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Topoisomerase III α resolves inter- and intra-molecular intertwinings during DNA replication

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Abstract

Resolution of topological stress is crucial for genome integrity. Vertebrate topoisomerase II α (TOP2 α) resolves catenanes to relieve topological stress and unlink daughter molecules during DNA replication. Topoisomerase III α (TOP3 α) can also resolve catenanes, but its direct role during DNA replication, substrate specificity, and relevant binding partners remain unclear. Here we show that TOP3 α directly resolves replication-generated DNA intertwinings in *Xenopus* egg extracts. When TOP2 α function is compromised, TOP3 α promotes replication fork progression and daughter strand unlinking preferentially during replication termination. TOP3 α can carry out this role independently of its binding partners RMI1-RMI2 and the BLM helicase by acting on lagging-strand single-stranded DNA (ssDNA). Strikingly, elevated lagging-strand ssDNA drives formation of intramolecular ssDNA intertwinings even when TOP2 α is present, and TOP3 α resolves these structures. Thus, lagging-strand ssDNA provides a substrate for TOP3 α to promote replication termination but can also generate intramolecular intertwinings that require TOP3 α for their resolution.

Introduction

DNA replication generates topological stress that manifests as supercoils ahead of the fork and pre-catenanes behind the fork¹⁻⁴. These structures must be quickly resolved by topoisomerases to avoid buildup of topological stress that would otherwise stall DNA replication⁵⁻¹⁵. Pre-catenanes that persist after replication is complete ('catenanes')^{16,17} link the daughter DNA molecules and must be removed for faithful chromosome segregation¹⁸. During eukaryotic DNA replication topoisomerase I (TOP1) can resolve supercoils^{19,20}, while topoisomerase II (TOP2) can resolve supercoils and pre-catenanes²¹. In contrast, the role of topoisomerase III is much less clear²²⁻²⁴. Addressing its role is important because topoisomerase function is essential for cell viability and targeted during cancer therapy, and because TOP3A defects underlie human disease characterized by growth restriction, microcephaly, and genome instability^{25,26}.

Topoisomerase III acts by creating a transient single-stranded DNA break through which an adjacent DNA strand, or DNA duplex, is passed²⁷⁻³⁰. This activity is crucial to resolve hemicatenanes that arise from homologous recombination^{22,31}. Bacterial topoisomerase III (topo III) can resolve catenanes³² and can operate during elongation^{9,33} and termination^{27,34,35}. However, yeast topoisomerase III (Top3) cannot act during termination³⁶ and has no defined role in unperturbed replication other than at sites of fork stalling³⁷. The vertebrate replicative topoisomerase III, TOP3 α , is important for DNA replication³⁸ but also resolves transcription-replication collisions³⁹ and is required for mitochondrial DNA segregation⁴⁰⁻⁴³. It therefore remains to be established whether TOP3 α promotes vertebrate fork progression by directly unlinking daughter DNA or indirectly through its roles in homologous recombination, transcription-replication collisions, and mitochondrial DNA segregation²¹⁻²⁴.

The mechanism of topoisomerase III function during replication remains uncertain (Fig. S1A). Topoisomerase III may resolve pre-catenanes by acting on single-stranded DNA within the lagging

strand template³³, but this has not been directly tested and is unexplored for the eukaryotic topoisomerase III paralogs. Single-stranded DNA can also arise on the leading strand template when the leading strand polymerase stalls but the replicative helicase continues to unwind ('uncoupling')^{44,45}, which is frequent^{46,47} and thus could also be the substrate topoisomerase III acts upon (as noted in²⁴). Consistent with this, TOP3 α accumulates on DNA when uncoupling is induced⁴⁸. Topoisomerase III may also resolve pre-catenanes by acting on double-stranded DNA, which it can also act on⁴⁹, although less efficiently than single-stranded DNA⁵⁰. Beyond questions about substrate specificity, eukaryotic Top3/ TOP3 α also interacts with additional proteins to form distinct complexes that direct its nuclear^{27,51,52}, but not mitochondrial⁴⁰, function and whose roles are incompletely understood, and TOP3 α can also function non-enzymatically within these complexes^{53,54}. For TOP3 α these are the non-catalytic RMI1-RMI2 to form the 'TRR' complex^{55,56} and the BLM helicase to form the 'BTRR' complex⁵⁷. Finally, topoisomerase III can resolve intramolecular DNA linkages^{50,58,59} but it is unclear whether this activity operates during replication. Thus, the substrate for topoisomerase III during DNA replication, the complexes that direct it, and the range of linkages it can resolve are all open questions.

Here, we characterized the role of TOP3 α during vertebrate DNA replication using *Xenopus* egg extracts^{60,61}. This approach allowed us to perform a detailed biochemical analysis of TOP3 α using a complete vertebrate nuclear proteome but independent of transcription and mitochondrial DNA replication. We show that TOP3 α normally plays no detectable role during DNA replication but promotes fork merger and pre-catenane resolution when TOP2 α activity is lost. This function is independent of both RMI1-RMI2 and BLM, distinguishing it from canonical recombination-intermediate dissolution. Mechanistically, TOP3 α acts on lagging strand gaps to resolve pre-catenanes primarily during termination. Additionally, we find that high levels of lagging-strand single-stranded DNA drive formation of intramolecular single-stranded DNA intertwinings, which TOP3 α resolves. Overall, our data reveal that TOP3 α resolves intermolecular DNA intertwinings

- 67 (pre-catenanes) to compensate for loss of TOP2 α function while playing a unique role in resolving
68 intramolecular intertwinings.

Results

No role for TOP3 α during unperturbed DNA synthesis

To investigate the role of topoisomerase III α (TOP3 α) during vertebrate DNA replication, we first assessed whether loss of TOP3 α impacted DNA replication in *Xenopus* egg extracts ('extracts'). Plasmid DNA was replicated in extracts depleted of TOP3 α and radiolabeled nucleotides were included to monitor the extent of DNA synthesis and the products formed (Fig. S1D). Immunodepletion was effective and removed $\sim$ >99% of TOP3 α (Fig. S1E). In mock immunodepleted extracts, replication fork structures were initially detected (0s; Fig. S1F, lane 1) then converted to nicked and supercoiled circular monomeric products of DNA replication (nCMs, scCMs; Fig. S1F, lane 2) coincident with completion of DNA synthesis (Fig. S1G). These replication products were supercoiled because the plasmid DNA template is chromatinized in extracts⁶² and the chromatin is replicated⁶³, so removal of nucleosomes results in compensatory supercoiling when the reaction is stopped⁶⁴. Importantly, TOP3 α immunodepletion did not affect the DNA species formed (Fig. S1F), the rate of DNA synthesis (Fig. S1G), or the time taken to form circular monomeric products of DNA replication (Fig. S1H). Thus, TOP3 α does not ordinarily play a role during DNA replication in *Xenopus* egg extracts.

A TOP3 α complex facilitates fork merger in the absence of TOP2 α

Topoisomerase II α (TOP2 α) is important for catenane resolution during vertebrate DNA replication, but a low level of unlinking still takes place even in the absence of TOP2 α (Fig. S1B)⁶. We therefore investigated whether TOP3 α , whose orthologs can also resolve catenanes^{49,50}, might play a role in the absence of TOP2 α (Fig. S1C). To do so, we included a catalytic inhibitor of TOP2 activity (TOP2-i, ICRF-193; characterized previously in this system⁶) and once again replicated DNA in either mock- or TOP3 α -immunodepleted extracts (Fig. 1A). In mock-treated extracts, replication fork structures accumulated and persisted for tens of minutes (0s; Fig. 1A-B) before being converted to catenanes (cats; Fig. 1B) and a small population of high molecular weight species

(HMws; Fig. 1B) as previously described. TOP3 α -immunodepletion did not affect incorporation of radiolabeled nucleotides, indicating that the early stages of replication (initiation and elongation) were unaffected (Fig. 1C). However, replication fork structures persisted to the end of the reaction (Fig. 1B, lane 8). This persistence indicated that fork merger, which corresponds to complete unwinding of the parental DNA (Fig. 1A), was severely inhibited (Fig. 1D). Importantly, addition of recombinant TOP3 α as part of the TOP3 α -RMI1-RMI2 complex (TRR; Fig. S1I) rescued the fork merger defect (Fig. 1B, Fig. 1D), demonstrating that TOP3 α or some other part of the TRR complex (see Fig. 2) promotes fork merger. Overall, these data indicate that TOP3 α is required for residual fork merger upon TOP2 α inhibition.

TOP2-i treatment traps TOP2 on DNA, which exacerbates fork merger defects and induces aberrant DNA species (HMws; Fig. 1B)⁶. To test whether the TOP3 α requirement reflected loss of TOP2 α activity rather than TOP2 trapping, we immunodepleted TOP2 α (Fig. 1E). ICRF-193 was included to suppress residual TOP2 activity⁶. TOP2 α immunodepletion caused replication fork structures to persist (Fig. 1F, lanes 1-4) but to a lesser extent than following TOP2-i treatment (Fig. 1B, lanes 1-4), leading to efficient catenane formation (cats; Fig. 1F, lanes 1-4), as expected⁶. Total DNA synthesis was unaffected (Fig. 1G), consistent with TOP2 α immunodepletion interfering with termination and not earlier stages of DNA replication, as previously described⁶. Importantly, TOP3 α immunodepletion delayed fork merger and this was rescued by addition of recombinant TRR (Fig. 1F, lanes 5-12, Fig. 1H). Thus, TOP3 α promotes fork merger in the absence of TOP2 α , not just upon TOP2 α inhibition. The catenanes formed in TOP2 α -immunodepleted extracts included a subset of slower migrating species that contained fewer intertwinings (Cats-; Fig. 1F, lanes 1-4), also as previously found⁶. Importantly TOP3 α immunodepletion blocked formation of these species and this condition yielded only the faster-migrating catenanes with more intertwinings (Cats+; Fig. 1F, lanes 5-8). This change in catenane distribution was rescued upon re-addition of recombinant TRR (Fig. 1F, lanes 9-12), which

demonstrated that TOP3 α promoted unlinking of catenanes, either during or after DNA replication. The fork merger and unlinking defects were also observed using immunodepletion alone, without small-molecule inhibition (Fig. 4B, lanes 1-4 and 9-12). Overall, these data indicate that TOP3 α promotes fork merger and unlinking of daughter DNA duplexes in the absence of TOP2 α activity.

TOP3 α promotes fork merger independently of RMI1-RMI2-BLM

Our initial analysis (Fig. 1) indicated that TOP3 α or another component of the TRR complex promotes fork merger. Additionally, TOP3 α can play a non-enzymatic structural role^{53,54} and can also form the BLM-TOP3 α -RMI1-RMI2 (BTRR) complex⁵⁵⁻⁵⁷. We therefore investigated which TOP3 α functions and interacting partners were required to promote fork merger when TOP2 α activity is compromised (as in Fig. 1).

We first tested the requirement for the catalytic activity of TOP3 α . To this end we purified TRR complex containing the Y367F mutant of TOP3 α (Fig. S2A), which eliminates the conserved catalytic tyrosine residue⁶⁵, then tested its ability to rescue fork merger defects (Fig. 2A). The Wild Type TRR complex rescued fork merger (WT; Fig. 2B lanes 5-8, Fig. 2C) and did not impact DNA synthesis (Fig. S2B), as expected (Fig. 1A-D). In contrast, the catalytic mutant was indistinguishable from the control (Mut; Fig. 2B lanes 9-12, Fig. 2C). Thus, the ability of the TRR complex to promote fork merger was entirely dependent on the catalytic activity of TOP3 α .

We next tested whether TOP3 α itself could promote fork merger, or whether RMI1-RMI2 was required. We first immunodepleted RMI1 but found that this co-depleted TOP3 α (Fig. S2C), consistent with reports that TOP3 α forms a stable complex with RMI1-RMI2. This prevented us from independently assessing the contributions of RMI1-RMI2 by immunodepletion. We therefore tested the ability of TOP3 α alone to rescue fork merger defects, compared to the complete TRR complex. We were unable to obtain catalytically active *Xenopus* TOP3 α independent of RMI1-

RMI2. Instead we used human TOP3 α (hTOP3 α). Surprisingly, hTOP3 α alone was able to promote fork merger to the same extent as the *Xenopus* TRR complex (Fig. 2D-F, Fig. S2E). This was not due to the ability of residual *Xenopus* RMI1-RMI2 in the extract because xRMI1 was co-depleted using an xRMI1 antibody for this experiment (as in Fig. S2C). Thus, TOP3 α alone could promote fork merger. However, hTOP3 α only partially rescued formation of catenanes with fewer intertwinings (Cats-; Fig. 2E, Fig. S2D,F) suggesting RMI1-RMI2 play some role. Overall, these data show TOP3 α alone can support fork merger.

