The G-quadruplex (G4) resolvase DHX36 efficiently and specifically disrupts DNA G4s via a translocation-based helicase mechanism

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ABSTRACT

Single-stranded DNA (ssDNA) and RNA regions that include at least four closely spaced runs of three or more consecutive guanosines strongly tend to fold into stable G-quadruplexes (G4s). G4s play key roles as DNA regulatory sites and as kinetic traps that can inhibit biological processes, but how G4s are regulated in cells remains largely unknown. Here, we developed a kinetic framework for G4 disruption by DEAH-box helicase 36 (DHX36), the dominant G4 resolvase in human cells. Using tetramolecular DNA and RNA G4s with four to six G-quartets, we found that DHX36-mediated disruption is highly efficient, with rates that depend on G4 length under saturating conditions ($k_{cat}$) but not under subsaturating conditions ($k_{cat}/K_M$). These results suggest that a step during G4 disruption limits the $k_{cat}$ value and that DHX36 binding limits $k_{cat}/K_M$. Similar results were obtained for unimolecular DNA G4s. DHX36 activity depended on a 3′ ssDNA extension and was blocked by a polyethylene glycol linker, indicating that DHX36 loads onto the extension and translocates 3′–5′ toward the G4. DHX36 unwound dsDNA poorly compared with G4s of comparable intrinsic lifetime. Interestingly, we observed that DHX36 has striking 3′-extension sequence preferences that differ for G4 disruption and dsDNA unwinding, most likely arising from differences in the rate-limiting step for the two activities. Our results indicate that DHX36 disrupts G4s with a conventional helicase mechanism that is tuned for great efficiency and specificity for G4s. The dependence of DHX36 on the 3′-extension sequence suggests that the extent of formation of genomic G4s may not track directly with G4 stability.

Single-stranded DNA and RNA segments that include at least four closely-spaced runs of three or more consecutive guanosine nucleotides have a strong intrinsic propensity to fold into stable G-quadruplex structures (G4s) (1) (Fig. 1A, top left). The genomes of humans and many other eukaryotes are replete with putative G4-forming sequences, and pronounced, conserved patterns have been observed in their distribution, suggesting that G-quadruplex structures form at least transiently in cells and perform conserved functions (2-6). Supporting this hypothesis, G4s have been detected in cells for both DNA and RNA (7-11). To function as regulatory elements, G4s must be folded and disrupted in a regulated way. In addition, processes that require single-stranded DNA or RNA, such as replication and translation, require that G4s be temporarily disrupted. The high stability and long intrinsic lifetime of G4s suggest
that their efficient disruption requires the activity of ATP-dependent enzymes such as helicases. Supporting this view, incorporation of sequences predicted to form particularly stable G4s leads to genetic instability in yeast, suggesting that these G4 structures are not processed efficiently and pose blocks to replication (12). Indeed, several helicases have been shown to possess G4 disruption activity (13). While some of these helicases associate specifically with molecular machines such as the replisome, the DEAH/RHA family helicase DHX36 (a.k.a. RHAU and G4R1) is of particular interest for general G4 disruption activity because it is highly expressed, comprises the bulk of the DNA G4 disruption activity in a human cell lysate, and is one of only two helicases known to disrupt RNA G4s (14-16). DHX36 has been implicated as a regulator of RNA folding and assembly (17,18), RNA localization, translation (19), pre-mRNA processing (20), and transcription (21), most simply suggesting that it functions in both the cytoplasm and nucleus and interacts with both RNA and DNA G4s. Underscoring the importance of this protein and potentially G4s in vivo, DHX36 is an essential gene in mouse (22-24).

Considerable progress has been made toward understanding how DHX36 targets and disrupts G4s. DHX36 can efficiently disrupt both intramolecular and tetramolecular G4s (17,25,26). Disruption of a tetramolecular G4 DNA was shown to require ATP and a 3′ extension (25,27). The disruption rate was shown to be greater for a G4 with fewer G-quartets, suggesting that less stable G4s are disrupted more efficiently by DHX36 (26). A small N-terminal domain directs DHX36 to G4s by binding specifically to these structures (28-30).

Nevertheless, important aspects of the mechanism of G4 disruption by DHX36 remain unknown. DEAH/RHA family helicases (abbreviated herein as DEAH family) typically use a translocation-based mechanism for unwinding DNA or RNA helices, a mechanism in which ATP drives directional motion along a sequestered single strand, displacing the complementary strand (31). In contrast, a distinct family of helicase proteins known as DEAD-box proteins do not translocate but instead operate only on short helices, separating the strands by interacting directly with the dsRNA segment and using a single cycle of ATP-dependent conformational changes (31-34). Although the simplest model is that DHX36 would use the translocation-based, DEAH-family mechanism in keeping with its family affiliation, G4s are sufficiently compact that a single ATP-dependent step might suffice for their disruption. Further, the dependence of the G4 disruption rate on G4 stability, without a corresponding dependence of the ATPase rate on G4 stability, suggested that DHX36 may use a mechanism more reminiscent of DEAD-box proteins (26).

Here, we explored further how DHX36 disrupts G4s by performing detailed steady-state and single-turnover kinetics experiments. We found that DHX36 disrupts tetramolecular DNA G4s using a conventional DEAH family mechanism involving loading onto ssDNA followed by directional translocation. DHX36 disrupts G4 structures with high efficiency, even attaining a limited measure of catalytic perfection, and it is highly evolved to recognize and disrupt G4s in preference to DNA duplexes. Interestingly, the binding properties of DHX36 depend significantly on sequence features in the 3′ extension, raising the possibility of biological specificity for G4s that depends on both the sequence and accessibility of single-stranded DNA immediately 3′ of the G4 structure.

RESULTS

Kinetic analysis of tetramolecular DNA G4 disruption by DHX36

To probe how DHX36 disrupts DNA G4s, we built on a tetramolecular G4 system developed previously (26). Tetramolecular G4s are attractive models for monitoring DHX36 activity because they predominantly adopt the parallel conformation (26,35,36), which is preferentially recognized by the N-terminal domain of DHX36 (30). In addition, tetramolecular G4s form slowly (37), allowing their disruption to be monitored by an electrophoretic mobility shift assay (EMSA) without the need for a ‘chase’ oligonucleotide to prevent G4 reformation (14,26,38) (Fig. 1A,B).
The prior work showed that with a single set of protein and G4 concentrations (with 40-fold excess DNA G4 over DHX36), the steady-state G4 disruption rate by DHX36 was decreased by increasing the number of G-quartets in the G4 substrate (26). To probe G4 disruption by DHX36 further, we systematically varied the concentrations of an analogous series of G4s across saturating and subsaturating regimes. Consistent with the previous report, we found that DHX36-mediated disruption depended on ATP (Fig. 1C and S1) and that the $k_{\text{cat}}$ values decreased as the number of G-quartets increased, from 36 min$^{-1}$ for a G4 with four quartets to 0.6 min$^{-1}$ for a G4 with six quartets (Fig. 1C). The maximal rates are smaller for G4 structures with more G-quartets, presumably because these G4s are more stable (Fig. S2 and Table S1).

