

Mcm10 and RecQL4 Activate the Eukaryotic Replicative Helicase CMG in Metazoa

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ABSTRACT

Chromosomes are copied from thousands of origins. At each origin, two replicative DNA helicases are first assembled, then activated to begin unwinding DNA. Several replication proteins are subsequently recruited to the active helicase, forming a replisome. The helicase must undergo dramatic conformational changes during its activation, and this process **remains** poorly understood, especially in metazoa. How the metazoan replicative helicase is activated, and which proteins promote this essential process are long-standing questions. Using a combination of single-molecule imaging and ensemble biochemistry, we show that Mcm10 and RecQL4 act in a concerted manner to activate replicative helicases. Mcm10 first binds to inactive helicases, then recruits RecQL4, **and RecQL4 acts in tandem with** Mcm10 to promote helicase activation. **Like RecQL4**, Mcm10 is not incorporated into replisomes and dissociates from origins during replication initiation. In the absence of Mcm10, RecQL4 is recruited to origins via an interaction with the Mcm7 subunit of the helicase. Our data reveal that Mcm10 and RecQL4 play partially redundant roles during helicase activation, help resolve long-standing controversies about the roles of Mcm10 and RecQL4 in DNA replication, and reveal replication initiation defects caused by pathologic RecQL4 mutations.

INTRODUCTION

During cell division, eukaryotic genomes are replicated in parallel from thousands of replication origins. Each replication origin fires bidirectionally, ensuring that chromosomes are fully replicated regardless of the exact number or location of individual origins. Replication initiation is strictly regulated to maintain genome integrity, and dysregulation of origin firing is linked to genomic instability and replication stress, as reported in many cancers¹⁻⁴. Despite its importance in maintaining genome integrity, replication initiation remains poorly understood, especially in metazoa.

Origins are licensed for replication during late mitosis and G1, when two Mcm2-7 complexes (Mini Chromosome Maintenance proteins 2-7) are loaded onto double-stranded DNA (dsDNA), forming a pre-Replication Complex (preRC)^{5,6}. During S-phase, two copies of Cdc45 (Cell Division Control protein 45) and two copies of GINS (Go-Ichi-Ni-San, Japanese for 5-1-2-3, referring to subunits Sld5, Psf1, Psf2, and Psf3) are recruited to the preRC⁷. This completes the assembly of two replicative helicases, also known as CMG (Cdc45•Mcm2-7•GINS)⁸. Cdc45 and GINS recruitment is promoted by DDK (Dbf4-Dependent Kinase) and CDK2 (Cyclin-Dependent Kinase 2)⁷. In metazoa, several firing factors facilitate CMG assembly, including TopBP1 (Topoisomerase 2 Binding Protein 1), TRESLIN (TOPBP1-interacting, Replication-Stimulating protein), MTBP (MDM2 Binding Protein), and DONSON (protein Downstream Neighbor of SON)⁹⁻¹⁵. After assembly, the two CMGs remain in an inactive head-to-head configuration and encircle dsDNA. Two copies of DONSON are tightly bound to this inactive CMG dimer^{16,17}. We refer to this (CMG•DONSON)₂ complex as the “pre-activation complex”, **or preAC** (Fig. 1a).

To initiate replication, the two CMGs are activated in a multi-step process: DONSON dissociates from CMG¹⁷, the ATPase/helicase activity of each CMG is upregulated⁷, the two CMGs separate from each other^{18,19}, and each CMG transitions from encircling dsDNA to encircling single-stranded DNA (ssDNA). After their activation, the two CMGs **separate from each other** start unwinding DNA bidirectionally away from the origin, and several replication proteins

are recruited to each CMG to form a multi-subunit complex known as the replisome. To date, the molecular mechanism of CMG helicase activation remains one of the most poorly understood aspects of replication.

The best molecular characterization of CMG activation comes from yeast, where Mcm10 (Mini Chromosome Maintenance protein 10) promotes helicase activation by directly binding CMG, allosterically stimulating its ATPase/helicase activity, and facilitating the cracking of the Mcm2-7 ring^{18–22}. Degrading Mcm10 *in vivo* or omitting Mcm10 from *in vitro* replication reactions strongly suppresses replication initiation and leads to the accumulation of CMGs on chromatin^{23–25}.

By contrast, the mechanism of CMG activation in metazoa is poorly understood and remains controversial. In addition to Mcm10, RecQL4 (RecQ Like helicase 4) has been implicated in metazoan CMG activation. Both proteins are poorly conserved between yeast and vertebrates, especially RecQL4 – whose disordered N-terminus is distantly related to the yeast Sld2, while the structured C-terminus contains an ATPase/helicase domain unique to higher eukaryotes²⁶ (Extended Data Fig. 1a). It has been challenging to interrogate the role of Mcm10 and RecQL4 in replication initiation because both proteins are involved in other aspects of genome maintenance, including replication elongation, DNA damage response, and DNA repair^{27–30}.

There are several seemingly contradictory reports about the involvement of metazoan Mcm10 in replication initiation. For example, one study reported that depleting Mcm10 from *Xenopus* egg extracts strongly inhibited replication initiation³¹, while a more recent publication concluded that Mcm10 is important for replication elongation, but not for initiation³². Studies in human cells³³ and *Xenopus* egg extracts³¹ independently concluded that Mcm10 is required for CMG helicase assembly, in sharp contrast with reports from yeast that Mcm10 participates in CMG activation^{23,25,34}. Finally, there is a well-documented interaction between RecQL4's N-terminus and Mcm10 which is thought to be important for replication initiation³⁵. However, the exact purpose of this interaction remains unclear: it was proposed that Mcm10 may recruit RecQL4 to origins³⁶ or vice-versa³⁷.

Importantly, Mcm10 and RecQL4 are dysregulated in human diseases: both Mcm10^{28,38,39} and RecQL4^{30,40–42} are frequently over-expressed in rapidly proliferating tumors, and their expression levels often correlate with poor patient prognosis. Mcm10 deficiency leads to genomic instability, slower cell proliferation, and impaired cell survival^{43–45}. Mutations in RecQL4 are associated with three related but distinct autosomal recessive disorders: Rothmund-Thompson Syndrome (RTS), Baller-Gerold Syndrome (BGS), and RAPADILINO Syndrome (RS)^{46,47}. RTS, BGS, and RS patients exhibit skeletal abnormalities, short stature, poikiloderma, and a high predisposition to osteosarcoma⁴⁷. Notably, most patient mutations occur in the C-terminal portion of RecQL4 that is unique to metazoa⁴⁸. Since RecQL4 mutations linked to RTS, BGS, and RS are not embryonically lethal, any replication initiation defect caused by those pathogenic mutations must be relatively mild. Therefore, a highly sensitive replication initiation assay is needed to investigate the molecular basis of RecQL4 syndromes.

To interrogate the molecular mechanism of CMG activation in metazoa and dissect the roles of Mcm10 and RecQL4 in this process, we employed a combination of single-molecule imaging and ensemble biochemistry in *Xenopus* nuclear egg extracts. These extracts contain all the soluble nuclear proteins and efficiently recapitulate DNA replication and other aspects of genome maintenance^{49,50}.

By directly imaging fluorescently-labeled Mcm10 molecules on DNA during replication, we found that Mcm10 binds to origins and dissociates from chromatin shortly before origin firing. We found that Mcm10 is not stably incorporated into replisomes, contradicting previous reports that Mcm10 travels with replication forks^{28,51}. Contrary to previous reports that Mcm10 promotes CMG assembly in metazoa^{31,33}, our data indicates that Mcm10 participates in CMG activation. We showed that Mcm10 recruits RecQL4 to replication origins. In the absence of Mcm10, RecQL4 is recruited to origins via a previously unknown interaction between RecQL4 C-terminus and the winged-helix-domain of Mcm7. Finally, we showed that the presence of Mcm10 masks replication

initiation defects caused by mutations in RecQL4's C-terminus, where most pathogenic mutations are found. Our data [show](#) that Mcm10 and RecQL4 play partially redundant roles during CMG helicase activation, [reveal the distinct contribution of RecQL4's N-terminus and C-terminus to CMG activation](#), help resolve long-standing controversies about the function of Mcm10/RecQL4 in metazoan replication initiation, and provide valuable mechanistic insights for researchers studying RecQL4 syndromes.

RESULTS

Mcm10 Binds to and Dissociates from Replication Origins Before Origin Firing

To directly establish Mcm10's role in metazoan replication initiation, we visualized its recruitment during DNA replication in *Xenopus* egg extracts. Extract was used to replicate a linear λ DNA substrate stretched to ~85% of its contour length and immobilized via biotin-streptavidin linkages in a microfluidic flow cell as previously described (Fig. 1bi)^{17,52,53}. DNA was licensed for replication via incubation in High-Speed Supernatant (HSS), an extract that mimics G1 (Fig. 1bii). Replication was initiated by adding Nucleo-Plasmic Extract (NPE), an extract that mimics S-phase (Fig. 1biii)⁴⁹. Mcm10-depleted extract was supplemented with 10 nM of recombinant Mcm10 labeled with AlexaFluor 647 (Mcm10^{AF647}). An ensemble plasmid replication assay was used to verify that recombinant Mcm10 was functional (Extended Data Fig. 1b-e). A total internal reflection fluorescence (TIRF) microscope was used to visualize the recruitment of individual Mcm10^{AF647} molecules to replicating DNA (Fig. 1biv). We previously used [a similar](#) approach to visualize the dynamics of GINS, Cdc45, RecQL4, TopBP1, and DONSON during replication initiation and elongation^{17,53}. To monitor DNA synthesis, we supplemented egg extract with recombinant Fen1 fused to the fluorescent protein mKikGR (Fen1^{mKikGR}) as described previously (Fig. 1biii)⁵⁴. Fen1^{mKikGR} is recruited to DNA-bound PCNA, acting as a marker of nascent DNA (Fig. 1biv).

In ~90% of origin firing events, we detected Mcm10^{AF647} binding shortly before replication initiation (Fig. 1c, white arrows). Mcm10^{AF647} bound to the origin ~2 min before origin firing, remained bound at the origin for ~1 min, and dissociated from DNA ~1 min before origin firing (Fig. 1d). In 63 out of 69 origin firing events, Mcm10^{AF647} dissociated before the appearance of the Fen1^{mKikGR} signal (Fig. 1d). In only 6 out of 69 initiation events, Mcm10^{AF647} remained on DNA after origin firing but disappeared within ~5 min (due to dissociation or photobleaching). These findings indicate that in *Xenopus* egg extract, Mcm10 participates almost exclusively in replication initiation, and is not stably incorporated into the replisome. Consistent with this idea, replication fork speed was not altered in Mcm10-depleted (Δ Mcm10) extract (Fig. 1e). [This behavior is similar to that of RecQL4, which participates in replication initiation but not in replication elongation¹⁷. Just like Mcm10, depleting RecQL4 does not affect the speed of replication forks \(Extended Data Fig. 1f\), consistent with our previous finding that RecQL4 is not incorporated into the replisome¹⁷.](#)

In addition to productive Mcm10^{AF647} binding events which led to origin firing (Fig. 1c, white arrows), [we also observed non-productive Mcm10^{AF647} binding events \(~25% of all Mcm10^{AF647} binding events](#), see gray arrow in Fig. 1c). We attribute these non-productive binding events to failed origin firing attempts. Non-productive Mcm10^{AF647} binding events often exhibited two-step photobleaching (Fig. 1f, orange arrows), indicating that they correspond to a Mcm10 dimer. [Notably, productive Mcm10 binding events were too short-lived \(~1 min, see Fig. 1d\) to estimate the Mcm10 stoichiometry via photobleaching. Nevertheless, in reactions where CMG activation was rate-limiting \(in RecQL4-depleted extract, see Fig. 3 below\), productive Mcm10 binding events were sufficiently long to enable us to unambiguously detect Mcm10 dimers \(Fig. 1g\). Taken together, these data suggest that the oligomeric state of Mcm10 during initiation is a dimer, in agreement with two recent structural studies^{55,56}.](#)

Mcm10 is Recruited to Origins After CMG Assembly

To determine if Mcm10 participates in CMG assembly or CMG activation (Fig. 1a), we used single-molecule imaging to quantify Mcm10^{AF647} recruitment to DNA in mock-depleted or DONSON-depleted extract (Extended Data Fig. 1g). DONSON-mediated recruitment of GINS to Mcm2-7 is the last known step in CMG assembly¹⁷. Mcm10^{AF647} recruitment to licensed DNA was almost entirely abolished in DONSON-depleted extract, and this defect was robustly rescued by supplementing DONSON-depleted extract with recombinant DONSON (Fig. 1h). This indicates that Mcm10 is recruited to origins downstream of DONSON binding, i.e. after the assembly of the stable (CMG•DONSON)₂ pre-activation complex.

Mcm10 and RecQL4 Play Partially Redundant Roles During Replication Initiation

We previously showed that RecQL4 promotes CMG activation and the accompanying dissociation of DONSON from metazoan origins¹⁷. Here, we show that Mcm10 is also involved in CMG activation in metazoa (Fig. 1). This led us to hypothesize that Mcm10 and RecQL4 may play overlapping and redundant roles in CMG activation.

To dissect the division of labor between RecQL4 and Mcm10 during CMG activation, we replicated a plasmid DNA template in extract depleted of RecQL4 (Δ RecQL4), Mcm10 (Δ Mcm10), or both (Δ RecQL4+ Δ Mcm10) (Fig. 2a). Since depleting Mcm10 or RecQL4 does not alter the speed of replication forks (Fig. 1e and Extended Data Fig. 1f), any differences in replication kinetics/efficiency measured in the biochemical assay *must be* caused by differences in origin firing. DNA replication was strongly (but not completely) suppressed in RecQL4-depleted extract (Fig. 2b lanes 5-8, compare to mock in lanes 1-4; see quantification in Fig. 2c). In contrast, a stringent depletion of Mcm10 caused only a minor reduction in replication efficiency (Fig. 2b lanes 9-12, compare to lanes 1-4; see Fig. 2d for the corresponding immunoblots). Depleting both RecQL4 and Mcm10 completely abolished DNA replication (Fig. 2b lanes 13-16), consistent with our hypothesis that both proteins *promote* CMG helicase activation. Importantly, DNA replication in double-depleted extract was efficiently rescued by near-endogenous levels (100 nM) of recombinant RecQL4 (Fig. 2b, lanes 17-20; see Fig. 2d for the corresponding immunoblots). In contrast, replication in double-depleted extract was only partially rescued by near-endogenous levels (100 nM) of recombinant Mcm10 (Fig. 2b, lanes 21-24, see independent replicate in Extended Data Fig. 2a-c), suggesting that RecQL4 is a more potent CMG activator than Mcm10. Importantly, these findings were reproducible with different preparations of egg extract and of recombinant proteins (Extended Data Fig. 2d-f).

To more directly test the hypothesis that both RecQL4 and Mcm10 promote CMG activation, we performed a chromatin fractionation assay (also known as “chromatin spin-down”) as outlined in Fig. 2e. To this end, sperm chromatin was first licensed in Mcm10-and-RecQL4 depleted HSS. Licensed chromatin was then incubated in mock-depleted NPE or Mcm10-and-RecQL4 depleted NPE (Fig. 2f). To maximize the number of fired origins, the replication reaction was supplemented with caffeine, which inhibits the S-phase checkpoint (ATR/CHK1). Samples from each replication reaction were collected at various timepoints, chromatin and chromatin-bound proteins were pelleted via centrifugation, and unbound protein were washed away. Immunoblotting was used to quantify the amount of chromatin-bound replication elongation factors (PCNA and the PolA1 subunit of DNA polymerase alpha), CMG assembly factors (DONSON, Cdc45, and GINS), and CMG activation factors (Mcm10 and RecQL4).

As expected, in mock-depleted extract, replication elongation proteins bound to chromatin at early time-points (20-40 min), then gradually dissociated from chromatin as the DNA was replicated and terminating replisomes were unloaded (Fig. 2g, left half). Similarly, CMG assembly factors bound to chromatin at early timepoints, and their amounts decreased over time as chromatin was replicated.

In contrast, the Mcm10-and-RecQL4 depleted extract did not support the binding of elongation factors to chromatin above background levels (Fig. 2g, right half, compare with “+Geminin” and

the “-Chromatin” negative controls). This is consistent with our plasmid replication assay where depleting both RecQL4 and Mcm10 fully inhibits DNA replication (Fig. 2b, lanes 13-16). Strikingly, we observed a massive accumulation of CMG assembly factors on chromatin in the double-depleted extract (Fig. 2g, right half). This indicates that depleting both RecQL4 and Mcm10 prevents CMG activation, leading to a buildup of pre-activation complexes on chromatin. Taken together, the data presented in Fig. 2 indicates that both Mcm10 and RecQL4 promote CMG activation, but that they play partially redundant roles in this process.

In the Absence of RecQL4, Mcm10 Is a Weak CMG Activator

The ensemble plasmid replication assay suggests that Mcm10 alone is a less potent CMG activator than RecQL4 alone (Fig. 2b). To gain insights into why this is the case, we directly visualized the dynamics of fluorescently-labeled Mcm10 during replication initiation in mock-depleted or RecQL4-depleted extract (Fig. 3a). To this end, we stringently depleted all endogenous Mcm10 from extract, and supplemented this Mcm10-depleted extract with 5 nM of recombinant Mcm10^{AF647}. In this assay, depleting RecQL4 caused several distinct replication initiation defects.

First, depleting RecQL4 dramatically increased (>3x) Mcm10^{AF647}'s binding to chromatin (Fig. 3b) and sharply decreased the fraction of productive Mcm10^{AF647} binding events from ~75% in mock to ~25% in RecQL4-depleted extract (Fig. 3c). These findings indicate that in the absence of RecQL4, Mcm10 alone is very inefficient at promoting CMG activation, and that Mcm10 binds repeatedly to the preAC until it successfully triggers origin firing.

Second, depleting RecQL4 significantly prolonged (~3x) the time delay between Mcm10^{AF647} binding and origin firing (Fig. 3d) and substantially lengthened (~2.5x) the duration of productive Mcm10^{AF647} binding events (Extended Data Fig. Fig. 3a). This indicates that even when Mcm10 successfully activates CMGs at the origin, it takes Mcm10 longer to do so in the absence of RecQL4. By contrast, depleting RecQL4 did not alter the duration of non-productive Mcm10^{AF647} binding events (Extended Data Fig. Fig. 3b).

Importantly, all these replication initiation defects were robustly rescued by supplementing RecQL4-depleted extract with a near-endogenous concentration (100 nM) of recombinant wildtype RecQL4 (Fig. 4b-d, also see Extended Data Fig. Fig. 3a). Taken together, these data indicate that in the absence of RecQL4, Mcm10 alone is a weak activator of CMG, consistent with our ensemble plasmid replication assay (Fig. 2b).

Interestingly, we also found that depleting RecQL4 increased the time delay between Mcm10^{AF647} dissociation and origin firing (Extended Data Fig. Fig. 3c), suggesting that RecQL4 may be involved in an initiation step downstream of Mcm10 dissociation. This raises questions about the order in which Mcm10 and RecQL4 are recruited to the origin.

RecQL4 is Recruited to Origins After Mcm10

It was previously reported that Mcm10 can interact with the disordered N-terminus of RecQL4 and that this interaction is important for efficient origin firing in human cells³⁵ (see annotated disorder prediction for RecQL4 in Extended Data Fig. 3d). We independently verified that recombinant *Xenopus* RecQL4 and *Xenopus* Mcm10 interact *in vitro* (Extended Data Fig. 3e), and that this interaction is largely mediated by RecQL4's N-terminus (Extended Data Fig. 3f). However, the exact role of this interaction has been debated: one study concluded that Mcm10 helps recruit RecQL4 to origins³⁶, while another study concluded that RecQL4 recruits Mcm10 to origins³⁷.

To uncover the order in which Mcm10 and RecQL4 bind to the pre-activation complex, we immunodepleted endogenous Mcm10 and RecQL4 from egg extract, and supplemented this double-depleted extract with 3 nM of Mcm10^{AF647} and 3 nM of RecQL4^{AF546}. We directly visualized the binding of fluorescently-labeled Mcm10 and RecQL4 to origins using our single-molecule imaging assay. We analyzed only origin firing events where RecQL4^{AF546} and Mcm10^{AF647} signals overlapped before replication initiation (Fig. 3e). In over 90% of such origin firing events, Mcm10

arrived first, followed by RecQL4, with a median time delay of ~1 min (Fig. 3f). Interestingly, there was no defined dissociation order: in ~50% of cases RecQL4 dissociated before Mcm10 and vice-versa (Fig. 3g). The observation that Mcm10 arrives at origins before RecQL4 supports the hypothesis that Mcm10 helps recruit RecQL4 to origins (Fig. 3h). We systematically test this model below.

RecQL4's C-terminus Mediates a Previously Unknown Interaction with Mcm7's WHD

Although the Mcm10-RecQL4 interaction was previously shown to be important for efficient origin firing in human cells, experiments by us (Fig. 2b) and the Blow group³² indicate that depleting Mcm10 causes a mild replication defect in *Xenopus* egg extract. This suggests that in addition to the Mcm10-dependent recruitment mechanism, there must be a Mcm10-independent mechanism of recruiting RecQL4 to origins. Intriguingly, AlphaFold3 predicted that a previously unannotated portion of RecQL4's C-terminus may interact with the winged-helix domain (WHD) of Mcm7 (Fig. 4a-b, also see Extended Data Fig. 4a-c for human/mouse models and sequence alignments). We hypothesize that this interaction is responsible for the Mcm10-independent recruitment of RecQL4 to the pre-activation complex.

As reported previously, truncating the last 92 amino-acids from the human RecQL4 impairs its ability to bind ssDNA and unwind dsDNA *in vitro*⁵⁷. Therefore, truncating the extreme C-terminus of RecQL4 is not an acceptable way of specifically ablating the interaction with Mcm7. Guided by the AlphaFold3 prediction, we sought to disrupt the RecQL4-Mcm7 interaction by introducing two point-mutations (K1119A, K1120E) and deleting the last 9 amino-acids of RecQL4. We refer to this Mcm7-interaction deficient mutant as RecQL4^{AE-ΔC}. *In vitro*, recombinant CMG co-immunoprecipitated with purified RecQL4^{WT} but not with RecQL4^{AE-ΔC} (Fig. 4c), indicating that the RecQL4-Mcm7 interaction was effectively disrupted in this mutant.

To further validate this predicted RecQL4-Mcm7 interaction, we designed a bait construct consisting of the Mcm7 winged helix domain with a FLAG-SNAP tag at its N-terminus (see cartoons in Fig. 4d). We also designed a bait with four point-mutations in Mcm7's WHD aimed at disrupting its interaction with RecQL4: E701K, E703A, E704K, and N706A (see cartoons in Fig. 4d, also see Extended Data Fig. 4c for multiple-sequence alignments). We purified the wildtype bait (Mcm7-WHD^{WT}) and the mutant bait (Mcm7-WHD^{KAKA}) constructs from bacteria (see Coomassie gel in Fig. 4d), immobilized them on anti-FLAG magnetic beads, and incubated the beads with *Xenopus* egg extract (NPE). Immunoblotting revealed that endogenous RecQL4 co-immunoprecipitated with Mcm7-WHD^{WT}, but not with Mcm7-WHD^{KAKA} (Fig. 4d, left), corroborating AlphaFold's prediction.

