Bruce Chang-Gu, Devin Bradburn, Philip M. Yangyuoru and Rick Russell*

The DHX36-specific-motif (DSM) enhances specificity by accelerating recruitment of DNA G-quadruplex structures

<https://doi.org/10.1515/hsz-2020-0302>

Received September 6, 2020; accepted December 7, 2020;

published online December 16, 2020

Abstract: DHX36 is a eukaryotic DEAH/RHA family helicase that disrupts G-quadruplex structures (G4s) with high specificity, contributing to regulatory roles of G4s. Here we used a DHX36 truncation to examine the roles of the 13-amino acid DHX36-specific motif (DSM) in DNA G4 recognition and disruption. We found that the DSM promotes G4 recognition and specificity by increasing the G4 binding rate of DHX36 without affecting the dissociation rate. Further, for most of the G4s measured, the DSM has little or no effect on the G4 disruption step by DHX36, implying that contacts with the G4 are maintained through the transition state for G4 disruption. This result suggests that partial disruption of the G4 from the 3' end is sufficient to reach the overall transition state for G4 disruption, while the DSM remains unperturbed at the 5' end. Interestingly, the DSM does not contribute to G4 binding kinetics or thermodynamics at low temperature, indicating a highly modular function. Together, our results animate recent DHX36 crystal structures, suggesting a model in which the DSM recruits G4s in a modular and flexible manner by contacting the 5' face early in binding, prior to rate-limiting capture and disruption of the G4 by the helicase core.

Keywords: G4R1; G4 resolvase; G-quadruplex; helicase kinetics; RHAU; RNA helicase.

Introduction

G-quadruplexes (G4s) form by self-assembly of four guanosine tracts within one or more DNA or RNA strands. Hydrogen bonding by the Hoogsteen and base-pairing faces of four guanine bases form planar G-quartets, and cation-stabilized stacking of these G-quartets leads to the formation of G4s (Gellert et al. 1962). Putative G4-forming sequences are conserved and overrepresented in the promoter regions of human proto-oncogenes while underrepresented in tumor suppressor genes, suggesting key roles of G4s in regulation of gene expression and cancer (Hänsel-Hertsch et al. 2017). These sequences are also overrepresented in the 5' and 3' untranslated regions of mRNA, and RNA G4s have been suggested to play regulatory roles in translation and mRNA lifetime (Bugaut and Balasubramanian 2012; Beaudoin and Perreault 2013; Sauer et al. 2019). Further, both DNA and RNA G4s have been suggested to function broadly in cellular processes including telomere replication, transcription, and translation (Ambrus et al. 2006; Kumari et al. 2007; Siddiqui-Jain et al. 2002; Xu et al. 2010).

Although the direct detection of G4s *in vivo* remains challenging, G4-staining antibodies and stabilizing ligands have shown that G4s can form *in vivo* and have provided evidence for their biological functions (Biffi et al. 2014; Chen et al. 2018a; Henderson et al. 2014; Rodriguez et al. 2012; Varshney et al. 2020). Additionally, the presence and importance of G4-binding proteins and helicases provide further support for biological roles of G4s (Damerla et al. 2012; Mendoza et al. 2016; Murat et al. 2018; Nguyen et al. 2014; Sauer et al. 2019; Wu et al. 2008). One helicase of particular interest, DHX36 (also known as RHAU), is an ATP-dependent G4 resolvase that is highly expressed in mammals, essential for mouse development, and constitutes the bulk of the G4-resolving activity in human cell lysate (Creacy et al. 2008; Lai et al. 2012; Vaughn et al. 2005; Yangyuoru et al. 2018).

DHX36 is a DEAH/RHA family helicase that displays highly specific binding of G4s, with reported K_d values in the nanomolar to picomolar range (Creacy et al. 2008; Lattmann et al. 2010; Giri et al. 2011; Yangyuoru et al. 2018). In addition

***Corresponding author: Rick Russell**, Department of Molecular Biosciences, The University of Texas at Austin, Austin, TX 78712, USA, E-mail: rick_russell@cm.utexas.edu

Bruce Chang-Gu, Department of Molecular Biosciences, The University of Texas at Austin, Austin, TX 78712, USA.

<https://orcid.org/0000-0001-8617-0294>

Devin Bradburn, Department of Molecular Biosciences, The University of Texas at Austin, Austin, TX 78712, USA; and Department of Biology, Stanford University, Stanford, CA 94305, USA

Philip M. Yangyuoru, Department of Molecular Biosciences, The University of Texas at Austin, Austin, TX 78712, USA; and Department of Chemistry, Northern Michigan University, Marquette, MI 49855, USA

to a helicase core that is conserved among DEAH/RHA helicases, DHX36 features a flexible N-terminal domain (NTD) with a 13-amino-acid motif known as the DHX36-Specific Motif (DSM, also known as the RSM for RHAU-specific motif). The DSM is rich in aromatic residues and binds to the planar G4 face, contributing substantially to the affinity and presumably the specificity of G4 binding by DHX36 (Figure 1) (Heddi et al. 2015; Lattmann et al. 2010).

Mechanistic work has led to a simple model for G4 disruption by DHX36 (Creacy et al. 2008; Chen et al. 2018b; Yangyuoru et al. 2018). The helicase core binds to single stranded DNA or RNA on the 3' side of the G4, a structural element that is required for DHX36 activity, and the DSM binds to the G4 face (Chen et al. 2018b). The helicase core then undergoes ATP-dependent, 3' – 5' translocation into the G4 to disrupt it. It is known that the DSM contributes to G4 binding by DHX36 (Chen et al. 2018a; Chen et al. 2018b; Yangyuoru et al. 2018), and it was recently reported that the DSM contributes modestly to the maximal disruption rates of RNA G4s, and more substantially under subsaturating conditions (Srinivasan et al. 2020). However, it is not clear what kinetic steps are affected by the DSM or whether these effects are general for disruption of both RNA and DNA G4s.

There are interesting additional questions about the function of the DSM from biochemical and biophysical perspectives. For many G4s that have been studied, the process of DHX36-mediated disruption is limited by DHX36 binding (Yangyuoru et al. 2018). The implication of this result is that if the DSM tightened G4 binding by increasing the lifetime of the bound complex, this effect would not lead to enhanced G4 disruption, as only the rate constant for binding would determine the disruption efficiency. At a physical level, it is not clear how specific binding of the DSM to a G4 would accelerate disruption of the G4. Indeed, specific binding might most simply be imagined to stabilize the G4, potentially inhibiting the first-order steps of G4 disruption that are carried out by the helicase core.

To probe more deeply into the functional roles of the DSM, here we measured the binding and disruption kinetics of DNA G4s by wild-type DHX36 (DHX36_{WT}) and an N-terminally truncated mutant that lacks the DSM

(DHX36_{ΔDSM}) (Figure 1). Using a series of tetramolecular DNA G4s with varying numbers of G-quartets and 3'-tail sequences, we analyzed the role of the DSM in G4 binding and dissociation kinetics as well as G4 disruption. We find that the DSM contributes to G4 affinity by accelerating DHX36 binding kinetics and does not contribute significantly to the G4 disruption step itself. In the context of prior structural data, our results suggest a model in which the NTD of DHX36 acts as a tether, binding rapidly to the 5' face of G4s to recruit them into functional engagement with the helicase core. Once bound, the NTD maintains contact during G4 disruption until after the rate-limiting transition state is reached.