Finally, we examined the role of the BLM helicase, which binds TOP3 α -RMI1-RMI2 to form the BTRR complex. We used a previously validated antibody⁶⁶ to immunodeplete BLM from extracts (Fig. S2G). This antibody efficiently immunodepleted BLM and co-immunoprecipitated TOP3 α (Fig. S2G lanes 5-7), but left most TOP3 α in extracts (Fig. S2G lanes 1-4), indicating that most TOP3 α was not bound to BLM. We then examined fork merger in extracts immunodepleted of TOP3 α or BLM or both (Fig. 2G). Surprisingly, BLM immunodepletion did not appreciably impair fork merger in mock- or TOP3 α -depleted extracts (Fig. 2H-I). Thus, BLM helicase did not appear to be involved in the fork merger role we had identified for TOP3 α .

Altogether, these results (Fig. 2) indicate that the catalytic activity of TOP3 α promotes fork merger, independent of RMI1-RMI2 and BLM helicase.

TOP3 α acts primarily during replication termination

We previously found that TOP2 α unlinks DNA throughout replication (i.e. during elongation) to prevent buildup of topological stress that would otherwise stall termination⁶. Similarly, bacterial topo III has been reported to operate throughout replication^{9,33} as well as during termination²⁷. We therefore investigated whether TOP3 α operates throughout replication, during termination, or both (Fig. S3A).

173

174 To test when during replication TOP3 α functions, we wanted to inhibit TOP3 α at different stages
175 of replication. However, no inhibitors of TOP3 α were available. We therefore raised an antibody
176 targeting an extended region of TOP3 α and tested whether it could act as an inhibitor of TOP3 α
177 function.

178 To determine when TOP3 α acts during replication, we generated a blocking antibody targeting an
179 extended region of TOP3 α . Addition of this TOP3 α antibody had no effect on DNA replication
180 when TOP2 α was fully active (Fig. 3A, Fig. 3B, lanes 1-4, 9-12), as expected (Fig. S1D-H).
181 However, upon addition of TOP2-i the TOP3 α antibody blocked fork merger (Fig. 3B, lanes 5-8,
182 13-16), reproducing the immunodepletion effect (Fig. 1A-D). The antibody had no effect when
183 TOP3 α was absent (Fig. S3C-F), indicating that it was specific. Thus, this TOP3 α -blocking
184 antibody selectively inactivates TOP3 α in extracts, allowing its requirement to be tested at
185 different stages of replication.

186

187 To test whether TOP3 α acts primarily late during replication we added TOP3 α -blocking antibody
188 at 10 minutes, when most DNA synthesis was complete (~70% complete; Fig. S3B). Antibody
189 addition at this late time point reproduced most of the fork merger defect observed when the
190 antibody was added at the onset of replication (Fig. 3B, lanes 5-8, 13-20, Fig. 3C). We note that
191 this result likely underestimates the extent to which TOP3 α functions during termination because
192 the antibody may take time to fully inhibit TOP3 α . Still, these data show that most of the effect of
193 TOP3 α on fork merger can be attributed to it functioning late during replication.

194

195 To test this timing independently of antibody-mediated inactivation, we immunodepleted TOP2
196 and TOP3, then added back TRR either late during replication or at the onset of DNA replication
197 (Fig. 3D). Analogously to the previous result (Fig. 3A-C), we found that addition of TRR rescued
198 fork merger to the same extent, whether added at the onset of DNA replication (T=0'; Fig. 3E,

lanes 7-12, Fig. 3F) or late during replication (T=20'; Fig. 3E, lanes 13-18, Fig. 3F). Although immunodepletion resulted in nonspecific slowing of DNA synthesis (compare Fig. S3B to Fig. S3G), this did not affect our experimental interpretation because we added xTRR after most DNA synthesis was complete (20 minutes; Fig. S3G). These data indicate that most of the effect of TOP3 α on fork merger can be attributed to its role during termination.

Although fork merger was unaffected by the timing of TOP3 α add back, there was a small difference in the number of less intertwined catenanes (Cats-; Fig. 3D) produced when TRR was added back late during replication (Fig. 3E, lanes 7-18, Fig. S3H-I). We therefore added back TRR even later during replication (T=60'; Fig. 3E, lanes 19-24) when most fork merger was complete (Fig. 3F). Doing so eliminated most of the less intertwined catenanes (Fig. S3H-I) even though the extent of replication was essentially unaltered between the two time points (Fig. S3G). Thus, once forks merge the ability of TOP3 α to carry out unlinking is substantially diminished, suggesting that it is only able to act at forks and not on fully replicated DNA.

We previously showed that MCM10 and RTEL1 promote fork merger in the absence of TOP2 α and this reflects a generalized role for these proteins in overcoming replication fork stalling⁶⁷. To determine whether TOP3 α functions the same way, we assessed the impact of TOP3 α immunodepletion on fork progression through an array of LacR proteins, which stalls replication forks (Fig. S3J). Immunodepletion of TOP3 α had no effect on the ability of forks to progress through a LacR array (Fig. S3K-L). Thus, the role of TOP3 α in promoting fork merger is specific to topological stress and does not reflect a general role for TOP3 α in overcoming fork stalling.

Together, these data (Fig. 3) show that TOP3 α operates primarily at replication forks during replication termination, rather than throughout DNA replication or as a general response to fork stalling.

TOP3 α targets lagging strand ssDNA

TOP3 α may target lagging strand template ssDNA ('lagging ssDNA')^{24,33}, which should be present throughout replication (Fig. S4Ai). However, it could equally target ssDNA generated by uncoupling (Fig. S4Aii)⁴⁴⁻⁴⁷ or dsDNA given that it can unlink double-stranded DNA catenanes (Fig. S4Aiii)^{49,50}. We therefore set out to directly test the model that TOP3 α targets lagging strand ssDNA. The major prediction of this model is that increased lagging ssDNA should result in increased TOP3 α activity. In our experiment (Fig. 1) this would mean that increased lagging strand ssDNA should at least partially rescue the decatenation defect in TOP2 α mutants in a TOP3 α -dependent manner (Fig. S4B). To test this, we took advantage of the Pol α inhibitor CD437 (Pol α -i)⁶⁸, which we found to specifically inhibit lagging strand synthesis in *Xenopus* egg extracts (Fig. S4C-D), as in cells^{69,70}.

To test the effect of lagging ssDNA on catenane resolution, we replicated plasmid DNA in extracts immunodepleted for either TOP2 α or both TOP2 α and TOP3 α , in the presence or absence of a partially inhibitory dose of Pol α -i, and quantified the abundance of the less-catenated Cats- (Fig. 4A)⁶ as a read-out for catenane abundance. We omitted TOP2-i from the reaction, which would have severely limited formation of Cats-⁶. TOP3 α immunodepletion impaired disappearance of replication fork structures (θ s; Fig. 4B, lanes 1-4, 9-12) and inhibited formation of partially-decatenated species (Cats-; Fig. 4B, lanes 1-4, 9-12). Thus, the earlier fork merger and decatenation defects following loss of TOP3 α (Fig 1) were observed in the absence of ICRF-193. Addition of Pol α -i reduced the total amount of DNA synthesis (Fig. S4E), which is expected because Pol α -Primase activity is required for initial priming of the leading strand during DNA replication⁷¹. Strikingly, Pol α -i treatment greatly increased the proportion of less intertwined catenanes formed (Fig. 4B, lanes 1-8, Fig. 4C, Fig. S4F) in TOP2 α -immunodepleted extracts, which indicated that decatenation was increased by Pol α -i treatment. Importantly, this effect was

severely reduced in extracts immunodepleted of TOP2 α and TOP3 α (Fig. 4B, lanes 9-16, Fig. 4C, Fig. S4F). Thus, increased lagging ssDNA promotes decatenation in a manner that is dependent on TOP3 α .

Formation of less intertwined catenanes (Cats-; Fig. 4A-C) is an indirect readout of decatenation. To directly examine decatenation, we used 2-D gel electrophoresis to resolve daughter DNA circles into a discrete series of catenanes differing in linkage number (Fig. 4D-I and ⁶). We analyzed samples from the 30 minute time point of Fig. 4B when catenanes had initially formed (Fig. S4F) to prevent residual decatenation activity from remaining TOP2 α from confounding the results. Following TOP2 α immunodepletion the linkage median was approximately 14 (Fig. 4E, Fig. 4I). This was lower than we had previously reported ⁶, because omission of TOP2-i allowed some amount of unlinking to take place. Addition of Pol α -i shifted the distribution of linkages down, resulting in a reduction to approximately 4 (Fig. 4F, Fig. 4I), consistent with our assessment that Pol α -i reduces the number of intertwinings in the catenated products of replication (Fig. 4A-C). Immunodepletion of TOP3 α shifted the distribution of linkages up and the median was approximately 20 (Fig. 4G, Fig. 4I). Addition of Pol α -i once again reduced the linkage median to approximately 14 (Fig. 4H, Fig. 4I) resulting in a smaller reduction than when TOP3 α was present. There was no net effect on linkage median when TOP3 α was immunodepleted and Pol α -i was added, as evidenced by a linkage median of approximately 14 in both cases (Fig. 4E, Fig. 4H, Fig. 4I). Thus, increased lagging ssDNA reduces the number of intertwinings in the catenated products of replication through a mechanism that involves TOP3 α .

Collectively, our data (Fig. 4) provide direct evidence for the model that TOP3 α acts on lagging ssDNA.

TOP3 α resolves intramolecular ssDNA linkages

Our finding that TOP3 α specifically acts on lagging ssDNA raises the possibility that TOP3 α may be uniquely important when lagging strand DNA synthesis is inhibited. To test this possibility, we replicated plasmid DNA in the presence of a high concentration of Pol α -i using either mock- or TOP3 α -immunodepleted extracts (Fig. 5A). No major differences in total DNA synthesis were observed (Fig. S5A). However, we observed the formation of a unique DNA species, that migrated slightly faster than nicked circular monomers, specifically in TOP3 α -immunodepleted conditions (CM*; Fig. 5B, lanes 1-10, Fig. 5C). Thus, in the presence of elevated lagging ssDNA, TOP3 α suppresses the formation of a unique DNA species.

To gain additional information about the structure of the unique DNA species we performed 2-D gel electrophoresis (Fig. 5D-F). The species migrated within the area corresponding to monomers and migrated similarly to linear species. However, when the samples were nicked with a site-specific nicking enzyme the species was predominantly converted to nicked circular monomer (Fig. 5G-H), indicating that it was a topoisomer of covalently closed circular monomer. We thus named the species CM* to reflect its identity as an altered Circular Monomeric plasmid.

We noticed that nicking CM* led to formation of an arc of topoisomers emanating from the nicked circular monomers (linkages; Fig. 5H). This pattern would typically be interpreted as intramolecular double-stranded DNA (dsDNA) knots. However, we disfavored this possibility because TOP3 α appears to act on ssDNA during DNA replication (Fig. 3, Fig. 4), which made it difficult to envisage how its absence could lead to dsDNA knots. We reasoned that these species (linkages; Fig. 5H) could correspond to ssDNA linkages, in which case we would expect them to be resolved by nicking both DNA strands (i.e. by allowing both strands to freely rotate). Importantly, nicking both strands would not be expected to resolve dsDNA knots as breakage of both strands along with strand passage is required. Nicking both DNA strands did indeed resolve the linkages (Fig. S5B-C). Thus, the properties of CM* are consistent with the presence of some

intramolecular ssDNA linkages.

The best characterized substrate for TOP3 α is hemicatenanes²², which are ssDNA linkages between different DNA molecules (Fig. S5D). We reasoned that the intramolecular ssDNA linkages present in CM* (Fig. 5H) should exhibit similar behavior (Fig. S5D). We therefore treated CM* with purified TRR complex (Fig. 5I-J), which resolves hemicatenanes. TRR was able to directly resolve CM* (Fig. 5J, Fig. S5E) while having minimal effect on supercoiled circular monomers (Fig. S5F), supporting the idea that it contained hemicatenane-like structures. We reasoned that any such structures should also be hypersensitive to ssDNA endonucleases so we also tested whether they could be resolved by T7 endonuclease (Fig. 5I, Fig. 5K, Fig. S5G-H) or S1 endonuclease (Fig. 5I, Fig. 5L, Fig. S5I-J). CM* was resolved by both T7 endonuclease (Fig. 5K, compare lanes 3-4) and S1 endonuclease (Fig. 5L, compare lanes 3-4). Under these same conditions, supercoiled plasmids were unaffected indicating that this effect was specific to single-stranded DNA (scCMs; Fig. 5K, lanes 1-2; Fig. S5H; Fig. 5L, lanes 1-2; Fig. S5J). Thus, the properties of CM* suggest it contains intramolecular single-stranded linkages.