In the Absence of Mcm10, Mcm7's WHD Recruits RecQL4 to the PreAC

To more directly test our hypothesis that Mcm10-independent recruitment of RecQL4 to origins relies on the Mcm7-RecQL4 interaction, we employed the ensemble replication assay. Specifically, we sought to measure how well RecQL4^{AE-ΔC} can support origin firing in the presence or absence of endogenous Mcm10.

First, we stringently immunodepleted endogenous RecQL4 from egg extract (Extended Data Fig. 5a, left), and supplemented this RecQL4-depleted extract with 100 nM of RecQL4^{WT} or RecQL4^{AE-ΔC}. In this experiment, the RecQL4^{AE-ΔC} mutant supported replication nearly as well as wildtype (Fig. 4e, compare green and magenta curves, for raw data see Extended Data Fig. 5b). This result indicates that in the presence of endogenous Mcm10, disrupting the RecQL4-Mcm7 interaction does not impair RecQL4's ability to activate CMG.

Second, we stringently immunodepleted both RecQL4 and Mcm10 from egg extract, and supplemented this double-depleted extract with 100 nM of RecQL4^{WT} or RecQL4^{AE-ΔC} (Extended Data Fig. 5a, center). In the absence of endogenous Mcm10, RecQL4^{AE-ΔC} supported a negligible amount of replication compared to RecQL4^{WT} (Fig. 4f, compare dotted green and magenta curves,

for raw data see [Extended Data Fig. 5c](#)). This indicates that in the absence of endogenous Mcm10, disrupting the RecQL4-Mcm7 interaction ablates RecQL4's ability to activate CMG. These results were reproducible with a different preparation of egg extract (see [Extended Data Fig. 5d-f](#), and [Extended Data Fig. 5g-i](#))

Taken together, the data presented in [Fig. 4](#) strongly support our model that in the absence of Mcm10, RecQL4 is recruited to the pre-activation complex via its interaction with the Mcm7 winged-helix domain (see cartoons in [Fig. 4e-f](#)).

Depleting Mcm10 Unmasks the Role of RecQL4's Structured C-terminus in Initiation

Since RecQL4 and Mcm10 have partially redundant functions during origin firing, we hypothesized that replication initiation defects caused by RecQL4 mutations are partially masked by the presence of Mcm10.

To dissect the roles of RecQL4's disordered N-terminus and structured C-terminus in initiation, we measured plasmid replication upon depleting RecQL4 alone or depleting both RecQL4 and Mcm10. Depleted extracts were supplemented with 100 nM of the following recombinant proteins ([Fig. 5a](#)): wildtype RecQL4 (RecQL4^{WT}), a construct that contains only the disordered N-terminus (RecQL4¹⁻⁶⁶³), or ATPase-dead RecQL4 with a point mutation in the Walker B motif (RecQL4^{D856A}) which can bind ATP but cannot hydrolyze it⁵⁸. Unlike RecQL4^{WT}, which can unwind short (<20 nt) stretches of dsDNA, the Walker B mutant has no DNA helicase activity⁵⁹. Importantly, RecQL4^{D856A} mimics RTS patient mutations with impaired ATPase function, while RecQL4¹⁻⁶⁶³ mimics RTS patient mutations with a premature stop codon where the entire C-terminus of RecQL4 is missing⁴⁷.

In RecQL4-depleted extract, RecQL4^{WT} supported robust DNA replication, indistinguishable from mock ([Fig. 5b](#), compare green and black curves; for raw data see [Extended Data Fig. 6a](#)). In the same conditions, both RecQL4^{D856A} and RecQL4¹⁻⁶⁶³ exhibited a delay in replication kinetics (how fast DNA is replicated), and reduced replication efficiency (how much total DNA is replicated) ([Fig. 5b](#) blue and orange curves). These data indicate that RecQL4's C-terminus and its ATPase domain contribute to efficient origin firing, but are not strictly required for replication initiation, consistent with previous reports^{60,61}.

In RecQL4-and-Mcm10 depleted extract, RecQL4^{WT} supported robust DNA replication, though less efficient than mock-depleted extract ([Fig. 5c](#) compare dotted green curve to the solid black curve, for raw data see [Extended Data Fig. 6b-c](#)). We attribute this difference to the absence of Mcm10, which normally facilitates RecQL4's recruitment to origins and itself promotes CMG activation.

In RecQL4-and-Mcm10 depleted extract, RecQL4¹⁻⁶⁶³ did not support any detectable replication ([Fig. 5c](#), dotted orange curve) because this construct lacks the C-terminal Mcm7-interacting region and therefore cannot be recruited to origins in the absence of Mcm10 ([Fig. 5c](#), see cartoon with dotted orange border).

By contrast, in RecQL4-and-Mcm10 depleted extract, RecQL4^{D856A} supported some (~20%) DNA replication ([Fig. 5c](#), dotted blue curve). We attribute this to the fact that RecQL4^{D856A} retains its ability to interact with Mcm7, and therefore can be recruited to origins in the absence of Mcm10. However, RecQL4^{D856A} cannot use its ATPase domain to promote CMG activation, and therefore employs only its N-terminus to activate CMG ([Fig. 5c](#), see cartoon with a dotted blue border). This explains why RecQL4^{D856A} supports only low levels of origin firing in double-depleted extract ([Fig. 5c](#), dotted blue curve). These observations were reproducible with a different extract preparation (see [Extended Data Fig. 6d-l](#)).

Our data indicate that Mcm10 partially masks replication defects caused by RecQL4 mutants which mimic RTS patient mutations.

RecQL4's N-terminus and C-terminus Have Distinct CMG Activation Functions

Our data suggests that in the absence of Mcm10, RecQL4^{WT} is recruited to origins via its interaction with Mcm7 and that RecQL4^{WT} drives CMG activation in two distinct ways: the N-terminus moderately activates CMG, while the C-terminus weakly activates CMG (see cartoon with dotted green border in Fig. 5c). To better understand the roles of RecQL4's N-term and C-term domains, we sought to separately interrogate their function via the plasmid replication assay. To account for Mcm10's role in initiation, we measured plasmid replication in RecQL4-depleted or RecQL4-and-Mcm10-depleted extract supplemented with 100 nM of RecQL4¹⁻⁶⁶³ (N-term), RecQL4⁶⁶⁴⁻¹⁵⁰⁰ (C-term), RecQL4^{WT} (full-length), or buffer.

In RecQL4-depleted extract, RecQL4⁶⁶⁴⁻¹⁵⁰⁰ alone supported a moderate efficiency (~50%) of DNA replication (Fig. 5d, light blue curve, see raw data in Extended Data Fig. 7a-b) compared to a low replication efficiency (~30%) in the buffer control reaction (Fig. 5d, light gray curve). This is consistent with our hypothesis that RecQL4's C-term weakly activates CMG, and that it can be recruited to origins via its interaction with Mcm7 (independently of the Mcm10-RecQL4^{N-term} interaction). Importantly, the ability of RecQL4⁶⁶⁴⁻¹⁵⁰⁰ to support replication entirely depends on its ATPase activity (Fig. 5e, blue versus red curves, see raw data in Extended Data Fig. 7c-d).

In RecQL4-depleted extract, RecQL4¹⁻⁶⁶³ supported a higher replication efficiency (~70%), than RecQL4⁶⁶⁴⁻¹⁵⁰⁰ (~50%) (Fig. 5d, compare orange and blue curves), consistent with our model that RecQL4's N-terminus activates CMG more potently than its C-terminus.

Importantly, RecQL4-depleted extract supplemented with both RecQL4¹⁻⁶⁶³ and RecQL4⁶⁶⁴⁻¹⁵⁰⁰ supported replication nearly as well as full-length wildtype RecQL4 (Fig. 5d, compare magenta and green curves). This observation supports our model that the two halves of RecQL4 can be recruited to origins via independent mechanisms: Mcm10 recruits RecQL4¹⁻⁶⁶³, while Mcm7 recruits RecQL4⁶⁶⁴⁻¹⁵⁰⁰ (see cartoon with magenta outline in Fig. 5d).

Finally, in RecQL4-and-Mcm10-depleted extract, RecQL4⁶⁶⁴⁻¹⁵⁰⁰ did not support DNA replication (Fig. 5f, dotted blue curve, see raw data in Extended Data Fig. 7e), suggesting that its CMG stimulatory function alone is insufficient to promote origin firing. These observations were reproducible using a different extract preparation (Extended Data Fig. 7f-h).

Interestingly, double-depleted extract supplemented with both RecQL4¹⁻⁶⁶³ and RecQL4⁶⁶⁴⁻¹⁵⁰⁰ supported low replication efficiency (~20%), suggesting that RecQL4¹⁻⁶⁶³ can be recruited (albeit inefficiently) to origins even in the absence of Mcm10. We hypothesized that this recruitment in the absence of Mcm10 may be facilitated by an interaction between the N-term and the C-term of RecQL4. This idea is supported by separate experiments in which untagged RecQL4¹⁻⁶⁶³ co-immunoprecipitated with V5-tagged RecQL4⁶⁶⁴⁻¹⁵⁰⁰ (Extended Data Fig. 8a). These experiments also reveal that untagged RecQL4^{WT} co-IPed with V5-tagged RecQL4^{WT}, suggesting that RecQL4 forms dimers (or higher order oligomers) (Extended Data Fig. 8b), consistent with a previous study which analyzed human RecQL4 sedimentation on a glycerol gradient⁶². Similarly, we found that untagged RecQL4¹⁻⁶⁶³ co-IPed with V5-tagged RecQL4¹⁻⁶⁶³ (Extended Data Fig. 8c), suggesting that RecQL4's N-terminus can oligomerize. Finally, our co-IP experiments suggest that RecQL4⁶⁶⁴⁻¹⁵⁰⁰ does not oligomerize (Extended Data Fig. 8d).

Taken together, these data indicate that the N-term and the C-term portions of RecQL4 are separately able to promote CMG activation, with the N-terminus being the stronger activator, and the C-terminus being the weaker activator. Importantly, the ability of the C-terminus to promote CMG activation depends entirely on RecQL4's ATPase activity.

The RTS-Like RecQL4¹⁻⁶⁶³ Fragment Requires Several Binding Attempts to Activate CMG

The data presented above indicates that in the presence of Mcm10, the disordered N-terminal region of RecQL4 (RecQL4¹⁻⁶⁶³) is sufficient to support a moderate level of origin firing. Our working model is that Mcm10 first docks to the preAC, then recruits RecQL4¹⁻⁶⁶³, and the two proteins jointly activate CMG (see cartoon with orange outline in Fig. 5b). Since RecQL4¹⁻⁶⁶³ mimics RecQL4 truncations found in many RTS patients⁴⁸, we sought to directly visualize the replication initiation defect caused by RecQL4¹⁻⁶⁶³. To this end, we immunodepleted endogenous

RecQL4 from nuclear extract and supplemented this RecQL4-depleted extract with 10 nM of fluorescently-labeled RecQL4^{WT}, 10 nM of fluorescently-labeled RecQL4¹⁻⁶⁶³, or 10 nM of fluorescently-labeled RecQL4⁶⁶⁴⁻¹⁵⁰⁰. We then visualized the binding dynamics of these RecQL4 constructs using our single-molecule assay (Fig. 6a-c).

First, we found that in reactions containing RecQL4^{WT} or RecQL4¹⁻⁶⁶³, the majority (~85%) of origin firing events were preceded by the binding of the respective fluorescent RecQL4 construct (Fig. 6d, see examples in Fig. 6a and Fig. 6b). By contrast, fluorescently-labeled RecQL4⁶⁶⁴⁻¹⁵⁰⁰ binding preceded only ~15% of origin firing events (Fig. 6d, see examples in Fig. 6c). These data indicate that in the low concentration regime (10 nM), RecQL4^{WT} and RecQL4¹⁻⁶⁶³ (but not RecQL4⁶⁶⁴⁻¹⁵⁰⁰) are efficiently recruited to the preAC. This is consistent with our hypothesis that the primary mechanism of recruiting RecQL4 to the preAC relies on the interaction between RecQL4's N-terminus and origin-bound Mcm10. At low concentrations (10 nM), RecQL4⁶⁶⁴⁻¹⁵⁰⁰ is inefficiently recruited to origins via the weaker interaction between RecQL4's C-terminus and Mcm7's WHD. At the higher RecQL4 concentrations (100 nM) used in the biochemical assay, the weak RecQL4⁶⁶⁴⁻¹⁵⁰⁰-Mcm7 interaction is sufficient for recruiting RecQL4⁶⁶⁴⁻¹⁵⁰⁰ to origins and supporting a moderate level (~50%) of DNA replication (see blue curve in Fig. 5d).

Second, there was striking difference in the fraction of productive binding events (i.e. binding events that lead to origin firing): while ~50% of RecQL4^{WT} binding events were productive, only ~5% of RecQL4¹⁻⁶⁶³ were productive (Fig. 6e). In other words, the vast majority (~95%) of RecQL4¹⁻⁶⁶³ binding events did not promote origin firing (see examples in Fig. 6b). Notably, we observed "trains" of non-productive RecQL4¹⁻⁶⁶³ binding events at the same location on DNA: in some cases, the origin at that location eventually fired, but in other cases the origin never fired (Fig. 6b). These data indicate that each individual RecQL4¹⁻⁶⁶³ binding event is much less efficient at activating CMG than full-length RecQL4^{WT}, with RecQL4¹⁻⁶⁶³ requiring several binding events to fire a single origin.

Third, individual productive binding events for RecQL4¹⁻⁶⁶³ were shorter than for RecQL4^{WT} (Fig. 6f, violin plots with green dots). This difference was even more pronounced for non-productive binding events (Fig. 6f, violin plots with gray dots). These data suggest that RecQL4^{WT} binds the preAC more tightly than RecQL4¹⁻⁶⁶³. This is consistent with our hypothesis that RecQL4^{WT} interacts both with origin-bound Mcm10 and with Mcm7 (a preAC subunit), while RecQL4¹⁻⁶⁶³ interacts only with origin-bound Mcm10.

Next, we quantified the overall binding (in sec/kb) of fluorescently-labeled RecQL4^{WT}, RecQL4¹⁻⁶⁶³, and RecQL4⁶⁶⁴⁻¹⁵⁰⁰ to stretched DNA in the single-molecule experiments from Fig. 6a-c. We found that RecQL4¹⁻⁶⁶³ exhibited >3-fold more binding to licensed origins than full-length RecQL4^{WT} (Fig. 6g, the "+Geminin" control indicates that both RecQL4^{WT} and RecQL4¹⁻⁶⁶³ bind to licensed origins). It seems counterintuitive that RecQL4^{WT} (a more potent CMG activator) binds DNA less than RecQL4¹⁻⁶⁶³ (a less potent CMG activator). Nevertheless, this is consistent with our observation that RecQL4¹⁻⁶⁶³ exhibits many more binding events than RecQL4^{WT} (see examples in Fig. 6b versus Fig. 6a), and that ~95% of RecQL4¹⁻⁶⁶³ binding events are non-productive (Fig. 6e).

We also found that depleting Mcm10 dramatically reduced the binding of both RecQL4¹⁻⁶⁶³ and RecQL4^{WT} to licensed DNA (Fig. 6g), consistent with the hypothesis that Mcm10 plays a central role in recruiting RecQL4's N-terminus to the preAC. Importantly, the amount of RecQL4¹⁻⁶⁶³ binding in Mcm10-depleted extract was statistically indistinguishable from non-specific binding to unlicensed DNA (see +Geminin control in Fig. 6g), supporting our model that this RTS-like truncation is entirely dependent on Mcm10 for its recruitment to origins. This is not the case for RecQL4^{WT}, which exhibited more DNA binding in Mcm10-depleted extract than in the +Geminin control (Fig. 6g). This is consistent with our model that in the absence of Mcm10, RecQL4's C-terminus can be recruited to the preAC via its interaction with Mcm7. The same analysis revealed very little RecQL4⁶⁶⁴⁻¹⁵⁰⁰ binding to licensed DNA even in the presence of Mcm10 (Fig. 6g). However, due to the very low number of RecQL4⁶⁶⁴⁻¹⁵⁰⁰ binding events (see examples in Fig. 6c),

there was no statistical difference between the RecQL4⁶⁶⁴⁻¹⁵⁰⁰ binding to licensed or unlicensed DNA (Fig. 6g).

Finally, we measured the number of origins that fired per λ DNA molecule. In reactions containing 10 nM of RecQL4^{WT}, depleting Mcm10 reduced origin firing from ~2.5 origins/DNA to ~0.5 origins/DNA. This reduction is in part due to Mcm10's role in recruiting RecQL4 to origins, and in part due to Mcm10's own role in activating CMG. The low but statistically significant origin firing activity of 10 nM RecQL4^{WT} in Mcm10-depleted extract supports our model that RecQL4^{WT} can be recruited to the preAC independently of Mcm10 (namely by Mcm7). By contrast, in reactions containing 10 nM of RecQL4¹⁻⁶⁶³, depleting Mcm10 entirely abolished origin firing, consistent with our hypothesis that RecQL4¹⁻⁶⁶³ is entirely dependent on Mcm10 for its recruitment to origins.

Taken together, these data provide further support for our model of how RecQL4 and Mcm10 cooperate to activate CMG (see Fig. 7).

DISCUSSION

Proposed Model of CMG Helicase Activation in Metazoa

Based on the data presented here, we propose a new model of CMG activation in metazoa (Fig. 7). Fig. 7a-b depicts a model of the pre-activation complex containing two inactive CMGs encircling dsDNA, and a dimer of DONSON that “staples” them. We found that Mcm10 is recruited to the pre-activation complex first, as shown in Fig. 7c. AlphaFold3 predicted that the well-folded core of the *Xenopus* Mcm10 (containing a ssDNA binding domain⁶³) should bind to the N-terminus of Mcm4. The placement of Mcm10 agrees with a recent yeast CMG-Mcm10 structure¹⁹, and is consistent with cross-linking mass spectrometry data⁶⁴. Finally, this placement of Mcm10 agrees with cryo-EM structures of CMG-Mcm10 reported in two pre-prints^{55,56} posted to bioRxiv while our manuscript was in revision. Our data indicate that Mcm10 recruits RecQL4 to the pre-activation complex as shown in Fig. 7d, via the known interaction between Mcm10 and RecQL4's N-terminus³⁵.

We show that metazoan Mcm10 alone can promote CMG activation (albeit more weakly than RecQL4). By analogy to yeast Mcm10, which allosterically stimulates CMG's ATPase-helicase activity *in vitro*^{21,65}, we propose that metazoan Mcm10 stimulates CMG's ATPase-helicase activity, thus promoting DNA melting inside CMGs central channel (Fig. 7g).

We also show that the disordered N-terminus and the structured C-terminus of RecQL4 play distinct CMG activation functions, with the N-terminus being the more potent CMG activator. However, maximal CMG activation efficiency is achieved when both N-term and C-term regions are present, i.e. by the full-length wildtype protein. It is unclear how the N-terminus promotes CMG activation. By analogy to Mcm10, it is tempting to speculate that RecQL4's N-term allosterically stimulates CMG's ATPase-helicase activity.

AlphaFold3 consistently docked RecQL4's structured C-terminus to CMG's C-terminal side, in proximity to dsDNA (Fig. 7e). We show that RecQL4's C-terminus contributes to CMG activation, and this contribution is entirely dependent on RecQL4's ATPase activity. In light of a previous report that RecQL4 can unwind short DNA oligos *in vitro*⁶⁶, we speculate that RecQL4 promotes CMG activation by melting dsDNA on either side of the pre-activation complex (Fig. 7f). Alternatively, RecQL4 may promote CMG activation by generating extra torque to melt dsDNA enclosed in CMG's central channel. This could explain why RecQL4's ATPase activity is important for CMG helicase activation. In the absence of Mcm10, the RecQL4-Mcm7 interaction serves as a backup mechanism of recruiting RecQL4 to the pre-activation complex. This explains the findings of a previous study, which reported that depleting Mcm10 had a very modest effect on DNA replication, but attributed this mild defect to Mcm10's role in replication elongation and fork stability³².

During CMG activation, the lagging strand template must be expelled from CMG's central channel. We speculate that Mcm10's ssDNA binding domain captures the excluded strand and

stabilizes it long enough to facilitate the activation of both CMG complexes (Fig. 7h, white ovals). Finally, the two CMGs separate from each other, and this is accompanied by Mcm10, RecQL4, and DONSON dissociation. As proposed previously^{18,21}, the head-to-head CMG orientation ensures that each origin fires only when both CMGs are fully activated and can pass each other unobstructed on different DNA strands (Fig. 7i).

Division of Labor Between RecQL4 and Mcm10 During Replication Initiation

Although both RecQL4 and Mcm10 were previously implicated in metazoan replication initiation, the exact roles of these proteins have long been debated. In a recent study, we demonstrated that RecQL4 participates in CMG helicase activation¹⁷. Here, we show that Mcm10 also participates in CMG helicase activation, alongside RecQL4. Our data indicate that Mcm10 and RecQL4 are partially redundant during CMG activation, explaining why RecQL4-depleted *Xenopus* extracts still support significant levels of DNA replication¹⁷, and why some RecQL4-knockout cells maintain viability in cell culture⁶⁷. While our manuscript was under revision, a pre-print from the Kanemaki lab⁶⁸ and one from the Labib lab⁶⁹ were posted to bioRxiv, summarizing their findings that Mcm10 and RecQL4/SLD2 play redundant roles during CMG helicase activation in worms, mouse embryonic cells, and in human cells. Moreover, both studies found that depleting either Mcm10 or RecQL4 caused only mild replication defects, whereas depleting both proteins completely blocked CMG activation, in perfect agreement with our findings.

Why do metazoa employ two distinct initiation factors to drive CMG activation? It is possible that Mcm10 and RecQL4 may act on different classes of origins. For example, either protein alone may be sufficient to promote CMG activation at origins in euchromatin, whereas firing origins in heterochromatin may require both proteins acting in concert. Alternatively, Mcm10 or RecQL4 may be selectively recruited to specific loci. For example, RecQL4 can bind DNA secondary structures such as G4 quadruplexes, and this may regulate replication timing⁷⁰. Future studies mapping Mcm10-dependent versus RecQL4-dependent origins will further elucidate the division of labor between these two CMG activators.