Results

The DSM accelerates G4 binding by DHX36

To probe the roles of the DSM, we first measured the steady-state kinetics of DNA G4 disruption by DHX36_{WT} (amino acids 54–985) and DHX36_{ΔDSM} (amino acids 67–985). As in previous work (Chen et al. 2018c; Srinivasan et al. 2020; Yangyuoru et al. 2018), the DHX36 constructs excluded a low complexity, Gly-rich region at the natural N-terminus, which was shown to participate in subcellular localization but not biochemical activities of DHX36 (Chalupníková et al. 2008; Lattmann et al. 2010). Building on previous work, we used tetramolecular DNA G4s because they form parallel G4s, the preferred substrate of DHX36, and their slow reformation after disruption allowed easy detection of G4 disruption by electrophoretic mobility shift assay (EMSA) without the need for a 'chase' oligonucleotide to block G4 reformation (Figure 2A) (Chen et al. 2015; Yangyuoru et al. 2018).

Using a five-quartet G4 with a 3' extension of 15 adenosine nucleotides (5G-A₁₅; Table S1), we found that DHX36_{ΔDSM} disrupts subsaturating concentrations of this G4 with a second-order rate constant (k_{cat}/K_M) of $(7.3 \pm 0.7) \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$ (Figure 2B). In side-by-side measurements, DHX36_{WT} resolved the same G4 nearly 100-fold more efficiently, with a k_{cat}/K_M value of $(7.0 \pm 0.04) \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$, similar to a previously-published value (Yangyuoru et al. 2018). This decrease in $k_{\text{cat}}/$

Figure 1: Domain structure of DHX36. The DSM is a 13-amino-acid sequence (amino acids 54–66) within the N-terminal domain. DHX36_{WT} consists of amino acids 54–985. The DHX36_{ΔDSM} variant is N-terminally truncated and consists of amino acids 67–985.

Figure 2: DHX36-mediated disruption of G4s with A₁₅ tail sequences.

(A) Native gel image showing time dependent disruption of 4G-A₁₅ by DHX36 Δ DSM. (B) Steady-state disruption of A-tailed G4 substrates by DHX36 Δ DSM. Values of k_{cat}/K_M are $(4.3 \pm 0.1) \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$ for 4G-A₁₅, $(7.3 \pm 0.7) \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$ for 5G-A₁₅, and $(2.4 \pm 0.7) \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$ for 6G-A₁₅. Values of k_{cat} are $6.3 \pm 0.2 \text{ min}^{-1}$ for 5G-A₁₅ and $1.0 \pm 0.2 \text{ min}^{-1}$ for 6G-A₁₅. The absence of a clear plateau for 4G-A₁₅ prevented determination of a k_{cat} value. Results for disruption of 5G-A₁₅ by DHX36_{WT} are shown by filled black circles, giving a k_{cat}/K_M value of $(7.0 \pm 0.04) \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$. The black and blue dashed lines represent the maximal disruption rates (k_{cat}) by DHX36_{WT} for 5G-A₁₅ and 6G-A₁₅, respectively, as determined previously (Yangyuoru et al. 2018). Data are shown as the average and SEM from 2–3 independent measurements.

K_M upon removal of the DSM does not result from a decrease in the rate of G4 disruption itself, as the maximal rate observed with saturating G4 concentrations (k_{cat}) remains unchanged (Figure 2B) (Yangyuoru et al. 2018). Therefore, these results indicate that the reduction in the k_{cat}/K_M value is most likely due to a decrease in the rate of G4 binding, an increase in the rate of G4 dissociation, or both.

To further probe the roles of the DSM and to examine how changes in G4 stability impact disruption, we next tested G4s containing four and six quartets (4G-A₁₅ and 6G-A₁₅). As shown previously for DHX36_{WT} (Yangyuoru et al. 2018), the k_{cat}/K_M values did not depend on the length of the G4, as DHX36 Δ DSM disrupted 4G-A₁₅, 5G-A₁₅, and 6G-A₁₅ with the same efficiencies within error (Figure 2B). A dependence on

G4 length emerged with saturating substrate concentrations, as the k_{cat} values for disruption by DHX36 Δ DSM were $>6 \text{ min}^{-1}$ for 4G-A₁₅, $6.3 \pm 0.2 \text{ min}^{-1}$ for 5G-A₁₅, and $1.0 \pm 0.2 \text{ min}^{-1}$ for 6G-A₁₅. The dependence of the rate of G4 disruption on G4 stability under saturating but not subsaturating conditions was also observed previously for DHX36_{WT} and indicates that the overall reaction is limited by different steps in these two regimes (Yangyuoru et al. 2018). Under saturating conditions, the reaction is evidently limited by the disruption step and is therefore faster with less stable G4s. Under subsaturating conditions, the reaction is most likely limited by DHX36 Δ DSM binding to the G4s, explaining the lack of dependence on G4 length. G4 binding was found previously to limit the reaction for DHX36_{WT} under subsaturating conditions also (Yangyuoru et al. 2018), and thus we conclude that the 100-fold lower second-order rate constant for DHX36 Δ DSM arises because the DSM accelerates binding of these G4s to DHX36 by 100-fold. The maximal rate constants for G4 disruption by DHX36 Δ DSM mirror previous values determined for disruption by DHX36_{WT} (Yangyuoru et al. 2018), indicating that the DSM does not contribute to the disruption step for these G4s.

The DSM does not affect G4 dissociation kinetics

It has previously been reported that DHX36 binds to DNA G4s with thymidine-rich 3' extensions up to 1000-fold more tightly than those with adenosine-rich extensions (Yangyuoru et al. 2018). The maximal disruption rates of G4s with T-rich extensions are also much lower than for their A-tailed counterparts (Yangyuoru et al. 2018). Together, these results suggested that G4s with T-rich extensions might have relatively long lifetimes with bound DHX36 and therefore might be well-suited for measurements of G4 dissociation and perhaps binding by DHX36.

To understand which step or steps limit the rate of DHX36-mediated disruption of G4s with T₁₅ extensions, we first varied the length of the G4 from 4 to 6 quartets and measured disruption by DHX36_{WT} and DHX36 Δ DSM under conditions of excess DHX36. In contrast to the behavior observed with the A-tailed G4s, for DHX36_{WT} we found that increasing G4 length decreased the k_{cat}/K_M value (Figure 3A). Further, the variation in the k_{cat}/K_M value tracked closely with the variation in the maximal rate of disruption (k_{disrupt}) (Figure 3A). These results suggest that for disruption of T-tailed G4 substrates by DHX36_{WT}, the G4 disruption step is rate limiting under both saturating and subsaturating conditions. If G4 disruption limits the

Figure 3: DHX36-mediated disruption of G4s with T₁₅ extension sequences.