Overall, our data (Fig. 5) show that high levels of lagging ssDNA create a requirement for TOP3 α to suppress intramolecular ssDNA linkages even when TOP2 α is present.

DISCUSSION

We have identified a mechanism by which TOP3 α directly unlinks daughter DNA to support replication termination when TOP2 α activity is compromised (Fig. 6A-B). To do so, TOP3 α targets lagging single-stranded DNA (ssDNA) independent of BLM and RMI1-RMI2. We also found that TOP3 α plays an additional role in resolving intramolecular ssDNA intertwinings that can arise in the presence of high levels of lagging ssDNA (Fig. 6C). Thus, TOP3 α resolves both inter- and intramolecular intertwinings during vertebrate DNA replication.

TOP3 α function during DNA replication

Our finding that TOP3 α exerts no detectable effect during normal DNA replication in *Xenopus* egg extracts (Fig. S1D-H) differs from a previous study that found a role for TOP3 α ³⁸. We favor the idea that the cellular role for TOP3 α during replication reflects some facet of nuclear biology that is missing from the *Xenopus* egg extract system, such as transcription-replication collisions ³⁹ given that the extracts we use are transcriptionally inactive unless provided with altered DNA template or additional transcription factors ^{72,73}. In bacteria, topo III is present at replication forks, ³³ which could reflect a role in ongoing replication or fork-associated recombination. In contrast, we show that TOP3 α resolves pre-catenanes generated during replication (Fig. 4) and that TOP3 α alone can support this activity (Fig. 2), indicating a direct replication function independent of its recombination function involving BLM-RMI1-RMI2.

We previously reported that TOP2 α resolves pre-catenanes and loss of TOP2 α causes replication forks to stall during termination ⁶. Here we find that TOP3 α similarly resolves pre-catenanes when TOP2 α is absent and loss of TOP3 α exacerbates stalling during termination (Fig. 1). In contrast to TOP2 α , which resolves pre-catenanes throughout replication ⁶, TOP3 α predominantly resolves pre-catenanes during termination (Fig. 3). We favor a model whereby TOP2 α resolves most pre-catenanes and thus obviates a need for TOP3 α under normal conditions, but when TOP2 α

function is absent TOP3 α can act at termination to resolve any remaining pre-catenanes.

We previously found that the helicase RTEL1, recruited by the replisome component MCM10, promotes fork merger during termination⁶⁷. As for TOP3 α , MCM10-RTEL1 is crucial for fork merger when forks stall due to lack of TOP2 α . However, MCM10-RTEL1 promotes fork progression as part of a generalized response to stalling that is not specific to topological stress or termination⁶⁷. In contrast, we found that TOP3 α acts when forks stall during termination due to topological stress (Fig. 3) and not when forks are stalled at a replication barrier (Fig. S3J-L). Thus, MCM10-RTEL1 and TOP3 α can both support fork progression when forks stall during termination but operate through distinct mechanisms.

Loss of both TOP2 α and TOP3 α leads to a synergistic block to fork convergence (Fig. 1). In human cells, metaphase-specific TOP3 α depletion and TOP2 α inhibition synergistically increase ultrafine anaphase bridges⁵⁸. This mitotic requirement is distinct from the replication function identified here. Because anaphase bridges can arise from unreplicated stretches of DNA⁷⁴, TOP3 α may also suppress bridge formation by promoting replication completion before mitosis. Cells from patients with biallelic TOP3A mutations exhibit chromosome-segregation defects attributed to unresolved replication or recombination intermediates²⁵. Impaired resolution of replication-generated catenanes could therefore contribute to genome instability in TOP3A-associated disease, alongside defects in recombination-intermediate dissolution.

TOP3 α Independence from BLM and RMI1-RMI2

We identified a nuclear role for TOP3 α that is independent of BLM and RMI1-RMI2 (Fig. 2). Fork merger by TOP3 α was unaltered by the loss of BLM and TOP3 α was sufficient to rescue the TOP3 α immunodepletion effect. However, BLM and RMI1-RMI2 are clearly important for TOP3 α function, so this observation suggests that some facet of TOP3 α unlinking at replication forks

obviates the requirement. For example, the replicative CMG helicase may substitute for the unwinding activity of the BLM helicase and other protein-protein interactions may substitute for RMI1-RMI2 function. We note that the independence of RMI1-RMI2-BLM matches the situation during mitochondrial DNA replication⁴⁰, suggesting it could be generally true of TOP3 α function during DNA replication. Given that BLM and RMI1-RMI2 play crucial roles in cells^{56,75-77} and during other aspects of replication⁶⁶ it will be important to determine in which circumstances they are required for TOP3 α activity.

TOP3 α acts on Lagging Strands

Our data show that impaired lagging strand synthesis increased daughter strand unlinking and this increase was dependent on TOP3 α (Fig. 4). Thus, we provide direct evidence that TOP3 α targets lagging ssDNA. We found no ability of TOP3 α to resolve double-stranded DNA (dsDNA) intertwinings, despite its ability to do so in a range of circumstances^{32,49,50}. These data argue that under normal circumstances TOP3 α acts specifically on ssDNA structures to resolve DNA intertwinings.

A key question is why TOP3 α acts preferentially during termination even though lagging-strand ssDNA is present throughout replication. One possibility is that TOP3 α requires persistent ssDNA but during fork progression lagging-strand ssDNA is too transient to be acted upon and persists long enough only when forks stall during termination. Alternatively, TOP3 α may not be able to act on pre-catenanes during earlier stages of replication if they preferentially partition into the fully double-stranded regions of the daughter strands. Distinguishing these models will be an important goal of future work.

TOP3 α acts on intramolecular Intertwinings

An unexpected outcome of our investigations was the discovery that high levels of lagging-strand

399 ssDNA can give rise to intramolecular ssDNA intertwines (Fig. 5, Fig. 6C). To our knowledge, such
400 structures have not been previously described in the context of DNA replication, though a
401 potentially similar structure was speculated to arise from DNA looping ⁷⁸. The simplest explanation
402 for these structures is that they are essentially intramolecular hemicatenanes, which typically arise
403 from Holliday junctions formed during HR ²². However, we disfavor this as a source of
404 intramolecular ssDNA linkages because homologous recombination would be expected to favor
405 sister molecules, whereas we instead observe a preference for formation of these intertwines
406 within the same molecule. Thus, the origin of these structures is highly uncertain. Although these
407 events are rare, they could conceivably give rise to highly mutagenic extrachromosomal DNA ⁷⁹.
408 An important future direction is to establish the exact causes and consequences of these
409 intramolecular intertwines.

Methods

Xenopus Egg Extracts

Xenopus egg extracts were prepared from *Xenopus laevis* wild-type males and females (Nasco) as previously described⁸⁰. All experiments and protocols were approved by the Vanderbilt University Division of Animal Care (DAC) and Institutional Animal Care and Use committee (IACUC).

Plasmid Construction and Preparation

The construction of pJD152 (p[lacOx16]) and pJD156 (p[lacOx32]) was described previously⁸¹. pJD156 (p[lacOx32]) was used in Fig. S3J-L. All other experiments used pJD152(p[lacOx16]) as a template for replication.

Protein Purification

Biotinylated LacR protein was expressed in *Escherichia coli* bacteria cells and purified as described previously^{61,81}.

Recombinant wild-type (WT) and catalytic mutant (TOP3 α Y367F mutant) *Xenopus* TOP3 α -RMI1-RMI2 complex (xTRR) were expressed and purified by Genscript (U1353074G0 for WT; U062D412G0 for mutant). xTRR was expressed in insect cells as a complex, then purified via affinity purification against HIS and FLAG tags. In Fig. 1 WT xTRR was used at a final concentration of 42.68 μ g/ml. In Fig. 2 wild type xTRR was used at a final concentration of 5.12 μ g/ml while mutant xTRR was used at a final concentration of 13.35 μ g/ml. These concentrations of WT and mutant xTRR were selected quantitatively for having the same amount of TOP3 α protein (see Fig. S2A).

To purify human TOP3 α , Rosetta2 (DE3) pLysS *E. coli* cells (Novagen, Sigma) were transformed

with the pET29H2 plasmid⁸², which encodes N-terminally His-tagged hTOP3A, and grown in 2YT media supplemented with kanamycin (50 µg/ml) and chloramphenicol (20 µg/ml) to OD600 of 0.5. Cells were cooled down and IPTG was added to a final concentration of 1 mM. Expression was carried out overnight at 18°C with constant shaking. Cells were harvested by centrifugation, resuspended in lysis buffer (20 mM Hepes-NaOH pH = 7.5, 1 M NaCl, 0.5 mM TCEP, 10% v/v glycerol, 0.1% v/v Triton X-100, 30 mM imidazole, 1 mM PMSF, and Roche Complete EDTA-free protease inhibitor), and lysed by sonication. After removing cell debris by centrifugation (Sorvall SS34 rotor, 18k rpm, 40 min), the soluble fraction was loaded onto a 5 ml Nickel-NTA column (HisPur Ni-NTA resin, Thermo Fisher) equilibrated with lysis buffer. After loading, the column was washed with lysis buffer and a wash buffer (20 mM Hepes-NaOH pH = 7.5, 0.2 M NaCl, 0.5 mM TCEP), and the protein was eluted in wash buffer supplemented with 250 mM imidazole. The eluted sample was diluted 1:1 with wash buffer and loaded onto a 5 ml HiTrap Heparin HP (Cytiva) column equilibrated with the same buffer. Protein was eluted with a NaCl gradient of 0.2 M to 1 M in wash buffer. Elution fractions were run on an SDS-PAGE gel and samples containing TOP3A were pooled. The pooled sample was concentrated to ~1 mg/ml with a 50 kDa ultra centrifugation filter (Amicon) and dialyzed into storage buffer (20 mM Hepes-NaOH pH = 7.5, 0.2 M NaCl, 10% v/v glycerol, 1 mM DTT). Concentration was determined using UV absorbance and aliquots were frozen in an ethanol/dry ice bath and stored at -80°C. Human TOP3α was used at a final concentration of 240 nM in Fig. 2E–F.

Antibodies

For immunodepletions, a TOP3α antibody (NEP 3858) was generated in rabbits by New England Peptide using purified peptide of *Xenopus* TOP3α composed of the sequence CHQPGHNRTNSPQNR.

For Western Blotting and acute inactivation of TOP3α a second, function-blocking TOP3α

antibody was generated. The following HIS-tagged polypeptide composed of the sequence
MHHHHHHHHHHNRPPQAAPRESNHLQVNQTSNRMGNSGDGRGRQPQTKTLKPPVVKPSTS
RGAPPIPGNSESENEVVCNCGVTAVQLTVRKEGPNQGRPFYKCNGGACNFFLWADQEDGGGQ
NAPAGPSTGRPSNAFNNSRAPDSFRSGGGSNDPGSEGQAVVCMCNQPAVTRTVQKDGANKG
RQFHTCSKPREQQCGYFQWADENVPPGSSSGFSGSSGGSSFSGGSGAQRNSGKKNKRSN
SFSGPTAKKPRSCG was purified by Genscript (U8068HWUG0). This antigen was then used to
generate affinity purified TOP3 α antibody (NEP 5606) in rabbits by New England Peptide. For
acute TOP3 α inactivation, this antibody was used at a final concentration of 0.233 mg/ml in all
experiments.

Xenopus antibodies targeting TOP2 α and BLM were previously generated^{6,66}. RMI1 antibody was
from Proteintech (Cat#: 14630-1-AP).

Immunodepletions

To deplete TOP2 α , TOP3 α , BLM, and RMI1 from *Xenopus* egg extracts, 1.29 volumes Protein A-
coupled magnetic beads (Dynabeads Protein A (30 mg/mL)) were bound to target antibody or
control anti-KLH (Keyhole limpet hemocyanin) 'mock' antibody (0.5 μ g antibody per 1 μ l beads).
Antibody-bound beads were then incubated with 1 volume of NPE or 0.5 volumes HSS for 20 min
at room temperature with end-over-end rotation and this was repeated once. Depleted extracts
were then collected and used for DNA replication (as above). For co-depletion of two proteins,
beads were combined for each round of depletion and the number of depletion rounds remained
constant. In all experiments beads bound to control anti-KLH were used to ensure that the
concentration of beads and anti-KLH was identical for all conditions.