Mechanistic Differences and Similarities Between Yeast and Metazoa

Until our study and the two related pre-prints from the Labib and Kanemaki groups^{68,69}, the mechanism of CMG helicase activation had been best understood in yeast. It is widely accepted that yeast Mcm10 plays a central role in CMG activation¹⁸. Until recently, it was thought that Sld2 (the yeast homolog of RecQL4) participates exclusively in CMG assembly²⁴. Our data reveal that unlike yeast, metazoan CMG activation involves the concerted action of both Mcm10 and RecQL4. Importantly, we show that RecQL4's structured C-terminus is important for efficient origin firing, and becomes essential for origin firing in the absence of Mcm10. This fragment of RecQL4 is unique to metazoa (Extended Data Fig. 9a) and includes the ATPase domain, the DNA-binding domain (R4ZBD), and the Mcm7-interaction motif that we identified (Fig. 4a-b).

The role of Mcm10 and RecQL4 in metazoan replication initiation has been highly debated because these proteins are involved in several other aspects of genome maintenance^{27,28,71}. In hindsight, previous conflicting reports about the roles of RecQL4/Mcm10 can be explained by the fact that Mcm10 and RecQL4 are partially redundant during CMG activation, as shown here and in the two related Mcm10-RecQL4/SLD2 manuscripts^{68,69}. While our manuscript was under revision, the Costa and Diffley groups published a study⁷² that implicates yeast Sld2 in CMG activation, suggesting that the helicase activation mechanism is more conserved among eukaryotes than previously thought.

Mcm10 Does Not Travel with the Replication Fork During Unperturbed Replication

Mcm10 has been called "one of the more mysterious players in DNA replication and the cause of much controversy"⁷³ because it is implicated in numerous aspects of DNA replication, ranging from initiation, elongation⁷¹, termination⁷⁴, replication fork reversal⁷⁵, and replication fork

restart^{27,76}. These functions imply that Mcm10 travels with the replication fork. [Until now, it has been](#) widely accepted that Mcm10 is a constitutive replisome subunit, with supporting evidence coming from reports of Mcm10 enrichment at/near sites of active replication⁷¹. However, there are also studies reporting little to no enrichment of Mcm10 at replication forks²³. A recent single-molecule study with purified yeast proteins, found that Mcm10 travels with CMG, but those experiments did not recapitulate CMG loading/activation and did not contain fully assembled replisomes⁷⁷. So far, there have been no studies that directly visualized Mcm10 at active replication forks.

Our data indicates that Mcm10 is recruited to the pre-activation complex and that Mcm10 dissociates from the origin shortly before the start of DNA synthesis. In >90% of cases we do not observe Mcm10 travel with active replication forks ([Fig. 1c-d](#)). In the absence of any additional perturbations, we rarely observe Mcm10 transiently associating with active replisomes. We propose that following CMG activation, Mcm10 is displaced from its binding site at the front of CMG by other replisome subunits. For example, Claspin and [TIM-TIPIN](#) also bind at the front of the CMG complex and could prevent Mcm10 re-binding⁷⁸. It is also possible that Mcm10 does not associate with replisomes during unperturbed DNA replication but is conditionally recruited to CMG during replication stress. [This idea will be explored in future studies.](#)

Replication Initiation Defects Caused by Pathological RecQL4 Mutations

The field lacks a clear understanding of how mutations in RecQL4 lead to the phenotypes reported in patients with Rothmund-Thomson, Baller-Gerold, and RAPADILINO syndromes. Our data is consistent with the idea that RecQL4 disease phenotypes [can](#) be attributed to its role in replication initiation.

A large fraction of disease-associated RecQL4 mutants contain premature stop codons or large deletions predicted to result in low mRNA/protein levels⁴⁸. Similarly, many point mutations or small deletions may also destabilize RecQL4 folding/stability. For such hypomorphic mutations, the exact location of the mutation (Sld2-like domain, ATPase domain, R4ZBD, etc.) should be interpreted with caution. The one exception are premature stop codons in the last exon, which are usually not subject to nonsense-mediated decay⁷⁹. There are two such mutations reported in Rothmund-Thompson Syndrome patients: p.Gln1175*⁸⁰ and p.Thr1200Argfs*26⁴⁷. They are expected to produce almost full length RecQL4 at nearly-normal levels, yet are pathological⁴⁸. Here we report that the unannotated extreme C-terminus of RecQL4 mediates the interaction with Mcm7 ([Fig. 4a-b](#)). We propose that this interaction docks RecQL4's ATPase-helicase to the rear of CMG, proximal to dsDNA ([Fig. 7d-e](#)).

Our data show that all domains of RecQL4 play a role in replication initiation, therefore any patient-derived mutations that impair protein function or stability are expected to reduce origin firing efficiency. Since Mcm10 can partially compensate for the loss of RecQL4's ATPase activity/domain, we speculate that Mcm10 may be overexpressed in some patient tissues to compensate for pathologic RecQL4 mutations.

Limitations

All experiments were repeated at least twice with different extract preparations, [and](#) a representative experiment is shown in the [main](#) figures. Different extract preparations may exhibit some variations in DNA replication kinetics which could be caused by small changes in firing factor concentrations.

According to the OpenCell database⁸¹, the cellular concentrations of Mcm10 (~50 nM) and RecQL4 (~50 nM) are lower than in *Xenopus* egg extract (~250 nM for RecQL4 and ~100 nM for Mcm10 in our biochemical reactions). Plasmid replication reactions were performed with 50-100 nM of unlabeled recombinant Mcm10 or RecQL4, which was saturating for the ensemble assays ([for Coomassie gels of purified proteins see Extended Data Fig. 9b](#)).

The RecQL4-Mcm7 interaction is relatively weak, as stringent washes decrease the efficiency of co-immunoprecipitation *in vitro* (data not shown). This is consistent with our measurement of very low recruitment of RecQL4⁶⁶⁴⁻¹⁵⁰⁰ to origins in Mcm10-depleted extract (Fig. 6c and Fig. 6g). Single-molecule experiments were performed at 10 nM of Mcm10^{AF647} or RecQL4^{AF647} to maintain an acceptable signal-to-noise ratio. Although this concentration is lower than that of endogenous proteins, these experiments provided a sensitive way of quantifying the relative binding affinity for different mutants under different depletion conditions.

In single-molecule experiments conducted with 10 nM of RecQL4^{AF647}, origin firing efficiency was very sensitive to the absence/presence of Mcm10 (Fig. 6h) because Mcm10-independent recruitment of RecQL4 to origins was inefficient at this low concentration of RecQL4. By contrast, ensemble replication experiments conducted with either endogenous RecQL4 (~250 nM) or recombinant RecQL4 (100 nM), revealed only a minor replication defect upon Mcm10 depletion (Fig. 5b-c). This is consistent with the idea that at high RecQL4 concentrations, Mcm10-independent recruitment of RecQL4 to origins is not rate-limiting.

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Author Contributions

R.T. and G.C. conceived the project; G.C., wrote the manuscript with input from R.T.; R.T. carried out all ensemble and single-molecule experiments, with a few exceptions mentioned below; L.A.S. raised/validated RecQL4 custom antibodies, optimized the Mcm10 immunodepletion protocol, and cloned Mcm10 for expression via IVTT; D.S. raised/validated the Mcm10 and TopBP1 custom antibodies; E.H.H. performed co-IP experiments with the Mcm7^{WHD} (Fig. 4d); J.P. performed single-molecule experiments to simultaneously visualize Mcm10 and RecQL4 (Fig. 3e-h); B.D. optimized and performed chromatin fractionation experiments (Fig. 2e-g); G.C. wrote MATLAB code to analyze single-molecule experiments; G.C. and R.T. analyzed single-molecule data; G.C. performed AlphaFold3 modeling and bioinformatics analysis.

Declaration of Interests

Authors declare no conflicts of interest.

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Figure 1

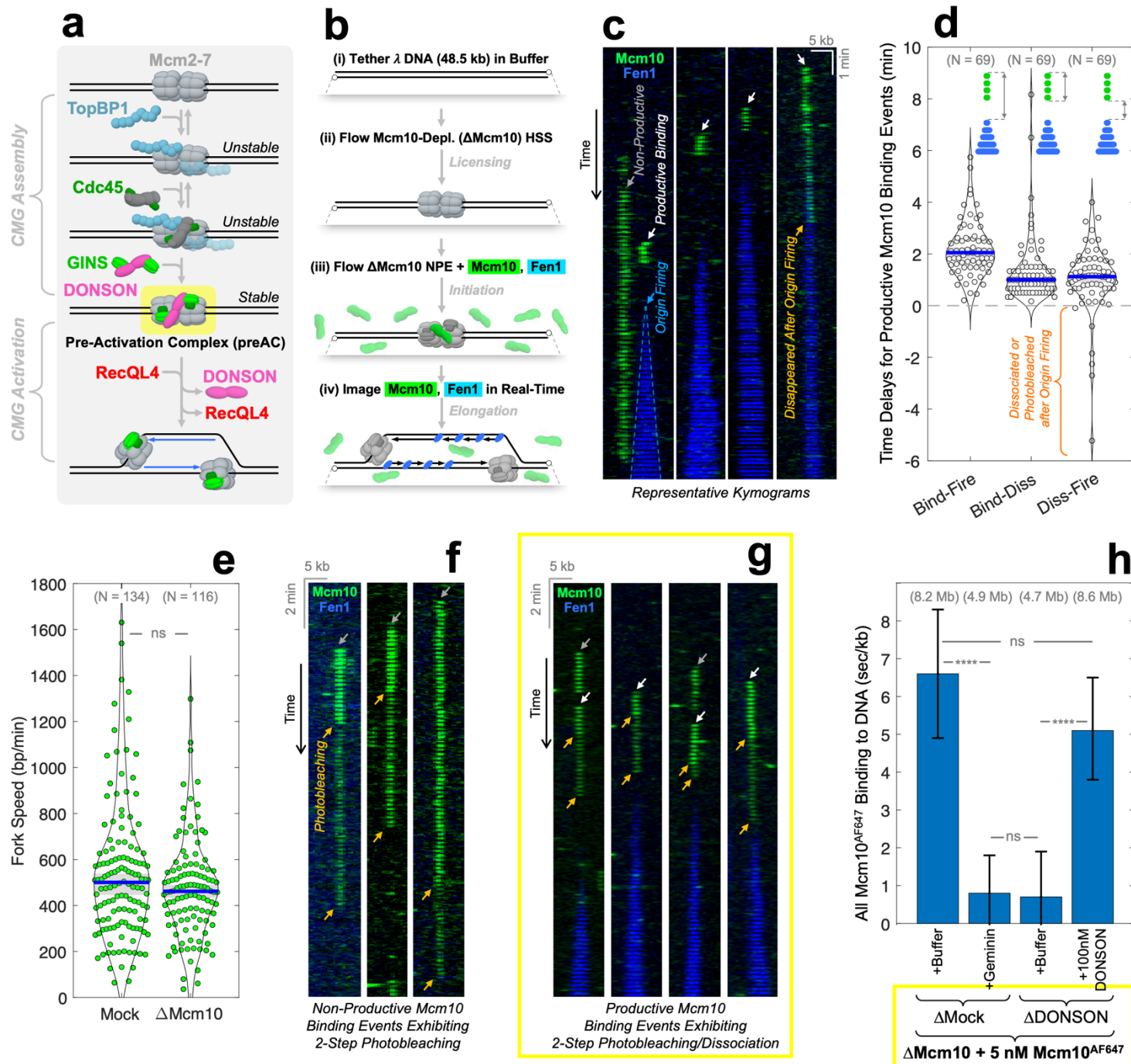
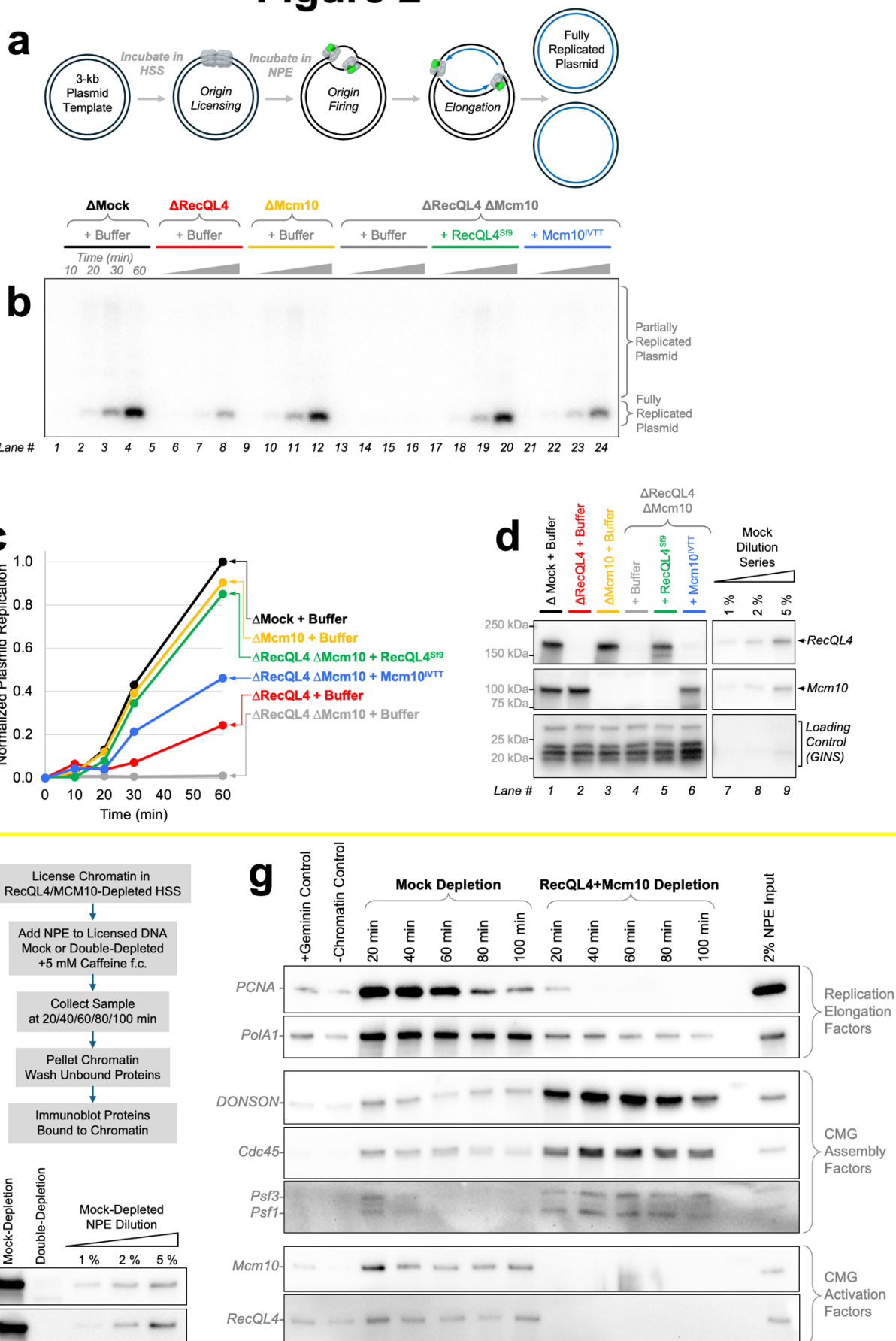


Figure 1: (a) Current model of replication initiation in metazoa. **(b)** Single-molecule experiment to visualize Mcm10^{AF647} (green) binding to DNA, Fen1^{mKikGR} (blue) acts as a marker of nascent DNA. **(c)** Representative kymograms illustrating Mcm10^{AF647} (green) binding to licensed DNA. Fen1^{mKikGR} (blue) marks nascent DNA. **(d)** Time delays for productive Mcm10^{AF647} binding events. Left: delay between Mcm10^{AF647} binding and origin firing. Center: delay between Mcm10^{AF647} binding and dissociation. Right: delay between Mcm10^{AF647} dissociation and origin firing (negative values mean that Mcm10^{AF647} dissociated/photobleached after origin firing). N is the number of productive Mcm10^{AF647} binding events. **(e)** Fork speed in mock-depleted versus Mcm10-depleted reactions. N is the number of replication forks. **(f)** Representative kymograms of non-productive Mcm10^{AF647} binding events exhibiting two-step

975 photobleaching (orange arrows). **(g)** Representative kymograms of productive Mcm10^{AF647} binding
976 events (white arrows) exhibiting two-step photobleaching/dissociation (orange arrows). Gray arrows
977 denote non-productive events. **(h)** Quantification of all Mcm10^{AF647} binding (productive and non-
978 productive) to DNA normalized to DNA length (Extended Data Fig. 1g illustrates how normalized binding
979 is measured). Geminin blocks licensing and serves as a negative control. Total DNA length analyzed for
980 each condition is reported in parentheses. Error-bars denote the 95% Confidence Interval (CI) estimated
981 via bootstrapping. In **(d)** and **(e)** blue bars represent median values, gray boxes mark the 95% CI. The
982 two-sample Kolmogorov-Smirnov test was used to compute p-values: ns (not significant, $p > 0.05$), * ($p <$
983 0.05), ** ($p < 0.01$), *** ($p < 0.001$), **** ($p < 0.0001$).

Figure 2



985 **Figure 2: (a)** Overview of the ensemble plasmid replication assay (dCTP^{P32} is added to the reaction and
 986 is incorporated into nascent DNA). **(b)** Autoradiograph of agarose gel showing the results of a plasmid
 987 replication assay performed in mock-depleted, RecQL4-depleted, Mcm10-depleted, or double-depleted
 988 extract (see Extended Data Fig. 2a-c for a biological repeat). Reactions were supplemented with buffer,
 989 100 nM of recombinant RecQL4 expressed in Sf9 insect cells (RecQL4^{Sf9}), or 100 nM of Mcm10 expressed
 990 using In Vitro Transcription-Translation (RecQL4^{IVTT}). Time-points were collected at 10, 20, 30, and 60
 991 minutes. **(c)** Quantification of the nascent DNA signal for each reaction shown in panel **(b)**. **(d)** Immunoblots
 992 of extract samples taken from reactions shown in panel **(b)**. Both Mcm10 and RecQL4 depletions are very
 993 stringent (less than 1% of endogenous protein remaining). GINS serves as a loading control. **(e)** Overview
 994 of the sperm chromatin fractionation assay. **(f)** Immunoblot of depleted extracts used for the chromatin
 995 fractionation assay. **(g)** Immunoblots of chromatin-bound proteins in mock depleted or RecQL4-and-
 996 Mcm10 depleted extracts. Samples were collected at 20, 40, 60, 80, 100 min timepoints. “-Chromatin” and
 997 “+Geminin” samples serve as negative controls of non-specific binding. In the mock, Mcm10 is bound to
 998 chromatin at late time-points, which may reflect its involvement in replication termination, and other aspects
 999 of genome maintenance.

Figure 3

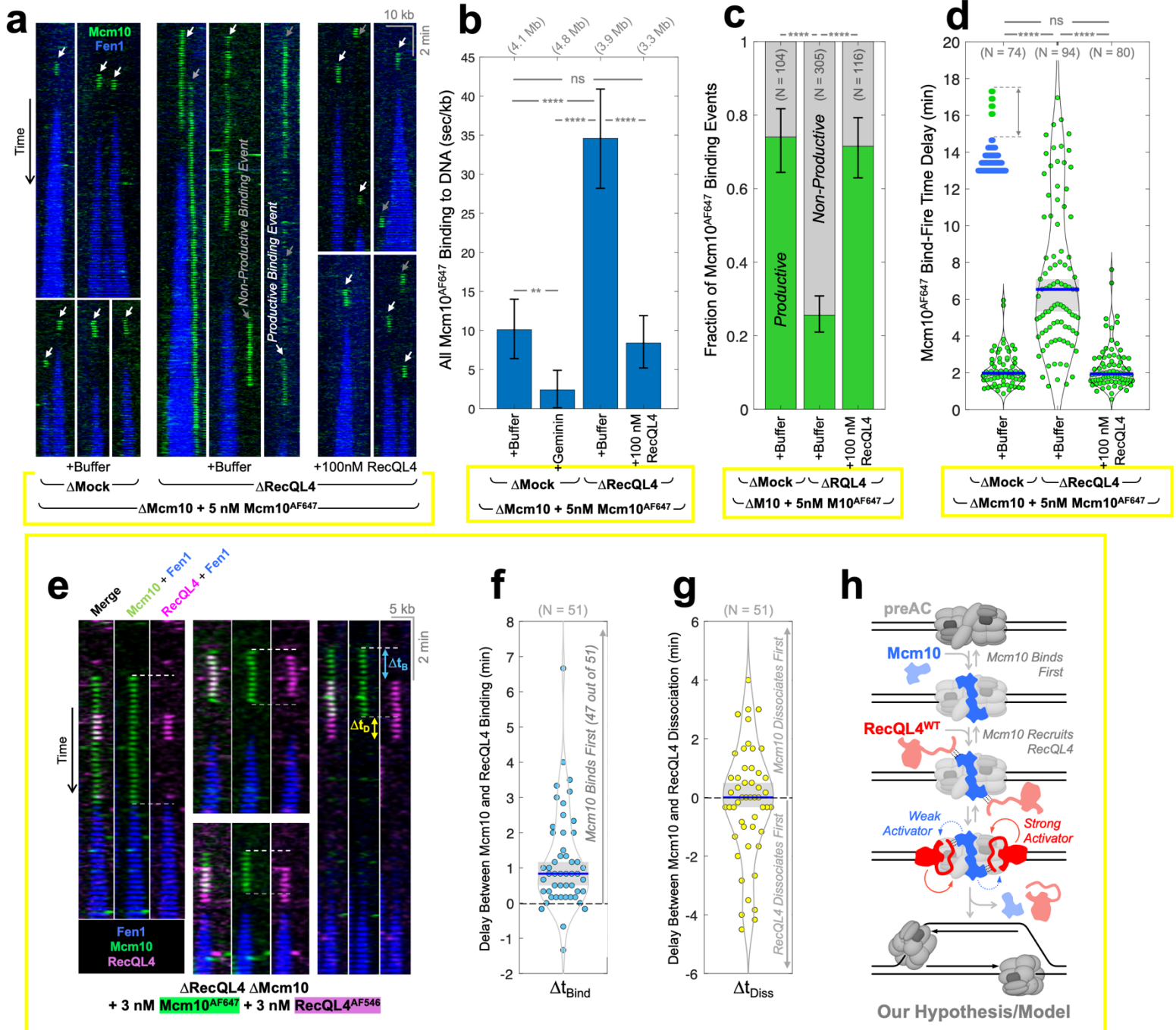


Figure 3: (a) Representative kymograms of Mcm10^{AF647} (green) binding licensed DNA. Fen1^{mKikGR} (blue) marks nascent DNA. White and gray arrows mark productive and non-productive Mcm10^{AF647} binding events. (b) Quantification of all Mcm10^{AF647} binding to DNA (productive and non-productive). Total DNA length analyzed is reported in parentheses. (c) Fraction of productive (green) versus non-productive (gray) Mcm10^{AF647} binding events. N denotes the number of binding events analyzed. (d) Time delay between Mcm10^{AF647} binding and origin firing. N is the number of productive events analyzed. See Extended Data Fig. 3a-c for the duration of productive events, non-productive events, and time delay between Mcm10^{AF647} dissociation and origin firing. (e) Representative kymograms of Mcm10^{AF647} (green) and RecQL4^{AF546} (magenta) binding origins before origin firing (marked by Fen1 in blue). Low concentrations (3 nM) of both CMG activators were used because at higher concentrations there were too many non-productive binding events, making it challenging to interpret the kymograms. (f) Time delay between Mcm10^{AF647} binding and RecQL4^{AF546} binding (Δt_{Bind}) before origin firing (positive values mean that Mcm10 bound before RecQL4).