(A) DHX36_{WT} concentration dependence for disruption of 4G-T₁₅ (red), 5G-T₁₅ (black), and 6G-T₁₅ (blue). These experiments gave k_{cat}/K_M and $k_{disrupt}$ values of $(1.4 \pm 0.1) \times 10^8 \text{ M}^{-1} \text{ min}^{-1}$ and $0.66 \pm 0.16 \text{ min}^{-1}$ for 4G-T₁₅; $(4.5 \pm 0.6) \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$ and $0.17 \pm 0.04 \text{ min}^{-1}$ for 5G-T₁₅; and $(2.7 \pm 0.5) \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$ and $0.02 \pm 0.004 \text{ min}^{-1}$ for 6G-T₁₅. (B) Concentration dependence of DHX36_{ΔDSM} for disruption of the same G4 substrates. These experiments gave k_{cat}/K_M values of $(2.2 \pm 0.1) \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$ for 4G-T₁₅, $(3.4 \pm 0.1) \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$ for 5G-T₁₅, and $(3.1 \pm 0.2) \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$ for 6G-T₁₅. Data represent the average $\pm$ SEM of 2–3 measurements.

observed rate under subsaturating conditions, the binding rate constant must be larger than the highest k_{cat}/K_M values; i.e. $>1.4 \times 10^8 \text{ M}^{-1} \text{ min}^{-1}$. It is likely that this change in behavior relative to the A-tailed G4 substrates arises because G4 disruption is slower for the T-tailed G4s, enabling equilibration of DHX36_{WT} binding and dissociation prior to G4 disruption.

For DHX36_{ΔDSM}, the k_{cat}/K_M values were substantially lower and did not vary systematically with G4 length [$(2.2 \pm 0.1) \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$ for 4G-T₁₅, $(3.4 \pm 0.1) \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$ for 5G-T₁₅, and $(3.1 \pm 0.2) \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$ for 6G-T₁₅; Figure 3B]. The result that the second-order rate did not depend on G4 length indicates either that binding is rate limiting for disruption by DHX36_{ΔDSM} under subsaturating conditions or that the disruption step is rate limiting and that, counterintuitively, the $k_{disrupt}$ value for DHX36_{ΔDSM} is the same for all of the tested G4s. The lack of saturation in single turnover experiments prevented direct determination of $k_{disrupt}$, and attempts to determine the maximal rate in steady-state experiments were unsuccessful because these T-tailed G4s were retained in the wells of native gels in steady-state reactions at the higher concentrations (data not shown).

Therefore, to test whether disruption of these T-tailed G4s by DHX36_{ΔDSM} is rate-limited by G4 binding or disruption, we determined whether the DHX36_{ΔDSM}-bound G4 is committed to being disrupted. Thus, we incubated DHX36_{ΔDSM} or DHX36_{WT} with the 6G-T₁₅ G4 and ATP to allow accumulation of the bound species (Figure 4A,B). We then added unlabeled G4 and monitored the conversion of the DHX36-G4 species to unbound G4 substrate or to the single-stranded oligonucleotide product. For both

DHX36_{WT} and DHX36_{ΔDSM}, we observed an accumulation of free G4 substrate, not oligonucleotide product, after the addition of the unlabeled chase G4 (Figure 4B–D). This result indicates that the rate constant for G4 dissociation is larger than that for G4 disruption of 6G-T₁₅ ($k_{off} > k_{disrupt}$), and thus that disruption of 6G-T₁₅ is rate-limited under subsaturating conditions by the disruption step rather than by binding. We infer that the disruption step is also rate-limiting for DHX36_{ΔDSM}-mediated disruption of 4G-T₁₅ and 5G-T₁₅, because the alternative model that binding becomes rate limiting for the shorter G4s would require slower binding of DHX36_{ΔDSM} to the shorter G4s than to 6G-T₁₅, coincidentally giving similar k_{cat}/K_M values for the three G4s. Thus, the uniform k_{cat}/K_M value indicates that the rate constant for disruption of these T-tailed G4s by DHX36_{ΔDSM} does not depend on G4 length from 4–6 quartets (see Discussion). These experiments also suggested that DHX36 dissociation is likely measurable by hand, as we observed accumulation of the free G4 substrate for both DHX36_{WT} and DHX36_{ΔDSM} on the timescale of approximately 1 min.

With these encouraging results, we next set out to determine whether the DSM contributes to the lifetime of the bound complex by measuring the G4 dissociation kinetics more systematically. Thus, we incubated DHX36_{WT} and DHX36_{ΔDSM} with radiolabeled, T-tailed G4 substrate (the ‘pulse’ phase), added an excess of unlabeled ‘chase’ G4 substrate, and then monitored dissociation of DHX36 using EMSA (Figure S1, top). To increase the signal for dissociation by preventing DHX36 from disrupting the G4 in the pulse phase, we used ADP-BeF_x, a non-hydrolysable ATP

Figure 4: The complex of DHX36-bound state is not committed to G4 disruption.

(A) Reaction schematic. We used the G4 substrate 6G-T₁₅ to allow for accumulation of the G4-DHX36 complex while minimizing disruption of the G4 prior to introduction of the unlabeled, chase G4. (B) Native gel depicting the time-dependent depletion of the shifted band representing G4 bound to DHX36 and concomitant accumulation of free G4. (C) Graphical depiction of the accumulation of free G4 (blue circles) over time as G4-DHX36 (green diamonds) was depleted. For comparison, the amount of ssDNA product remains the same (red squares). (D) The same experiment for DHX36_{ΔDSM}. For panels (C) and (D), the plots represent a single experiment, and duplicate experiments gave similar results.

analog that has been shown to provide a good mimic of the ATP-bound state for other helicases (Del Campo and Lambowitz 2009; Liu et al. 2008; Liu et al. 2014; Mallam et al. 2014). Using this experimental scheme, we found that 6G-T₁₅ dissociated from DHX36_{WT} and DHX36_{ΔDSM} with the same rate constant within error, $\sim 0.9 \text{ min}^{-1}$ (Figure 5A,B). By measuring dissociation from G4s of varying lengths (4–6 quartets) for both DHX36_{WT} and DHX36_{ΔDSM}, we found that the dissociation rates do not depend on G4 length over this range for either DHX36 protein (Figure 5A,B).

The results also showed that a fraction of the complex for the longer G4s (5G-T₁₅ and 6G-T₁₅) did not dissociate with the measured rate constant but remained formed at longer times, suggesting that DHX36 can transition slowly to a longer-lived binding mode in the presence of ADP-BeF_x. The fraction of DHX36 that populated this long-lived binding mode increased with increasing preincubation time, on the timescale of minutes and hours (Figure S2). The transition to this tight-binding mode was more efficient for DHX36_{ΔDSM} than for DHX36_{WT} but was significant for both, as $\geq 40\%$ of each protein was in the tight-binding mode after a 3 h

preincubation. The presence of a tight-binding mode depended on ADP-BeF_x and is reminiscent of a previous finding for several RNA helicases from the DEAD-box family (Liu et al. 2014). This tight-binding species, which is apparently not on the pathway for G4 disruption as its formation is slower than G4 disruption, likely accounts for the very high affinities reported previously for DHX36 in the presence of ADP-BeF_x (Yangyuru et al. 2018). Other reports of very high affinity, in the picomolar range, have come from experiments carried out in the absence of nucleotide (Creacy et al. 2008; Giri et al. 2011), and it is not clear whether the tighter binding in these reports reflects an analogous tight-binding state or differences in the G4 constructs and/or the solution conditions.

