DNA damage from one cell cycle to the next (Svendsen et al. 2009; Naim et al. 2013; Ying et al. 2013; Sarbajna et al. 2014). Presumably, the accumulation of DNA damage during consecutive cell cycles contributes to the high levels of mortality of these resolvase-deficient cells.

## CONCLUSION

In summary, our understanding of the mechanisms of HJ resolution in human cells has gathered pace since the identification of GEN1 and the SLX-MUS complex opened up the possibility of pathway analysis (Ip et al. 2008; Castor et al. 2013; Garner et al. 2013; Wyatt et al. 2013). Indeed, the work of several laboratories now provides us with a clear picture of the way that the BTR pathway dissolves double HJs to generate NCO products, whereas the resolution pathways cleave persistent double HJs, single HJs, and possibly other secondary structures in DNA that remain until mitosis (Fig. 6). However, although the resolvases might have been originally considered as backup pathways for BTR, accumulating evidence demonstrating reduced survival of resolvase-defective cells indicates that all three pathways are necessary for genome maintenance. Instead they should be thought of as essential factors that ensure chromosome segregation. That the dissolution and resolution pathways

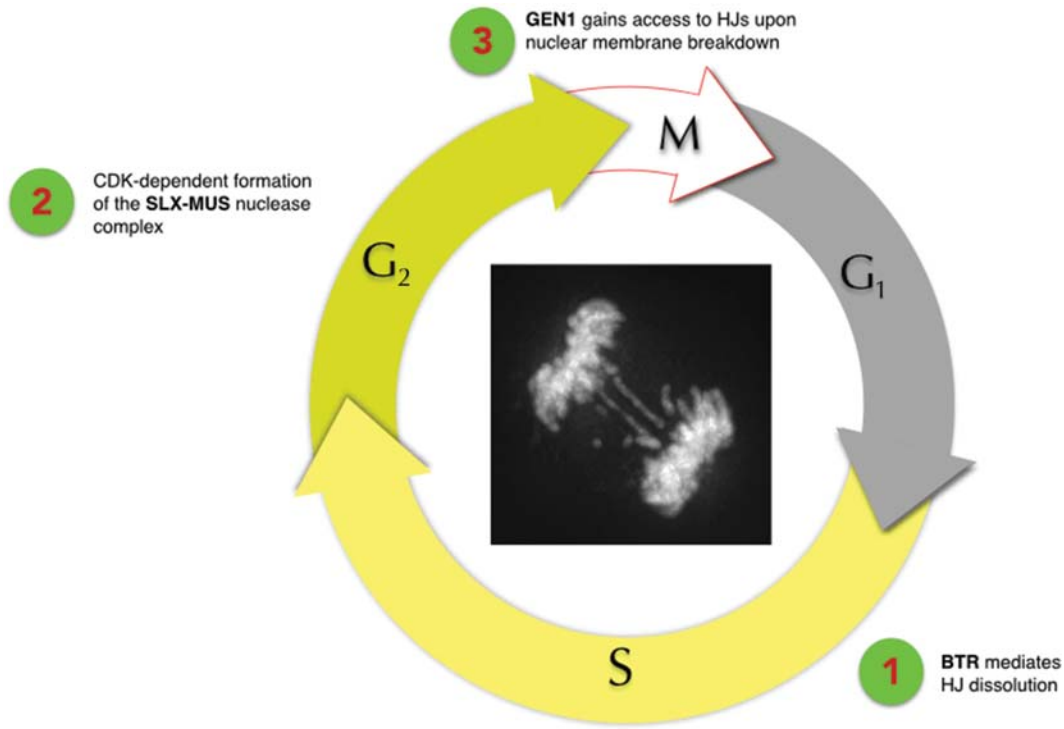


**Figure 6.** Schematic diagram indicating that the three human Holliday junction (HJ) processing pathways are regulated by temporal (cell cycle stage) and spatial (subcellular localization) control. Together, they ensure the resolution of DNA intermediates before chromosome segregation. Defects in these processing pathways lead to segregation problems that are manifest by the formation of anaphase bridges and the presence of lagging chromosomes (*center*).

are temporally regulated throughout the cell cycle most likely relates to the need to promote NCOs, rather than COs that could lead to a loss of heterozygosity and the ensuing dangerous elimination of tumor-suppressor functions.

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