1012 (g) Time delay between Mcm10^{AF647} dissociation and RecQL4^{AF546} dissociation (Δt_{Diss}) before origin firing
1013 (positive values mean that Mcm10 dissociated before RecQL4). In (f) and (g) N denotes the number of
1014 fired origins analyzed. In (b) and (c) error-bars denote the 95% CI estimated via bootstrapping. In (d), (f),
1015 and (g) blue bars denote the median while gray boxes show the 95% CI. Two-sample Kolmogorov-Smirnov
1016 test was used to compute p-values: ns (not significant, $p > 0.05$), * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$),
1017 **** ($p < 0.0001$).

Figure 4

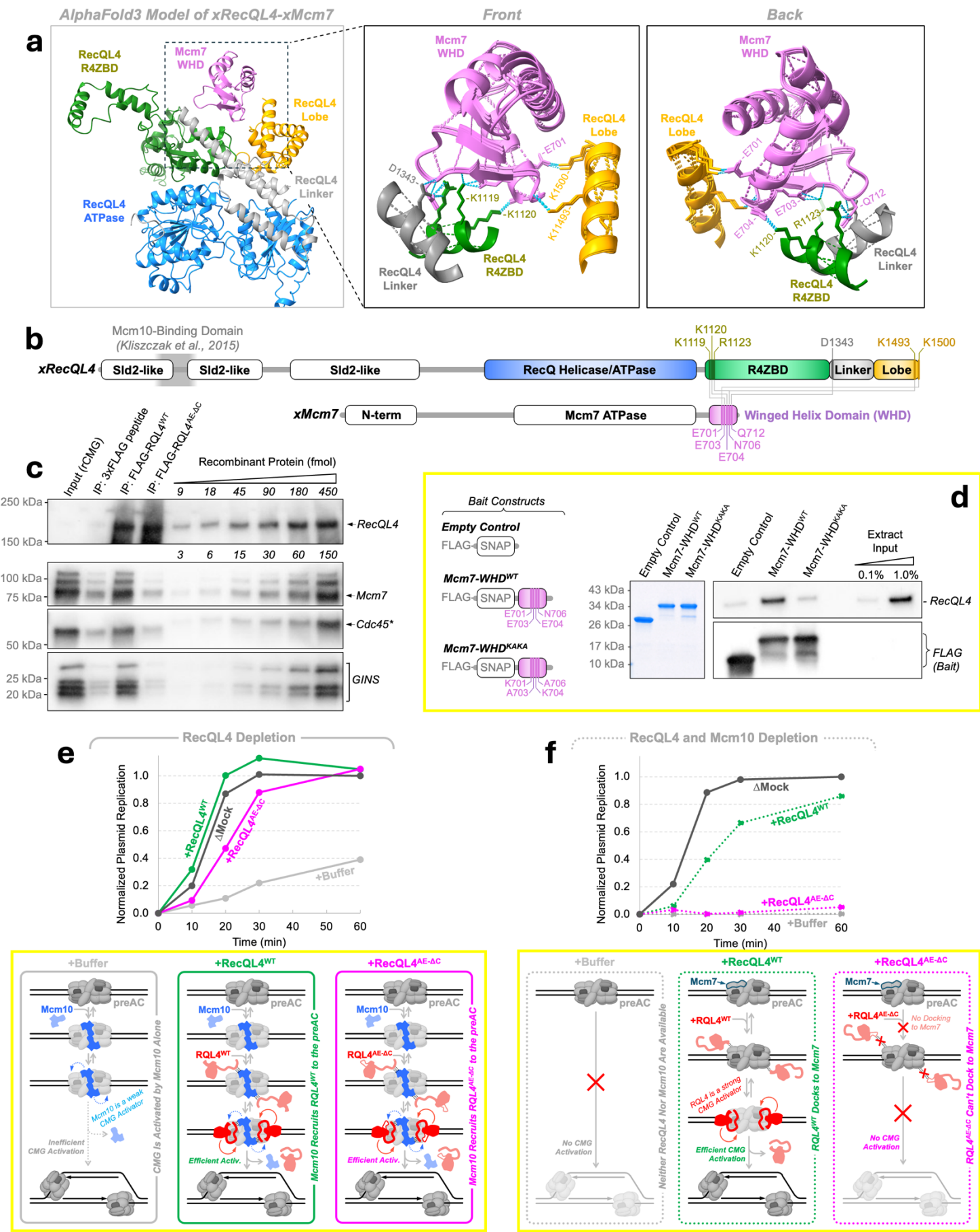


Figure 4: (a) Left: AlphaFold3-predicted structure of *Xenopus* Mcm7's winged-helix-domain (WHD, magenta) bound to the structured portion of RecQL4. [Extended Data Fig. 4a-b](#) shows similar predictions for human and mouse orthologs. Middle/Right: magnified view of the top three predicted structures (overlaid), highlighting key interactions. For clarity, some parts of the structure are not shown. (b) Diagram of *Xenopus* RecQL4 and Mcm7 highlighting residues involved in AF3-predicted interactions, using the same color scheme as in (a). (c) Immunoblot showing the results of the co-immunoprecipitation experiment between recombinant frog CMG (untagged) and recombinant FLAG-tagged RecQL4 (wildtype, or RecQL4^{AE-ΔC} - a mutant predicted to no longer interact with Mcm7). (*) For the recombinant protein input titration, we used recombinant Mcm2-7 (with a N-terminal tag on Mcm3), recombinant Cdc45 (with a C-terminal tag and linker), and recombinant GINS (with a C-terminal tag and linker on Psf3). The tags/linkers on tagged subunits leads to a small shift in molecular weight. (d) Results of co-immunoprecipitation experiments between Mcm7^{WHD} and RecQL4. Left: diagram of FLAG-tagged bait constructs containing empty, wildtype, or mutant Mcm7^{WHD}. Center: Coomassie-stained protein gel showing purified bait constructs. Right: Immunoblot showing how much endogenous RecQL4 co-IPed from extract with different bait constructs. The FLAG immunoblot shows how much bait was bound to magnetic beads. (e) Results of a plasmid replication assay performed in RecQL4-depleted extract supplemented with buffer or 100 nM of recombinant RecQL4^{WT} (green) or RecQL4^{AE-ΔC} (magenta). [Cartoons below illustrate our interpretation.](#) (f) Same as (e), but using extract depleted of both Mcm10 and RecQL4. See [Extended Data Fig. 5a-c](#) for raw autoradiographs and immunoblots corresponding to panels (e) and (f). See [Extended Data Fig. 5d-i](#) for experimental replicates.

Figure 5

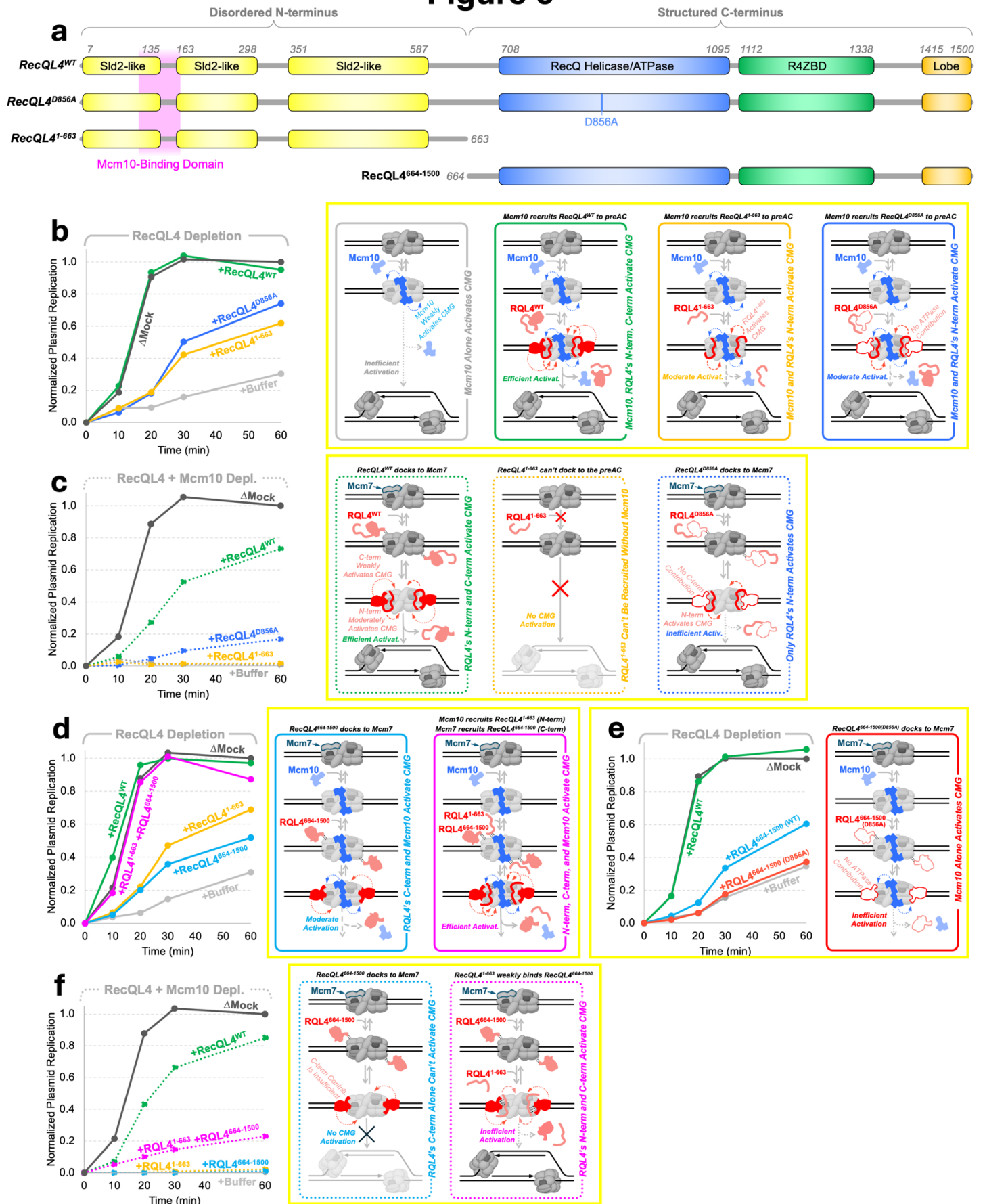


Figure 5: (a) Domain diagram for *Xenopus laevis* RecQL4 constructs used in this study. See Extended Data Fig. 1a for domain diagrams of wildtype human, mouse, and yeast orthologs. **(b)** Quantification of a plasmid replication assay performed in mock-depleted or RecQL4-depleted extract supplemented with buffer, or 100 nM of recombinant RecQL4^{WT}, RecQL4^{D856A}, or RecQL4¹⁻⁶⁶³. See raw autoradiograph and corresponding immunoblots in Extended Data Fig. 6a, c. See experimental repeats in Extended Data Fig. 6d-i. **(c)** Same as **(b)**, but using extract depleted of both Mcm10 and RecQL4. See raw autoradiograph in Extended Data Fig. 6b. See experimental repeats in Extended Data Fig. 6j-l. **(d)** Quantification of a plasmid replication assay performed in RecQL4-depleted extract supplemented with buffer or 100 nM of recombinant RecQL4^{WT}, RecQL4¹⁻⁶⁶³, RecQL4⁶⁶⁴⁻¹⁵⁰⁰, or both. See raw autoradiograph and corresponding immunoblots in Extended Data Fig. 7a-b. See experimental repeat in Extended Data Fig. 7c-d. **(e)** Same as **(d)**, but using extract depleted of both Mcm10 and RecQL4. See raw autoradiograph in Extended Data Fig. 7a and corresponding immunoblots in Extended Data Fig. 7b. See experimental repeat in Extended Data Fig. 7f-h. In panels **(b)** through **(f)** cartoons depict our interpretation of the results.

Figure 6

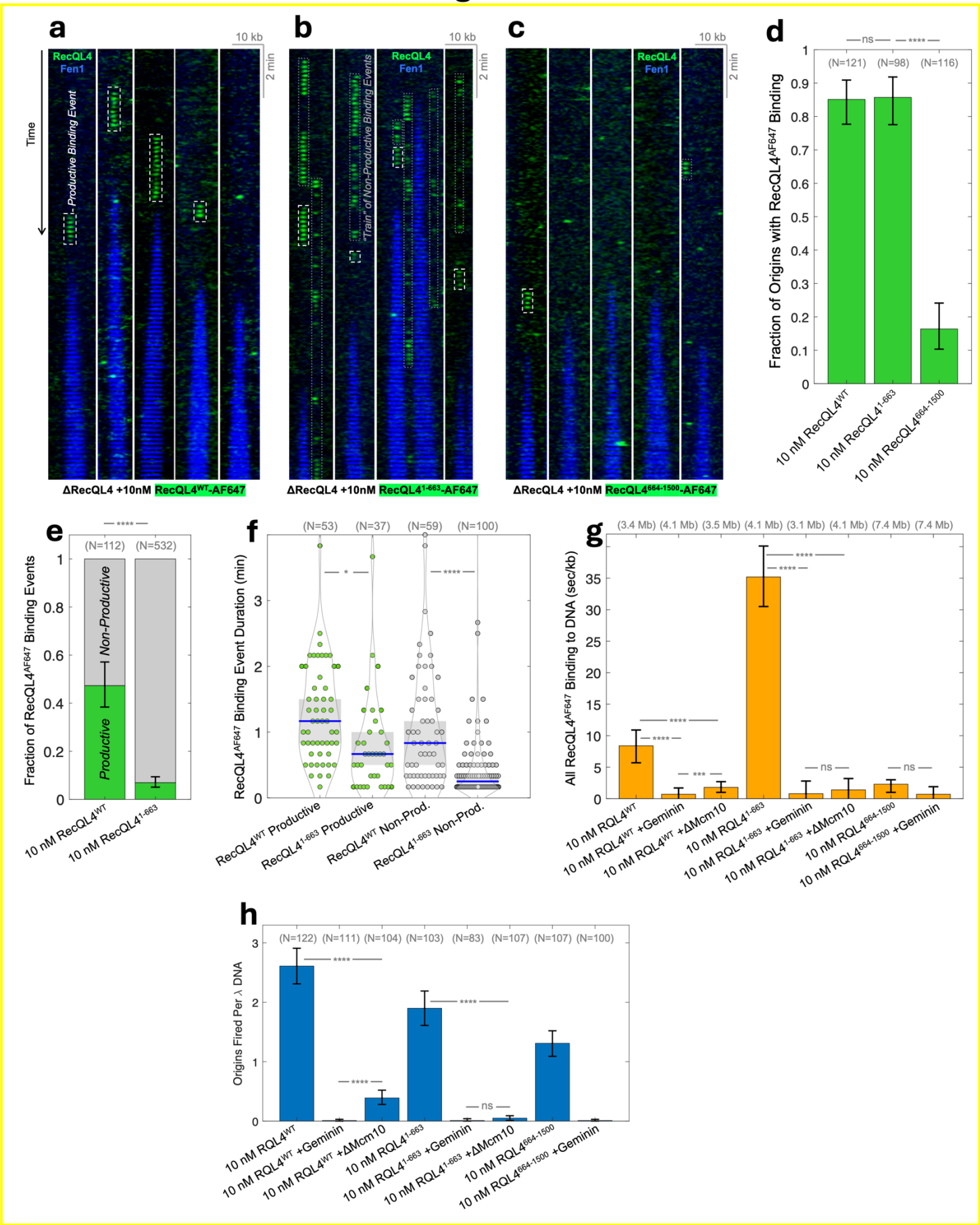


Figure 6: Representative kymograms illustrating the binding of AF647-labeled (green) RecQL4^{WT} (a), RecQL4¹⁻⁶⁶³ (b), and RecQL4⁶⁶⁴⁻¹⁵⁰⁰ (c) to licensed DNA. Binding events lasting a single frame were not scored, as they may represent non-specific binding. In (a) through (c) Fen1^{mKikGR} (blue) marks nascent DNA; dashed white boxes denote productive binding events and dotted gray boxes denote non-productive binding. (d) Fraction of fired replication origins (measured in the Fen1 channel) that were preceded by a AF647-labeled RecQL4^{WT}, RecQL4¹⁻⁶⁶³, or RecQL4⁶⁶⁴⁻¹⁵⁰⁰ binding event. N is the number of fired origins analyzed. (e) Fraction of productive (green) versus non-productive (gray) AF647-labeled RecQL4^{WT} or RecQL4¹⁻⁶⁶³ binding events. RecQL4⁶⁶⁴⁻¹⁵⁰⁰ was not included in this analysis, because the number of observed binding events was too low. N is number of all binding events analyzed. (f) Measured durations of productive (green dots) and non-productive (gray dots) RecQL4^{WT} and RecQL4¹⁻⁶⁶³ binding events. N is the number of binding events analyzed. Blue bars and gray boxes denote the median and the 95% CI estimated via bootstrapping. (g) Quantification of AF647-labeled RecQL4 binding to DNA (normalized in seconds per kb). This includes both productive and non-productive binding. “+Geminin” experiments represent negative controls where origins were not licensed. Total DNA length analyzed is reported in parentheses. (h) The number of origins fired per DNA molecule. N is number of λ DNA molecules analyzed. In (d), (e), (g), and (h) error-bars denote the 95% CI estimated via bootstrapping. The two-sample Kolmogorov-Smirnov test was used to compute p-values: ns (not significant, $p > 0.05$), * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), **** ($p < 0.0001$).

Figure 7

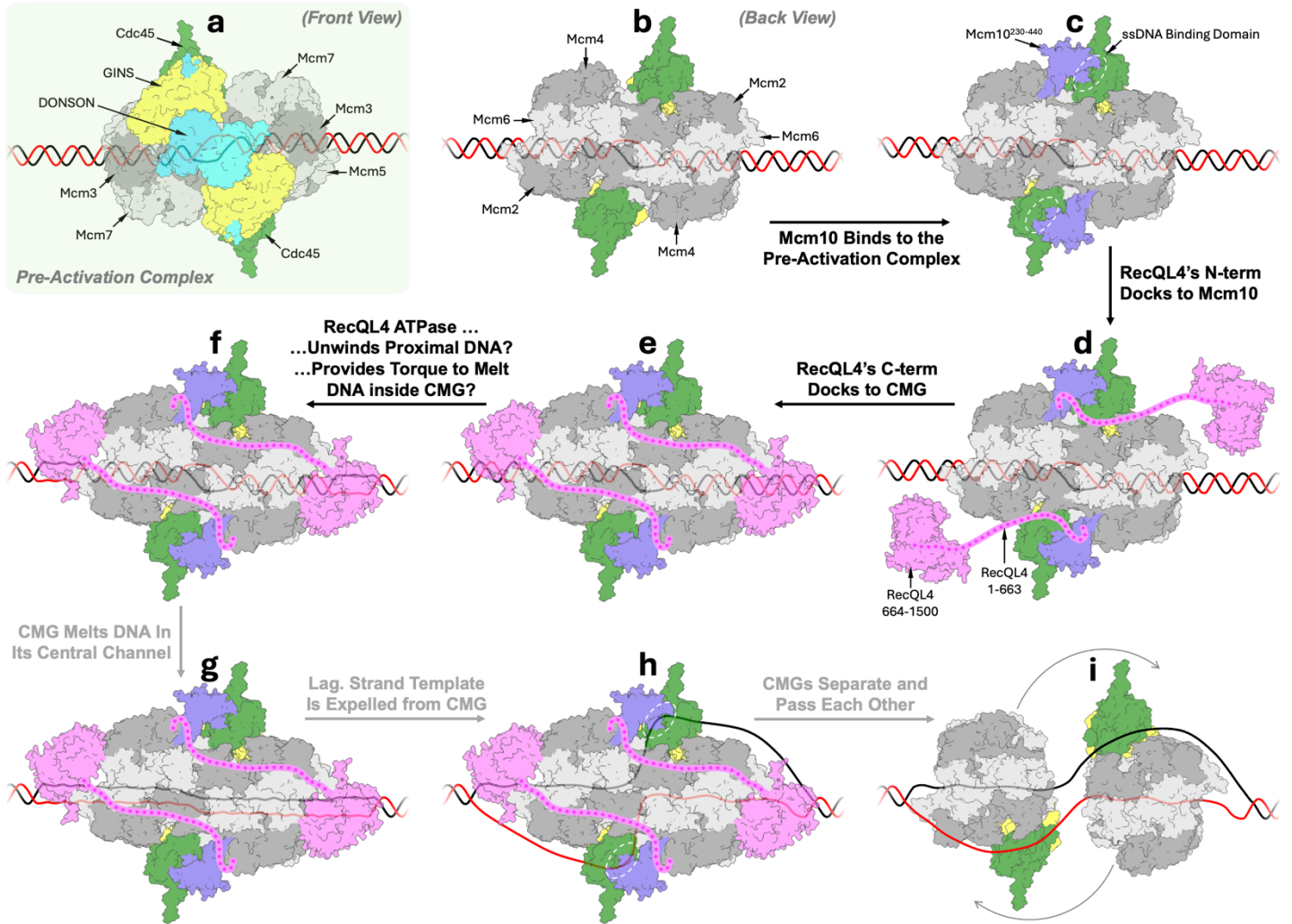
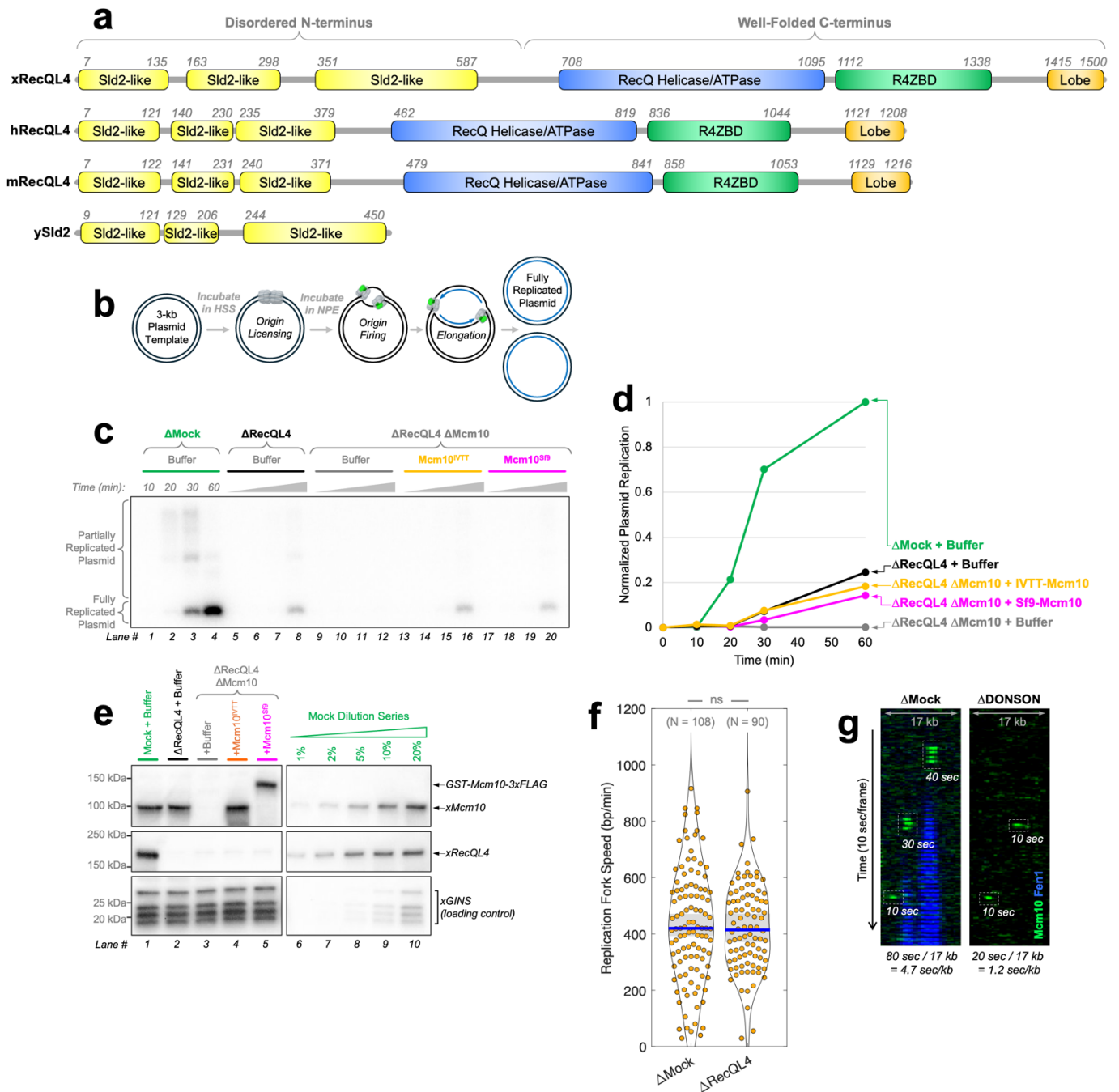