To further probe G4 dissociation and to examine whether the identity of the bound nucleotide impacts the dissociation rate, we used a fluorescence-quencher assay (Figure S1, bottom). We used the 6G-T₁₅ construct for these experiments to minimize the amount of G4 disruption in the ‘pulse’ phase of the experiment and included a 3’ Cy3 moiety, which we found did not affect DHX36-mediated disruption (data not shown). Binding of DHX36_{WT} or DHX36_{ΔDSM} to 6G-T₁₅-Cy3 did not change the fluorescence value significantly (data not shown). We then added an excess of an oligonucleotide with a quencher moiety and sequence complementarity to the 3’ extension of G4s, which resulted in a time-dependent decrease in fluorescence. This decrease was much slower than simple binding of the quencher oligonucleotide in the absence of protein, and it was independent of the oligonucleotide concentration (data not shown), indicating that the rate of the fluorescence decrease reflects the DHX36 dissociation rate. Using this assay, the measured dissociation rate constants for the G4 substrate 6G-T₁₅-Cy3 were $0.48 \text{ min}^{-1} \pm 0.22 \text{ min}^{-1}$ for DHX36_{WT} and $0.68 \pm 0.23 \text{ min}^{-1}$ for DHX36_{ΔDSM} in the presence of ADP-BeF_x (Figure 5C, left). These results are similar to those measured using EMSA, with the 2-fold rate decrease most likely due to the lower temperature in the fluorescence measurements ($\sim 28^\circ\text{C}$).

We next used this fluorescence assay to test whether the DSM impacted the lifetime of the bound G4 with other forms of bound nucleotide. For both DHX36_{WT} and DHX36_{ΔDSM}, we found that the G4 dissociation rate was unaffected by the nucleotide bound, as the k_{off} values were essentially the same with saturating concentrations (2 mM) of ADP-BeF_x, ATP, or no nucleotide (Figure 5C, middle and right). In conclusion we find that, in contrast with the large increase in the G4 binding rate constant in the presence of the DSM, the dissociation rate is not affected by the DSM. Additionally, we find that the bound nucleotide does not affect the rate of G4 dissociation from DHX36.

Figure 5: G4 dissociation of T-tailed G4s from DHX36.

(A) EMSA measurements of k_{off} for DHX36_{WT}, which gave values of $1.1 \pm 0.1 \text{ min}^{-1}$ for 4G-T₁₅ (red circles), $0.5 \pm 0.1 \text{ min}^{-1}$ for 5G-T₁₅ (black circles), and $0.9 \pm 0.3 \text{ min}^{-1}$ for 6G-T₁₅ (blue circles). (B) EMSA measurements of k_{off} for DHX36_{ΔDSM}. Rate constants were $1.0 \pm 0.2 \text{ min}^{-1}$ for 4G-T₁₅ (red squares), $1.0 \pm 0.1 \text{ min}^{-1}$ for 5G-T₁₅ (black squares), and $0.9 \pm 0.05 \text{ min}^{-1}$ for 6G-T₁₅ (blue squares). Plots represent a single time course and reported values are the average $\pm$ SEM of at least three independent measurements. (C) Measurements of dissociation of 6G-T₁₅Cy3 from DHX36_{WT} (blue) or DHX36_{ΔDSM} (red) using a fluorescence assay. For DHX36_{WT}, values of k_{off} were $0.48 \pm 0.22 \text{ min}^{-1}$ and $0.28 \pm 0.07 \text{ min}^{-1}$ in the presence of ADP-BeF_x and ATP-Mg, respectively, and 0.30 min^{-1} in the absence of nucleotide. For DHX36_{ΔDSM}, values of k_{off} were $0.68 \pm 0.23 \text{ min}^{-1}$ and $0.35 \pm 0.05 \text{ min}^{-1}$ in the presence of ADP-BeF_x and ATP-Mg, respectively, and 0.33 min^{-1} in the absence of nucleotide. Values represent the averages $\pm$ SEM of three independent determinations except for measurements without nucleotide, which were performed once and are reported without error estimates.

The role of the DSM is specific for G4 recognition

We next tested whether the DSM contributes to the relatively inefficient DHX36-mediated unwinding of a DNA duplex. We used a 20-base-pair duplex with a single-stranded 3'-T₁₅ extension (Comp20-T₁₅), which has a lifetime comparable to that of 5G-T₁₅ and undergoes detectable unwinding by DHX36 (Yangyuoru et al. 2018). In contrast to the results for G4 disruption, we found that removal of the DSM did not affect the second-order rate constant for unwinding of this duplex under single-turnover conditions ($4.0 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$; Figure 6), suggesting that the contribution of the DSM is specific to G4s. Further, because the intrinsic lifetimes of Comp20-T₁₅ and 5G-T₁₅ are similar, a comparison of the $k_{\text{cat}}/K_{\text{M}}$ values for DHX36-mediated disruption of the two substrates provides insight into the level of specificity for G4 substrates relative to simple helices. DHX36_{WT} exhibits a 160-fold preference for the G4 substrate, and interestingly DHX36_{ΔDSM} retains a 10-fold preference for the G4. The residual specificity in the absence of the DSM most likely

arises at least in part from specific contacts of the G4 with the helicase core (Chen et al. 2018b). In addition, DHX36 may not undergo the multiple cycles of translocation efficiently that are likely to be necessary to unwind the 20-bp duplex.

The DSM does not contribute to G4 binding at low temperature

To extend our analysis of the DSM to other conditions that may impact DHX36 kinetics and specificity, we used EMSA to measure DHX36 binding kinetics using 6G-T₁₅ at 5 °C. Surprisingly, we found that G4 binding was very slow and was not accelerated significantly by the DSM (Figure 7A). The protein concentration dependences of the binding rates hinted at upward curvature, which could be caused by cooperative binding of DHX36 under these conditions, but additional data will be required to explore this question. For simplicity, we applied linear fits to the data, which gave indistinguishable second-order rate constants of $(2.0 \pm 0.5) \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$ and $(3.0 \pm 0.7) \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$ for DHX36_{WT} and DHX36_{ΔDSM}, respectively (Figure 7A).

Figure 6: Duplex unwinding by DHX36_{WT} and DHX36_{ΔDSM}.

Left: The observed rate constant for unwinding is plotted as a function of the concentration of excess DHX36_{WT} (blue circles) or DHX36_{ΔDSM} (red squares). The second-order rate constants for DHX36_{WT} and DHX36_{ΔDSM} are $(4.0 \pm 0.3) \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$ and $(4.0 \pm 0.2) \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$ respectively. Right: Cartoon schematic of duplex unwinding assays. DHX36-mediated duplex disruption was measured using a 20-bp duplex. Re-annealing was blocked by the addition of excess unlabeled complementary oligonucleotide (0.5 μM or 1 μM , which gave indistinguishable results).