On the other hand, calculation of the $k_{\text{cat}}/K_M$ values from hyperbolic fits of the data in Fig. 1C suggested that this parameter does not depend strongly on G4 length from four to six G-quartets ($\sim 2 \times 10^8$ M$^{-1}$ min$^{-1}$). To probe the process further under subsaturating conditions, we carried out single-turnover experiments by varying the DHX36 concentration in the presence of a trace amount of radiolabeled G4 substrate (Fig. 1D and S3). The second-order rate constants were similar to the values from the steady-state experiments, and there was at most a very small dependence on G4 length (Fig. 1E). The relative insensitivity of the $k_{\text{cat}}/K_M$ value to G4 length suggests that the G4 is not disrupted in the rate-limiting transition state under these subsaturating conditions, most simply suggesting that DHX36 binding limits the reaction rate under subsaturating conditions.

These results imply that once bound under these conditions, DHX36 disrupts the G4 more frequently than it dissociates nonproductively. To test this idea directly, we pre-bound DHX36 to the same series of radiolabeled G4s and then added ATP together with an excess of unlabelled ‘chase’ G4 substrate. As expected, we observed rapid bursts of G4 disruption for all three substrates, consistent with rate-limiting binding, and the magnitude of the bursts did not depend systematically on the number of G-quartets (Fig. S4). The bursts were incomplete, perhaps reflecting that some of the DHX36 protein is inactive for G4 disruption or that nucleotide-free DHX36 dissociates more rapidly than ATP-bound protein. Thus, in the ATP-bound form, DHX36 is partially or fully committed to G4 disruption without dissociating from these substrates.

For comparison, we also measured the single-turnover and steady-state disruption by DHX36 of a comparable tetramolecular RNA G4, r5G-A15 (Fig. S5A, B). The levels of activity in both the saturating and subsaturating regimes were similar to those for the DNA, with a small increase in the $k_{\text{cat}}/K_M$ value, probably due to faster binding of DHX36, and a modest decrease in $k_{\text{cat}}$ value ($\sim 2.6$-fold less), most likely reflecting that the RNA G4 is more stable than its DNA counterpart (Fig. S2A,B).

**Disruption of unimolecular G4s by DHX36**

To extend our analysis and to test the generality of rate-limiting DHX36 binding for G4 disruption, we investigated unimolecular DNA G4s, which are more likely to be encountered by DHX36 in vivo. To modulate the stability of the G4, we included loop sequences of one or two T nucleotides (TTA(G3T)3G3-A15 and TTA(G3TT)3G3-A15). Previous work showed that the G4 with single-nucleotide loops is highly stable and adopts the parallel topology (39,40), while the G4 with two-nucleotide loops is less stable and adopts primarily the parallel topology (39). We monitored disruption of the unimolecular G4s by trapping the unfolded G4 with a ‘chase’ oligonucleotide complementary to the G4-forming region (Fig. 2A).

We first measured unfolding of the unimolecular G4s in the absence of DHX36. As expected from previous results, a G4 construct with 1-nt loops unfolded much slower than the construct with 2-nt loops ($\sim 100$-fold, Fig. 2B). We then performed DHX36-mediated disruption assays by varying DHX36 concentration under subsaturating, single-turnover conditions as above (Fig. 2B). Interestingly, we observed robust disruption, with similar $k_{\text{cat}}/K_M$ values for these two unimolecular constructs ($\sim 1.0 \times 10^8$ M$^{-1}$ min$^{-1}$), despite their large difference in intrinsic unfolding rates (Fig.
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2B,C) and their large stability difference (Fig. S6 and refs 39,41). The lack of dependence of the $k_{\text{cat}}/K_M$ value on G4 stability suggests that DHX36 binding limits the rate of disruption for these unimolecular G4s under subsaturating conditions.

The $k_{\text{cat}}/K_M$ values were ~5-fold larger than those for the tetramolecular G4s with the same A15 extension, suggesting that DHX36 is able to bind faster to these unimolecular constructs. We tested whether this difference was due to an inhibitory effect of the four A15 extensions in the tetramolecular G4 substrate by measuring disruption of a tetramolecular G4 in which 3 of the extensions were shorter (A3) while only one was A15. The $k_{\text{cat}}/K_M$ value for DHX36-mediated disruption of this construct was $1.5 \times 10^8$ M$^{-1}$ min$^{-1}$ (Fig. S7), essentially unchanged from the standard tetramolecular construct. Thus, there is not a large effect of changing the number of 3' extensions, and the somewhat faster binding to the unimolecular constructs may reflect more efficient interactions with the G4 structure or with the loops that connect the G runs.

Efficient DHX36 activity requires a single-stranded 3' extension of greater than 5 nucleotides

We next examined the dependence of G4 disruption by DHX36 on the length of the single-stranded 3' extension. A distinguishing feature of DEAH family helicases is that they typically require such an extension because the protein loads onto the single-stranded segment and then translocates 3' to 5' for helix unwinding. It was shown recently that DHX36 indeed requires a 3' extension for efficient G4 disruption (27,42). However, little is known about the dependence of G4 disruption efficiency on the length of this 3' extension, and the mechanistic origin of this requirement has not been systematically explored.

Therefore, we varied the length of the 3' extension from 3 to 25 nt (A3 to A25) on tetramolecular G4s and measured single-turnover G4 disruption rates (Fig. 3). We observed robust G4 disruption for substrates with 25- and 15-nt extensions, with no significant difference between them. There was only a small reduction in second-order rate constant for a 10-nt extension. However, activity was not detected for extensions of 3 or 5 nt (<1 $\times$ 10$^5$ M$^{-1}$ min$^{-1}$). The requirement for the extension to be approximately 10 nt for optimal activity is consistent with a model in which its role is in loading of the helicase core. A recently-solved crystal structure of the related helicase MLE suggests a binding region for single-stranded nucleotides of approximately this length (43).

Using the single-turnover G4 disruption assay, we also tested whether a 5' extension influences DHX36 activity. For a DNA G4 substrate that included A15 at the 3' end, we observed no effect of adding a 5' A15 extension (Fig. S8). As expected from previous results, we observed no DHX36 activity for a G4 lacking the 3' extension, regardless of the presence or absence of a 5' extension. The requirement for a 3' extension and lack of dependence on a 5' extension are also consistent with the general properties of DEAH family helicases and inconsistent with the properties of DEAD-box helicases, which do not require single-stranded extensions for productive interactions (31).