Figure 7: Proposed Model of CMG Activation in Metazoa. Thick black arrows highlight events supported by our data. Thich gray arrows indicate events known to occur during CMG activation. **(a)** Front view of the pre-activation complex consisting of two head-to-head CMGs on dsDNA bound by a DONSON dimer (light blue). Model was obtained by aligning a human CMG structure (PDB 6XTX) to a structure of frog (CMG•DONSON)₂ which lacks the Mcm2-7 ATPase domains (PDB 8Q6O). The central channels of CMGs are offset in the PDB 8Q6O structure, suggesting that the DNA inside the pre-activation complex *may be* partially melted. **(b)** Back view of the pre-activation complex model (DONSON and GINS are not visible here). **(c)** AF3-predicted docking of Mcm10's OB-fold domain known to bind ssDNA (white oval). AF3 did not consistently predict the docking of other Mcm10 domains. **(d)** The disordered N-terminus of RecQL4 (magenta) docks to Mcm10 (blue). **(e)** The structured C-terminus of RecQL4 (magenta) docks to CMG (AF3 prediction). **(f)** RecQL4's ATPase/helicase activity *may* melt dsDNA at the periphery of the pre-activation complex. *Alternatively, RecQL4's ATPase/helicase may generate extra torque, aiding in melting the DNA enclosed in CMGs central channel.* **(g)** We hypothesize that Mcm10 and RecQL4's N-terminus allosterically stimulate CMG's ATPase/helicase activity. We hypothesize that the two CMGs in a head-to-head orientation exert force/torque on dsDNA inside their central channels, which ultimately leads to the melting of origin DNA (similar to a model proposed by the O'Donnell Lab for yeast CMG activation²¹). **(h)** The lagging-strand template is expelled from CMG's central channel. **(i)** The two CMGs separate from each other and begin translocating along different template DNA strands.

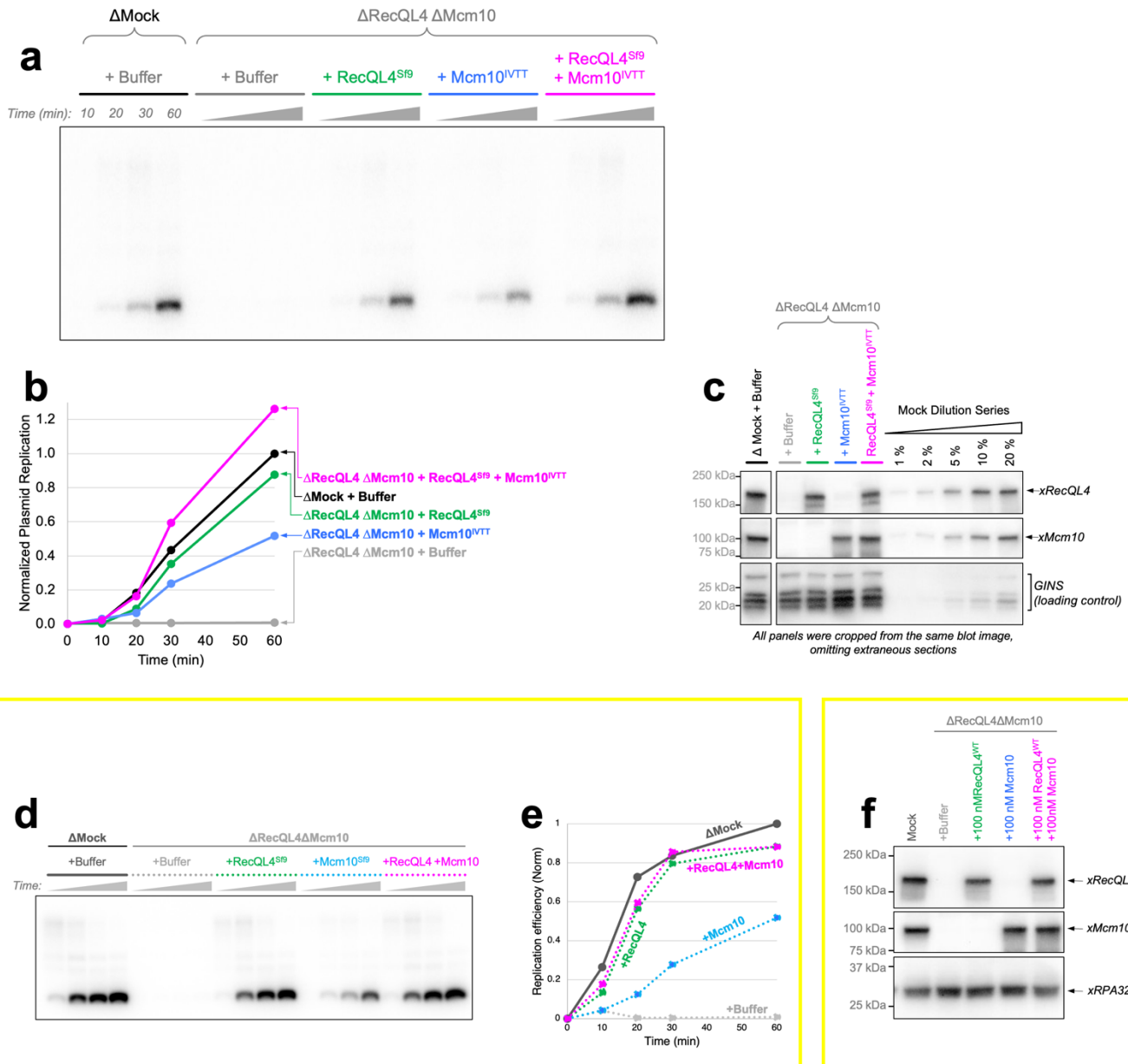
Extended Data Figure 1



Extended Data Figure 1: (a) Domain diagram for frog (x), human (h), and mouse (m) RecQL4 compared to yeast (y) Sld2. ATPase (blue) and R4ZBD (green) domain boundaries for human RecQL4 are taken from a structural study the Kisker lab⁵⁷ and extrapolated to its orthologs using multiple-sequence alignment. Sld2-like region boundaries (yellow) are defined here based on sequence alignment and serve as a rough indicator of sequence conservation. C-terminal lobe boundaries (orange) were defined based on AlphaFold3 predictions and multiple-sequence alignment. **(b)** Diagram illustrating the workflow of the ensemble plasmid replication assay (dCTP^{P32} is added to the reaction and is incorporated into nascent DNA). **(c)** Autoradiograph of agarose gel showing the results of a plasmid replication assay performed in mock-depleted, RecQL4-depleted, or Mcm10-and-RecQL4 depleted extract. Reactions were supplemented with buffer, 100 nM of recombinant Mcm10 expressed in insect cells (Mcm10^{Sf9}), or 100 nM of Mcm10 expressed using In Vitro Transcription-Translation (Mcm10^{IVTT}). Time-points were collected at 10,

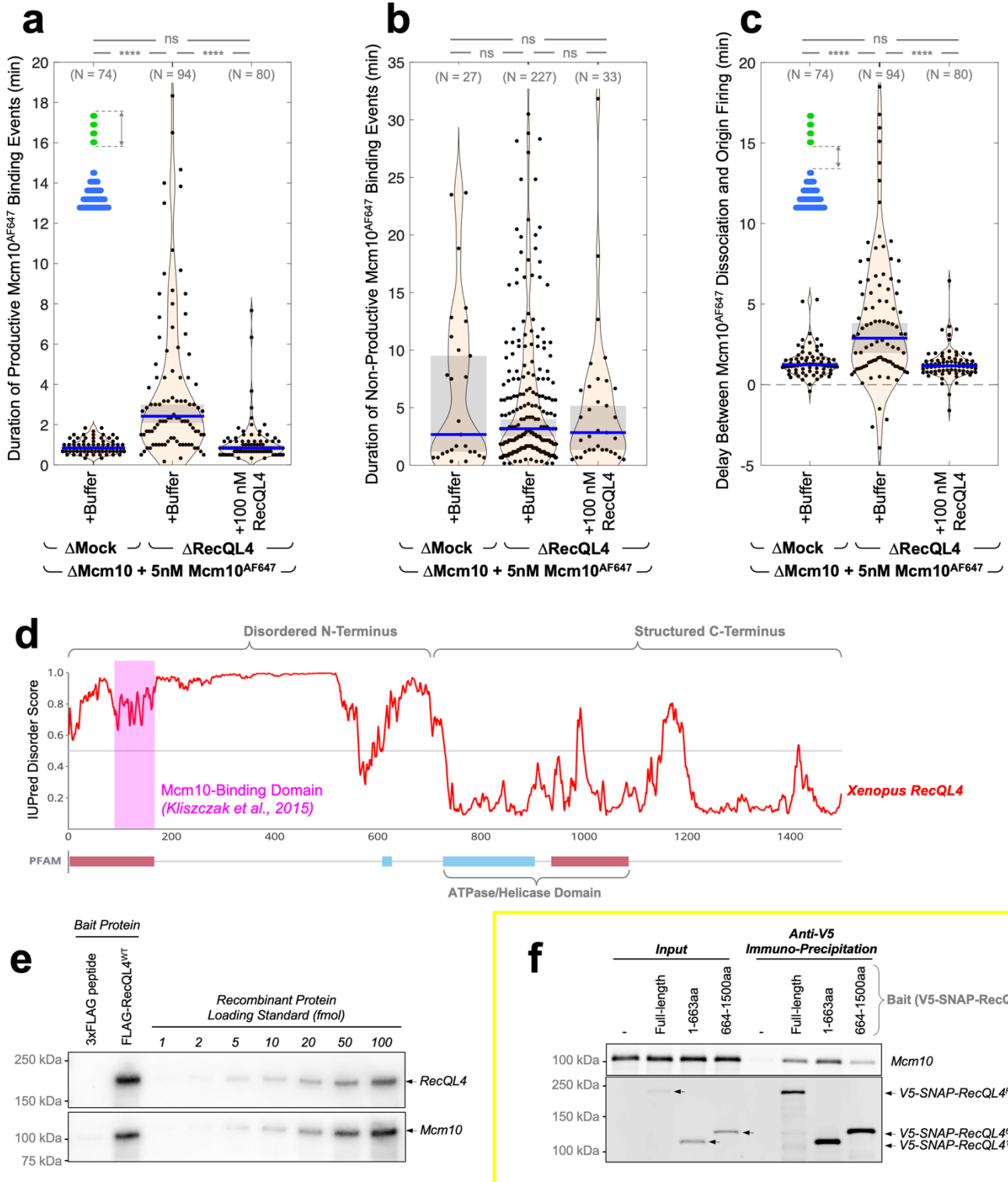
20, 30, and 60 minutes. **(d)** Quantification of the nascent DNA signal for each reaction shown in panel **(c)**. **(e)** Immunoblots of samples taken from reactions shown in panel **(c)**. Both Mcm10 and RecQL4 depletions are very stringent (less than 1% of endogenous protein remaining). GINS serves as a loading control. Both Mcm10^{Sf9} and Mcm10^{IVTT} support similar levels of DNA replication as RecQL4-depleted extract, indicating that recombinant Mcm10 has similar biochemical activity as endogenous Mcm10. **(f)** Distribution of fork speeds measured in mock-depleted extract or RecQL4-depleted extract. N is the number of forks analyzed. Blue bars and gray boxes denote the median and 95% CI estimated via bootstrapping. The two-sample Kolmogorov-Smirnov test was used to compute p-values: ns (not significant, $p > 0.05$). **(g)** Kymograms illustrating Mcm10^{AF647} binding to licensed DNA in mock-depleted or DONSON-depleted extract. To calculate the Mcm10 binding time on DNA, the durations of all binding events are added, and normalized by the length of DNA analyzed. This is done for several stretched λ DNA molecules (typically 100-200 DNA molecules). This measurement is corrected for non-specific binding of Mcm10^{AF647} off the DNA by performing a similar analysis in regions of interest that contain no DNA molecules, as previously described¹⁷.

Extended Data Figure 2



Extended Data Figure 2: (a-c) Replicate of the experiment shown in Fig. 2b, focusing on the double-depletion reactions. **(a)** Autoradiograph of agarose gel showing the results of the plasmid replication assay performed in mock-depleted, or Mcm10-and-RecQL4 depleted extract. Reactions were supplemented with buffer, 100 nM of recombinant RecQL4 expressed in Sf9 insect cells (RecQL4^{Sf9}), 100 nM of Mcm10 expressed using In Vitro Transcription-Translation (RecQL4^{IVTT}), or both. **(b)** Quantification of the nascent DNA signal for each reaction shown in panel (a). **(c)** Immunoblots of extract samples taken from reactions shown in panel (a). **(d-f)** Replicate of the experiment shown in Extended Data Fig. 2a-c using different preparations of recombinant proteins. Notably, this replicate used recombinant Mcm10 expressed in insect cells.

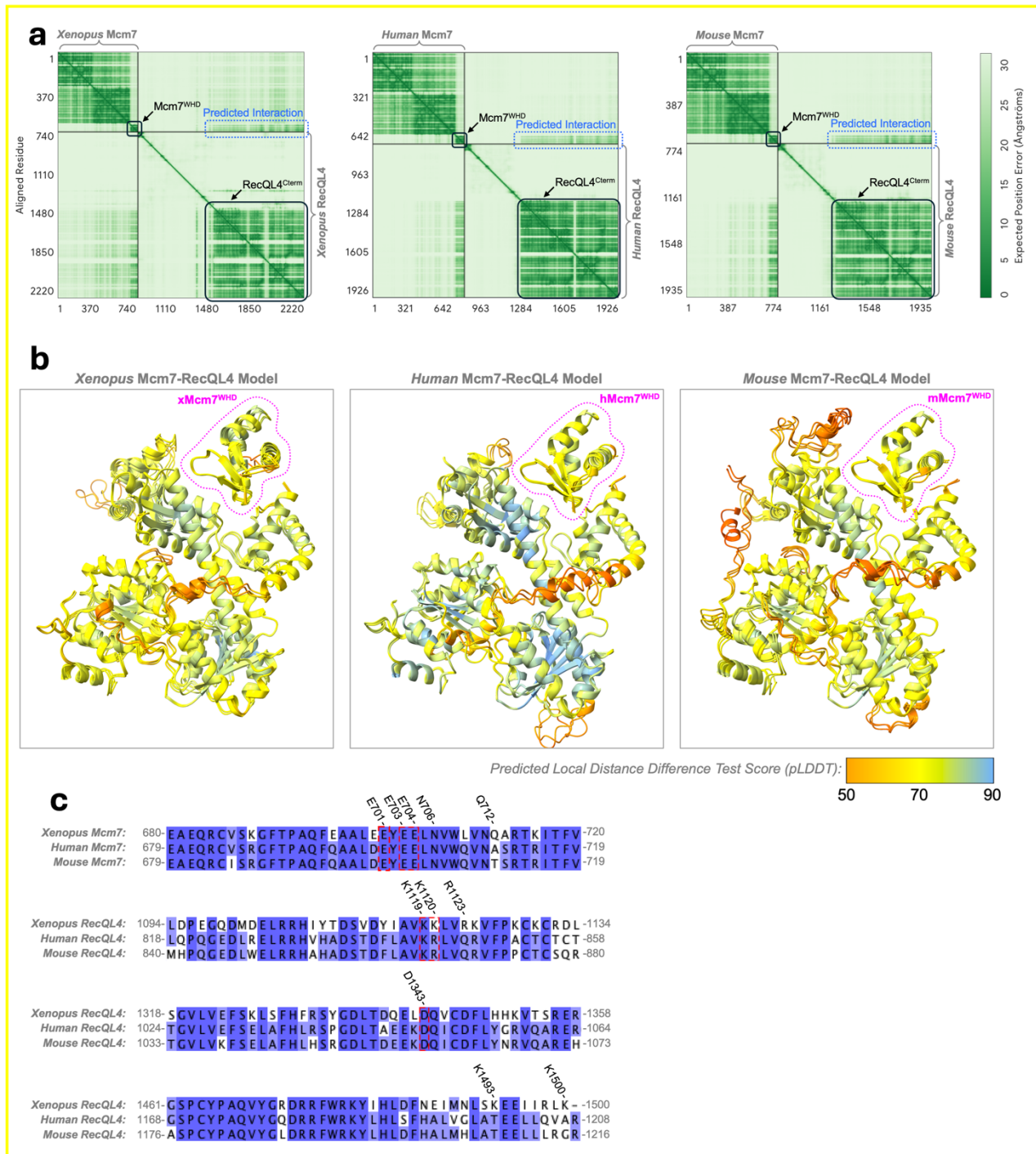
Extended Data Figure 3



Extended Data Figure 3: (a-c) Additional analysis of Mcm10^{AF647} binding events from experiments shown in Fig. 3a. (a) Duration of productive Mcm10^{AF647} binding events. (b) Duration of non-productive Mcm10^{AF647} binding events. (c) Time delay between Mcm10^{AF647} dissociation and origin firing. In (a-c), N denotes number of binding events analyzed; blue bars and gray boxes denote the median and 95% CI estimated via bootstrapping; two-sample Kolmogorov-Smirnov test was used to compute p-values: ns (not significant, $p > 0.05$), * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), **** ($p < 0.0001$). (d) Predicted disorder score for *Xenopus* RecQL4 calculated using

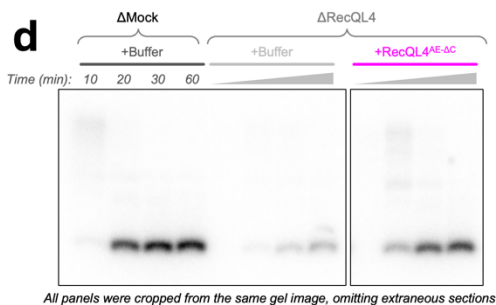
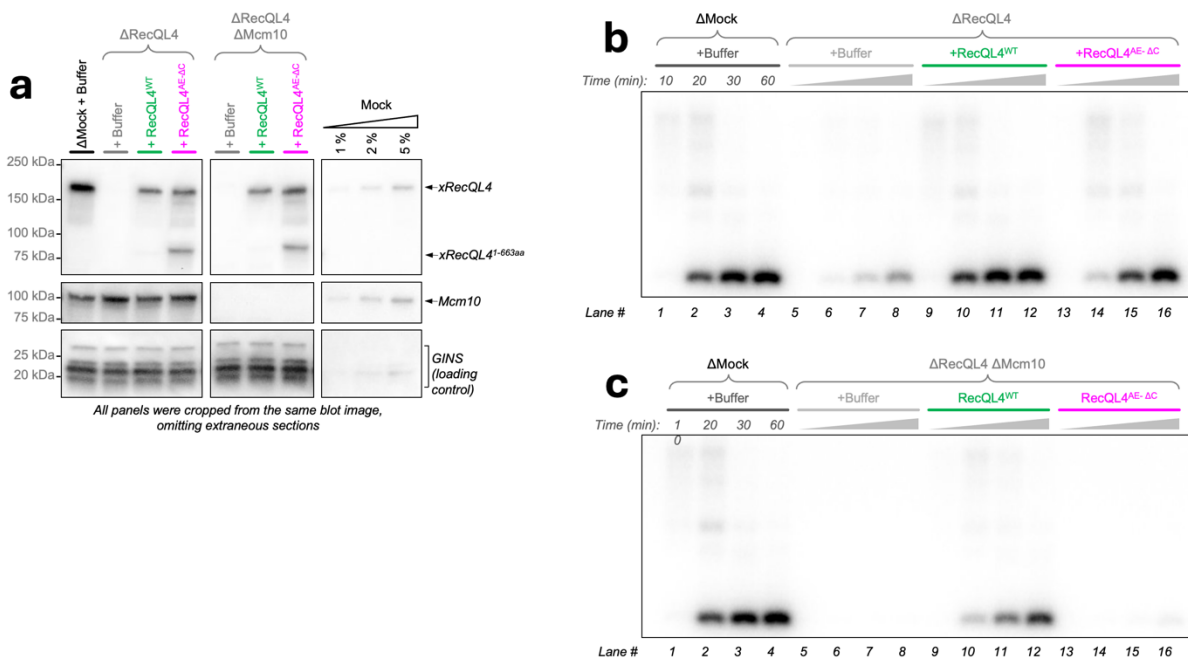
the AIUPred server⁸³. The Mcm10 binding region identified previously³⁵ is shown in magenta. **(e)** Immunoblot showing the results of a co-immunoprecipitation experiment between recombinant *Xenopus* Mcm10 (untagged) and recombinant FLAG-tagged RecQL4. 3xFLAG peptide was used as a negative control. A serial titration of both proteins was performed to estimate co-IP efficiency. **(f)** Immunoblots showing the result of a co-immunoprecipitation experiment using untagged Mcm10 and V5-tagged RecQL4 constructs expressed via IVTT (full-length wildtype, 1-663 aa N-terminal fragment, and 664-1500 aa C-terminal fragment). The RecQL4 membrane was blotted with an anti-V5 primary antibody. In the negative control, an empty vector was mock-expressed in IVTT (this control accounts for the non-specific binding of untagged Mcm10 to magnetic beads). Note that for a comparable amount of RecQL4¹⁻⁶⁶³ and RecQL4⁶⁶⁴⁻¹⁵⁰⁰ bait, RecQL4¹⁻⁶⁶³ co-IPed more Mcm10.