DNA Replication in *Xenopus* egg extracts

DNA replication was performed as previously described⁶¹. High Speed Supernatant (HSS) was

incubated with nocodazole (3 ng/μl) and ATP regenerating system('ARS,' 20 mM phosphocreatine, 2 mM ATP and 5 ng/μl phosphokinase) for five minutes at room temperature. When indicated, LacR protein was bound to *lacO* repeats on plasmid DNA for one hour at room temperature before licensing DNA. To license plasmid DNA, one volume of 'licensing mix' was prepared by incubating plasmid DNA with HSS (final concentration: 6.75-13.5 ng/μl DNA) at room temperature for 30 minutes. NucleoPlasmic Extract (NPE) was supplemented with ARS, and DTT (final concentration: 2 mM). To initiate replication, 2 volumes of NPE mix were added to 1 volume of licensing mix. If applicable, replication forks that were stalled at a LacR-bound *lacO* array were released by IPTG addition as described previously^{61,81}. Samples were withdrawn into Replication Stop Solution (8 mM EDTA, 0.13% phosphoric acid, 10% ficoll, 5% SDS, 0.2% bromophenol blue, 80 mM Tris, pH 8) then vigorously mixed and treated with Proteinase K (929 ng/μl). Where indicated, ICRF-193 (TOP2-i) was dissolved in DMSO and used at a nominal final concentration of 200 μM. NPE mixtures were preincubated with ICRF-193 before replication initiation, typically for 10–15 minutes. Polymerase α inhibitor (Polα-i, CD-437) was dissolved in DMSO and used at a final concentration of 6 μM for Fig. 4, 200 μM for Fig. S4C-D, and 50 μM for Fig. 5. Polα-i was used with a final concentration of 4% (v/v) DMSO in the reaction. ICRF-193 was included in the TOP2-inhibited reactions shown in Figs. 1-3 and S3C-F, including after TOP2α immunodepletion to suppress residual TOP2 activity. ICRF-193 was omitted from all reactions in Figs. 4 and 5. Vehicle-treated reactions in Fig. 4 contained neither ICRF-193 nor CD437.

Analysis of replication intermediates

Replication intermediates were separated by native agarose gel electrophoresis and detected by autoradiography. Quantification was performed using ImageJ (NIH). To quantify total DNA synthesis, total lane signal was measured and normalized to the maximum signal across all time points and conditions (AU). The abundance of supercoiled monomers, linears, and other indicated species are expressed as a percentage of total lane signal (%).

514

515 **2-D Gel Electrophoresis**

516 Nicked 2-D catenane gels (Fig. 4) were performed as described previously ⁶. Briefly, purified DNA
517 was digested with 0.02 U/ μ L Nb.BtsI (New England Biolabs) and then separated on a 0.4%
518 agarose gel at 1 V/cm for 21.5 hours. The gel was stained with 0.3 μ g/ml ethidium bromide then
519 lanes were excised and recast in a 1.2% gel containing 0.3 μ g/ml ethidium bromide before being
520 separated at 4.8 V/cm for 12 hours. Radiolabeled DNA was detected by phosphorimaging. Total
521 catenane signal was quantified as a percentage of signal from the entire 2D gel autoradiograph.
522 To calculate linkage median, the signal from every 5 catenanes (e.g., species with 1-5 linkages,
523 species with 6-10 linkages, etc.) was measured and then expressed as a percentage (%) of total
524 catenanes signal.

525

526 For Fig. 5, purified DNA was either left uncut or digested with 0.4 U/ μ L Nb.BssSI (New England
527 Biolabs) to cut one strand only or 0.2 U/ μ L Nb.BtsI (New England Biolabs) to cut both strands.
528 Digests were performed in rCutsmart buffer for 1 hour at 37°C. DNA was then separated on a
529 0.4% agarose gel at 1 V/cm for 21.5 hours. Lanes were excised and recast in a 1.2% gel
530 containing 0.3 μ g/ml ethidium bromide before being separated at 4.5 V/cm for 12 hours.
531 Radiolabelled DNA was detected by phosphorimaging.

532

533 **DNA Enzyme Digests**

534 For all DNA enzyme digests, DNA samples were first purified via phenol-chloroform extraction
535 and then precipitated by ethanol precipitation before resuspension in 10mM Tris (pH 8.0) buffer.
536 For S1 Nuclease (Thermo Scientific, Cat#: EN0321) digestion, S1 nuclease was diluted with 1x
537 S1 Nuclease Reaction Buffer (Thermo Scientific Cat#: EN0321) and then approx. 10 ng of DNA
538 was treated with S1 Nuclease with a final reaction concentration as indicated (Fig. 5) in 1x S1
539 Nuclease Reaction Buffer at 23°C for 30 minutes. Reactions were stopped by addition of 1 μ l of

0.5 M EDTA and heated at 70°C for 10 minutes, as per manufacturer's instructions. 3 µl of 6x SDS Loading DNA Buffer (15% Ficoll-400, 66.5 mM EDTA, 20 mM Tris-HCl pH 8.0, 0.1% SDS, 0.09% Bromophenol blue) was added to each sample reaction before loading onto a 1% agarose TBE native gel. DNA products were separated at 5 V/cm for approximately 2 hours.

For T7 Endonuclease I (New England Biolabs, Cat#: M0302S) treatment, approximately 10 ng of DNA was treated with 3 U/ml T7 Endonuclease I (Fig. 5) in 1x Buffer 2 (New England Biolabs) at 23°C for 30 minutes. Reactions were stopped by addition of 2 µl of 6x SDS Loading DNA Buffer (15% Ficoll-400, 66.5 mM EDTA, 20 mM Tris-HCl pH 8.0, 0.1% SDS, 0.09% Bromophenol blue). DNA was loaded onto a 1% agarose TBE native gel and products were separated at 5 V/cm for approximately 2 hours.

Topoisomerase Treatment

For *Xenopus* Topoisomerase III α -RMI1-RMI2 (xTRR) (Genscript) protein treatment, approximately 10 ng of DNA was treated with a final concentration of 2.24 µg/ml xTRR protein at 37°C for 15 minutes in a buffer containing 35 mM Tris-HCl, pH 7.5, 3 mM MgCl₂, 60 mM NaCl, 20% glycerol, 1 mM dithiothreitol (DTT), 2 mM ATP, and 20 mM phosphocreatine. 1 unit of Creatine Phosphokinase was added last to each reaction. During the incubation, TopSTOP buffer was prepared from 20 µL Proteinase K (929 ng/µl), 80 µL filtered MilliQ water, and 100 µL 6% SDS. After incubation, a volume of TopSTOP buffer equivalent to a third of the reaction was added and then the reaction was incubated for a further 15 minutes at 37°C. Reactions were stopped by addition of 3 µl of 6x SDS Loading DNA Buffer (15% Ficoll-400, 66.5 mM EDTA, 20 mM Tris-HCl pH 8.0, 0.1% SDS, 0.09% Bromophenol blue). DNA was loaded onto a 1% agarose TBE native gel and products were separated at 5 V/cm for approximately 2 hours.

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Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions

JMD conceptualized and supervised the project and wrote the manuscript. SVR performed all experimental work and analysis except where otherwise noted. LVC made the initial discovery of synergy between inactivation of TOP2 α and TOP3 α . GMH purified human TOP3 α with supervision from KCN. DRH performed the experiment in Fig. S4C-D. SND performed the experiment in Fig. S3F. WGD supplied BLM antibody.

Figure Legends

Figure 1: TOP3 α promotes fork merger in the absence of TOP2 α

(A) Plasmid DNA was replicated using *Xenopus* egg extracts in the presence of [α - ^{32} P]dATP to label newly-synthesized DNA strands under TOP2-inhibited conditions (TOP2-i, ICRF-193), with or without TOP3 α immunodepletion and addition of recombinant *Xenopus* Topoisomerase III α -RMI1-RMI2 (xTRR) complex. The expected DNA structures (identified in ^{6,81}) are indicated.

(B) Replication was performed as in (A). Samples were separated on a native agarose gel and visualized by autoradiography. See also Supplemental Fig. S1D,I.

(C) Quantification of total DNA synthesis from (B).

(D) Quantification of fork merger from (B).

(E) Plasmid DNA was replicated using *Xenopus* egg extracts in the presence of [α - ^{32} P]dATP to label newly-synthesized DNA strands under TOP2-immunodepleted conditions, with or without TOP3 α immunodepletion and addition of recombinant Topoisomerase III α -RMI1-RMI2 (TRR) complex. ICRF-193 was included to suppress residual TOP2 activity after immunodepletion.

(F) Replication was performed as in (E). Samples were separated on a native agarose gel and visualized by autoradiography.

(G) Quantification of total DNA synthesis from (F).

(H) Quantification of fork merger from (F).

Figure 2: TOP3 α promotes fork merger independently of RMI1-RMI2 and BLM

(A) Plasmid DNA was replicated using *Xenopus* egg extracts in the presence of [α - ^{32} P]dATP to label newly-synthesized DNA strands under TOP2-inhibited (TOP2-i) and TOP3 α immunodepleted conditions supplemented with wild type (WT) xTRR complex or catalytic mutant (Mut).

(B) Replication was performed as in (A). Samples were separated on a native agarose gel and visualized by autoradiography. See also Supplemental Fig. S2A-B.

(C) Quantification of fork merger from (B).

(D) Plasmid DNA was replicated using *Xenopus* egg extracts in the presence of [α - ^{32} P]dATP to label newly-synthesized DNA strands under conditions co-depleted of TOP2 α , TOP3 α , and RMI1 with addition of recombinant xTRR complex or human TOP3 α (hTOP3 α). ICRF-193 was included to suppress residual TOP2 activity after immunodepletion.

(E) Replication was performed as in (D). Samples were separated on a native agarose gel and visualized by autoradiography.

(F) Quantification of fork merger from (E). See also Supplemental Fig. S2D. Quantification of an experimental replicate is shown in Fig. S2E-F.

(G) Plasmid DNA was replicated using *Xenopus* egg extracts in the presence of [α - ^{32} P]dATP to label newly-synthesized DNA strands under TOP2 inhibited (TOP2-i) conditions and immunodepleted of TOP3 α , BLM, or both.

(H) Replication was performed as in (G). Samples were separated on a native agarose gel and visualized by autoradiography. See also Supplemental Fig. S2G.

(I) Quantification of fork merger from (H).

Figure 3: TOP3 α acts on replication forks during termination

(A) Plasmid DNA was replicated using *Xenopus* egg extracts in the presence of [α - ^{32} P]dATP to label newly-synthesized DNA strands in the presence of vehicle (veh) or TOP2-i. At different time points extracts were supplemented with a TOP3 α -blocking antibody (Ab-TOP3 α) or a control (Ab-Control).

(B) Replication was performed as in (A). Samples were separated on a native agarose gel and visualized by autoradiography.

(C) Quantification of fork merger from (B). See also Supplemental Fig. S3B.

(D) Plasmid DNA was replicated using *Xenopus* egg extracts in the presence of [α - ^{32} P]dATP to label newly-synthesized DNA strands in extracts that were immunodepleted of TOP2 α and TOP3 α . ICRF-193 was included to suppress residual TOP2 activity after immunodepletion. At different time points recombinant xTRR was added back to the reaction.

(E) Replication was performed as in (D). Samples were separated on a native agarose gel and visualized by autoradiography. See also Supplemental Fig. S3G-I.

(F) Quantification of fork merger from (E).

Figure 4: TOP3 α acts on lagging strand template ssDNA

(A) Plasmid DNA was replicated using *Xenopus* egg extracts in the presence of [α - ^{32} P]dATP to label newly-synthesized DNA strands in extracts that were depleted of TOP2 α with or without co-depletion of TOP3 α . Extracts were also treated with either a small molecule inhibitor of POL α (POL α -i, CD437) or vehicle control (veh). ICRF-193 was omitted from all reactions, so vehicle-treated samples contained neither ICRF-193 nor CD437.

(B) Replication was performed as in (A). Samples were separated on a native agarose gel and visualized by autoradiography.

(C) Quantification of the lower mobility species of catenanes (Cats-) in (B). See also Fig. S4 E-F.

(D) Schematic of the different species of catenated plasmid dimers detected by 2-D gel electrophoresis following nicking of plasmid DNA to relieve supercoiling. Each spot on the indicated arc corresponds to a species of catenanes with a distinct number of linkages. Species with 21 or more linkages cannot be discerned from each other because the individual positions are too close to be resolved.

(E) The sample from Lane 3 in (B) was separated by 2-D gel electrophoresis and visualized by autoradiography. Note that only the section of the gel containing catenated species was analyzed.

(F) The sample from lane 7 in (B) was analyzed as in (E).

(G) The sample from lane 11 in (B) was analyzed as in (E).

(H) The sample from lane 15 in (B) was analyzed as in (E).

(I) Quantification of the median number of linkages for the catenated species detected in (E)-(H).