Translocation from the 3' extension is required for G4 disruption by DHX36

To directly test the hypothesis that DHX36 loads onto the 3' extension and disrupts G4 structures by translocating in the 3'-to-5' direction, we introduced a polyethylene glycol (PEG) linker region between the 3' extension and the G4 (Fig. 4A). We found that an 18-atom hexaethylene glycol linker (sp$_{18}$) or a shorter 9-atom linker (sp$_9$) completely blocked detectable DHX36-mediated G4 disruption, most likely by blocking translocation of DHX36 (Fig. 4B). To test whether the PEG linker instead prevented loading of DHX36, we measured the ability of the G4 with the PEG linker to inhibit DHX36-mediated G4 disruption, most likely by blocking translocation of DHX36 (Fig. 4C). We found that the G4 with the PEG linker is a robust inhibitor (Fig. 4C), eliminating the possibility that the PEG linker prevents DHX36 loading and indicating that DHX36 uses directional translocation to disrupt G4s.
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Together, the results here and in the preceding sections indicate that DHX36 uses a conventional DEAH family helicase mechanism to disrupt G4 structures by loading onto a single-stranded 3’ extension and then using ATP to translocate from 3’ to 5’, disrupting the G4 in the process. This mechanism differs from a previous proposal that DHX36 uses a DEAD-box-like mechanism (26,42), which would imply disruption by direct binding of DHX36 to the G4 and the absence of DHX36 translocation. We find that the G4 disruption process is highly efficient, so much so that DHX36 binding to the 3’ extension limits the rate of disruption under subsaturating conditions.

Detectable but low DNA duplex unwinding activity by DHX36

We next investigated whether DHX36 possesses DNA duplex unwinding activity. We were interested in this question for two reasons. First, the findings that DHX36 uses a conventional DEAH family helicase mechanism to disrupt G4s suggests that it would have some duplex unwinding activity, as directional translocation along ssDNA would be expected to result in displacement of a complementary strand with at least some efficiency. Second, if DHX36 functions on DNA G4s in biology, they will likely be flanked by dsDNA, so we were interested to know whether DHX36 has the potential to unwind intervening dsDNA to reach a G4 structure.

To compare duplex unwinding and G4 disruption activities of DHX36 for substrates of comparable lifetime, we determined the intrinsic unwinding rates of DNA duplexes of varying lengths to identify a construct with a similar intrinsic lifetime as the G4 with five G-quartets (5G-A15), used extensively in the experiments above. The spontaneous unwinding rate constant of a 20-bp helix with an A15 3’ extension (Comp20-A15) was similar to that for spontaneous unfolding of 5G-A15 (1.5 ± 0.3) x 10^-4 min^-1 and 5.6 ±0.7) x 10^-4 min^-1, respectively; Fig. S9A). Surprisingly, we were unable to detect DHX36 acceleration of unwinding of this 20-bp helix (Fig. S9B). However, DHX36 showed detectable, albeit very weak unwinding activity of 15-bp and 20-bp duplexes that included a 3’ extension of T15 instead of A15 (Fig. 5A and B). DHX36-mediated unwinding of these duplexes gave kcat/Km values that depended only weakly on duplex length (2.4 x 10^5 M^-1 min^-1) and were approximately 1000-fold lower than the second-order rate constants for disruption of G4 substrates. The apparent preference of DHX36 for a T15 extension relative to A15 is probed further below.

To test whether the low activity for duplex unwinding was an artifact arising from inhibition of DHX36 by binding to the chase oligonucleotide, we added up to 5 µM of the same oligonucleotide to G4 disruption experiments. We observed no inhibition of the DHX36-mediated disruption activity of the G4 formed by 5G-A15 (Fig. S9C), ruling out sequestration of DHX36 in our duplex unwinding experiments.

To ask how the stability of the duplex impacts DHX36 unwinding activity, we also tested a 12-bp duplex with a T15 extension (Fig. 5B). DHX36 was also inefficient for unwinding this duplex, with no acceleration detected beyond the uncertainty generated by the significant intrinsic unwinding rate.

Together, our results indicate that DHX36 is highly specific for disrupting G4 structures and is inefficient at unwinding duplexes of comparable intrinsic lifetime. This preference is presumably due at least in part to the N-terminal domain of DHX36, which binds directly to G4s and is thought to direct DHX36 to these structures (29,30).

Efficiency of G4 disruption by DHX36 depends on the 3’ extension sequence

The observation above that DNA duplex unwinding by DHX36 was detectable with a substrate that includes a 3’ extension of T15 but not A15 led us to investigate the effect of 3’ extension sequence on G4 disruption. Extension sequences have been shown to influence equilibrium binding to G4 DNA structures (25,27), but potential effects of these sequences on G4 disruption kinetics have not been explored.

Therefore, we designed G4 substrates with five G-quartets and 15-nt 3’ extensions of varying sequence (Table S2). We first established that substrates with 3’ extensions of A15 or T15 had indistinguishable stabilities and lifetimes (Fig. S6 and S9A). We then measured DHX36-
mediated G4 disruption of this series in single-turnover and steady-state assays (Fig. 6A, B). We found that DHX36 is most active on G4s with A-rich extensions and least active on T-rich extensions, strikingly different from the results for duplex unwinding. Single-turnover experiments showed that the $k_{cat}/K_M$ value decreased as Ts were introduced, with a 9-fold decrease for $T_{15}$ relative to $A_{15}$ (Fig. 6A and Table S2). A similar preference for $A_{15}$ over $T_{15}$ was observed for unimolecular DNA G4s (Fig. S10A) and for $A_{15}$ over $U_{15}$ for tetramolecular RNA G4s (Fig. S10B, C). In the steady state, the $k_{cat}$ value for the tetramolecular DNA G4 was also larger for A-rich extensions, with $A_{15}$ 3′ extension resulting in the largest $k_{cat}$ value (Fig. 6B). While there was an overall correlation between the relative values of $k_{cat}$ and $k_{cat}/K_M$ for a given substrate, the decrease in $k_{cat}$ value became prominent with fewer Ts than required for a comparable decrease in $k_{cat}/K_M$ value (Fig. 6C). Together these data suggest that the sequence of the 3′ extension plays an important role in governing the level of DHX36 activity on G4 structures.

A unified model for disparate effects of 3′ extension sequence

The results above give what seems at first glance to be a perplexing set of effects of 3′ extension sequences for DHX36 disruption of G4s compared to unwinding of duplexes. For G4s, the greatest activity was observed with an $A_{15}$ extension, while $T_{15}$ was the preferred extension sequence for duplex unwinding. A simple model that reconciles these seemingly disparate results is that the two activities have two different rate-limiting steps for $k_{cat}/K_M$, such that a single set of underlying binding properties is manifest in different ways for the two different activities. Specifically, we considered a model in which DHX36 binding is rate limiting for G4 disruption, due to the high efficiency of this process, whereas DHX36 binding is in rapid equilibrium for duplex unwinding because of a slow unwinding and/or fast dissociation (see Discussion).