Extended Data Figure 4

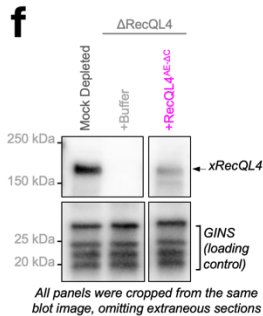
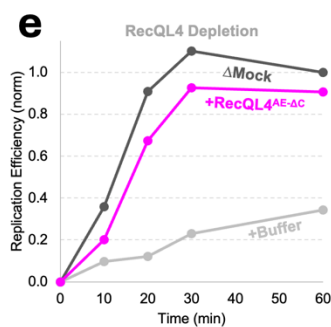


Extended Data Figure 4: (a) PAE (predicted alignment error) score matrix for co-folding frog, human, and mouse Mcm7 with RecQL4 using AlphaFold3. Low PAE corresponds to a higher confidence model. RecQL4's structured C-terminus and Mcm7's WHD are outlined in black rectangles. The predicted interaction between RecQL4 and Mcm7 is outlined with a dotted blue rectangle. **(b)** The top 3 models for each organism are shown overlaid. For clarity only the Mcm7^{WHD} and RecQL4^{C-term} are displayed. The color represents the pLDDT score calculated by AlphaFold3 (see color bar): pLDDT scores above 70 indicate a high model confidence, scores below 50 reflect a low model confidence. **(c)** Multiple-sequence alignment of frog/human/mouse Mcm7 and RecQL4 highlighting regions and residues involved in the AlphaFold3-predicted interaction (generated using Clustal Omega⁸⁴).

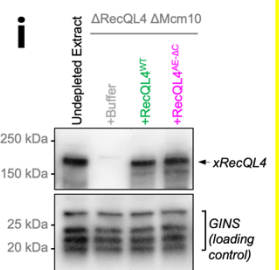
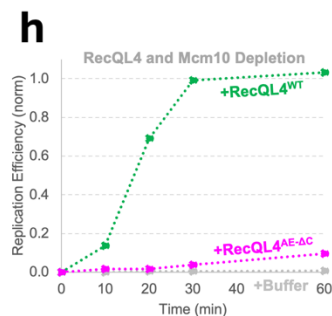
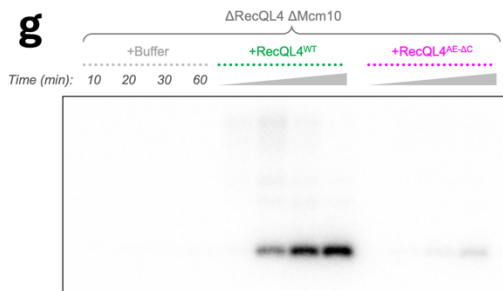
Extended Data Figure 5



All panels were cropped from the same gel image, omitting extraneous sections

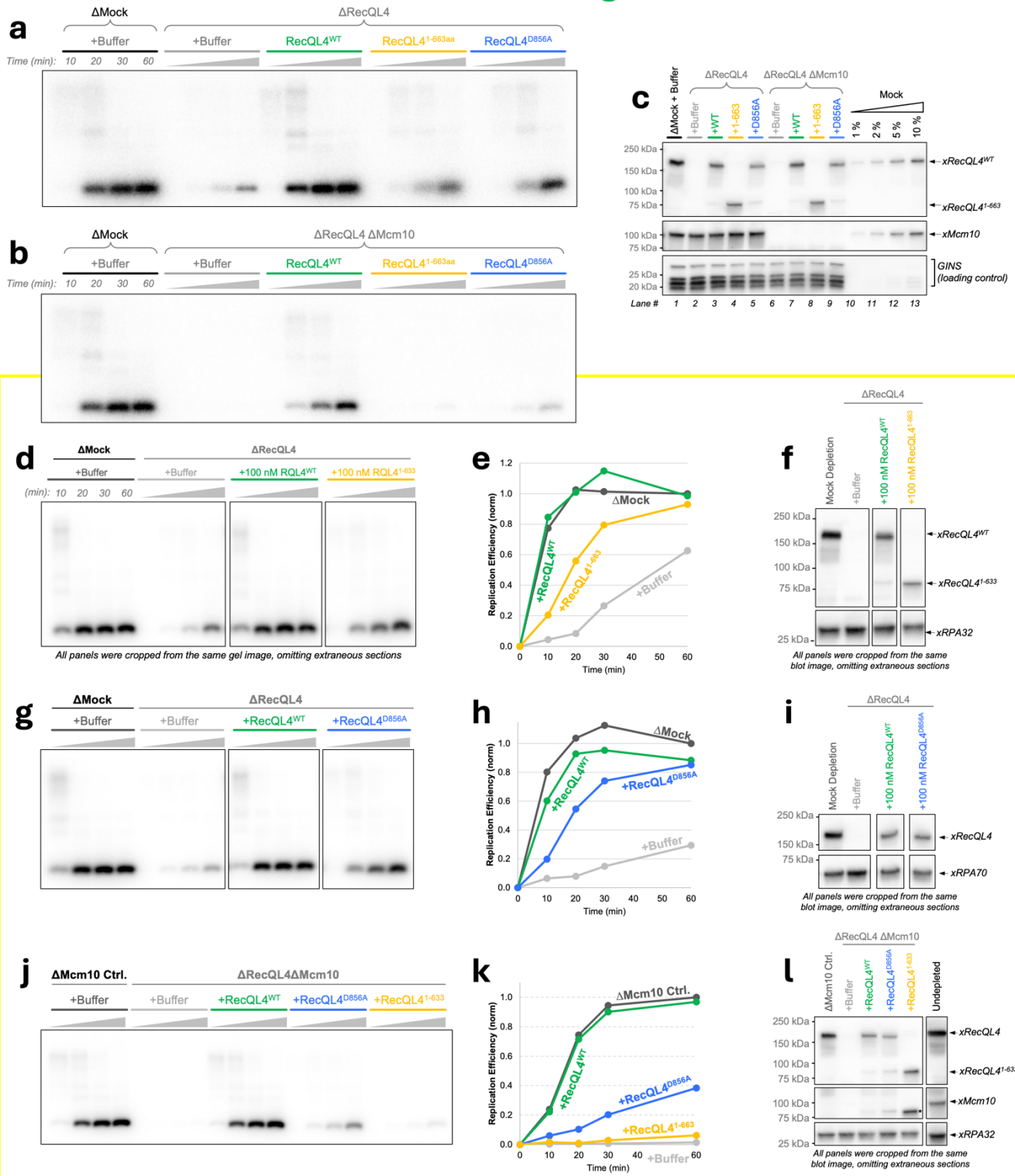


All panels were cropped from the same blot image, omitting extraneous sections



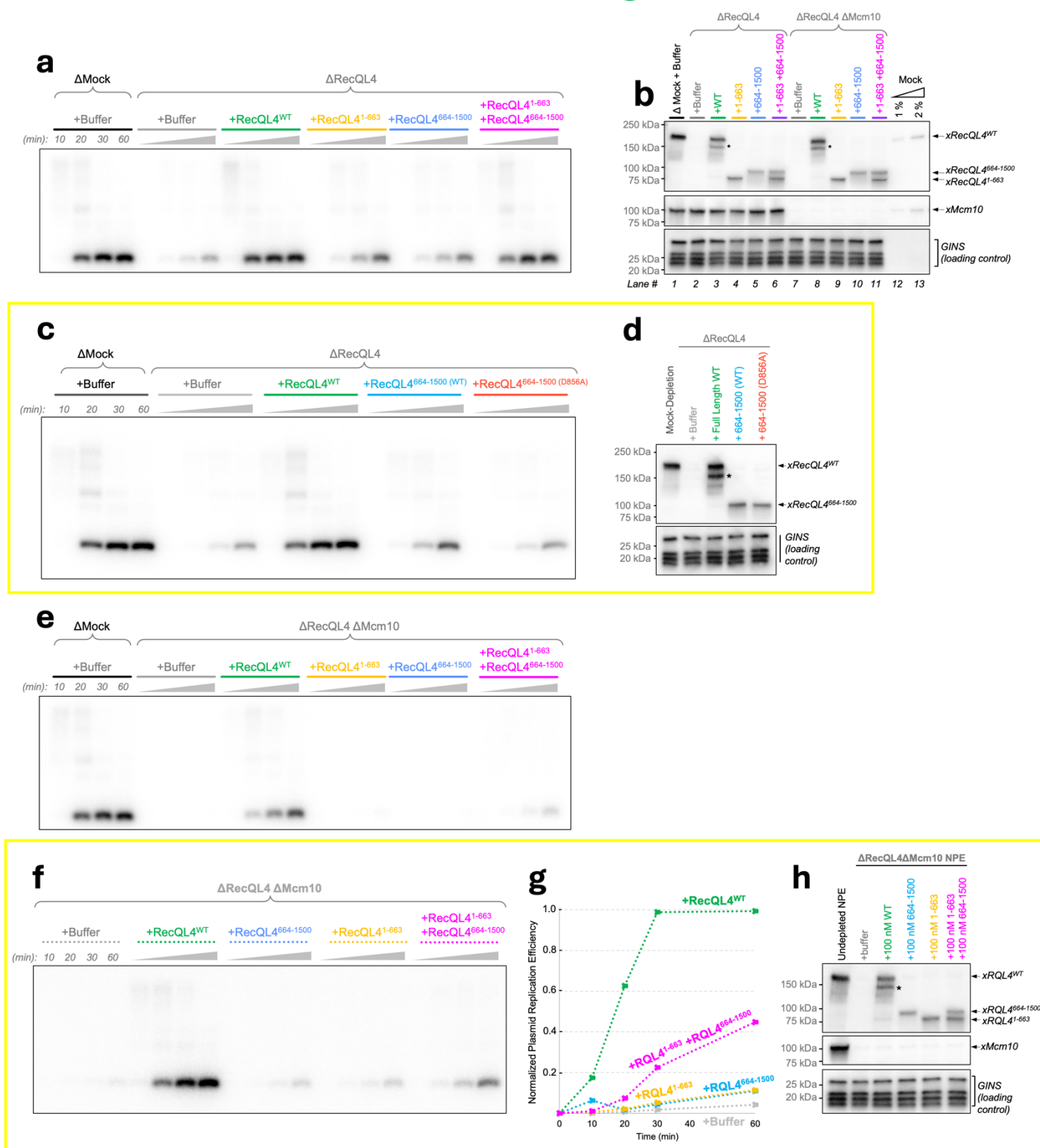
Extended Data Figure 5: (a) Immunoblots corresponding to data shown in Fig. 4e-f. (b) Autoradiograph corresponding to data shown in Fig. 4e. (c) Autoradiograph corresponding to data shown in Fig. 4f. In (b-c) 100 nM of recombinant RecQL4^{WT} or 100 nM of RecQL4^{AE-ΔC} was added to depleted extract. (d-f) Replicate of the experiment testing the addback of 100 nM of RecQL4^{AE-ΔC} to RecQL4-depleted extract. (d) Raw autoradiograph of the plasmid replication reaction. (e) Normalized signal from the plasmid replication reaction shown in panel (d). (f) Immunoblots corresponding to reactions shown in panel (d). (g-i) Replicate of the experiment testing the addback of 100 nM of RecQL4^{AE-ΔC} to RecQL4-and-Mcm10 depleted extract. 100 nM of RecQL4^{WT} serves as a positive control. (g) Raw autoradiograph of the plasmid replication reaction. (h) Normalized signal from the plasmid replication reaction shown in panel (g). (i) Immunoblots corresponding to reactions in panel (g).

Extended Data Figure 6



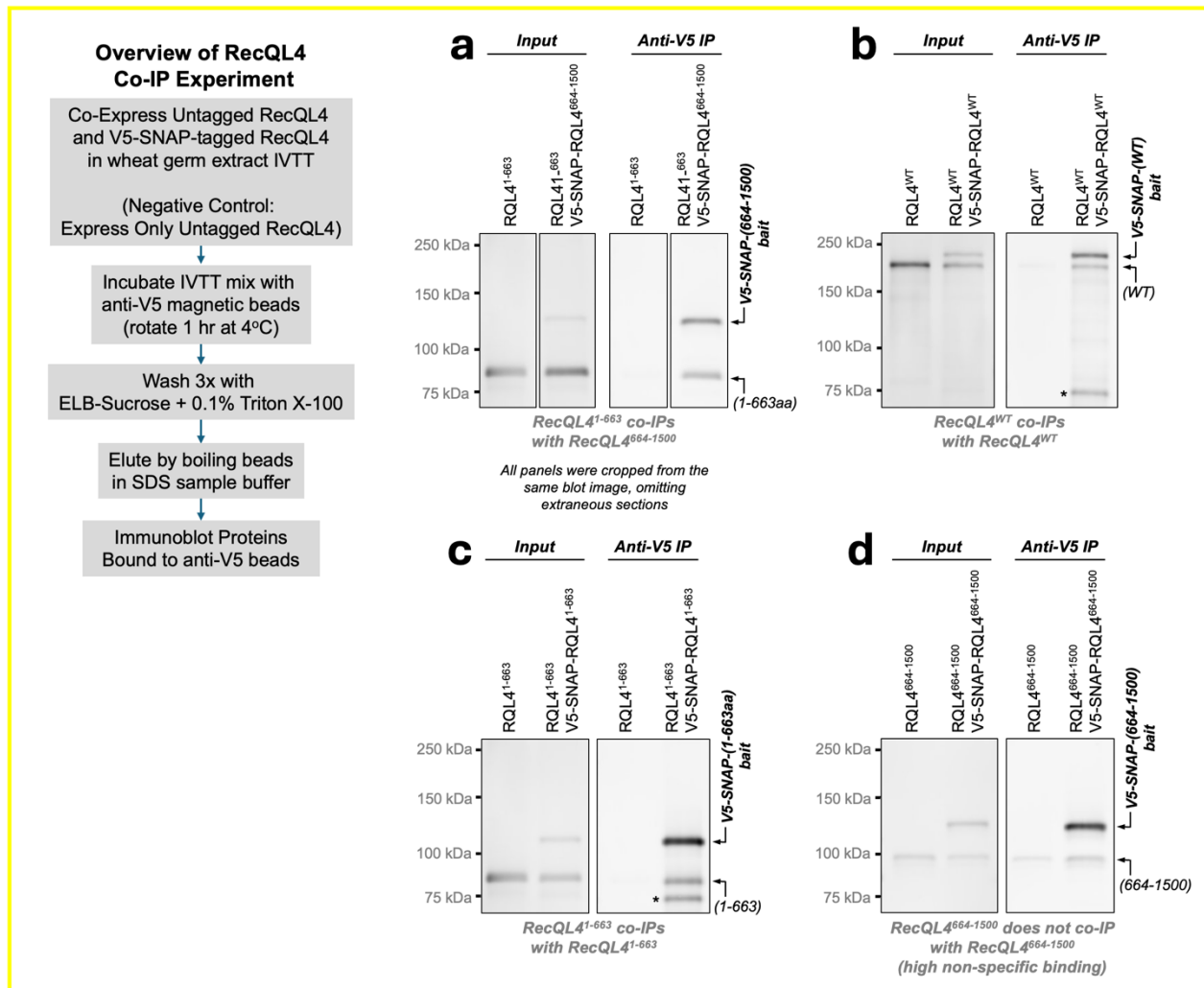
Extended Data Figure 6: (a) Raw autoradiograph corresponding to data shown in Fig. 5b. (b) Raw autoradiograph corresponding to data shown in Fig. 5c. (c) Immunoblots of extract samples taken from reactions shown in panels (a) and (b). (d-f) Replicate experiment (for Fig. 5b) comparing how well 100 nM of RecQL4^{WT} or RecQL4¹⁻⁶⁶³ support replication in RecQL4-depleted extract. (g-i) Replicate experiment (for Fig. 5b) comparing how well 100 nM of RecQL4^{WT} or RecQL4^{D856A} support replication in RecQL4-depleted extract. (j-l) Replicate experiment (for Fig. 5c) comparing how well 100 nM of RecQL4^{WT}, RecQL4¹⁻⁶⁶³, or RecQL4^{D856A} support replication in RecQL4-and-Mcm10 depleted extract.

Extended Data Figure 7



Extended Data Figure 7: (a) Autoradiograph corresponding to data shown in Fig. 5d. (b) Immunoblots of extract samples taken from reactions shown in panels (a). (c-d) Autoradiograph (c) and immunoblots (d) corresponding to experiment shown in Fig. 5e. (e) Autoradiograph corresponding to data shown in Fig. 5f. For the corresponding immunoblots see the right side of panel (b). (f-h) Replicate experiment (for Fig. 5f) comparing how well 100 nM of RecQL4^{WT}, RecQL4⁶⁶⁴⁻¹⁵⁰⁰, RecQL4¹⁻⁶⁶³, or both support replication in RecQL4-and-Mcm10 depleted extract.

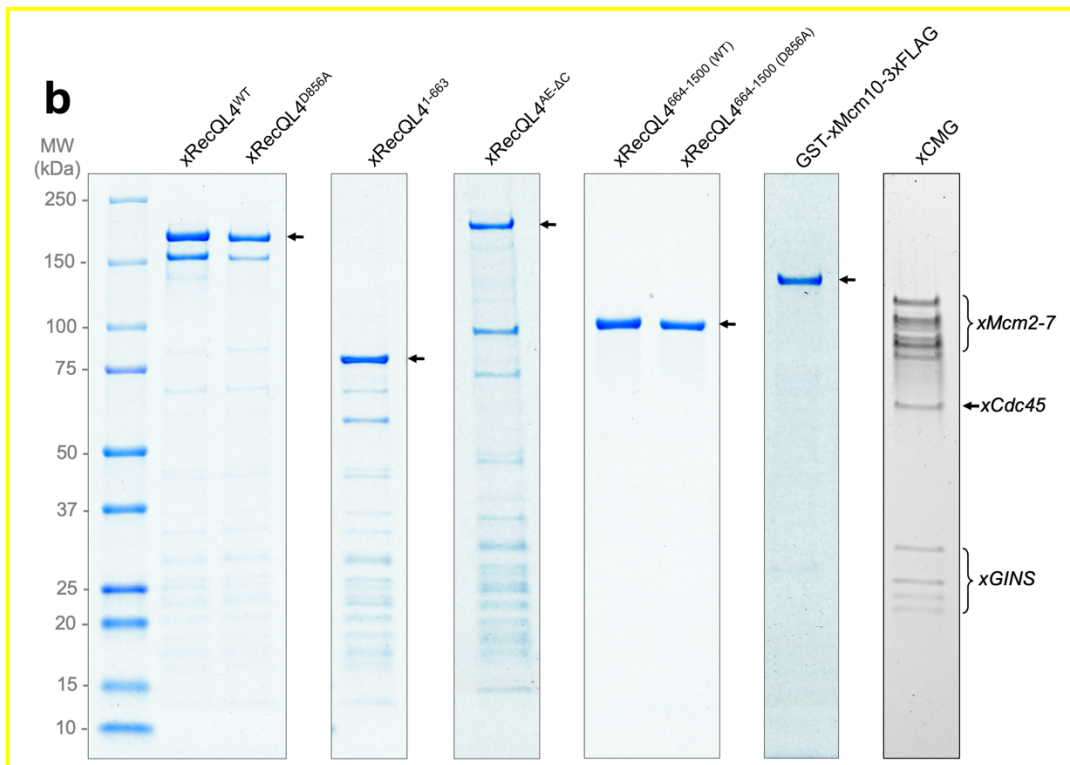
Extended Data Figure 8



Extended Data Figure 8: (a-d) Immunoblots showing the results of RecQL4 co-immunoprecipitation experiments using different tagged and untagged *Xenopus* RecQL4 fragments expressed via IVTT. The membrane was blotted with a mixture of anti-RecQL4 primary antibodies raised against N-terminal or C-terminal antigens. **(a)** Untagged RecQL4¹⁻⁶⁶³ was expressed alone (negative control) or was co-expressed with V5-SNAP tagged RecQL4⁶⁶⁴⁻¹⁵⁰⁰. Untagged RecQL4¹⁻⁶⁶³ expressed alone did not bind to anti-V5 magnetic beads. By contrast, untagged RecQL4¹⁻⁶⁶³ co-expressed with V5-SNAP-RecQL4⁶⁶⁴⁻¹⁵⁰⁰ did bind to anti-V5 magnetic beads, suggesting that RecQL4¹⁻⁶⁶³ interacts with RecQL4⁶⁶⁴⁻¹⁵⁰⁰. **(b)** Untagged RecQL4^{WT} was expressed alone (negative control) or was co-expressed with V5-SNAP-RecQL4^{WT}. RecQL4^{WT} was captured by anti-V5 magnetic beads only when co-expressed with V5-SNAP-RecQL4^{WT}, suggesting that RecQL4^{WT} forms oligomers. **(c)** Untagged RecQL4¹⁻⁶⁶³ was expressed alone (negative control) or was co-expressed with V5-SNAP-RecQL4¹⁻⁶⁶³. RecQL4¹⁻⁶⁶³ was captured by anti-V5 magnetic beads only when co-expressed with V5-SNAP-RecQL4¹⁻⁶⁶³, suggesting that RecQL4¹⁻⁶⁶³ forms oligomers. In **(b-c)** the asterisk (*) marks a prominent RecQL4 degradation band. **(d)** Untagged RecQL4⁶⁶⁴⁻¹⁵⁰⁰ was expressed alone (negative control) or was co-expressed with V5-SNAP-RecQL4⁶⁶⁴⁻¹⁵⁰⁰. Although untagged RecQL4⁶⁶⁴⁻¹⁵⁰⁰ bound to anti-V5 beads when it was co-expressed with V5-SNAP-RecQL4⁶⁶⁴⁻¹⁵⁰⁰, a similar amount of untagged RecQL4⁶⁶⁴⁻¹⁵⁰⁰ was captured by anti-V5 magnetic beads in the negative control (due to non-specific binding). This suggests that RecQL4⁶⁶⁴⁻¹⁵⁰⁰ does not oligomerize.