Figure 7: Binding and dissociation of DHX36 from 6G-T₁₅ at 5 °C.

(A) Concentration dependence of the observed rate constant for G4 binding by DHX36_{WT} (red squares, $(2.0 \pm 0.5) \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$) and DHX36_{ΔDSM} (blue circles, $(3.0 \pm 0.7) \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$). Each point represents the average $\pm$ SEM of duplicate measurements. (B) Representative progress curves of G4 dissociation from DHX36_{WT} (red squares, $2.0 \pm 0.5 \times 10^{-2} \text{ min}^{-1}$) and DHX36_{ΔDSM} (blue circles, $2.0 \pm 0.7 \times 10^{-2} \text{ min}^{-1}$). Reported values reflect the average $\pm$ SEM of three independent determinations.

We next determined whether the DSM affects the DHX36 dissociation rate at the lower temperature. For these experiments, we overcame the slow binding at low temperature by preincubating trace radiolabeled 6G-T₁₅ with DHX36_{WT} or DHX36_{ΔDSM} at 37 °C for ~10 min. After allowing the complex to form, we measured dissociation at 5 °C in the presence of excess unlabeled 6G-T₁₅. As expected, the presence of the DSM did not affect G4 dissociation, with both versions of DHX36 giving k_{off} values of 0.02 min⁻¹ (Figure 7B). Together, these results indicate that at 5 °C, the DSM does not contribute to G4 binding, most simply suggesting that it does not contact the G4 in the DHX36-bound state (see Discussion).

Discussion

The DSM, a 13-amino-acid sequence at the N-terminus of DHX36, has been shown previously to bind to the face of

G4s using hydrophobic and charged residues and to contribute to the high affinity of DHX36 for these structures. Here, we used steady-state and pre-steady-state kinetics approaches to probe how the DSM contributes to the mechanism and specificity of DNA G4 disruption by DHX36. We found that the dominant effect of the DSM is to accelerate binding of G4s by DHX36. As described below, this acceleration contributes to specificity –even for G4s that are processed efficiently by DHX36 with rate-limiting binding– and suggests a physical mechanism for the DSM in G4 recruitment by DHX36.

The DSM accelerates G4 binding

The effects of the DSM on G4 binding and disruption are schematized in the free energy profiles of Figure 8. For all of the G4s tested, the presence of the DSM results in a substantial acceleration of binding, as reflected in the

Figure 8: Model for G4 recruitment by the DHX36 DSM.

Top: a free energy diagram depicting the energetic contribution of the DSM. The black curve represents the reaction of DHX36_{WT} and the red curve represents the free energy profile for DHX36_{ΔDSM}. Bottom: The cartoon illustrates a physical model for the contribution of the DSM. Each diagram depicts the ground state that corresponds in horizontal position to the free energy profile. The DSM lowers the overall free energy for G4 binding ($\Delta\Delta G_{\text{binding}}$) by separating it into two steps, with the DSM first forming contacts with the G4 face to generate a bound intermediate. The helicase core then binds adjacent to the G4 in a second step, which is much faster than the equivalent step in the absence of the DSM because the G4 is already localized by the contacts with the DSM. The free energy stabilization contributed by the DSM is then maintained in the bound state and in the transition state for G4 disruption, as indicated by the constant difference between the black and red curves and illustrated by vertical arrows. Note that the experiments do not probe the relative free energy of the final product state, as depicted by the break in the free energy curve between the transition state for G4 disruption and the energy well corresponding to the free products.

larger k_{cat}/K_M values for G4 disruption for DHX36_{WT} than for DHX36_{ΔDSM} (Figure S3). This acceleration is depicted with a lower free energy barrier for overall G4 binding in the presence of the DSM. In contrast, the DSM has no effect on the rate of G4 dissociation from DHX36, and therefore the entire contribution of the DSM to G4 affinity, depicted by the lower free energy of the G4-bound state, is generated from an increase in the binding kinetics. The simplest interpretation for the effects of the DSM exclusively on binding kinetics is that contacts between the DSM and G4 are formed early in binding, such that the stabilization from these contacts is fully expressed before the overall rate-limiting transition state is reached.

Considered in the context of recent structural evidence, the kinetic effects of the DSM on G4 binding suggest the physical model shown as a series of corresponding cartoons below the free energy profile in Figure 8. In this model, the DSM is the first part of DHX36 to contact the G4,

explaining the enhancement in G4 binding kinetics. To account for the very large increase in binding rate (~100-fold), we suggest that the DSM, while tethered to the body, may be mobile and free to explore space. When it encounters a G4, the DSM contacts the G4 transiently [as suggested by the relatively weak binding exhibited by the DSM alone (Heddi et al. 2015; Lattmann et al. 2010; Meier et al. 2013)], allowing DHX36 to rapidly sample G4s and localize them in proximity to the helicase core. Despite the high reversibility of these contacts, a fraction of these binding events leads to the DSM-bound G4 entering the helicase core, where it forms more stable contacts in the overall rate-limiting step for binding.

In terms of the specificity of DHX36 for G4 disruption, the result that the DSM increases G4 affinity through an increase in binding kinetics, not a decrease in dissociation, is critically important because an acceleration of G4 binding results in a contribution to specificity even when binding is rate limiting for G4 disruption. This point is illustrated in Figure 8. For the G4s with A₁₅ extensions, disruption of the G4 is faster than DHX36 dissociation, indicated by the higher peak in free energy for dissociation than G4 disruption. Thus, the efficiency of disruption for these G4s (reflected in the k_{cat}/K_M value) depends only on changes to the binding kinetics, the highest overall peak in free energy. Because specificity—the efficiency with which one substrate is processed relative to another—depends only on the k_{cat}/K_M value, the acceleration of G4 binding by the DSM contributes to specificity even for G4s that are acted upon with rate-limiting binding.

The bound state of DHX36 and G4 disruption

For most of the G4s measured, the maximal rates of disruption are equivalent for DHX36_{WT} and DHX36_{ΔDSM}. While previous work suggested a minor role of the DSM in accelerating the first-order steps of G4 disruption and helix unwinding (2-3-fold) (Srinivasan et al. 2020), we did not observe such a role of the DSM. It is possible that the minor difference between our results and this previous work arises from the nature of the G4s (DNA vs RNA previously) or differences in solution conditions.

It is noteworthy that for most of the G4s for which we were able to measure maximal rates of G4 disruption, the DSM did not hinder this step. This result is interesting because the specific binding of the DSM to G4 structures would most simply be expected to stabilize the G4 and therefore decrease the maximal disruption rate for DHX36_{WT}. The result that the DSM does not decrease the

disruption rate suggests that its contacts with the G4 remain formed in the transition state for G4 disruption.