We tested three predictions of this model. First, we tested whether the G4 substrates with a T-rich tail bind DHX36 more tightly than the corresponding substrates with an A-rich tail despite being disrupted more slowly. Using a native gel shift approach, we found that the tetramolecular G4 substrate 5G-$T_{15}$ binds DHX36 with an apparent $K_d$ value of 60 pM, nearly 100-fold tighter than 5G-$A_{15}$ (Fig. 6D). We observed comparable effects for RNA G4s (Fig. 6D) and for unimolecular DNA G4s (Fig. S11). The second prediction is that under conditions that dramatically slow the first-order steps of G4 disruption by DHX36, the rate-limiting step for $k_{cat}/K_M$ could change from binding to G4 disruption. Under such conditions, the preference for an $A_{15}$ extension relative to a $T_{15}$ extension should be eliminated. To test this prediction, we decreased the ATP concentration to decrease the G4 disruption rate. Indeed, we found that the preference for $A_{15}$ was decreased and ultimately eliminated at low ATP concentrations (Fig. 6E). Third, we tested whether a dependence of the $k_{cat}/K_M$ value for G4 disruption on the number of G quartets would be unmasked at low ATP concentration. Indeed, we found that the $k_{cat}/K_M$ value was significantly larger for 4G-$A_{15}$ than for 5G-$A_{15}$ with 25 µM ATP (Fig. 6F). The difference was relatively modest, approximately 3-fold, perhaps because binding remains rate limiting for the shorter G4 but not for the longer one, such that only a fraction of the inherent difference is unmasked.

DISCUSSION

Our results show that DHX36 is highly efficient for disruption of G4s and that it disrupts them using a conventional mechanism characteristic of DEAH-box helicase proteins. DHX36 initially binds to single-stranded segments and then uses ATP binding and hydrolysis to translocate directionally, 3′ to 5′, resulting in disruption of an adjacent G4. The basic features of this mechanism are the same as determined for other members of the DEAH-box family (31,33). These basic features, including a requirement for translocation, probably reflect the presence in DEAH-box proteins of C-terminal domains that surround nucleic acid substrates and are likely important for translocation (33,43-45), as well as conserved sequence motifs within the
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helicase core. While we established this mechanism using principally DNA G4s, analogous RNA constructs were processed by DHX36 with similar rates, indicating that DHX36 likely uses the same mechanism to disrupt DNA and RNA G4s.

Our data additionally lead to a kinetic model for G4 disruption by DHX36. Under standard conditions, disruption of G4s by DHX36 is so efficient that the rate is limited under subsaturating conditions by DHX36 binding to the G4 construct. This binding presumably includes contacts of the helicase core with the single-stranded DNA extension, as shown in Fig. 7, resulting in an unwinding-competent state. It may also include contacts of the NTD with the G4. Rate-limiting binding is indicated by the lack of dependence of $k_{\text{cat}}/K_M$ on G4 length or stability, suggesting that the G4 remains fully formed in the rate-limiting transition state, as well as the $K_M$ values being much larger than the corresponding $K_T$ values (Fig. 1C, 6D; refs 15,25,27). With rate-limiting substrate binding, DHX36 can be described as having achieved a limited definition of ‘catalytic perfection’ for disrupting these G4s, as any further improvements in the rates of first-order steps would not increase the reaction efficiency or the level of specificity (46). The binding rate constants of $2 \times 10^8$ M$^{-1}$ min$^{-1}$ for tetramolecular G4s and $1 \times 10^9$ M$^{-1}$ min$^{-1}$ for unimolecular G4s are 2-3 orders of magnitude below the encounter frequency, most likely owing to the complexity of accommodating the 3′ extension within a crevice of the protein that may be only transiently accessible (45).

Our model provides a simple explanation for what initially seemed to be a complex and confusing set of sequence preferences in the 3′ extension. A-rich extensions are preferred over T-rich sequences for G4 disruption under standard conditions, which we infer arises from more rapid binding of DHX36 to A-rich sequences (Fig. 7, solid curves). It is possible that the greater ability of adenines to adopt stacked conformations accelerates binding by pre-aligning the A$_{15}$ extension in a conformation that is competent for binding. In contrast to the preference for A-rich sequences under standard conditions, if the conditions are altered such that the first-order structure disruption step is slow, here by lowering ATP concentration or by measuring duplex unwinding instead of G4 disruption, the sequence preferences are altered such that A-rich extension sequences are no longer preferred for $k_{\text{cat}}/K_M$, and indeed T-rich sequences can be preferred. In the model in Fig. 7, this change arises because when structure disruption is slow, the rate-limiting step changes from DHX36 binding to structure disruption (dashed curves). Thus, T-rich sequences are preferred because despite their slow binding, they bind much tighter than A-rich sequences (Fig. 6D), indicating that they dissociate more slowly. Interestingly, the MLE helicase has an analogous sequence preference, binding tightly to U-rich ssRNAs (43). The maximal rate constant for DHX36-mediated disruption of a G4 with a T$_{15}$ extension is also lower than that for a G4 with an A$_{15}$ extension, as shown in the model in Fig. 7, perhaps because the initiation of translocation is slower on the strongly interacting T-rich extension.

Several other helicase proteins possess ATP-dependent disruption activity toward DNA G4s, including RecQ, BLM, WRN, FANCJ, and Pif1 (13). Like DHX36, all of these proteins are thought to bind to ssDNA and translocate directionally during the course of ATP-dependent G4 disruption. For most of these proteins, G4 disruption may simply be a consequence of efficient translocation on ssDNA, an activity that is also used during duplex unwinding. Along with DHX36, two of these helicases are targeted to G4s by binding through a distinct site. BLM, a RecQ family helicase, binds through its HRDC domain (47), while FANCJ binds through a sequence that is related to a key sequence within the N-terminal domain of DHX36 (48). Despite these similarities, our work now sets DHX36 apart by showing that it disrupts a G4 ~1000-fold more efficiently than a duplex of comparable stability. Further work will be required to determine whether the preference of DHX36 for G4 disruption arises solely from specific binding of the N-terminal domain or whether additional features of DHX36 are evolved to tune DHX36 for G4 recognition and disruption.

Our results and model have important implications for the potential recognition and disruption of DNA G4s by DHX36 in vivo. In
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In general, genomic G4s are likely flanked by dsDNA regions, with at most short stretches of ssDNA immediately adjacent to the G4. The relatively poor unwinding of dsDNA by DHX36 suggests a strict requirement for ssDNA immediately adjacent to the G4. Upon binding to such a ssDNA segment, our results most simply suggest that the G4 will be disrupted efficiently by DHX36, presumably rendered irreversible by base pair formation with the complementary strand of DNA. This highly efficient G4 disruption, coupled with the relatively high expression of DHX36, may result in a steady-state level of G4 formation that is less than would be present at equilibrium. Interestingly, such underrepresentation of G4 structures has been observed on a genome-wide basis for mRNAs (49), which may also be substrates for DHX36, although the origin of this depletion is currently unclear.

In addition, the steady-state levels may depend in important and potentially complex ways on the sequence flanking the G4. For G4s that are readily accessible, we expect A-rich sequences to be more efficiently disrupted, whereas the tighter binding and slower transition from binding to translocation for T-rich sequences may result in preferential co-localization of DHX36, with these G4s remaining intact. On the other hand, in a genomic context in which G4s are stabilized, e.g. by G4-binding proteins, the disruption rate may be slowed such that it becomes rate limiting. In this setting, G4s with T-rich flanking sequences would become the preferred targets for G4 disruption by DHX36. Further work will be necessary to elucidate these effects in the cellular contexts, but it is reasonable to imagine that properties of DNA G4s beyond their intrinsic stability are important determinants of their relative populations and functional effects, both as regulatory elements and as kinetic traps that inhibit cellular processes.