Figure 5: TOP3 α resolves intramolecular ssDNA intertwinings

(A) Plasmid DNA was replicated using *Xenopus* egg extracts in the presence of [α - ^{32} P]dATP to label newly-synthesized DNA strands in extracts that were treated with POL α -i with or without immunodepletion of TOP3 α .

(B) Replication was performed as in (A). Samples were separated on a native agarose gel and visualized by autoradiography.

(C) Quantification of CM*, which is the circular monomer species migrating between supercoiled (scCMs) and nicked (nCMs) circular monomer species, in (B). See also Supplemental Fig. S5A.

(D) Schematic of the migration of different monomeric species of circular monomers following 2-D gel electrophoresis.

(E) The sample from lane 5 in (B) was separated by 2-D gel electrophoresis and visualized by autoradiography. Note that only the section of the gel containing monomeric species was analyzed.

(F) The sample from lane 10 in (B) was analyzed as in (E).

(G) The sample from lane 5 in (B) was nicked using a site-specific nicking enzyme then analyzed as in (E).

(H) The sample from lane 10 in (B) was nicked using a site-specific nicking enzyme then analyzed as in (E).

(I) Plasmid DNA was replicated using *Xenopus* egg extracts in the presence of [α - ^{32} P]dATP to label newly-synthesized DNA strands in extracts that were treated with POL α -i and mock- or TOP3 α -immunodepleted. Replication products were then purified and treated with recombinant xTRR, T7 nuclease, or S1 nuclease.

(J) Replication was performed as in (I). The purified products of replication in TOP3 α -immunodepleted extracts were treated with either purified xTRR or buffer control then separated on a native agarose gel and visualized by autoradiography. Quantification is shown in Supplemental Fig. S5E-F.

(K) Replication was performed as in (I). Purified replication products were treated with either T7 nuclease or buffer control then separated on a native agarose gel and visualized by autoradiography. Quantification is shown in Supplemental Fig. S5G-H.

(L) Replication was performed as in (I). Purified replication products were treated with either S1 nuclease or buffer control then separated on a native agarose gel and visualized by autoradiography. Quantification is shown in Supplemental Fig. S5I-J.

Figure 6: Model for distinct functions of TOP3 α during vertebrate DNA replication.

(A) In the absence of TOP2, pre-catenanes accumulate and cause converging replication forks to stall, leading to slowed fork merger. TOP3 α resolves pre-catenanes by acting on lagging template ssDNA during termination, contributing to residual fork merger and decatenation.

(B) In the absence of both TOP2 and TOP3 pre-catenanes persist, resulting in prolonged replication fork stalling.

(C) In the presence of high levels of lagging ssDNA intramolecular ssDNA intertwines can form and these can be resolved by TOP3 α .

Supplemental Figure Legends

Supplemental Figure S1: Roles of TOP3 α during vertebrate DNA replication

(A) Cartoon depicting the different stages of vertebrate DNA replication during which TOP3 could act: elongation (i) and the fork merger stage of termination (ii) at lagging strand template ssDNA; and (iii) the decatenation stage of termination by acting on dsDNA catenanes.

(B) Cartoon depicting the role of TOP2 during vertebrate DNA replication termination. Under unperturbed conditions TOP2 primarily resolves catenanes behind replication forks to promote fork merger.

(C) Cartoon depicting a potential role for TOP3 during vertebrate DNA replication termination. In the absence of TOP2 catenanes accumulate, causing converging replication forks to stall. However, forks ultimately overcome stalling and limited decatenation takes place, which could in part reflect the activity of TOP3.

(D) Plasmid DNA was replicated using *Xenopus* egg extracts in the presence of [α - ^{32} P]dATP to label newly-synthesized DNA strands with or without TOP3 α immunodepletion.

(E) TOP3 α -immunodepleted extracts from (D) were analyzed by western blotting for TOP3 α alongside a dilution series of mock-immunodepleted extracts. TOP3 α is visible in the 100-fold dilution of mock-immunodepleted extract (lane 6) but not in the undiluted TOP3 α -immunodepleted extract, suggesting >99% of TOP3 α was removed.

(F) Replication was performed as in (D). Samples were separated on a native agarose gel and visualized by autoradiography.

(G) Quantification of total DNA synthesis from (F). Because DNA replication is asynchronous in this system, DNA synthesis reflects the rate of both initiation and elongation. No detectable difference was observed between mock- and TOP3 α -immunodepleted extracts, indicating that TOP3 α does not play a major role in initiation or elongation.

(H) The fork merger stage of termination was quantified from (F). No detectable difference was

observed between mock- and TOP3 α -immunodepleted extracts, indicating that TOP3 α does not play a major role in fork merger.

(I) Purified *Xenopus* TOP3 α -RMI1-RMI2 complex was analyzed by SDS-PAGE to assess purity. Three distinct bands are visible, corresponding to the different complex subunits and no other bands are discernable, indicating a high level of purity of the prep.

Supplemental Figure S2: Analysis of TOP3 α -interacting proteins

(A) Purified wild type (WT) and catalytic mutant (Mut) of *Xenopus* TOP3 α -RMI1-RMI2 complex were analyzed by SDS-PAGE to assess evenness of loading. Even levels of TOP3 α are detected for both conditions (~120 kDa band) but the mutant preparation contains higher levels of the RMI1 and RMI2 components (~80 kDa and ~16 kDa bands).

(B) Quantification of total DNA synthesis from Fig. 2B.

(C) Extracts were immunodepleted of RMI1 and then analyzed by western blotting for RMI1 and TOP3 α . Arrows indicate expected band positions. RMI1 immunodepletion removed >95% of both RMI1 and TOP3 α (compare lanes 1 and 5) indicating that TOP3 α co-depletes with RMI1.

(D) Quantification of Cats- species from Figure 2E.

(E) Quantification of an experimental replicate of Figure 2F.

(F) Quantification of an experimental replicate of (D).

(G) Mock- and BLM-immunodepleted Extracts from Figure 2G were analyzed by western blotting for BLM and TOP3 α . Arrows indicate expected band positions. * indicates background band adjacent to BLM that was not immunodepleted.

Supplemental Figure S3: Analysis of TOP3 α activity during different stages of DNA replication

(A) Cartoon model depicting different stages of vertebrate DNA replication during which TOP3 α acts.

(B) Quantification of total DNA synthesis from Fig. 3B.

(C) To assess the specificity of the TOP3 α -blocking antibody (Ab-TOP3 α), plasmid DNA was replicated using *Xenopus* egg extracts in the presence of [α -³²P]dATP to label newly-synthesized DNA strands under TOP2 α -immunodepleted conditions, with or without TOP3 α immunodepletion and addition of anti-TOP3 α . ICRF-193 was included to suppress residual TOP2 activity after immunodepletion. As a control, antibody targeting keyhole limpet hemocyanin (Ab-Control) was used.

(D) Samples from (C) were separated on a native agarose gel and visualized by autoradiography. Ab-TOP3 α reproduced the fork merger defect of TOP3 α immunodepletion (compare lanes 5-8 to 9-12) and there was no effect of Ab-TOP3 α when TOP3 α was immunodepleted (compare lanes 9-12 to 13-16). Thus, Ab-TOP3 α specifically inhibits TOP3 α .

(E) Fork merger was quantified from (D).

(F) Experimental replicate of (E).

(G) Quantification of total DNA synthesis from Fig. 3E.

(H) Quantification of the lower mobility species of catenanes (Cats-) in Fig. 3E.

(I) Bar Chart of the 120 minute time point from (H).

(J) To assess the role of TOP3 α during replication fork stalling, plasmid DNA containing a *lacO* array was bound with LacR then was replicated using *Xenopus* egg extracts in the presence of [α -³²P]dATP to label newly-synthesized DNA strands with or without TOP3 α immunodepletion. Upon encountering the LacR barrier forks stall and then gradually progress through the *lacO* array until the two converging forks encounter each other and undergo fork merger, which acts as a read out for fork progression following stalling. Residual LacR binding interferes with decatenation and creates a range of complex topoisomers so to directly monitor fork merger, DNA was purified then restriction digested to monitor the conversion of high molecular weight replication fork structures (Double Ys) to low molecular weight linear molecules (linears).

(K) Samples from (J) were separated on a native agarose gel and visualized by autoradiography.

(L) Fork merger was quantified from (K). There was no difference in the progression of stalled forks between mock- and TOP3 α -immunodepleted extracts, indicating that TOP3 α does not play a general role in promoting stalled fork progression.

Supplemental Figure S4: Analysis of TOP3 α activity on different replication fork structures

(A) Cartoon depicting the different replication fork structures TOP3 α could act upon: lagging strand template ssDNA at replication forks (i); template ssDNA on both leading and lagging strands exposed by replication fork uncoupling (ii); and duplex DNA behind the replication fork (iii).

(B) Cartoon depicting the experimental test from Figure 4 of the model that TOP3 α acts on lagging template ssDNA to resolve catenanes. Loss of TOP2 (Δ TOP2) results in accumulation of catenanes due to insufficient TOP2 activity. If TOP3 α acts on lagging ssDNA template to resolve catenanes then increasing the amount of lagging ssDNA template by inhibition of Pol α -Primase would be expected to reduce the number of catenanes and this reduction would be expected to depend on the presence of TOP3 α .

(C) Cartoon depicting the experimental setup used to test the specificity of (Pol α -i, CD437) for inhibition of lagging strand synthesis. Plasmid DNA containing a *lacO* array was bound with LacR then was replicated using *Xenopus* egg extracts in the presence of [α -³²P]dATP to label newly-synthesized DNA strands. Once forks were stalled at the array, they were restarted by addition of IPTG along with either vehicle control or Pol α -i.

(D) Replication was performed as in (C). At different time points, replication intermediates from different time points were purified, restriction digested, then analyzed by denaturing alkaline gel electrophoresis to monitor the nascent DNA strands from the leftward moving fork in (C). Prior to IPTG addition a single population of nascent DNA strands existed at a specific location, corresponding to forks stalled at the LacR barrier (lanes 1, 8), as expected. Following IPTG and Vehicle addition nascent strands increased in size as forks restarted (lanes 2-4) then were

converted to high molecular weight ligated products once leading strands were ligated to downstream lagging strands (lanes 4-7). Following IPTG and Pol α -i addition two populations of nascent strands were observed: a persistent stalled species that did not increase in size, corresponding to the expected stalled lagging strands (stalled lag; lanes 9-11); and a slower-migrating species that increased in size, corresponding to the expected restarted leading strands (restarted lead; lanes 9-11). Both species quickly resolved and were converted to ligated products (lanes 11-14) as expected if leading strand synthesis proceeded unhindered until encountering the downstream lagging strands then triggered ligation as normal. Thus, Pol α -i results in stalling of a subset of nascent strands while leaving another subset unaffected, as expected for inhibition of lagging but not leading strand DNA synthesis.

(E) Quantification of total DNA synthesis from Figure 4B

(F) Quantification of Cats- from Figure 4B.

Supplemental Figure S5: Characterization of TOP3 α function during replication in the presence of high levels of lagging ssDNA

(A) Quantification of total DNA synthesis from Fig. 5B.

(B) The sample from lane 5 in Fig. 5B was nicked on both strands using a site-specific nicking enzyme then analyzed as in Fig. 5E.

(C) The sample from lane 10 in Fig. 5B was nicked on both strands using a site-specific nicking enzyme then analyzed as in Fig. 5E.

(D) Cartoon depicting the composition of intramolecular ssDNA intertwinings that could explain the results in Fig. 5A-H.

(E) Quantification of CM* from Fig. 5J.

(F) Quantification of scCMs from Fig. 5J.

(G) Quantification of CM* from Fig. 5K.

(H) Quantification of scCMs from Fig. 5K.