But how can specific contacts with the G4 remain formed while the G4 is being disrupted? This is a general issue for helicase proteins that recognize specific RNA or DNA structures, as the recognition by specific binding is inherently linked to stabilization of the structure, not its disruption. To understand why specific G4 binding by the DSM may not slow G4 disruption by DHX36, it may be useful to consider the spatial arrangement of the helicase core and the DSM when DHX36 is bound to a G4. Structural and kinetic evidence has shown that in the bound state prior to G4 disruption, the helicase core binds at the 3' end of the G4 while the DSM contacts the 5' face. Destabilization of the G4 can begin with ATP-independent, repetitive extrusion of one guanosine nucleotide from the 3' end (Chen et al. 2018b). ATP binding and hydrolysis then promote DHX36 translocation from 3' to 5' to fully disrupt the G4 (Lattmann et al. 2010; Srinivasan et al. 2020; Vaughn et al. 2005; Yangyuru et al. 2018; You et al. 2017), perhaps with further extrusion of the strand that is engaged by DHX36 in a mechanism that is reminiscent of the 'winching' found for the spliceosomal DEAH-box helicases Prp16 and Prp22 (Gilman et al. 2017; Semlow et al. 2016). Because this activity begins from the 3' end of the G4, the weakening and disruption of the structure also most likely begin at the 3' end. For most of the G4s tested, the overall transition state for disruption is apparently reached before the structure disruption reaches the 5' end of the G4. Our finding that the maximal rate constant is larger for G4s with fewer quartets suggests that the transition state is reached during disruption of the G-quartets, and that a larger number of G-quartets must be disrupted to reach the transition state of a larger and more stable G4. Nevertheless, for all of the G4s tested, the transition state is apparently reached before the disruption propagates all the way to the 5' end. Thus, it appears that the transition state for DHX36-mediated disruption of these G4s is reached sufficiently early that the specific contacts with the DSM do not impact the disruption step.

Note that for one of the G4s tested, 6G-T₁₅, the DSM indeed decreases the maximal disruption rate by approximately 10-fold (see Figure 3). Thus, for this G4, some contacts of the DSM may indeed be lost prior to the rate-limiting transition state for disruption. However, this substantial effect does not appear to be present for shorter T-tailed G4s. The result that the disruption step is rate-limiting under subsaturating conditions for the T-tailed G4s places an upper limit on their disruption rates by DHX36_{ΔDSM} of $<1 \text{ min}^{-1}$, which is not much larger than the corresponding disruption rates for DHX36_{WT}. It is

noteworthy that the rate constant of the disruption step for DHX36_{ΔDSM} appears not to depend on G4 length (Figure S3), most simply suggesting that the transition state for the disruption step itself is reached while the G4 remains intact. Related to this interpretation, it is also noteworthy that the G4 disruption step (k_{disrupt}) is much slower for T-tailed substrates than A-tailed substrates, both for DHX36_{WT} and DHX36_{ΔDSM}. We speculate that this difference arises because the tighter binding of the DHX36 core to the T-tail sequence, as suggested by the slower dissociation observed here and consistent with previous data on DHX36 and the related helicase MLE (DHX9) (Prabu et al. 2015), results in slower DHX36 translocation from the T-tail. Further work will be necessary to fully understand the differences in DHX36 binding and disruption of these G4 substrates.

The DSM as a modular regulator of DHX36 activity

Surprisingly, we found that the contributions of the DSM to the kinetics and thermodynamics of G4 binding are completely abrogated at the low temperature of 5 °C. The simplest interpretation of these results is that the DSM does not contact the G4 when it is bound to DHX36 under these conditions. In the context of the physical model above, a straightforward interpretation is that the modular, tethered DSM is able to make contacts with the helicase core of DHX36, and at low temperature these interdomain contacts are more stable than the DSM contacts with the G4. Consequently, even when the helicase core binds adjacent to a G4, the DSM retains its contacts with the helicase core instead of contacting the G4. Supporting this model, G4 binding to DHX36_{WT} is weaker at 5 °C than at 37 °C (K_d values of $100 \pm 36 \text{ nM}$ and $7 \pm 4 \text{ nM}$, respectively, calculated from k_{on} and k_{off} values).

These results highlight that the DSM functions as an element of modular structure, a regulator of activity that can be disabled by conditions that block contacts with the G4. In our experiments, it was the low temperature that prevented these interactions, but *in vivo* it is possible that a protein or small molecule regulator could stabilize the interdomain contacts of the DSM or otherwise block the DSM from interacting with G4s. Such an interaction would decrease the specificity of DHX36 for G4 structures by approximately 100-fold, effectively inactivating DHX36 for high-specificity G4 disruption. Further work will be required to determine how DHX36 activity and its potential regulation impact the lifetimes and spatial and genomic distributions of G4s in cells.

Materials and methods

Cloning and mutagenesis

A plasmid encoding His6-SUMO-DHX36 (54–985) was used to express DHX36_{WT}. A variation on QuickChange site-directed mutagenesis was used to remove the DSM, producing His6-SUMO-DHX36 (67–985), which was used to express DHX36_{ΔDSM}. Sequence changes to DHX36 were confirmed by Sanger sequencing.

Protein expression and purification

DHX36_{WT} and DHX36_{ΔDSM} were expressed in Rosetta 2 DE3 cells (EMD Millipore) that were grown at 37 °C to an O.D.₆₀₀ value of 0.8–1. The temperature was lowered to 18 °C and protein expression was induced using 0.2% lactose for 48 h. Cells were harvested by centrifugation and suspended in lysis buffer (50 mM Tris-Cl, pH 8.0, 13% glycerol, 12 mM imidazole, 350 mM NaCl), frozen in liquid nitrogen and stored at –80 °C.

DHX36_{WT} and DHX36_{ΔDSM} were purified as described previously for DHX36_{WT} (Yangyuru et al. 2018). Briefly, cells were thawed and lysed by sonication in lysis buffer (with 1 mM DTT, 100 mM PMSF, and 1x Roche Protease inhibitor cocktail). The lysate was cleared by centrifugation, and nucleic acids were precipitated using 0.1% poly-ethylenimine and cleared by centrifugation. The supernatant was flowed through a Ni-column equilibrated with buffer containing 50 mM Tris-Cl, pH 8.0, 20% glycerol, 20 mM imidazole, 350 mM NaCl, and 1 mM DTT. Bound DHX36 was eluted using the same buffer with 0.5 M imidazole. Eluted fractions containing DHX36 were dialyzed overnight at 4 °C in buffer containing 50 mM Tris-Cl, pH 8.0, 30% glycerol, 20 mM imidazole, 350 mM NaCl, and 1 mM DTT and then digested with ULP1 protease to remove the N-terminal tags. Cleaved His6-SUMO tags were subsequently resolved using a Ni-column. Flow-through fractions containing DHX36 were dialyzed in 25 mM Tris-Cl, pH 7.5, 50% glycerol, 150 mM NaCl, 1 mM DTT, snap frozen in liquid nitrogen, and stored at –80 °C. DHX36 concentrations were determined by Bradford assay (Bio-Rad).