EXPERIMENTAL PROCEDURES

G4 Formation

Tetramolecular G4s were formed by adding 0.1 mM of a G4-forming oligonucleotide (Integrated DNA Technologies) to TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) containing 100 mM KCl. The solution was heated to 99 °C for 10 min in a thermal cycler, slow cooled to 0 °C, and maintained for 10 min. A final annealing step was then performed at 55 °C for 16 hrs. Aliquots of the formed G4 were 5'-³²P]-end labeled using [γ-³²P]ATP (Perkin-Elmer) and T4 polynucleotide kinase (New England Biolabs) and purified by 12% native PAGE, eluted into 200 µL of 10 mM Tris-Cl, 1 mM EDTA, pH 7.5, and 100 mM KCl and then stored at -20 °C. The radiolabeled G4 was subsequently diluted to a trace concentration in reaction buffer (50 mM Na-MOPS, pH 7.0 containing 100 mM KCl).

A tetramolecular G4 with one 3' extension was formed by combining a trace amount of radiolabeled G4-forming sequence with the 3' extension with a large molar excess (100 µM) of an unlabeled G4-forming sequence lacking the extension and thermal cycled as above to form the tetramolecular G4 with one 3' A₁₅ or T₁₅ nucleotide extension (G4 denoted as 5G-A₁₅-3A₃ or 5G-T₁₅-3A₃).

Unimolecular G4s were formed by first 5'-³²P]-end labeling and PAGE purifying the single-stranded G4-forming sequence as above. The trace radiolabeled G4-forming sequence was diluted into reaction buffer (50 mM Na-MOPS, pH 7.0, 100 mM KCl). The solution was heated to 95 °C for 5 min in a thermal cycler, and slow cooled to 0 °C to form the G4.

DHX36 Protein Expression and Purification

The cDNA sequence of human DHX36 was PCR amplified using Phusion® Hot Start Flex DNA polymerase (NEB, Ipswich, MA) and inserted into pSMT3 vector (a generous gift from Dr. Christopher Lima, MSK Cancer Center) between NotI restriction sites. Primers 5'-GTTGTTGGATCCaaaagccgcctacgatgtgccgg-3' (forward) and 5'-GTTGTTGCGGCGCGCCTactttgtcatcagttaatgctaac-3' (reverse) were used in PCR to add the restriction sites (underlined).

Human DHX36 (54-985) was expressed as His6-SUMO fusion proteins in BL21-Codonplus (DE3)-RIPL cells. The cells were lysed by sonication and nonspecific nucleic acid contaminants were removed by 0.1% polyethyleneimine (PEI) precipitation. The
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A soluble protein was purified through immobilized metal affinity chromatography (IMAC) followed by Ulp1 protease digestion to remove the SUMO tag, and a second IMAC step. Some preparations were further purified through ion-exchange and size-exclusion chromatography. These latter two steps did not significantly affect the level of activity. Purified DHX36 (>80% pure) was stored in 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 50% glycerol, and 1 mM DTT at -80 °C. A summary gel is shown in Fig. S12.

DNA G4 disruption

For all experiments monitoring G4 disruption, trace radiolabeled G4 was used (~25 pM). For steady-state experiments, the radiolabeled G4 was mixed with various concentrations of unlabeled G4 that was prepared in the same manner but without the labelling step. Reactions were initiated by adding DHX36 to a solution containing the pre-formed G4 in 50 mM Na-MOPS, pH 7.0, 100 mM KCl and 2 mM ATP-Mg2+ at 37 °C. For experiments in which the ATP concentration was varied, the Mg2+ concentration was varied correspondingly. Experiments measuring binding of G4 constructs by inhibition were performed identically except that various concentrations of unlabelled inhibitor G4 were added to the reaction prior to DHX36. Pulse-chase experiments were performed by pre-incubating trace labeled G4 with 10 nM DHX36 (7 min) in the absence of ATP. ATP was then added with a large molar excess of unlabeled G4 to trap dissociated DHX36. At various time points, reaction aliquots were quenched in gel loading dye containing 1.5 mg/ml proteinase K (AMRESCO) and 1% SDS and electrophoresed on a 12% native gel at 4 °C. Gels were dried, exposed on a phosphorimager screen overnight, and scanned using a Typhoon FLA 9500 (GE Healthcare). Data were quantified using ImageQuant 5.2 (GE Healthcare) and analysed using Igor Pro 6.3.

Equilibrium binding of DHX36 to G4s

Experiments were performed by incubating 10 pM of 32P-labeled G4 substrate with varying concentrations of DHX36 at 37 °C for 30 minutes in the reaction buffer under standard conditions (50 mM Na-MOPS, pH 7.0 containing 100 mM KCl and supplemented with 2 mM ADP-BeF3-Mg2+). Following incubation, bound and free G4s were separated by 12% native PAGE as above.

UV Thermal melting experiments

G4 stability was probed using a Varian Cary UV-Vis spectrophotometer (Agilent Technologies) with a Peltier temperature control system. Preformed G4s were diluted to 2 µM in the standard reaction buffer in a 1 cm quartz cuvette. Thermal denaturation was achieved by increasing the temperature at 0.5 °C/min from 25-100 °C while monitoring absorbance at 240 nm.

DNA duplex unwinding

For duplex unwinding experiments, complementary oligonucleotides were designed such that one strand included a 3′ poly A or poly T extension. The short strands lacking the extension were 32P-labeled as described above. Duplexes were formed by adding a trace amount of the labeled short strand to 100 nM of the complementary strand with the 3′ extension. The labeled duplex was then added to the reaction buffer under standard conditions (50 mM Na-MOPS, pH 7.0 containing 100 mM KCl and 2 mM ATP-Mg2+) followed by addition of 1 µM of the unlabeled short strand as a chase and then addition of DHX36 to initiate the unwinding reaction at 37 °C. Aliquots were quenched at various times in gel loading dye solution containing 1.5 mg/ml Proteinase K and 1% SDS and processed as above by 12% native PAGE.