- 835 (I) Quantification of CM* from Fig. 5L.
- 836 (J) Quantification of scCMs from Fig. 5L.
- 837 (K) Depiction of possible structure of CM* species from Fig. 5

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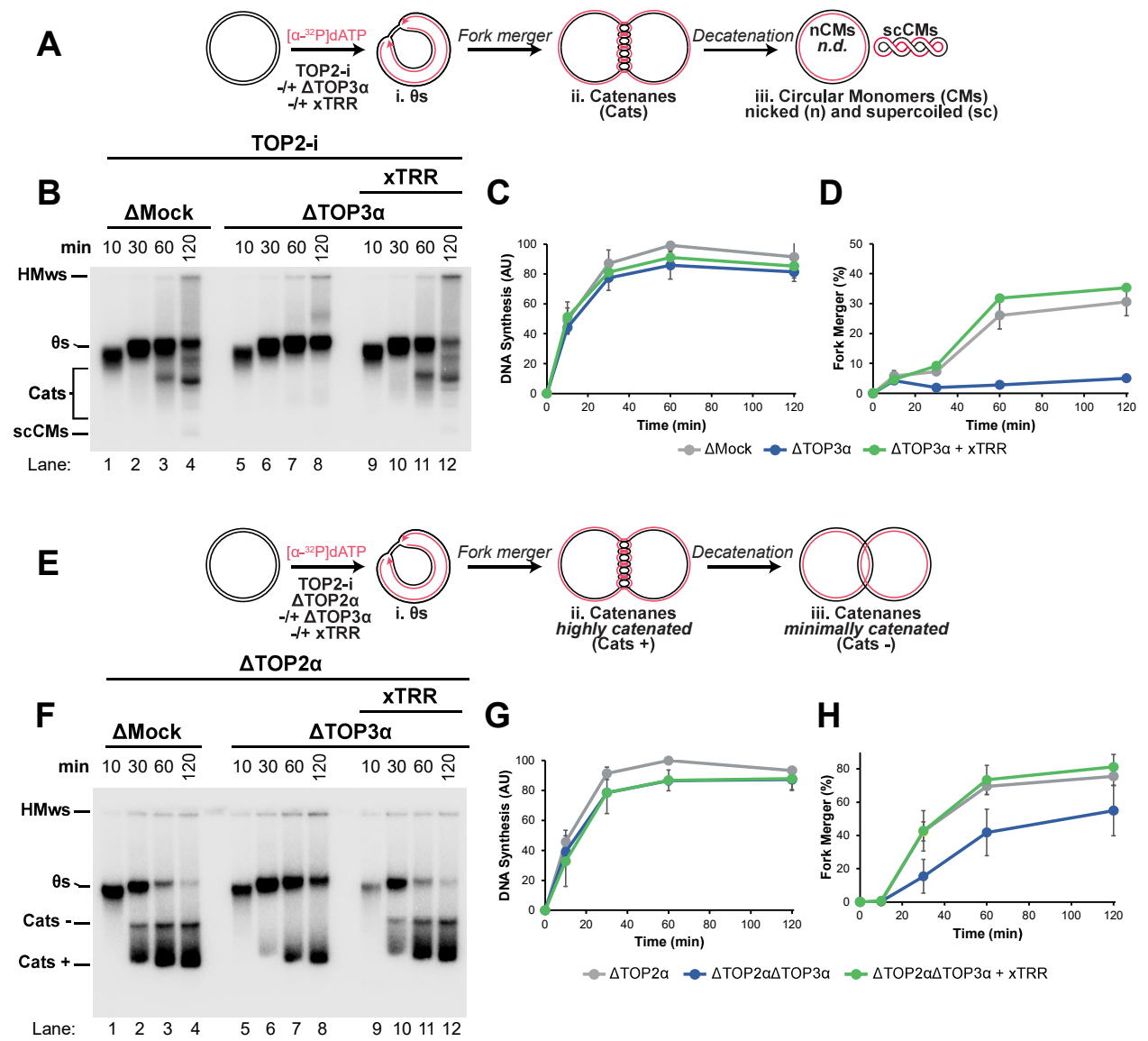
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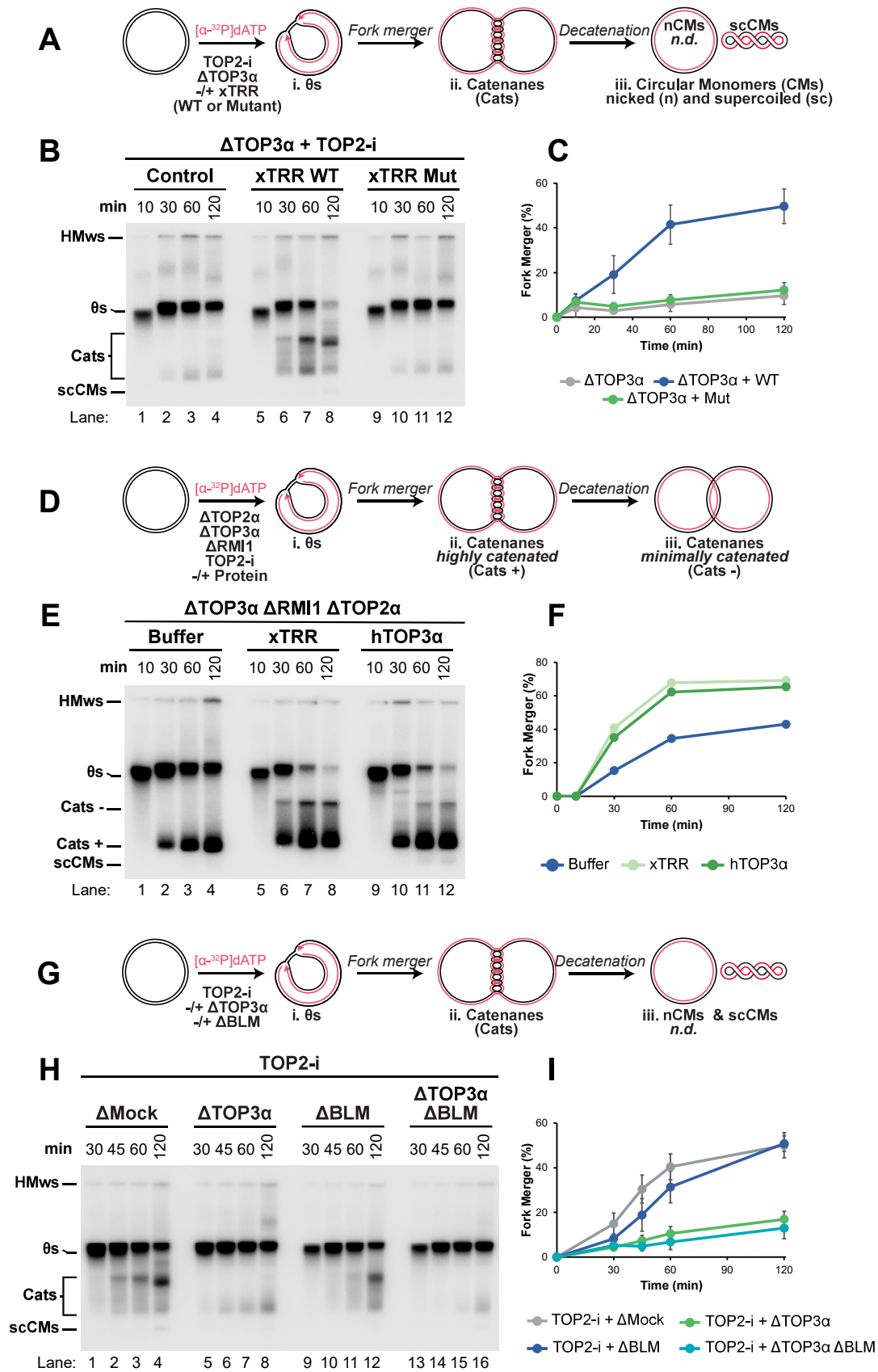
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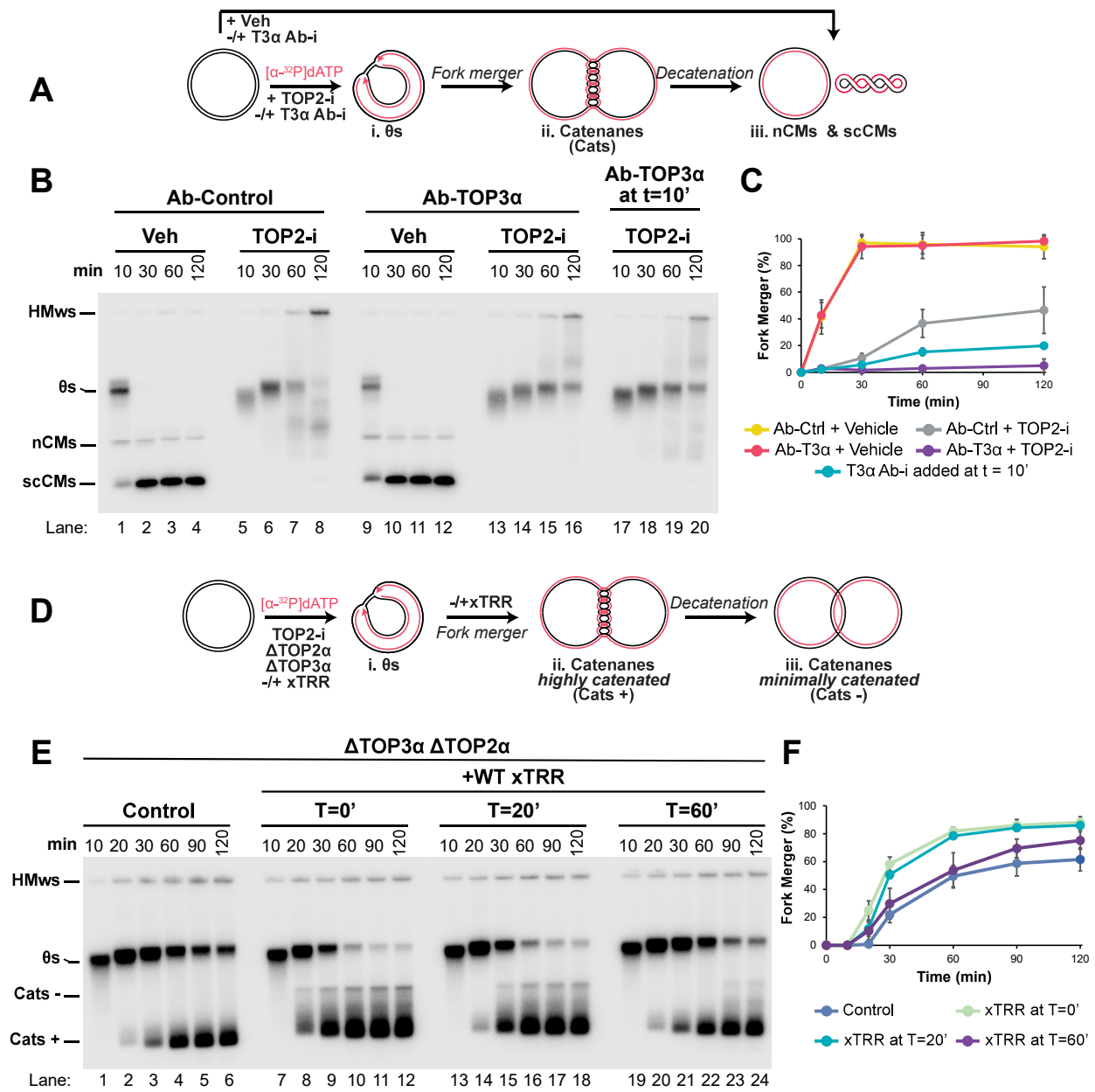
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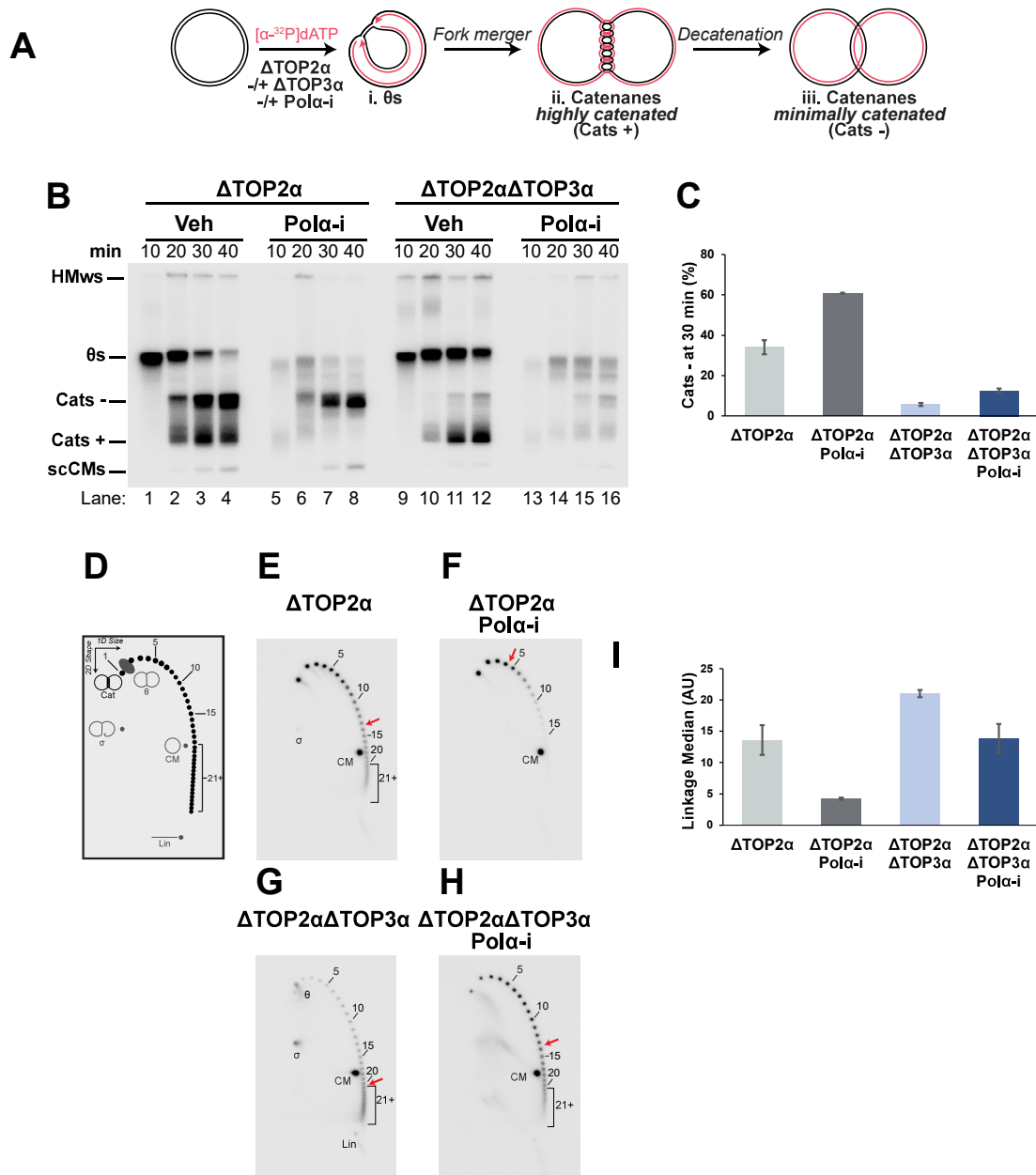
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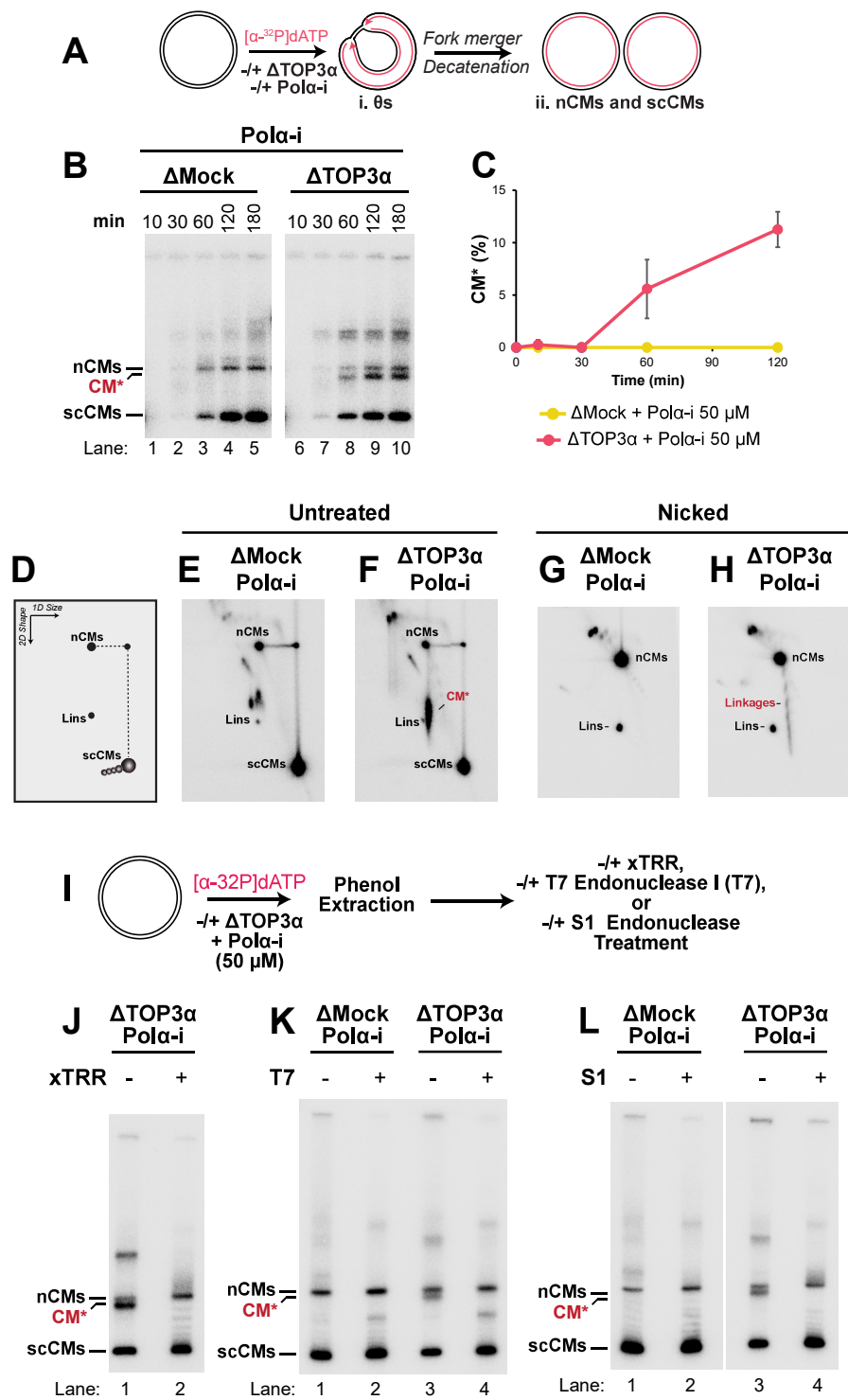
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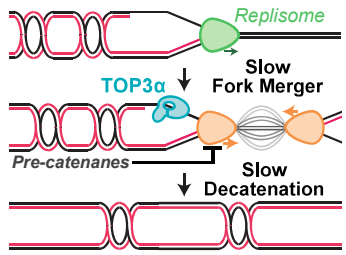