Extended Data Figure 9

xRecQL4/1-1500	1	MERYNEKVLKKWETARLQKRRKNNLTVESASNETOELYKFKS	KKLEQDILSDTDVTFPKSQGVMTSSPKPTHRADAQSGESASV	HLNRSATVPK	SKERDSLKASQAQYGMKGLAN	126											
xRecQL4/1-1216	1	MERLRDVRRLQKQWETARLQKRRKNNLTVESASNETOELYKFKS	KKLEQDILSDTDVTFPKSQGVMTSSPKPTHRADAQSGESASV	HLNRSATVPK	SKERDSLKASQAQYGMKGLAN	126											
ySLD2/1-453	1	MERLATVRARLQKQWETARLQKRRKNNLTVESASNETOELYKFKS	KKLEQDILSDTDVTFPKSQGVMTSSPKPTHRADAQSGESASV	HLNRSATVPK	SKERDSLKASQAQYGMKGLAN	113											
		1MYSFEELDKLIKELTKWETARLQKRRKNNLTVESASNETOELYKFKS	KKLEQDILSDTDVTFPKSQGVMTSSPKPTHRADAQSGESASV	HLNRSATVPK	SKERDSLKASQAQYGMKGLAN	112											
xRecQL4/1-1500	127	LGCAIRLEHVSLLKSSFTTPFRPLDSDPVKLNKNLASLSDTPSSPLALQDQ	SEFDTLTVSHVSLKEPFLVMNPPQTQKIKGHO	QAMVYGCOR	LNQELQRLERLNGVEMVETAAEQRTV	254											
xRecQL4/1-1208	113	LGCTIQACAPLGRCPWPGRASS	KASTPK	PGDTGTPSPFAEKVSDEHPQFL	QPRPRLQHQ	QASLRQCSLDQGLQWRCHSEVDF	LGAPK	206									
mRecQL4/1-1216	114	LKNTTGTGTGPTSRKLLQKRRSL	TVPAIR	PGCKSTGTPCEDADALPRVF	RPRLQLQQL	RSSLRRSLT	QDGLWICRHNRRVLD	LEVPG	207								
ySLD2/1-453	113	INDVNNSD	TI-SNESPR	KTILKSSAPDRTLVA	EIS	SVKRLNFMQNLAS	STRTPTSST	KNRNGLV	EI	184							
xRecQL4/1-1500	255	NRQQCNTMREALGEGAI	EENKESVKVPGNNDELTHSTOHTVAS	QKQVQAYTPVFS	PHAAAPSLPLTFGRQDTR	ENPKLKAEEISDFGRSSSR	SGNDNLVSPAIR	REIWDPEHQ	LKRRS	256							
xRecQL4/1-1208	207	NRNFRPCYLRLKCVLRERL	ACRPDLSSND	LLPGESAVLCPG	AG	SGQPEAFV	QEVSS	IRVCS	251								
mRecQL4/1-1216	208	NRNFRPCYLRLKCVLRERL	ACRPDLSSND	LLPGESAVLCPG	AG	SGQPEAFV	QEVSS	IRVCS	251								
ySLD2/1-453	185	NRNFRPCYLRLKCVLRERL	ACRPDLSSND	LLPGESAVLCPG	AG	SGQPEAFV	QEVSS	IRVCS	251								
xRecQL4/1-1500	378	PLRKQICGES	KMSSEAGQAVALDKGPM	SEHSKRWDQDTSERAMK	NRTGQOMPS	QLEVDMSQENIAAEQVSV	LAKGKRKK	QRETSASE	PDVTVSPKSVKKRRKTRNSPD	489							
xRecQL4/1-1208	352	PLRKQICGES	KMSSEAGQAVALDKGPM	SEHSKRWDQDTSERAMK	NRTGQOMPS	QLEVDMSQENIAAEQVSV	LAKGKRKK	QRETSASE	PDVTVSPKSVKKRRKTRNSPD	489							
mRecQL4/1-1216	352	PLRKQICGES	KMSSEAGQAVALDKGPM	SEHSKRWDQDTSERAMK	NRTGQOMPS	QLEVDMSQENIAAEQVSV	LAKGKRKK	QRETSASE	PDVTVSPKSVKKRRKTRNSPD	489							
ySLD2/1-453	267	PLRKQICGES	KMSSEAGQAVALDKGPM	SEHSKRWDQDTSERAMK	NRTGQOMPS	QLEVDMSQENIAAEQVSV	LAKGKRKK	QRETSASE	PDVTVSPKSVKKRRKTRNSPD	489							
xRecQL4/1-1500	490	SSNSSVRKRKKKARCMQDSDPAMESPA	EQVTKAVSDELFCLE	EELPEQRSSVS	LVNTFPMQHQ	YVIRNLLKKS	SHVKKVCA	LNHRRLRKQWKQKQKKEG	CGGCKHFNRS	608							
xRecQL4/1-1208	313	SPNS	PRYHL	SPSSQA	ACKAGETAP	KHIFPLRLHGRGVN	YVIRNLLKKS	SHVKKVCA	LNHRRLRKQWKQKQKKEG	CGGCKHFNRS	608						
mRecQL4/1-1216	313	SPNS	PRYHL	SPSSQA	ACKAGETAP	KHIFPLRLHGRGVN	YVIRNLLKKS	SHVKKVCA	LNHRRLRKQWKQKQKKEG	CGGCKHFNRS	608						
ySLD2/1-453	383	SPNS	PRYHL	SPSSQA	ACKAGETAP	KHIFPLRLHGRGVN	YVIRNLLKKS	SHVKKVCA	LNHRRLRKQWKQKQKKEG	CGGCKHFNRS	608						
xRecQL4/1-1500	609	KDTCFCRCGCMHWASQ	CPSPVYVVRVQDAVEETEEVLS	LPTLEDVARM	TLRPNTEGLSGWCQD	PVTVTVNVRVYEP	TPPPMPQ	LFLPCDGR	VKFTSEVDNLE	SELGVRS	FSRSGQ	QVM	736				
xRecQL4/1-1208	609	KDTCFCRCGCMHWASQ	CPSPVYVVRVQDAVEETEEVLS	LPTLEDVARM	TLRPNTEGLSGWCQD	PVTVTVNVRVYEP	TPPPMPQ	LFLPCDGR	VKFTSEVDNLE	SELGVRS	FSRSGQ	QVM	736				
mRecQL4/1-1216	609	KDTCFCRCGCMHWASQ	CPSPVYVVRVQDAVEETEEVLS	LPTLEDVARM	TLRPNTEGLSGWCQD	PVTVTVNVRVYEP	TPPPMPQ	LFLPCDGR	VKFTSEVDNLE	SELGVRS	FSRSGQ	QVM	736				
ySLD2/1-453	392	KDTCFCRCGCMHWASQ	CPSPVYVVRVQDAVEETEEVLS	LPTLEDVARM	TLRPNTEGLSGWCQD	PVTVTVNVRVYEP	TPPPMPQ	LFLPCDGR	VKFTSEVDNLE	SELGVRS	FSRSGQ	QVM	736				
xRecQL4/1-1500	737	RILSGFSSGIC	STGCMCKSLYQLPAPYMYKRSRE	SLVLSPLVLSLMDMDQVSG	SLPKLACAH	HSNMTRKQREAAI	LGRQCKVH	LVLLSP	EALVGGGFS	SLCPPL	ADQLPPVAFACIDEAH	CVSEW	864				
xRecQL4/1-1208	737	RILSGFSSGIC	STGCMCKSLYQLPAPYMYKRSRE	SLVLSPLVLSLMDMDQVSG	SLPKLACAH	HSNMTRKQREAAI	LGRQCKVH	LVLLSP	EALVGGGFS	SLCPPL	ADQLPPVAFACIDEAH	CVSEW	864				
mRecQL4/1-1216	737	RILSGFSSGIC	STGCMCKSLYQLPAPYMYKRSRE	SLVLSPLVLSLMDMDQVSG	SLPKLACAH	HSNMTRKQREAAI	LGRQCKVH	LVLLSP	EALVGGGFS	SLCPPL	ADQLPPVAFACIDEAH	CVSEW	864				
ySLD2/1-453	508	RILSGFSSGIC	STGCMCKSLYQLPAPYMYKRSRE	SLVLSPLVLSLMDMDQVSG	SLPKLACAH	HSNMTRKQREAAI	LGRQCKVH	LVLLSP	EALVGGGFS	SLCPPL	ADQLPPVAFACIDEAH	CVSEW	864				
xRecQL4/1-1500	865	SHNFRPCYLRLKCVLRERL	EMVNC	LLGLTATATATATADVAH	HLQVSPGEC	PIVRMASVP	NHLHVSMDR	NDRDQALVT	LKGER	FGCLDSV	IVYCTRE	TQRISAL	RTR	LGQVTS	VANS	HLNSQEQ	992
xRecQL4/1-1208	865	SHNFRPCYLRLKCVLRERL	EMVNC	LLGLTATATATATADVAH	HLQVSPGEC	PIVRMASVP	NHLHVSMDR	NDRDQALVT	LKGER	FG							



Extended Data Figure 7: (a) Full-length multiple-sequence alignment of *Saccharomyces cerevisiae* (y) Sld2 versus RecQL4 from *Xenopus laevis* (x), human (h), mouse (m) generated using Clustal Omega⁸⁴. It highlights high sequence conservation among metazoa, and poor sequence conservation between metazoa and yeast. **(b)** SDS-PAGE gels of recombinant protein preparations used in this study. Except for the CMG prep (stained with Sypro Ruby), all gels were stained with Coomassie Blue.

SUPPLEMENTARY INFORMATION

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Gheorghe Chistol (chistol@stanford.edu).

Materials availability

All newly generated reagents generated in this study are available upon request from the lead contact.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report any original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Xenopus Laevis Model System

Eggs from adult lab bred female *Xenopus laevis* frogs (Xenopus 1, Cat# 4800) were used to prepare egg extracts. Testes from adult lab bred male *Xenopus laevis* frogs (Xenopus 1, Cat# 4293) were used to prepare sperm chromatin. All animals were healthy, not subjected to previous procedures, and no animal husbandry was performed. Frogs were housed at the Boswell Small Amphibians Research Facility at Stanford School of Medicine in compliance with Institute Animal Care and Use Committee (IACUC) regulations and all experiments involving frogs were approved by the Stanford University APLAC (Administrative Panel on Laboratory Animal Care).

Insect Cell Lines

Sf9 cells (Expression Systems, Cat# 94-001S) and Tni cells (Expression Systems, Cat# 94-002S) were cultured in ESF 921 insect cell culture medium (Expression Systems, Cat# 96-001-01) at 27°C for baculovirus production and protein overexpression.

Bacterial Cell Lines

DH10EMBacY (Geneva Biotech, Cat# DH10EMBacY), DH5 α (New England Biorabs (NEB), Cat# C2987H), and Rosetta (DE3) pLysS (Novagen, Cat# 70956-4) bacteria were cultured in LB broth (Thermo Fisher Scientific, Cat# BP9723) at 37°C for plasmid production and protein overexpression.

IVTT System

IVTT reactions were performed using TnT SP6 High-Yield Wheat Germ Protein Expression System (Promega, Cat# L3261) according to the manufacturer's instructions. Proteins were not purified after their production by IVTT. The IVTT reaction was directly added replication reactions as the source of a given protein. The optimal concentration of the template plasmid was titrated for each protein.

Preparation of DNA Substrates

All single molecule experiments used doubly tethered λ phage DNA. The 48.5 kb DNA substrate as purchased from NEB (#N3011S) has 12-nt 5' ssDNA overhangs on both ends. We used the Klenow Fragment (3' to 5' exo-) polymerase (NEB, Cat# M0212S) to fill in the overhangs with biotin-dCTP (Thermo Fisher Scientific, Cat# 19518018) and biotin-dGTP (PerkinElmer, Cat# NEL541001EA). The product was separated via electrophoresis and electro-eluted from the gel

before use in tethering. A step-by-step detailed protocol for preparing biotinylated λ DNA is available in our dedicated methods paper⁵².

Xenopus Egg Extract Preparation

Xenopus laevis egg extracts were prepared following published protocols^{85,86}. Frogs were primed with 75 IU of human chorionic gonadotropin (CHORULON) (Merck, Cat# 133754) 5-7 days before extract preparation. To induce ovulation, 660 IU of CHORULON was injected 20-21 hr before extract preparation. High-speed supernatant (HSS) was prepared from 5-6 adult female frogs, and nucleoplasmic extract (NPE) was prepared from 15-20 frogs. Sperm chromatin was purified from adult male frog testes and used for NPE preparation.

Ensemble Replication Assay

To validate antibodies and recombinant proteins, ensemble DNA replication experiments were performed following a detailed protocol described in a recent methods article⁵². Since all proteins of interest (POIs) in this study are dispensable for Mcm2-7 double-hexamer loading, all licensing reactions were performed with HSS depleted of the POI.

Protein Co-Immunoprecipitation Assays

For the co-immunoprecipitation experiment with recombinant FLAG-RecQL4 and untagged Mcm10, untagged *Xenopus laevis* Mcm10 was expressed in IVTT from [pRT81]pSP64-xMcm10 at 25°C for 2 hours. Anti-FLAG M2 Magnetic Beads (Millipore #M8823) were pelleted and washed three times with binding buffer (25 mM HEPES-KOH pH 7.5, 150 mM NaCl, 5% glycerol, 1 mM DTT) and aliquots with 3 μ L packed beads were made. As a negative control reaction, 6 μ L of 300 nM 3xFLAG peptide (APExBio, Cat# A6001) and 3 μ L of IVTT reaction containing 600 nM Mcm10 were added to 3 μ L of washed anti-FLAG M2 Magnetic Beads. To co-immunoprecipitate Mcm10 with FLAG-RecQL4, 6 μ L of 300 nM FLAG-RecQL4 and 3 μ L of IVTT reaction containing 600 nM Mcm10 were added to 3 μ L of washed anti-FLAG M2 Magnetic Beads. These mixtures were incubated for 2 hours at 25°C with gentle rotation. Next, beads were washed three times with IP buffer (25 mM HEPES-KOH pH 7.5, 100 mM KCl, 10% glycerol, 0.1 mg/mL BSA, 1 mM DTT). Finally, beads were resuspended in 6 μ L of IP buffer and transferred to a fresh tube. Bound proteins were eluted by boiling beads in 12 μ L 1x Laemmli buffer (50mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue, 5% β -mercaptoethanol). To carefully quantify the co-immunoprecipitation efficiency, recombinant protein standards were loaded alongside eluted samples on the acrylamide gel.

For the co-immunoprecipitation experiment with recombinant FLAG-RecQL4 and untagged CMG complex, anti-FLAG M2 Magnetic Beads were pelleted and washed three times with high-salt binding buffer (25 mM HEPES-KOH pH 7.5, 500 mM NaCl, 5% glycerol, 1 mM DTT) and aliquots with 3 μ L of packed beads were made. 3 μ L of washed anti-FLAG M2 Magnetic Beads were incubated with 9 μ L of 200 nM 3xFLAG peptide, 200 nM FLAG-RecQL4^{WT}, or 200 nM FLAG-RecQL4^{AE- Δ C} for 2 hours at 4°C with gentle rotation. Beads were washed twice with high-salt binding buffer, and then, washed twice with IP buffer. 3 μ L of pre-incubated packed beads were incubated with 6 μ L of 100 nM CMG complex purified from Tni cells infected with [pGC299-b2] for 1 hour at 4°C with gentle rotation. 9 μ L of IP reaction was overlaid to 400 μ L of IP-0.5M Sucrose cushion (25 mM HEPES-KOH pH 7.5, 100 mM KCl, 0.5 M Sucrose, 0.1 mg/mL BSA, 1 mM DTT) in a 0.75-mL tube. Beads were immediately pelleted by centrifugation at 1,2000 xg for 2 min. Beads were resuspended in 6 μ L of IP buffer and transferred to a fresh tube. Bound proteins were eluted by boiling beads in 12 μ L 1x Laemmli buffer (50mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue, 5% β -mercaptoethanol). To carefully quantify the co-immunoprecipitation efficiency, recombinant protein standards were loaded alongside eluted samples on the acrylamide gel.

Protein co-immunoprecipitations using Mcm7-WHD as bait were performed using anti-FLAG M2 Magnetic Beads (Millipore, Cat# M8823). FLAG-tagged Mcm7-WHD^{WT} and Mcm7-WHD^{KAKA} were purified as described below and immobilized on the anti-FLAG magnetic beads by incubating 5 μ L of beads (binding capacity ~33 pmol FLAG peptide) with 200 pmol of protein at 10 μ M final concentration. The beads with the immobilized protein were washed four times (twice with 1x ELB-Sucrose + 0.05% NP-40, and twice with 1x ELB-Sucrose without detergent), then incubated with a *Xenopus* egg extract mixture of nucleoplasmic extract and high-speed supernatant (in a 1:1 ratio) diluted five-fold in 1x ELB-sucrose buffer. The beads were incubated with the extract for one hour at room temperature on a rotator. The beads were then washed four times (twice with 1x ELB-Sucrose + 0.05% NP-40, and twice with 1x ELB-Sucrose without detergent). The bound proteins were eluted from the beads by resuspending in SDS sample buffer and boiling at 98°C for five minutes. The beads were pelleted by spinning at 3000 g for 30 seconds, and the supernatant was collected for analysis. Eluted proteins and input samples were resolved by SDS-PAGE and analyzed by western-blotting with antibodies against RecQL4 and FLAG.

Western Blotting and Coomassie Staining

All samples were boiled in 1x Laemmli buffer and run on 4-15% Mini-PROTEAN TGX Precast Protein Gels (Bio-Rad #4561086) using Tris-Glycine-SDS Running Buffer (Boston BioProducts #BP-150). EZ-Run Prestained Rec Protein Ladder (Fisher Scientific #BP36031) or Precision Plus Protein Dual Color Standard (Bio-Rad #1610374EDU) was run alongside samples to infer protein band sizes. For Coomassie staining, protein gels were incubated with Optiblot Blue InstantBlue Coomassie Protein Stain (Abcam #ab119211) for at least 1 hour at room temperature with gentle shaking. For immunoblotting, proteins were transferred from the gel onto a PVDF membrane (VWR #10061-492) in transfer buffer (Fisher Scientific #NC9297917). Membranes were blocked in 1x PBST (1x Phosphate Buffered Saline (PBS) (Fisher Scientific, Cat# NC9140736), 0.05% Tween 20) containing 5% (w/v) nonfat milk for 1 hour at room temperature with gentle shaking. Following a wash in 1xPBST, the membrane was incubated with primary antibodies diluted in 1x PBST containing 1% (w/v) BSA overnight at 4°C with gentle shaking. Membranes were washed three times with 1x PBST and incubated with secondary antibody diluted in 1x PBST + 5% nonfat milk for 1 hour at room temperature with gentle shaking. Membranes were washed three times with 1x PBST, developed using SuperSignal West Pico PLUS Chemiluminescent Substrate (Fisher Scientific, Cat# PI34577), and imaged using Azure c600 (Azure Biosystems).

Single Molecule Replication Assay

All single molecule experiments were conducted following a previously published protocol⁵², except for the following modifications. In the λ DNA tethering step, 20 pM of streptavidin^{AF647} (Thermo Fisher Scientific, Cat# S21374) was added to the streptavidin solution. Streptavidin^{AF647} spots were used as a fiducial marker for focusing immediately before starting imaging. After λ DNA tethering, DNA was licensed in HSS for 10 minutes. In the original KEHRMIT protocol, after a brief pulse of replication initiation at high GINS^{AF647} concentration (~200 nM), the flow cell was flushed with GINS-depleted extract to prevent further origin firing and to minimize background^{52,53}. In this study, Replication Mix (10 μ L of NPE, 10 μ L of HSS, 6 μ L of ELB-Sucrose, 1 μ L of 600 ng/ μ L pBlueScript carrier plasmid DNA, 1 μ L of ATP regeneration Mix (67 mM ATP, 0.17 mg/mL creatine phosphokinase, 0.67 M phosphocreatine), 1 μ L of 60 μ M Fen1^{mKikGR}, and 1 μ L of 150-300 nM fluorescently labeled protein of interest) was flowed into the microfluidic chamber. The reaction was then imaged continuously (10 sec/frame) without washing out the free fluorescently labeled protein. Thus, fluorescent protein (5-10 nM) was present in the replication reaction during the entire duration of the experiment. Optimal replication efficiency was achieved when the single-molecule replication reaction contained equal volumes of NPE, HSS, and buffer⁸⁷. The concentrations of proteins examined in this study were estimated using quantitative western blots (see table below).

<i>Protein of Interest</i>	Concentration in HSS (nM)	Concentration in NPE (nM)	Concentration in the Replication Reaction (nM)
RecQL4	~80 nM	~700 nM	~250 nM
Mcm10	~20 nM	~300 nM	~100 nM

For two-color imaging, Fen1^{mKikGR} (488 nm laser excitation) and the POI labeled with Alexa Fluor 647 (640 nm excitation) were used. The following settings were used for imaging Alexa Fluor 647: TIRF arm set to 9000 (~67°), 500 ms exposure, 10 s/frame, 20% 640 nm laser power (~250 μ W power out of the objective, ~2.5 W/cm² power density), 100 EM Gain. Fen1^{mKikGR} was imaged using the following settings: TIRF arm set to 9000 (~67°), 200 ms exposure, 10 s/frame, 8% 488 nm laser power (~70 μ W power out of the objective, ~0.7 W/cm² power density), 100 EM Gain. Given these exposure durations, 4 fields of view (FOVs) could be sequentially imaged during the 10 s/frame interval.

Cloning and Plasmids

To prepare an antigen consisting of *Xenopus laevis* Mcm10 amino acids 128-240 with a His6 tag at the C-terminus, the geneblock GC601gb was inserted into pET-28b(+) linearized via digestion with NcoI and SacI via Gibson Assembly, yielding the plasmid [pLS002] pET-28b(+)-xMcm10¹²⁸⁻²⁴⁰-His6.

A plasmid encoding the full-length frog *mcm10* gene was a generous gift from Justin Sparks. It was used as a PCR template to clone [pLS049]pFastBac-His6-FLAG-HRV3C-xMcm10.

To express Mcm10 in IVTT, the *mcm10* gene was PCR amplified from [pLS049] using oLAS006 and oLAS007. The resulting untagged *mcm10* gene product was then digested with Sall and SacI and cloned into the Sall-SacI region of pSP64 Poly(A), resulting in the plasmid [pLAS003]pSP64-xMcm10. To add a 10aa linker, a SNAP tag, and a FLAG tag to the C-terminus of Mcm10, the *mcm10* gene fragment containing a 10aa linker sequence was PCR amplified from [pLAS003] using primers oRT117 and oRT190, while the tandem 10aa-SNAP-FLAG linker sequence was PCR amplified from [pRT065]pSP64-xDONSON-SNAP-FLAG using primers oRT093 and oRT094. The two resulting PCR products were fused by overlap extension PCR using primers oRT117 and oRT094. The resulting xMcm10-10aa-SNAP-FLAG fragment was digested with Sall-HF and cloned into the Sall-SmaI site of pSP64 Poly(A), yielding the plasmid [pRT079]pSP64-xMcm10-SNAP-FLAG.