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CONFLICT OF INTEREST
The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS
P.M.Y. designed experiments, performed experiments, analyzed data and wrote the manuscript; D.B. expressed and purified protein, performed experiments and analyzed data; Z.L. expressed and purified protein; T.S.X. coordinated protein expression and purification and wrote the manuscript; R.R. conceived, designed, coordinated, developed the study and wrote the manuscript. All authors approved the final version of the manuscript.
REFERENCES

1. Gellert, M., Lipsett, M. N., and Davies, D. R. (1962) Helix formation by guanylic acid. Proc Natl Acad Sci U S A 48, 2013-2018

2. Huppert, J. L., and Balasubramanian, S. (2007) G-quadruplexes in promoters throughout the human genome. Nucleic Acids Res 35, 406-413

3. Huppert, J. L., Bugaut, A., Kumari, S., and Balasubramanian, S. (2008) G-quadruplexes: the beginning and end of UTRs. Nucleic Acids Res 36, 6260-6268

4. Eddy, J., and Maizels, N. (2008) Conserved elements with potential to form polymorphic G-quadruplex structures in the first intron of human genes. Nucleic Acids Res 36, 1321-1333

5. Mullen, M. A., Olson, K. J., Dallaire, P., Major, F., Assmann, S. M., and Bevilacqua, P. C. (2010) RNA G-Quadruplexes in the model plant species Arabidopsis thaliana: prevalence and possible functional roles. Nucleic Acids Res 38, 8149-8163

6. Maizels, N., and Gray, L. T. (2013) The G4 genome. PLoS Genet 9, e1003468

7. Schaffitzel, C., Berger, I., Postberg, J., Hanes, J., Lipps, H. J., and Pluckthun, A. (2011) In vitro generated antibodies specific for telomeric guanine-quadruplex DNA react with Stylonychia lemnae macronuclei. Proc Natl Acad Sci U S A 98, 8572-8577

8. Biffi, G., Di Antonio, M., Tannahill, D., and Balasubramanian, S. (2014) Visualization and selective chemical targeting of RNA G-quadruplex structures in the cytoplasm of human cells. Nat Chem 6, 75-80

9. Biffi, G., Tannahill, D., McCafferty, J., and Balasubramanian, S. (2013) Quantitative visualization of DNA G-quadruplex structures in human cells. Nat Chem 5, 182-186

10. Henderson, A., Wu, Y., Huang, Y. C., Chavez, E. A., Platt, J., Johnson, F. B., Brosh, R. M., Jr., Sen, D., and Lansdorp, P. M. (2014) Detection of G-quadruplex DNA in mammalian cells. Nucleic Acids Res 42, 860-869

11. Hansel-Hertsch, R., Di Antonio, M., and Balasubramanian, S. (2017) DNA G-quadruplexes in the human genome: detection, functions and therapeutic potential. Nat Rev Mol Cell Biol 18, 279-284

12. Piazza, A., Adrian, M., Samazan, F., Heddi, B., Hamon, F., Serero, A., Lopes, J., Teulade-Fichou, M. P., Phan, A. T., and Nicolas, A. (2015) Short loop length and high thermal stability determine genomic instability induced by G-quadruplex-forming minisatellites. EMBO J 34, 1718-1734

13. Mendoza, O., Bourdoncle, A., Boule, J. B., Brosh, R. M., Jr., and Mergny, J. L. (2016) G-quadruplexes and helicases. Nucleic Acids Res 44, 1989-2006

14. Vaughn, J. P., Creacy, S. D., Routh, E. D., Joyner-Butt, C., Jenkins, G. S., Pauli, S., Nagamine, Y., and Akman, S. A. (2005) The DEXH protein product of the DHX36 gene is the major source of tetramolecular quadruplex G4-DNA resolving activity in HeLa cell lysates. J Biol Chem 280, 38117-38120

15. Creacy, S. D., Routh, E. D., Iwamoto, F., Nagamine, Y., Akman, S. A., and Vaughn, J. P. (2008) G4 resolvase 1 binds both DNA and RNA tetramolecular quadruplex with high affinity and is the major source of tetramolecular quadruplex G4-DNA and G4-RNA resolving activity in HeLa cell lysates. J Biol Chem 283, 34626-34634

16. Chakraborty, P., and Grosse, F. (2011) Human DHX9 helicase preferentially unwinds RNA-containing displacement loops (R-loops) and G-quadruplexes. DNA Repair (Amst) 10, 654-665

17. Booy, E. P., Meier, M., Okun, N., Novakowski, S. K., Xiong, S., Stetefeld, J., and McKenna, S. A. (2012) The RNA helicase RHAU (DHX36) unwinds a G4-quadruplex in human telomerase RNA and promotes the formation of the P1 helix template boundary. Nucleic Acids Res 40, 4110-4124
Mechanism of G4 disruption by DHX36

18. Sexton, A. N., and Collins, K. (2011) The 5’ guanosine tracts of human telomerase RNA are recognized by the G-quadruplex binding domain of the RNA helicase DHX36 and function to increase RNA accumulation. *Mol Cell Biol* 31, 736-743

19. Booy, E. P., Howard, R., Marushchak, O., Ariyo, E. O., Meier, M., Novakowski, S. K., Deo, S. R., Dzananovic, E., Stetefeld, J., and McKenna, S. A. (2014) The RNA helicase RHAU (DHX36) suppresses expression of the transcription factor PITX1. *Nucleic Acids Res* 42, 3346-3361

20. Newman, M., Sfaxi, R., Saha, A., Monchaud, D., Teulade-Fichou, M. P., and Vagner, S. (2017) The G-Quadruplex-Specific RNA Helicase DHX36 Regulates p53 Pre-mRNA 3’-End Processing Following UV-Induced DNA Damage. *J Mol Biol* 320, 3121-3131

21. Huang, W., Smaldino, P. J., Zhang, Q., Miller, L. D., Cao, P., Stadelman, K., Wan, M., Giri, B., Lei, M., Nagamine, Y., Vaughn, J. P., Akman, S. A., and Sui, G. (2012) Yin Yang 1 contains G-quadruplex structures in its promoter and 5'-UTR and its expression is modulated by G4 resolvase 1. *Nucleic Acids Res* 40, 1033-1049

22. Lai, J. C., Ponti, S., Pan, D., Kohler, H., Skoda, R. C., Matthias, P., and Nagamine, Y. (2012) The DEAH-box helicase RHAU is an essential gene and critical for mouse hematopoiesis. *Blood* 119, 4291-4300

23. Gao, X., Ma, W., Nie, J., Zhang, C., Zhang, J., Yao, G., Han, J., Xu, J., Hu, B., Du, Y., Shi, Q., Yang, Z., Huang, X., and Zhang, Y. (2015) A G-quadruplex DNA structure resolvase, RHAU, is essential for spermatogonia differentiation. *Cell Death Dis* 6, e1610

24. Nie, J., Jiang, M., Zhang, X., Tang, H., Jin, H., Huang, X., Yuan, B., Zhang, C., Lai, J. C., Nagamine, Y., Pan, D., Wang, W., and Yang, Z. (2015) Post-transcriptional Regulation of Nkx2-5 by RHAU in Heart Development. *Cell reports* 13, 723-732

25. Giri, B., Smaldino, P. J., Thys, R. G., Creacy, S. D., Routh, E. D., Hantgan, R. R., Lattmann, S., Nagamine, Y., Akman, S. A., and Vaughn, J. P. (2011) G4 resolvase 1 tightly binds and unwinds unimolecular G4-DNA. *Nucleic Acids Res* 39, 7161-7178

26. Chen, M. C., Murat, P., Abecassis, K., Ferre-D'Amare, A. R., and Balasubramanian, S. (2015) Insights into the mechanism of a G-quadruplex-unwinding DEAH-box helicase. *Nucleic Acids Res* 43, 2223-2231