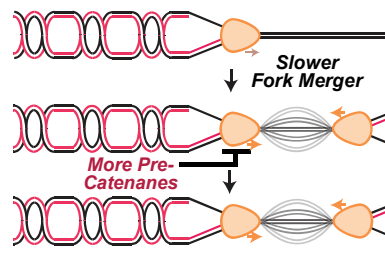




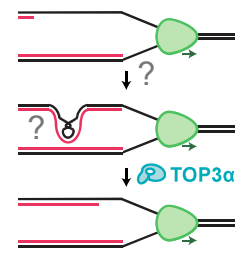
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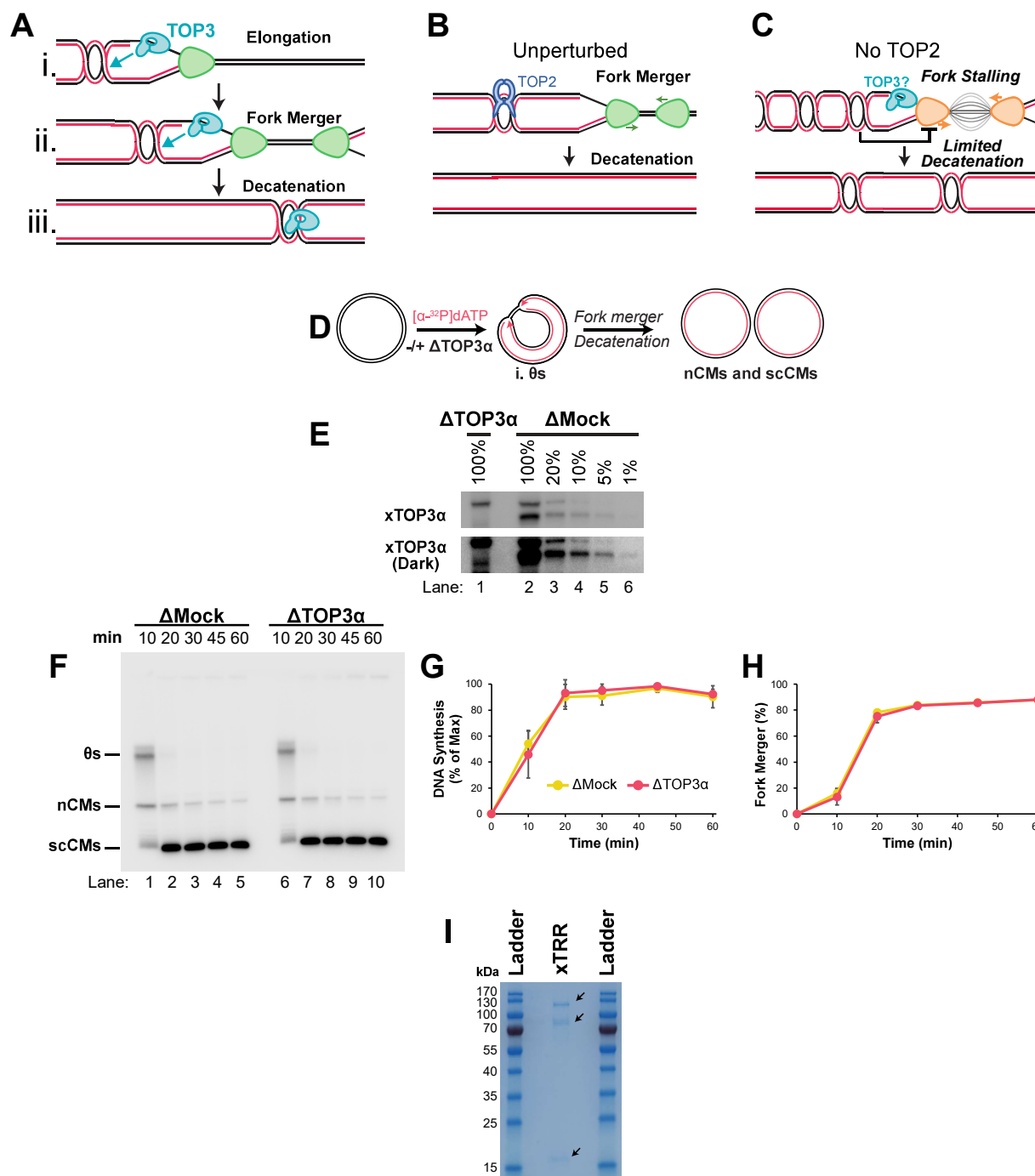