To express N-terminally FLAG tagged RecQL4, the untagged RecQL4 fragment was digested from [pRT025]pFastBac-xRecQL4 by NdeI and HindIII-HF, and inserted into the NdeI-HindIII site of [pRT027]pFastBac-FLAG-HRV3C-xRecQL4-10aa-sort-His6, yielding the plasmid [pRT074]pFastBac-FLAG-HRV3C-xRecQL4.

To express N-terminally tandem V5-SNAP-10aa tagged RecQL4 in IVTT, the NdeI-PvuI region of [pRT033]pSP64-V5-SNAP-xRecQL4-FLAG was substituted with the NdeI-PvuI region of [pRT030]pSP64-xRecQL4, yielding the plasmid [pRT082]pSP64-FLAG-SNAP-xRecQL4.

To clone the plasmid needed for expressing FLAG-HRV3C-RecQL4¹⁻⁶⁶³, a fragment of the *recql4* gene was PCR amplified from pRT027 using phosphorylated primers oRT118 and oRT188. The resulting PCR product was circularized by ligation, yielding the plasmid [pRT077]pFastBac-FLAG-HRV3C-xRecQL4¹⁻⁶⁶³.

To clone the plasmid needed for expressing FLAG-HRV3C-RecQL4^{AE- Δ C}, the gene fragments were amplified by PCR using primer pairs, oRT117 and oRT195, and oRT196 and oRT197, using pRT033 as a template. The two PCR fragments were fused by overlap-extension PCR with primers oRT117 and oRT197. The FLAG-HRV3C-xRecQL4^{AE- Δ C} fragment was digested with ApaI and XbaI and cloned into the same sites in pRT074 resulting in [pRT093]pFastBac-FLAG-HRV3C-xRecQL4^{AE- Δ C}.

To clone the plasmid needed for expressing FLAG-HRV3C-RecQL4⁶⁶⁴⁻¹⁵⁰⁰, a fragment of the *recql4* gene was PCR amplified from pRT074 using phosphorylated primers oRT198 and oRT199. The resulting PCR product was circularized by ligation, yielding the plasmid [pRT094]pFastBac-FLAG-HRV3C-xRecQL4⁶⁶⁴⁻¹⁵⁰⁰.

To clone the plasmid needed for expressing FLAG-HRV3C-RecQL4^{664-1500(D856A)}, a fragment of the ATPase-dead *recql4* gene was PCR amplified from [pRT076]pFastBac-FLAG-HRV3C-xRecQL4^{D856A} using phosphorylated primers oRT198 and oRT199. The resulting PCR product was circularized by ligation, yielding the plasmid [pRT095]pFastBac-FLAG-HRV3C-xRecQL4^{664-1500(D856A)}.

To express V5-SNAP-RecQL4¹⁻⁶⁶³ in IVTT, a truncated *recql4* gene fragment was PCR amplified from pRT033 using phosphorylated primers oRT117 and oRT118. The resulting PCR product was digested by BsrGI-HF and inserted into the BsrGI-SmaI site of pSP64 Poly(A), yielding the plasmid [pRT049]pSP64-V5-SNAP-xRecQL4¹⁻⁶⁶³.

To express V5-SNAP-RecQL4⁶⁶⁴⁻¹⁵⁰⁰ in IVTT, a truncated *recql4* gene fragment was PCR amplified from pRT025 using primers oRT86 and oRT200, while the V5-SNAP-10aa sequence was PCR amplified from the gene block gRT88 using primers oRT089 and oRT090. The two resulting PCR products were fused by overlap extension PCR using primers oRT089 and oRT086. The resulting V5-SNAP-xRecQL4⁶⁶⁴⁻¹⁵⁰⁰ fragment was digested with BmgBI and XmaI and cloned into the BmgBI-XmaI site of pRT082, yielding the plasmid [pRT085]pSP64-V5-SNAP-xRecQL4⁶⁶⁴⁻¹⁵⁰⁰.

To express frog CMG in insect cells from a single baculovirus, we used the MultiBac⁸⁸ cloning strategy to generate [pGC299] – a single plasmid encoding all 11 subunits of *Xenopus laevis* CMG as previously described^{89,90} with one difference: TwinStrep-HRV3C-Mcm3 was cloned in [pGC299] instead of FLAG-Mcm3 in [pGC187].

Antibody Preparation

Custom polyclonal antibodies were generated as previously described⁵². Briefly, the His6 tagged antigen sequence was cloned into pET28b(+) (see Cloning and Plasmids). The antigen was expressed in Rosetta (DE3) pLysS (Novagen, Cat# 70956-4) upon induction with 0.5-1.0 mM IPTG for 3 hrs at 37°C or overnight at 16°C. Soluble antigens were purified in native buffer, whereas insoluble antigens were purified in denaturing buffer (see table and 'Recombinant Protein Expression and Purification' below). In most cases, the Ni-NTA eluate (still containing 400 mM imidazole) was used to immunize rabbits. For some soluble antigens, additional chromatography was used to polish the protein preparation. All antibodies used in this study were raised by Cocalico Biologicals.

All antibodies used in this study were affinity purified. 1-5 mg of purified antigen was cross-linked to 1 mL of AminoLink Plus Coupling Resin (Fisher Scientific, Cat# PI20501) according to the manufacturer's protocol. 10-30 mL of serum was incubated with the affinity resin for 30 minutes at room temperature. The resin was washed twice with 10 mL of 1x PBS + 500 mM NaCl, and twice again with 10 mL of 1x PBS. Next, antibodies bound to the affinity resin were eluted 5-6 times with 1.0 mL of 200 mM glycine-HCl pH2.5. Each elution was collected into a tube containing 0.1 mL of 1M Tris-HCl pH9.0, which immediately neutralized the glycine. Eluted fractions were pooled and dialyzed against 1 L of 1x TBS (50 mM Tris-HCl pH8.0, 150 mM NaCl) + 10% (w/v) sucrose. The IgG concentration was measured using a Nanodrop, normalized to 1 mg/mL via dilution or spin-concentration, aliquots were flash frozen in liquid nitrogen, and stored at -80°C.

Target Protein (Rabbit #)	Antigen	Plasmid (expression)	Antigen Purification	Depletion Condition μg IgG per μL extract	Primary Antibody Concentration for WB
Anti-xGINS (SU289)	whole GINS complex Psf2-10aa-sort-His6	pGC127 (insect)	Native	2:1	0.2 μg/mL
Anti-Cdc45 (SU294)	(1-361 aa)-His6	pGC283 (bacteria)	Denaturing	1:1	0.2 μg/mL
Anti-xRecQL4 (SU307)	(56-165 aa)-His6	pSEB014 (bacteria)	Denaturing	1:1	0.2 μg/mL
Anti-xRecQL4 (SU305)	(1145-1319 aa)-His6	pSEB011 (bacteria)	Denaturing	N/A	0.2 μg/mL
Anti-xDONSON (SU351)	His6-(155-579 aa)	pRT057 (bacteria)	Native	1:1	0.2 μg/mL
Anti-xMcm10 (SU255)	(128-240 aa)-His6	pLS002 (bacteria)	Native	1:1	0.2 μg/mL
Anti-xMcm7 (SU347)	His6-(400-720 aa)	pRT053 (bacteria)	Denaturing	N/A	0.2 μg/mL

Recombinant Protein Expression, Purification

Previously published protocols were used to purify recombinant Cdc45¹⁷, DONSON¹⁷, Geminin⁹¹, GINS⁵³, RecQL4¹⁷, and Fen1-mKikGR⁵⁴.

A bacmid was generated by transforming DH10EMBaY cells with [pRT074]pFastBac1-FLAG-HRV3C-xRecQL4^{WT}, [pRT077] pFastBac-FLAG-HRV3C-xRecQL4¹⁻⁶⁶³, [pRT093] pFastBac-FLAG-HRV3C-xRecQL4^{AE-ΔC}, [pRT094] pFastBac-FLAG-HRV3C-xRecQL4⁶⁶⁴⁻¹⁵⁰⁰, [pRT095] pFastBac-FLAG-HRV3C-xRecQL4^{664-1500(D856A)} and the resulting bacmid was purified with the ZR BAC DNA Miniprep Kit (Zymo Research, Cat# D4049). The baculovirus was amplified in three stages (P1, P2, P3) in Sf9 cells.

RecQL4¹⁻⁶⁶³, RecQL4⁶⁶⁴⁻¹⁵⁰⁰, and RecQL4^{664-1500(D856A)} were purified following the same protocol¹⁷ as RecQL4^{WT} and RecQL4^{D856A}. Briefly, proteins were expressed in 250 mL of Sf9 suspension culture by infection with the P3 baculovirus for 48 hrs. Cells were harvested, washed with PBS, pelleted by centrifugation, and flash frozen in liquid nitrogen. Cell pellets were thawed and resuspended in four volumes of buffer L (25 mM HEPES pH7.5, 300 mM NaCl, 10% glycerol, 1 mM DTT) containing 1X Pierce Protease Inhibitor EDTA-free (ThermoFisher Scientific, Cat# A32965). Lysate was cleared by ultracentrifugation at 35,000 rpm for 40 min at 4°C in a Beckman Type 70 Ti rotor (Beckman Coulter). Cleared lysate was incubated with 200 μL of ANTI-FLAG M2 Affinity Gel for 1 hr at 4°C. The protein was eluted from the resin by cutting the HRV3C site with 50 μg/mL HRV3C protease in buffer L for 2 hours at 4°C. The eluted RecQL4 was separated from the HRV3C protease via size exclusion chromatography on a Superdex 200 Increase 10/300 GL column (Cytiva, Cat# 28990944). Peak fractions were pooled, concentrated, and snap frozen in LN₂ as small aliquots, and stored at -80°C.

FLAG-RecQL4^{WT} and FLAG-RecQL4^{AE-ΔC} were purified as follows: Proteins were expressed in 250 mL of Sf9 suspension culture by infection with the P3 baculovirus for 48 hrs. Cells were harvested, washed with PBS, pelleted by centrifugation, and flash frozen in liquid nitrogen. Cell pellets were thawed and resuspended in four volumes of buffer F (25 mM HEPES pH7.5, 500 mM NaCl, 10% glycerol, 1 mM DTT) containing 1X Pierce Protease Inhibitor EDTA-free. Lysate was cleared by ultracentrifugation at 35,000 rpm for 40 min at 4°C in a Beckman Type 70 Ti rotor. Cleared lysate was incubated with 200 μL of ANTI-FLAG M2 Affinity Gel for 1 hr at 4°C. The protein was eluted from the FLAG-M2 resin with 100 μg/mL 3X FLAG peptide (APExBio, Cat# A6001) in buffer F containing 1X Pierce Protease Inhibitor EDTA-free. The eluted protein was clarified via size exclusion chromatography on a Superdex 200 Increase 10/300 GL column. The

peak fractions were pooled and concentrated, snap frozen in liquid nitrogen as small aliquots, and stored at -80°C.

Recombinant frog CMG complex was purified as follows: A bacmid was generated by transforming DH10EMBacY cells with pGC299 and the resulting bacmid was purified with the ZR BAC DNA Miniprep Kit. The baculovirus was amplified in three stages (P1, P2, P3) in Sf9 cells. The protein was expressed in 1 L of Tni suspension culture by infection with the P3 baculovirus for 48 hrs. Cells were harvested, washed with PBS, pelleted by centrifugation, and flash frozen in liquid nitrogen. Cell pellets were thawed and resuspended in four volumes of buffer S (25 mM HEPES pH7.5, 300 mM NaOAc, 5 mM MgCl₂, 10% glycerol, 0.1% Tween-20, 1 mM DTT) containing 1X Pierce Protease Inhibitor EDTA-free. Lysate was cleared by ultracentrifugation at 35,000 rpm for 40 min at 4°C in a Beckman Type 70 Ti rotor. Cleared lysate was incubated with 350 µL of StrepTactin XT 4Flow resin (IBA Lifesciences, Cat#2-5010-002) for 1 hr at 4°C. The CMG complex was eluted from the resin by cutting the HRV3C site with 50 µg/mL HRV3C protease in buffer S for 2 hours at 4°C. The eluate was dilute threefold in dilution buffer (25 mM HEPES pH7.5, 3 mM MnCl₂, 10% glycerol, 1 mM DTT), and loaded onto 1 mL of Nuvia S resin (Biorad, Cat#1560311) packed into a 10-mL Poly-Prep chromatography column (Biorad, Cat#7311550) (final salt concentration was 100 mM NaOAc). The flowthrough from the Nuvia S column was incubated with 2.5 units/µL of Lambda protein phosphatase (NEB Cat#P0753S) for 30 min at room temperature, and loaded onto a Capto HiRes Q 5/50 (Cytive, Cat#29275878). The CMG complexes bound to the Capto HiRes Q column were eluted over a gradient of 100-700 mM KCl in buffer A (25 mM HEPES pH7.5, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 0.02% Tween-20, 1 mM DTT). Fractions corresponding to the CMG peak were collected and diluted threefold in buffer A (final salt concentration was 150 mM KCl). The diluted CMG pool was concentrated by binding to 30 µL of Nuvia Q resin (Biorad, Cat#1560411) for 30 min at 4°C and then eluting with 70 µL of 600 mM KCl in buffer A. The eluates were flash-frozen in 2.5-µL aliquots in liquid nitrogen, and stored at -80°C.

The native purification of antigens was performed as follows. The Rosetta (DE3) pLysS cell pellet overexpressing the His6-tagged antigen was thawed in Lysis buffer containing 1 mM PMSF, and sonicated. The lysate was cleared by ultracentrifugation at 35,000 rpm for 40 min at 4°C in a Beckman Type 70 Ti rotor. Cleared lysate was incubated with 1 mL of Ni-NTA resin for 1 hr at 4°C. The protein was eluted with 400 mM imidazole in Lysis buffer containing 1 mM PMSF.

The denaturing purification of antigens was performed as follows. The Rosetta (DE3) pLysS cell pellet overexpressing His6-tagged antigen was thawed in Denaturing Lysis buffer (50 mM HEPES pH7.5, 500 mM NaCl, 10 mM imidazole, 8 M Urea) containing 1 mM PMSF, and sonicated. The lysate was cleared by ultracentrifugation at 35,000 rpm for 40 min at room temperature in a Beckman Type 70 Ti rotor. Cleared lysate was incubated with 1 mL of Ni-NTA resin for 1 hr at room temperature. The protein was eluted with 400 mM imidazole in denaturing elution buffer (50 mM HEPES pH7.5, 500 mM NaCl, 6 M Urea, 1 mM PMSF).

Mcm7-WHD^{WT} and Mcm7-WHD^{KAKA} were purified using the following protocol. Rosetta (DE3) pLysS cells overexpressing each protein were harvested and the cell pellets were resuspended in Lysis buffer (500 mM NaCl, 50 mM HEPES pH 7.5, 15 mM Imidazole, 10% Glycerol, and 1 mM PMSF). Cells were lysed by sonication, and the lysate was cleared by ultracentrifugation at 35,000 rpm for 1 hr at 4°C using a Beckman Type 70 Ti rotor. The cleared lysate was incubated with 0.5 mL of Ni-NTA resin for 1 hr at 4°C. Bound protein was eluted with Elution buffer identical to the Lysis buffer except that it contained 400 mM Imidazole. The eluate was dialyzed into storage buffer (300 mM NaCl, 50 mM HEPES pH 7.5, 0.5 mM DTT, and 10% glycerol), flash-frozen, and stored at -80°C.

Chromatin Fractionation Assay

For chromatin fractionation assays, a final concentration of sperm chromatin of 1,000 sperm/µL was used. Sperm chromatin was licensed in Mcm10-and-RecQL4 depleted HSS for 30 min at

room temperature. Replication reactions were initiated by adding mock-depleted or Mcm10-and-RecQL4 depleted NPE, followed by incubation at room temperature. Each reaction was supplemented with caffeine at a final concentration of 5 mM. Timepoint samples were collected at 20, 40, 60, 80, and 100 min. Reactions were stopped by adding ice-cold quenching buffer (1x ELB salts, 0.25 M sucrose, 0.2% Triton X-100) and immediately overlaid onto a cushion of 1x ELB salts with 0.5 M sucrose. Samples were then centrifuged at 15,000 x g for 1 min at 4°C in a swing-bucket rotor to pellet the chromatin. To wash away unbound proteins, each chromatin pellet was washed twice with ice-cold 1x ELB salts with 0.25 M sucrose. Each time, the supernatant was carefully aspirated, leaving behind 15 µL of liquid (this is done to avoid accidentally aspirating the chromatin pellet). After washing, chromatin pellets were resuspended in SDS loading buffer containing β-mercapto-ethanol and boiled. Samples were resolved by SDS-PAGE electrophoresis, transferred to PVDF membranes, and analyzed by western blotting using the primary antibodies against the replication proteins of interest.

Quantification of Proteins Expressed in IVTT

The concentration of proteins expressed in IVTT reactions was determined by semi-quantitative western blotting. Typically, 0.05 µL of the IVTT reaction expressing the protein of interest (POI) was loaded on an SDS-PAGE gel, and a dilution series (5-200 fmol) of purified recombinant POI was loaded alongside. After transfer onto a PVDF membrane, the membrane was blotted with specific antibody against the POI. The concentration of POI in the IVTT reaction was estimated by comparing its band intensity to those from the dilution series of purified proteins.

Fluorescent Labeling using SNAP

After expressing RecQL4 or Mcm10 in the IVTT lysate, the reaction was kept on ice, and the concentration of the target protein was determined via quantitative western blotting. The SNAP labeling reagent was used in a two-fold molar excess relative to the target protein. SNAP-Surface Alexa Fluor 647 was added to the IVTT reaction containing the target protein and reacted overnight at 4°C with gentle end-over-end rotation. The final reaction was aliquoted, snap frozen in liquid nitrogen, and stored at -80°C. Fluorescent labeling efficiency was estimated by loading the IVTT reaction on a PAGE-Gel alongside a titration series of purified fluorescent protein standards (GINS-AF647). The labeled proteins were not purified after the SNAP-labeling reaction. The IVTT reaction was directly added replication reactions as the source of a given protein.

Data Reporting

Details of the statistical analyses performed are indicated in the figure legends. Since some quantities measured in this study did not have symmetric distributions, we reported the median of the distribution. Unless otherwise specified in the figure captions, error bars represent the 95% confidence interval for the median. This was calculated using MATLAB's built in bootstrapping function. The exact meaning of the number 'N' reported in each figure is indicated in the figure captions, it usually refers to the number of DNA molecules, the number of replication origins, or the number of binding events. We typically used N~100 events to report the duration of an intrinsically stochastic event (for example the duration of a productive binding event). p-values were computed using the two-sample Kolmogorov-Smirnov test function built into MATLAB; 'n.s.' denotes not significant, * = p value <0.05, ** = p value <0.01, *** = p value <0.001, **** = p value ≤0.0001.

Reproducibility and Blinded Analysis

All experiments were repeated at least twice, with independent extract preparations. The data presented in the main figures comes from one of these preparations. Treatment series (mock, depletion, addback) were performed with the same extract for consistency. All the single-molecule data was analyzed in a blinded manner. Specifically, raw data from different experiments in the

same treatment series (mock-depletion, Mcm10/RecQL4-depletion, recombinant protein add-back) were loaded into our custom-written data analysis package, the order of individual DNA molecules was scrambled, and the exact experiment corresponding to each DNA molecule was unknown to the user performing the analysis. The results were then unscrambled using custom MATLAB code written in-house.

Single-Molecule Data Analysis

Each replication initiation experiment imaged 4-6 fields of view (FOVs) over the course of 45 min with a resolution of 10 seconds/frame. Each FOV typically contained 50-200 DNA molecules stretched to 80-90% of their contour length. A suite of MATLAB scripts written in house were used to select and analyze data as described in our recent methods article⁹². Below we summarize the data analysis pipeline specific for replication initiation experiments.

Selecting DNA Molecules

Regions of interest (ROIs) corresponding to individual DNA molecules were selected manually based on the previews of DNA stained with SYTOX Green (these were acquired before the start of the experiment). Only well-separated DNA molecules were used in the analysis.

Quantifying the Fen1 Signal

Custom MATLAB scripts written in house were used to generate kymograms corresponding to each selected DNA molecule. Origin firing was monitored by visualizing the binding of Fen1^{mKikGR} to nascent DNA. To measure fork speed, the raw Fen1 signal was automatically thresholded using OTSU's method via a built-in MATLAB function. The OTSU threshold binary image was used to fit straight lines to the edges of the replication bubble, and the fork speed value was extracted from the slope of the line (accounting for pixel size, the time resolution of the experiment, and the extent to which DNA was stretched). [In our single-molecule experiments, replication forks undergo spontaneous stalling even in the absence of any exogenous replication stress, as we discussed in another recent study⁸². When quantifying fork speed, we measure the speed of active forks before they undergo spontaneous stalling.](#)

To accurately determine the time when an origin fired, the raw Fen1 signal was integrated over time, and the integrated signal was fit by two straight line segments: the first segment represents the background noise before origin firing, while the second segment corresponds to a linear increase in Fen1 signal after the start of DNA synthesis. The intersection of the two segments corresponds to the time of origin firing. This estimate agrees well with the origin firing time prediction from the OTSU threshold analysis.

Analyzing Mcm10^{AF647} and RecQL4^{AF647} Binding/Dissociation

To analyze the binding and dissociation of Mcm10/RecQL4, we employed the Generalized Likelihood Ratio Test (GLRT). GLRT was used to convert noisy single-molecule images into binary images that were then segmented into binding events. The GLRT algorithm uses a sliding window (we used a window with a 5-pixel radius) to determine whether the signal at a given pixel is consistent with noise or is significantly different from noise. This approach is superior to simple image thresholding because it accounts for the intrinsic properties of background noise. We adapted GLRT code written in MATLAB from a previous publication⁹³. When analyzing productive/non-productive binding events, we did not score any events that lasted less than two frames because we could not be confident that they represented bona-fide binding.

Global Protein Binding Analysis

To quantify the binding of RecQL4^{AF647} and Mcm10^{AF647} to the origins in mock versus depleted extracts, we devised a global binding analysis. First, ROIs with and without DNA were selected based on the SYTOX Green images. Next, kymograms were generated for both regions and the

1715 GLRT algorithm was employed to identify binding events (including single-frame events). The total
1716 duration of all binding events was added and normalized to DNA length (sec/kb) as shown in
1717 Extended Data Fig. 1g. Matched selections without DNA were used to subtract non-specific
1718 background binding (which may vary between experiments).