27. Smaldino, P. J., Routh, E. D., Kim, J. H., Giri, B., Creacy, S. D., Hantgan, R. R., Akman, S. A., and Vaughn, J. P. (2015) Mutational Dissection of Telomeric DNA Binding Requirements of G4 Resolvase 1 Shows that G4-Structure and Certain 3’-Tail Sequences Are Sufficient for Tight and Complete Binding. *PLoS One* 10, e0132668

28. Lattmann, S., Giri, B., Vaughn, J. P., Akman, S. A., and Nagamine, Y. (2010) Role of the amino terminal RHAU-specific motif in the recognition and resolution of guanine quadruplex-RNA by the DEAH-box RNA helicase RHAU. *Nucleic Acids Res* 38, 6219-6233

29. Meier, M., Patel, T. R., Booy, E. P., Marushchak, O., Okun, N., Deo, S., Howard, R., McEleney, K., Harding, S. E., Stetefeld, J., and McKenna, S. A. (2013) Binding of G-quadruplexes to the N-terminal recognition domain of the RNA helicase associated with AU-rich element (RHAU). *J Biol Chem* 288, 35014-35027

30. Heddi, B., Cheong, V. V., Martadinata, H., and Phan, A. T. (2015) Insights into G-quadruplex specific recognition by the DEAH-box helicase RHAU: Solution structure of a peptide-quadruplex complex. *Proc Natl Acad Sci U S A* 112, 9608-9613

31. Jarmoskaite, I., and Russell, R. (2014) RNA helicase proteins as chaperones and remodelers. *Annu Rev Biochem* 83, 697-725

32. Hilbert, M., Karow, A. R., and Klostermeier, D. (2009) The mechanism of ATP-dependent RNA unwinding by DEAD box proteins. *Biol Chem* 390, 1237-1250
Mechanism of G4 disruption by DHX36

33. Fairman-Williams, M. E., Guenther, U. P., and Jankowsky, E. (2010) SF1 and SF2 helicases: family matters. *Curr Opin Struct Biol* **20**, 313-324

34. Russell, R., Jarmoskaite, I., and Lambowitz, A. M. (2013) Toward a molecular understanding of RNA remodeling by DEAD-box proteins. *RNA Biol* **10**, 44-55

35. Cheong, C., and Moore, P. B. (1992) Solution structure of an unusually stable RNA tetraplex containing G- and U-quartet structures. *Biochemistry* **31**, 8406-8414

36. Deng, J., Xiong, Y., and Sundaralingam, M. (2001) X-ray analysis of an RNA tetraplex (UGGGGU)(4) with divalent Sr(2+) ions at subatomic resolution (0.61 A). *Proc Natl Acad Sci U S A* **98**, 13665-13670

37. Mergny, J. L., De Cian, A., Ghelab, A., Sacca, B., and Lacroix, L. (2005) Kinetics of tetramolecular quadruplexes. *Nucleic Acids Res* **33**, 81-94

38. Harrington, C., Lan, Y., and Akman, S. A. (1997) The identification and characterization of a G4-DNA resolvase activity. *J Biol Chem* **272**, 24631-24636

39. Hazel, P., Huppert, J., Balasubramanian, S., and Neidle, S. (2004) Loop-length-dependent folding of G-quadruplexes. *J Am Chem Soc* **126**, 16405-16415

40. Rachwal, P. A., Findlow, I. S., Werner, J. M., Brown, T., and Fox, K. R. (2007) Intramolecular DNA quadruplexes with different arrangements of short and long loops. *Nucleic Acids Res* **35**, 4214-4222

41. Bugaut, A., and Balasubramanian, S. (2008) A sequence-independent study of the influence of short loop lengths on the stability and topology of intramolecular DNA G-quadruplexes. *Biochemistry* **47**, 689-697

42. You, H., Lattmann, S., Rhodes, D., and Yan, J. (2017) RHAU helicase stabilizes G4 in its nucleotide-free state and destabilizes G4 upon ATP hydrolysis. *Nucleic Acids Res* **45**, 206-214

43. Prabu, J. R., Muller, M., Thomae, A. W., Schussler, S., Bonneau, F., Becker, P. B., and Conti, E. (2015) Structure of the RNA Helicase MLE Reveals the Molecular Mechanisms for Uridine Specificity and RNA-ATP Coupling. *Mol Cell* **60**, 487-499

44. Walbott, H., Mouffok, S., Capeyrou, R., Lebaron, S., Humbert, O., van Tilbeurgh, H., Henry, Y., and Leulliot, N. (2010) Prp43p contains a processive helicase structural architecture with a specific regulatory domain. *EMBO J* **29**, 2194-2204

45. Tauchert, M. J., Fourmann, J. B., Luhrmann, R., and Ficner, R. (2017) Structural insights into the mechanism of the DEAH-box RNA helicase Prp43. *eLife* **6**, 1-25

46. Albery, W. J., and Knowles, J. R. (1976) Evolution of enzyme function and the development of catalytic efficiency. *Biochemistry* **15**, 5631-5640

47. Chatterjee, S., Zagelbaum, J., Savitsky, P., Struzzenegger, A., Huttner, D., Janscak, P., Hickson, I. D., Gileadi, O., and Rothenberg, E. (2014) Mechanistic insight into the interaction of BLM helicase with intra-strand G-quadruplex structures. *Nat Commun* **5**, 5556

48. Wu, C. G., and Spies, M. (2016) G-quadruplex recognition and remodeling by the FANCJ helicase. *Nucleic Acids Res* **44**, 8742-8753