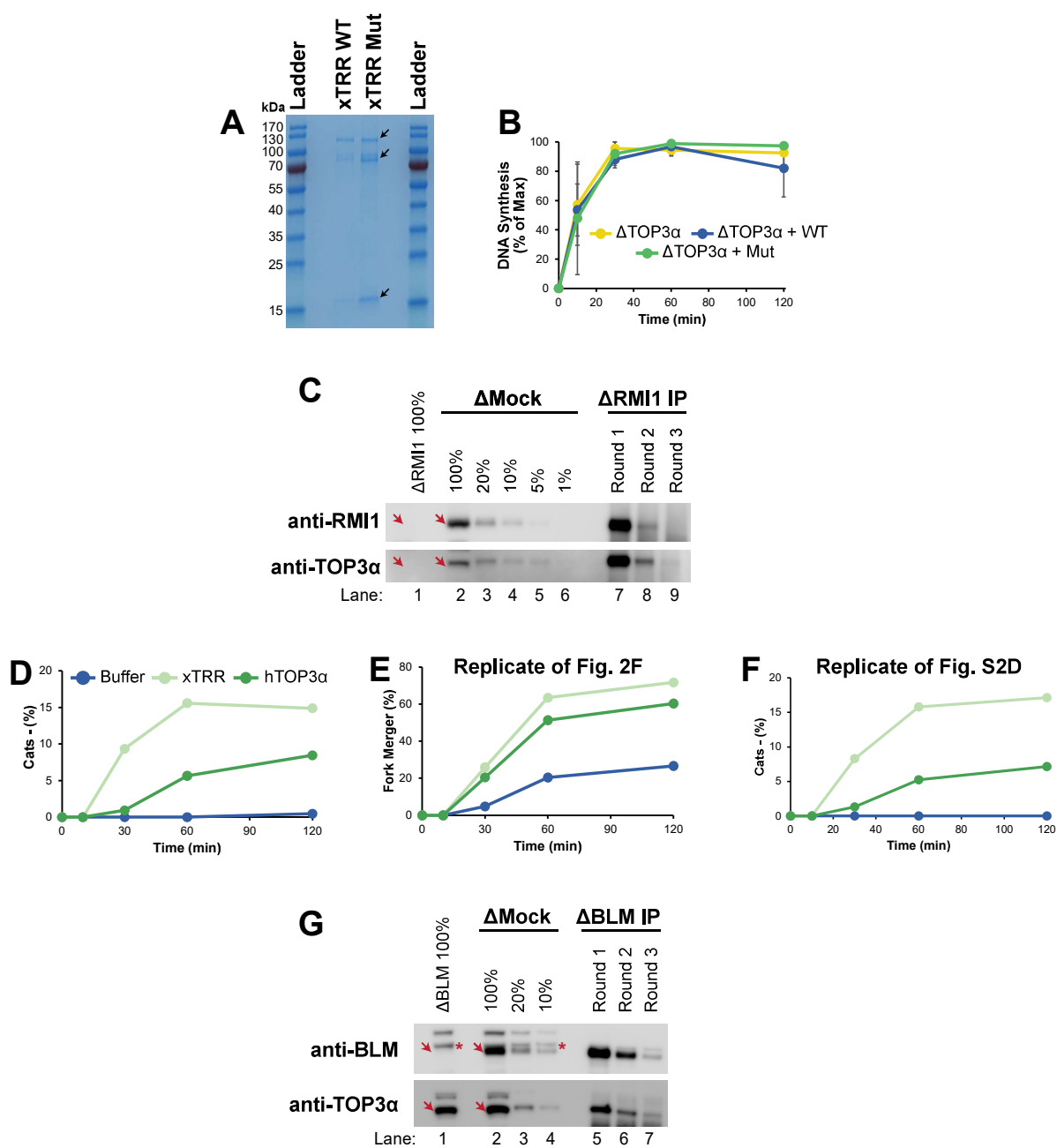
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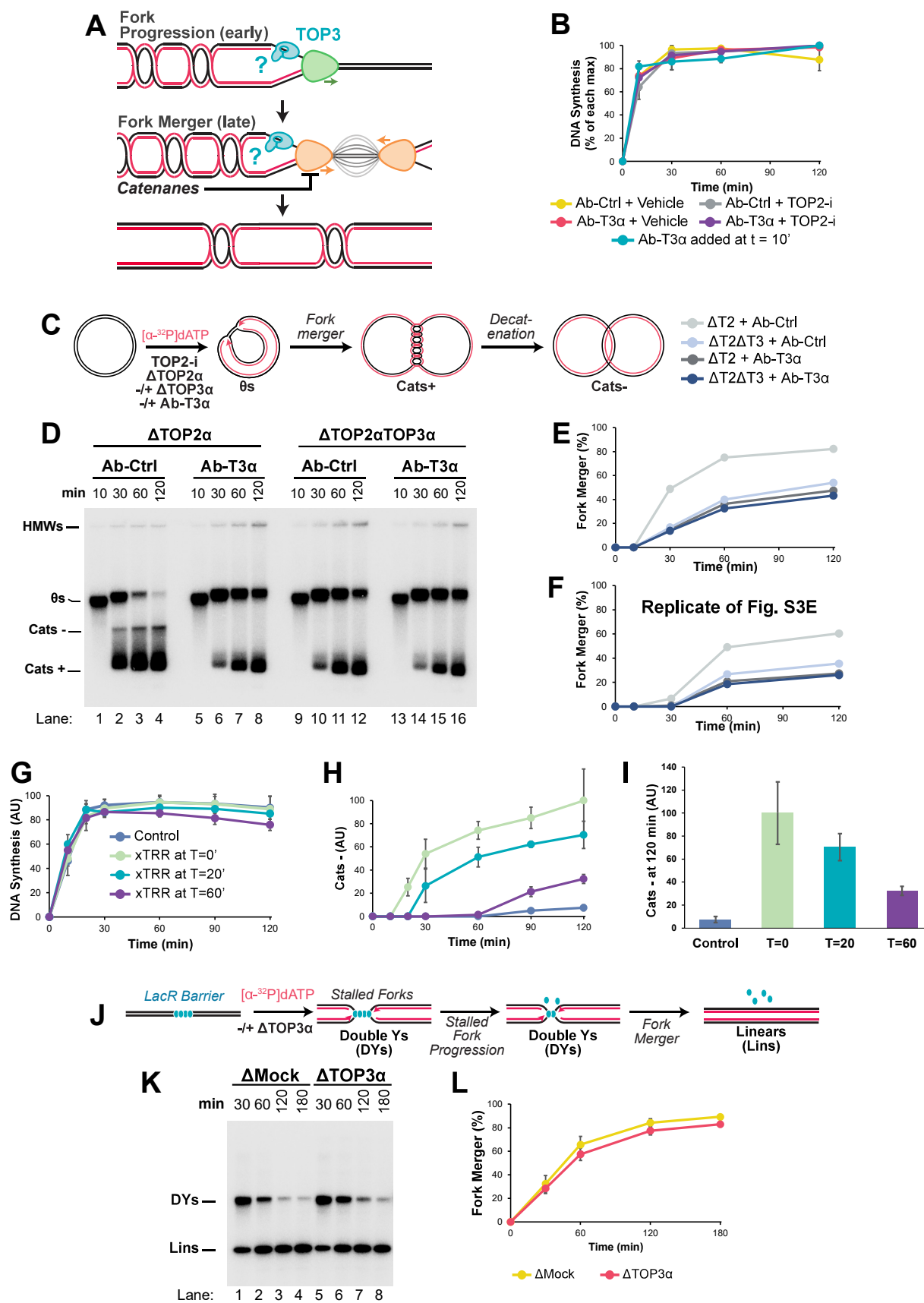


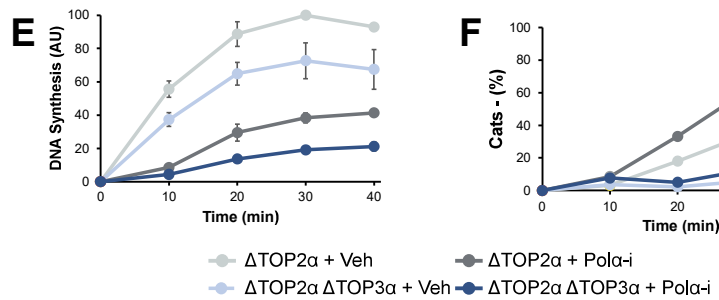
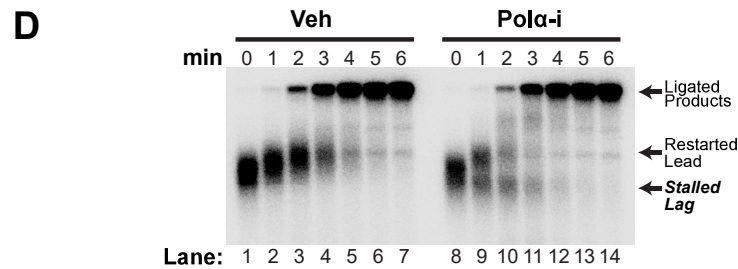
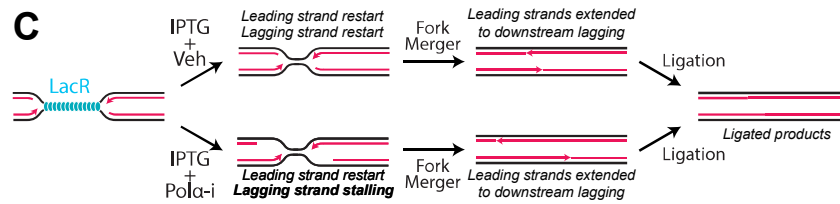
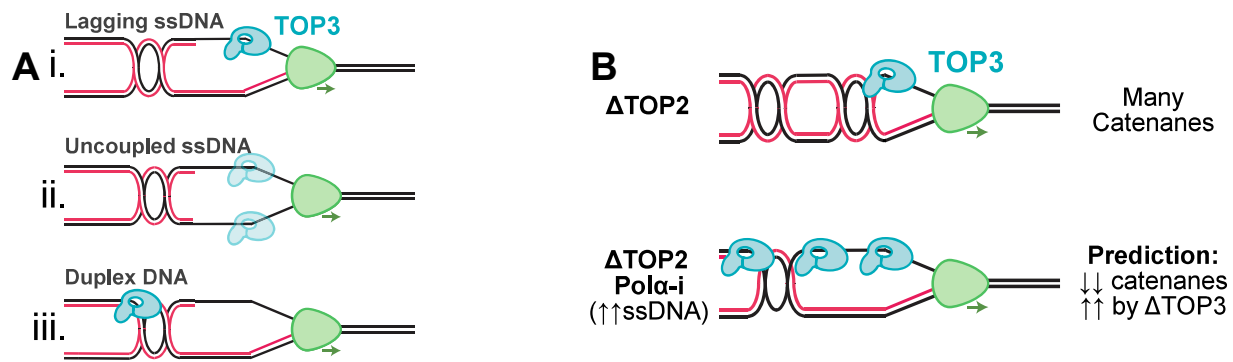
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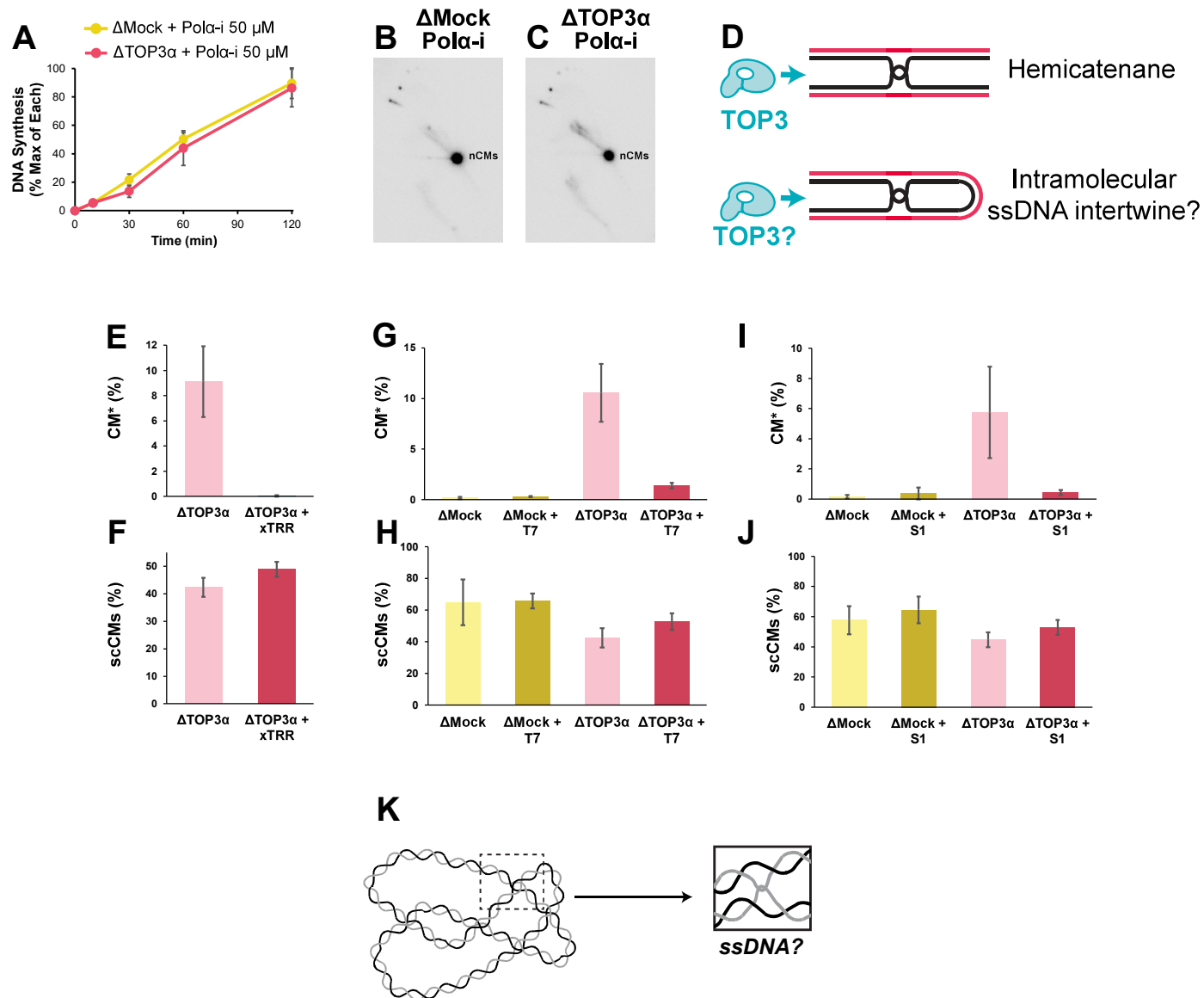












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