G4 formation

Tetramolecular G4s for EMSA assays were formed by adding 0.1 mM of the G4-forming oligonucleotide (Integrated DNA Technologies) in single-stranded form to TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) containing 100 mM KCl. The oligonucleotide was heated to 99 °C for 10 min in a thermal cycler before being slow cooled to 0 °C for 10 min and then incubated at 55 °C for 16 h. Tetramolecular G4s for fluorescence quencher assays were formed with 10 μM of T-tailed, Cy3-labeled G4 (6G-T₁₅Cy3 oligonucleotide) and an excess (100 μM) of 6G-T₅ oligonucleotide to ensure a nearly homogenous population of G4s with only one T₁₅ extension. Solution and G4 forming conditions were identical as above. For EMSA assays, G4s were radiolabeled using [γ -³²P]ATP (Perkin-Elmer) and T4 polynucleotide kinase (New England Biolabs). G4s were subsequently purified by 12% native PAGE, eluted into 200 μL of 10 mM Tris-Cl, pH 7.5, 1 mM EDTA, and 100 mM KCl and stored at –20 °C.

G4 disruption kinetics

G4 disruption assays of A-tailed substrates were carried out under steady state conditions. A trace amount of radiolabeled G4 (<25 pM) was mixed with excess unlabeled G4. The reaction was initiated by adding DHX36 to a solution containing 50 mM Na-MOPS, pH 7.0, 100 mM KCl and 2 mM ATP-Mg²⁺ with various G4 concentrations at 37 °C. At various times up to 30 min, aliquots were quenched in buffer containing 22.5% glycerol, 1.5 mg/mL proteinase K, 1% SDS, and 0.03% xylene cyanol. Samples were analyzed using 12% native PAGE run at 60 W for 30 min in a temperature-controlled apparatus (~4 °C). Gels were dried and visualized using a phosphorimager (GE Healthcare). Data were quantified using Image Quant 5.2 (GE Healthcare) and analyzed using KaleidaGraph (Synergy Software).

G4 disruption assays of T-tailed G4 substrates were carried out under single turnover conditions under the same buffer conditions (50 mM Na-MOPS, pH 7.0, 100 mM KCl and 2 mM ATP-Mg²⁺). Reactions included trace radiolabeled G4 (<25 pM) and were initiated by addition of various concentrations of DHX36. Gel-based separation and analysis were performed as described above.

Duplex unwinding kinetics

Duplex unwinding measurements were carried out under single-turnover conditions as described previously (Yangyuru et al. 2018). The duplex was generated by annealing 100 nM unlabeled Comp20-T₁₅ and a trace amount of a radiolabeled complementary oligonucleotide on ice. For duplex unwinding measurements, a trace concentration of the radiolabeled duplex was added to an excess of the unlabeled complementary oligonucleotide (0.5 μM or 1 μM) to form additional duplex and to function as a chase in the unwinding experiment. Unwinding reactions were initiated by the addition of excess DHX36, with conditions as described above for G4 disruption measurements. Reaction progress was followed over 30 min by native polyacrylamide gel electrophoresis as described above for G4 disruption measurements.

DHX36 dissociation kinetics

Measurements using native gel separations were performed by first preincubating 100 nM DHX36 with trace radiolabeled G4 (<25 pM) for 45 min in a solution containing 50 mM Na-MOPS, pH 7.0, 100 mM KCl and 2 mM ADP-BeF₃ at 37 °C. DHX36 dissociation from the radiolabeled G4 was made irreversible by the addition of excess unlabeled G4 (1 μM). Samples were taken over the course of 30 min, transferred into 6X-loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water), and analyzed by 12% native PAGE. Time points were loaded immediately on the gel, such that the later time points were run for a shorter period of time than the early time points. Analysis and quantitation were performed as described above.

Fluorescence measurements for dissociation kinetics were performed analogously, by first preincubating 100 nM DHX36 with 3'-Cy3-labeled 6G-T₁₅ (50 nM) for 45 min in a solution containing 50 mM Na-MOPS, pH 7.0, 100 mM KCl with 2 mM of ATP-Mg²⁺ or

nucleotide analog. The dissociation reaction was initiated by the addition of excess complementary quencher oligonucleotide (1 μ M). The reactions were performed in a 96-well Corning Costar plate, and fluorescent measurements (with excitation and emission wavelengths of 485 and 570 nm, respectively) were made as a function of time with a Spark 10 M fluorimeter (Tecan). The instrument was not temperature controlled, and measurements were taken at approximately 28 °C. Progress curves were fit by a single-exponential decay curve (Kaleidagraph) with the endpoint set to the level of background fluorescence determined independently.

Low temperature G4 binding and dissociation kinetics

Measurements of k_{on} and k_{off} between DHX36 and 6G-T₁₅ were performed by EMSA at 5 °C in the presence of ATP. Measurements of k_{on} began with the addition of varying DHX36 concentrations with trace radiolabeled 6G-T₁₅ (<25 pM) in a solution containing 50 mM Na-MOPs, pH 7.0, 100 mM KCl and 2 mM ATP at 5 °C. Samples were taken over 30 min, transferred into 6X-loading dye, and analyzed by 12% native PAGE. Gel preparation and analysis were performed as described above. EMSA measurements of k_{off} began with a preincubation period of 10 min with 10 nM DHX36 and trace radiolabeled 6G-T₁₅ under the same solution conditions as in the k_{on} measurements but at 37 °C, allowing the complex to form rapidly and completely. Dissociation from the radiolabeled G4 was made irreversible by the addition of excess unlabeled G4 (1 μ M) at 5 °C. Samples were taken over the course of 80 min, transferred into 6X-loading dye, and processed and analyzed as described above.

Acknowledgments: We thank Tanya Paull for use of her fluorometer for G4 dissociation experiments, and members of the Russell Lab for comments on the manuscript. This work was funded by grants to R.R. from NIGMS (R35 GM131777) and the Welch Foundation (F-1563) and a University of Texas Undergraduate Research Fellowship to B.C.

Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Research funding: None declared.

Conflict of interest statement: The authors declare no conflicts of interest regarding this article.