49. Guo, J. U., and Bartel, D. P. (2016) RNA G-quadruplexes are globally unfolded in eukaryotic cells and depleted in bacteria. *Science* **353**, aaf5371
Figure 1. DHX36-mediated disruption of tetramolecular DNA G4s under steady-state and single-turnover conditions. (A) Illustration showing disruption of the tetramolecular G-quadruplex substrate 5G-A_{15}, which includes five G-quartets and a 3’ single-stranded extension consisting of fifteen adenosine nucleotides (left), into single strands (right). (B) Native gel image showing time-dependent disruption of 5G-A_{15} (upper band) to single strands (lower band) in steady-state experiments with 10 nM DHX36 and G4 concentrations as indicated. In lane C1, the same ssDNA is shown with the G4-formation step omitted. In lane C2, the G4 formation step was performed with LiCl replacing KCl. (C) Initial rates for steady-state disruption of G4s with varying numbers of G-quartets: 4G-A_{15} (blue, $k_{cat} = 36 \pm 3$ min$^{-1}$ and $K_M = 180 \pm 6$ nM), 5G-A_{15} (red, $k_{cat} = 6.0 \pm 0.2$ min$^{-1}$ and $K_M = 40 \pm 2$ nM), and 6G-A_{15} (black, $k_{cat} = 0.6 \pm 0.1$ min$^{-1}$ and $K_M = 4.5 \pm 0.1$ nM). Solid blue squares show reactions of DHX36 with 4G-A_{15} in the absence of ATP. (D) Progress curves showing time-dependent disruption of the 5G-A_{15} G-quadruplex under single-turnover experiments with no DHX36 (triangles) and with 0.1 nM (squares) or 1 nM (diamonds) DHX36. (E) Plot of observed rate constants versus DHX36 concentration for disruption of 4G-A_{15} (2.8 $\pm$ 0.3 x 10$^8$ M$^{-1}$ min$^{-1}$), 5G-A_{15} (2.4 $\pm$ 0.2 x 10$^8$ M$^{-1}$ min$^{-1}$), and 6G-A_{15} (1.6 $\pm$ 0.2 x 10$^8$ M$^{-1}$ min$^{-1}$) under single-turnover conditions (with the same color code as panel C). Reactions here and in subsequent figures were performed under standard conditions unless otherwise indicated (see Experimental Procedures, DNA G4 disruption).
Figure 2. DHX36-mediated disruption of unimolecular DNA G4s. (A) Progress curves showing spontaneous unfolding of (G₃TT)₃G₃A₁₅ in the absence of DHX36 and with varying chase oligonucleotide concentration. (B) Progress curves showing unfolding of (G₃TT)₃G₃A₁₅ (purple) and (G₃T)₃G₃A₁₅ (green) in the presence and absence of DHX36 under standard conditions. Solid symbols show reactions in the presence of 0.5 nM DHX36 and 2 mM ATP-Mg²⁺ and open symbols show reactions in the absence of DHX36. Observed rate constants in the absence of DHX36 were 4.0 (±0.3) × 10⁻² min⁻¹ and 4.5 (±0.1) × 10⁻⁴ min⁻¹ for unfolding of (G₃TT)₃G₃A₁₅ and (G₃T)₃G₃A₁₅, respectively. (C) Plot of observed rate constants versus DHX36 concentration for unfolding of (G₃TT)₃G₃A₁₅ (purple, 1.3 (±0.2) × 10⁹ M⁻¹ min⁻¹) and (G₃T)₃G₃A₁₅ (green, 1.1 (±0.1) × 10⁹ M⁻¹ min⁻¹).
Figure 3. The effect of 3′-extension length on G4 disruption efficiency by DHX36. The plot shows observed rate constants versus DHX36 concentration for disruption of G4s with varying 3′-extension lengths in single-turnover reactions. Substrates and their measured second-order rate constants were: 5G-A25, 3.1 (±0.2) × 10^8 M⁻¹ min⁻¹; 5G-A15, 2.4 (±0.2) × 10^8 M⁻¹ min⁻¹; 5G-A10, 8.5 (±0.2) × 10^7 M⁻¹ min⁻¹; 5G-A5, < 1.2 × 10^5 M⁻¹ min⁻¹; and 5G-A3, < 1.2 × 10^5 M⁻¹ min⁻¹.
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Figure 4. Incorporation of a PEG linker between the G4 and the 3’ extension blocks G4 disruption by DHX36. (A) Illustration depicting DHX36 loading onto the 3’ extension (A_{15}) of a G4 substrate with a triethyleneglycol linker (5Gsp_{9}-A_{15}). For clarity, the PEG linker and 3’-extension are shown for just one strand, but the experiment included these features on all four strands of the G4. (B) Plot of G4 fraction versus time under single-turnover conditions for 2.5 nM DHX36 with 5Gsp_{9}-A_{15} (black) or 5Gsp_{18}-A_{15} (blue). Disruption of a comparable substrate that lacks the PEG linker was measured side-by-side and is included for comparison (red). (C) DHX36 binds strongly to the G4s with a PEG linker. The plot shows the observed rate constants for disruption of a standard G4 substrate 5G-A_{15} in the presence of various concentrations of the G4 that includes the 9-atom PEG linker (black curve, $K_i = 12$ (±1) nM) or the 18-atom PEG linker (blue curve, $K_i = 14$ (±2) nM).
Figure 5. DHX36 unwinding of DNA duplexes. (A) Progress curves of 15-bp DNA helix (Comp15-T₁₅) unwinding in the absence of DHX36 (circles, black) or in the presence of 20 nM (blue), 50 nM (red) or 84 nM DHX36 (green). (B) Plot of observed rate constants for DNA duplex unwinding vs DHX36 concentration for duplex constructs of 12 bp (blue), 15 bp (red), and 20 bp (black) with a T₁₅ 3’ extension. Second-order rate constants were 4.1 (±0.8) × 10⁵ M⁻¹ min⁻¹ for the 15-bp duplex and 2.4 (±0.9) × 10⁵ M⁻¹ min⁻¹ for the 20-bp duplex.
Figure 6. Effect of 3′-extension sequence on G4 disruption by DHX36. (A,B) Plots of observed rate constant versus concentration for disruption of G4s with various 3′-extension sequence (see Table S2) under single-turnover conditions (panel A) or steady-state conditions (panel B). (C) A plot of normalized $k_{cat}$ and $k_{cat}/K_M$ values measured in panels A and B above. Panels A-C use the same color scheme. (D) Equilibrium binding measurements for tetramolecular DNA and RNA G4s: 5G-A$_{15}$ (red), r5G-A$_{15}$ (gray), 5G-T$_{15}$, (black), and r5G-U$_{15}$ (orange). The indicated dissociation constants for 5G-T$_{15}$ and r5G-U$_{15}$ are likely to represent upper limits because of very slow dissociation of DHX36 from these substrates. (E) 3′-extension sequence preference at low ATP concentration. The plot shows second-order rate constants for G4 disruption for 5G-A$_{15}$ and 5G-T$_{15}$ versus ATP concentration. (F) G4 disruption rate depends on G4 stability at low ATP concentration. The plot shows the observed rate constant for disruption of 4G-A$_{15}$ (blue) and 5G-A$_{15}$ (red) versus DHX36 concentration with 25 µM ATP. These data gave apparent second-order rate constant values of $2.4 \pm 0.1 \times 10^8$ M$^{-1}$ min$^{-1}$ and $7.5 \pm 0.3 \times 10^7$ M$^{-1}$ min$^{-1}$ for disruption of 4G-A$_{15}$ and 5G-A$_{15}$, respectively.
Figure 7. Kinetic model for G4 disruption by DHX36 and effects of 3′-extension sequence. (A) Free energy profile showing DHX36 binding and disruption of G4 substrates under $k_{cat}/K_M$ (subsaturating) conditions. The red curves depict disruption of G4 substrates with an A$_{15}$ extension and the black curves depict disruption of G4 substrates with a T$_{15}$ extension. Solid curves depict reactions at high ATP concentrations. Dashed curves depict reactions at low ATP concentrations, such that the rate-limiting step shifts from binding to G4 disruption (see Discussion). (B) Measured rate constants for binding and disruption of G4 substrates with five G-quartets and 3′-extension sequences of 5G-A$_{15}$ (red, top) and 5G-T$_{15}$ (black, bottom) under conditions of saturating ATP. The positions of each state correspond to the free energy profiles above, as indicated by the gray shading. For simplicity, the illustration of DHX36 (light blue) depicts only the helicase core. The NTD of DHX36 likely forms additional contacts with these G4 structures, contributing to the high-affinity binding of DHX36.
The G-quadruplex (G4) resolvase DHX36 efficiently and specifically disrupts DNA G4s via a translocation-based helicase mechanism
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