References

- Ambrus, A., Chen, D., Dai, J., Bialis, T., Jones, R.A., and Yang, D. (2006). Human telomeric sequence forms a hybrid-type intramolecular G-quadruplex structure with mixed parallel/antiparallel strands in potassium solution. *Nucleic Acids Res.* 34: 2723–2735.
- Beaudoin, J.-D. and Perreault, J.-P. (2013). Exploring mRNA 3'-UTR G-quadruplexes: evidence of roles in both alternative polyadenylation and mRNA shortening. *Nucleic Acids Res.* 41: 5898–5911.
- 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.
- Bugaut, A. and Balasubramanian, S. (2012). 5'-UTR RNA G-quadruplexes: translation regulation and targeting. *Nucleic Acids Res.* 40: 4727–4741.
- Chalupníková, K., Lattmann, S., Selak, N., Iwamoto, F., Fujiki, Y., and Nagamine, Y. (2008). Recruitment of the RNA helicase RHAU to stress granules via a unique RNA-binding domain. *J. Biol. Chem.* 283: 35186–35198.
- Chen, M.C., Murat, P., Abecassis, K., Ferré-D'Amaré, A.R., and Balasubramanian, S. (2015). Insights into the mechanism of a G-quadruplex-unwinding DEAH-box helicase. *Nucleic Acids Res.* 43: 2223–2231.
- Chen, M.C., Tippiana, R., Demeshkina, N.A., Murat, P., Balasubramanian, S., Myong, S., and Ferré-D'Amaré, A.R. (2018b). Structural basis of G-quadruplex unfolding by the DEAH/RHA helicase DHX36. *Nature* 558: 465–469.
- Chen, W.-F., Rety, S., Guo, H.-L., Dai, Y.-X., Wu, W.-Q., Liu, N.-N., Auguin, D., Liu, Q.-W., Hou, X.-M., Dou, S.-X., et al (2018c). Molecular mechanistic insights into *Drosophila* DHX36-mediated G-Quadruplex unfolding: a structure-based model. *Structure* 26: 403–415.
- Chen, X.-C., Chen, S.-B., Dai, J., Yuan, J.-H., Ou, T.-M., Huang, Z.-S., and Tan, J.-H. (2018a). Tracking the dynamic folding and unfolding of RNA G-quadruplexes in live cells. *Angew. Chem. Int. Ed.* 57: 4702–4706.
- 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.
- Damerla, R.R., Knickelbein, K.E., Strutt, S., Liu, F.-J., Wang, H., and Opresko, P.L. (2012). Werner syndrome protein suppresses the formation of large deletions during the replication of human telomeric sequences. *Cell Cycle* 11: 3036–3044.
- Del Campo, M. and Lambowitz, A.M. (2009). Structure of the yeast DEAD box protein Mss116p reveals two wedges that crimp RNA. *Mol. Cell* 35: 598–609.
- Gellert, M., Lipsett, M.N., and Davies, D.R. (1962). Helix formation by guanylic acid. *Proc. Nat. Acad. Sci. U. S. A.* 48: 2013–2018.
- Gilman, B., Tijerina, P., and Russell, R. (2017). Distinct RNA-unwinding mechanisms of DEAD-box and DEAH-box RNA helicase proteins in remodeling structured RNAs and RNPs. *Biochem. Soc. Trans.* 45: 1313–1321.
- 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.
- Hänsel-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.
- 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. Nat. Acad. Sci. U. S. A.* 112: 9608–9613.
- Henderson, A., Wu, Y., Huang, Y.C., Chavez, E.A., Platt, J., Johnson, F.B., Brosh, R.M., Sen, D., and Lansdorp, P.M. (2014). Detection

- of G-quadruplex DNA in mammalian cells. *Nucleic Acids Res.* 42: 860–869.
- Kumari, S., Bugaut, A., Huppert, J.L., and Balasubramanian, S. (2007). An RNA G-quadruplex in the 5' UTR of the NRAS proto-oncogene modulates translation. *Nat. Chem. Biol.* 3: 218–221.
- 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.
- 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.
- Liu, F., Putnam, A., and Jankowsky, E. (2008). ATP hydrolysis is required for DEAD-box protein recycling but not for duplex unwinding. *Proc. Nat. Acad. Sci. U. S. A.* 105: 20209–20214.
- Liu, F., Putnam, A.A., and Jankowsky, E. (2014). DEAD-box helicases form nucleotide-dependent, long-lived complexes with RNA. *Biochemistry* 53: 423–433.
- Mallam, A.L., Sidote, D.J., and Lambowitz, A.M. (2014). Molecular insights into RNA and DNA helicase evolution from the determinants of specificity for a DEAD-box RNA helicase. *eLife* 3: e04630.
- Meier, M., Patel, T.R., Booy, E.P., Marushchak, O., Okun, N., Deo, S., Howard, R., McEleney, K., Harding, S.E., Stetefeld, J., et al (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.
- Mendoza, O., Bourdoncle, A., Boulé, J.-B., Brosh, R.M., and Mergny, J.-L. (2016). G-quadruplexes and helicases. *Nucleic Acids Res.* 44: 1989–2006.
- Murat, P., Marsico, G., Herdy, B., Ghanbarian, A., Portella, G., and Balasubramanian, S. (2018). RNA G-quadruplexes at upstream open reading frames cause DHX36- and DHX9-dependent translation of human mRNAs. *Genome Biol.* 19: 229.
- Nguyen, G.H., Tang, W., Robles, A.I., Beyer, R.P., Gray, L.T., Welsh, J.A., Schetter, A.J., Kumamoto, K., Wang, X.W., Hickson, I.D., et al (2014). Regulation of gene expression by the BLM helicase correlates with the presence of G-quadruplex DNA motifs. *Proc. Nat. Acad. Sci. U. S. A.* 111: 9905–9910.
- Prabu, J.R., Müller, M., Thomae, A.W., Schüssler, 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.
- Rodriguez, R., Miller, K.M., Forment, J.V., Bradshaw, C.R., Nikan, M., Britton, S., Oelschlaegel, T., Xhemalce, B., Balasubramanian, S., and Jackson, S.P. (2012). Small-molecule-induced DNA damage identifies alternative DNA structures in human genes. *Nat. Chem. Biol.* 8: 301–310.
- Sauer, M., Juranek, S.A., Marks, J., De Magis, A., Kazemier, H.G., Hilbig, D., Benhalevy, D., Wang, X., Hafner, M., and Paeschke, K. (2019). DHX36 prevents the accumulation of translationally inactive mRNAs with G4-structures in untranslated regions. *Nat. Commun.* 10: 2421.
- Semlow, D.R., Blanco, M.R., Walter, N.G., and Staley, J.P. (2016). Spliceosomal DEAH-box ATPases remodel pre-mRNA to activate alternative splice sites. *Cell* 164: 985–998.
- Siddiqui-Jain, A., Grand, C.L., Bearss, D.J., and Hurley, L.H. (2002). Direct evidence for a G-quadruplex in a promoter region and its targeting with a small molecule to repress c-MYC transcription. *Proc. Nat. Acad. Sci. U. S. A.* 99: 11593–11598.
- Srinivasan, S., Liu, Z., Chuenchor, W., Xiao, T.S., and Jankowsky, E. (2020). Function of auxiliary domains of the DEAH/RHA helicase DHX36 in RNA remodeling. *J. Mol. Biol.* 432: 2217–2231.
- Varshney, D., Spiegel, J., Zyner, K., Tannahill, D., and Balasubramanian, S. (2020). The regulation and functions of DNA and RNA G-quadruplexes. *Nat. Rev. Mol. Cell Biol.* 21: 459–474.
- 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.
- Wu, Y., Shin-ya, K., and Brosh, R.M. (2008). FANCI helicase defective in Fanconi anemia and breast cancer unwinds G-quadruplex DNA to defend genomic stability. *Cell Biol.* 28: 4116–4128.
- Xu, Y., Suzuki, Y., Ito, K., and Komiyama, M. (2010). Telomeric repeat-containing RNA structure in living cells. *Proc. Nat. Acad. Sci. U. S. A.* 107: 14579–14584.
- Yangyuru, P.M., Bradburn, D.A., Liu, Z., Xiao, T.S., and Russell, R. (2018). The G-quadruplex (G4) resolvase DHX36 efficiently and specifically disrupts DNA G4s via a translocation-based helicase mechanism. *J. Biol. Chem.* 293: 1924–1932.
- 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.

Supplementary Material: The online version of this article offers supplementary material (<https://doi.org/10.1515/hsz-2020-0302>).