

# Loquacious-PD facilitates *Drosophila* Dicer-2 cleavage through interactions with the helicase domain and dsRNA

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Edited by Rachel Green, Johns Hopkins University, Baltimore, MD, and approved August 13, 2017 (received for review April 27, 2017)

**Loquacious-PD (Loqs-PD) is required for biogenesis of many endogenous siRNAs in *Drosophila*. In vitro, Loqs-PD enhances the rate of dsRNA cleavage by Dicer-2 and also enables processing of substrates normally refractory to cleavage. Using purified components, and Loqs-PD truncations, we provide a mechanistic basis for Loqs-PD functions. Our studies indicate that the 22 amino acids at the C terminus of Loqs-PD, including an FDF-like motif, directly interact with the Hel2 subdomain of Dicer-2's helicase domain. This interaction is RNA-independent, but we find that modulation of Dicer-2 cleavage also requires dsRNA binding by Loqs-PD. Furthermore, while the first dsRNA-binding motif of Loqs-PD is dispensable for enhancing cleavage of optimal substrates, it is essential for enhancing cleavage of suboptimal substrates. Finally, our studies define a previously unrecognized Dicer interaction interface and suggest that Loqs-PD is well positioned to recruit substrates into the helicase domain of Dicer-2.**

Dicer | RNAi | dsRNA binding protein | protein–protein interaction | endo-siRNA

There are two Dicer genes in *Drosophila melanogaster*, Dcr-1 and Dcr-2, that produce micro-RNAs (miRNAs) and short interfering RNAs (siRNAs), respectively (1, 2). Dcr-2 is required to initiate antiviral RNA interference (RNAi), in which viral double-stranded RNA (dsRNA) is cleaved to produce siRNAs capable of silencing viral gene expression (3, 4). In vitro studies indicate Dcr-2 recognizes dsRNA termini and exhibits termini-dependent cleavage (5, 6). For example, dsRNA with blunt (BLT) termini are cleaved processively in an ATP-dependent manner, while dsRNA with 2-nt 3'overhanging (3'ovr) termini elicit distributive cleavage that occurs in the absence of ATP. Dcr-2's helicase domain plays an important role in termini discrimination (5, 6) and is required to mount an antiviral response (7, 8), suggesting that the termini preferences of Dcr-2 likely arose to distinguish between viral and cellular dsRNA.

Loquacious-PD (Loqs-PD), a dsRNA-binding protein (dsRBP), is required for the biogenesis of a subset of endogenous-siRNAs (endo-siRNAs) (9, 10) but is not required for antiviral RNAi (8). Early studies found that endo-siRNAs map to dsRNA originating from convergent transcription, inverted repeats, and transposons (11–16). Given the sensitive termini dependence of Dcr-2, many endo-siRNA precursors are predicted to be poor substrates. We recently showed that, in vitro, Loqs-PD minimizes the termini dependence of Dcr-2 and facilitates cleavage of suboptimal substrates, including predicted endo-siRNA precursors (6). This suggests Loqs-PD evolved to expand the range of Dcr-2 endogenous substrates; however, the mechanism by which Loqs-PD modulates Dcr-2 substrate recognition and processing is unknown.

Loqs-PD is one of four protein isoforms encoded by the gene *loqs*. Loqs-PA and Loqs-PB, homologs of TRBP, interact with Dcr-1 during miRNA biogenesis (9, 17–20). Loqs-PC is rarely expressed and has no known function (9). Loqs-PD is the only Loqs isoform capable of facilitating Dcr-2-dependent endo-siRNA biogenesis (19, 20). It contains two dsRNA-binding motifs (dsRBMs) separated by a short linker, with the rest of the

protein predicted to be largely unstructured. Only the C-terminal 22 amino acids are unique to the PD isoform (Fig. 1*A* and *C*), and studies performed in S2 cells indicate they are important for endo-siRNA silencing (21) and for interactions with Dcr-2 (21, 22). However, studies monitoring the interaction between Loqs-PD and Dcr-2 by immunoprecipitation have noted varying degrees of association (9, 10, 23), and so far, studies with purified proteins have not been performed. During RISC assembly, Dcr-2 interacts with another dsRBP, R2D2, and it is unclear whether R2D2 and Loqs-PD compete for the same binding site (23) or bind to unique sites (22).

Using purified components, we performed a series of biochemical experiments to investigate the mechanism by which Loqs-PD modulates Dcr-2 activity. We show that Loqs-PD directly interacts with Dcr-2 in an RNA-independent manner, and this interaction, as well as Loqs-PD binding to dsRNA, are both required for Loqs-PD function. We discovered the first dsRBM of Loqs-PD is uniquely required to enhance cleavage of suboptimal substrates but not an optimal substrate. Finally, we report an unrecognized Dicer–dsRBP interaction interface and describe its potential implication for the function of Loqs-PD.

## Results

**Purification of Loqs-PD Truncations.** We previously showed that Loqs-PD modifies Dcr-2 cleavage activity, but the mechanism by which Loqs-PD accomplishes this is unknown. To identify regions of Loqs-PD required to alter Dcr-2 activity, we designed and purified a series of N- and C-terminal truncations (Fig. 1*A* and *B*). By precedent (24), each construct was named based on domains or features it contained. For example, the smallest construct, LR<sub>2</sub>C, contained the linker region between dsRBMs

## Significance

*Drosophila melanogaster* use RNA interference to respond to a viral infection. Dicer-2 cleaves viral double-stranded RNA (dsRNA), producing siRNAs that silence viral gene expression. Dicer-2 recognizes the ends of dsRNA, and this property likely evolved to distinguish between viral and cellular dsRNA. Loquacious-PD (Loqs-PD), a dsRNA binding protein, is not required for Dicer-2's antiviral activity. However, by allowing Dicer-2 to cleave in a termini-independent manner, Loqs-PD facilitates cleavage of endogenous substrates with more complex termini. Our studies are significant because they provide a mechanistic basis for how Loqs-PD modulates Dicer-2 activity. For example, they reveal a previously unrecognized protein–protein interaction interface on the helicase domain of Dicer-2.

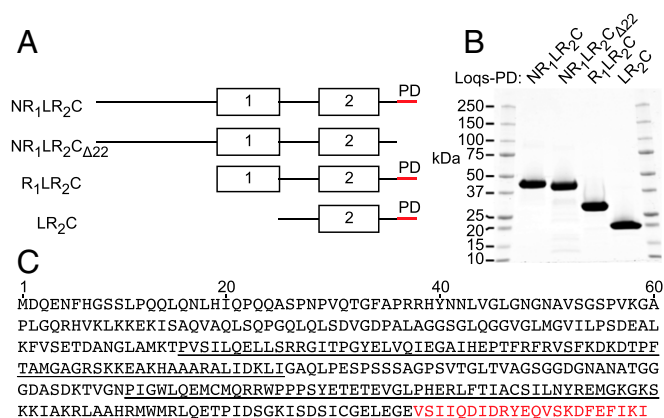
Author contributions: K.D.T. and B.L.B. designed research; K.D.T. and D.M.E. performed research; K.D.T., N.K.S., and S.E.A. contributed new reagents/analytic tools; K.D.T., D.M.E., and B.L.B. analyzed data; and K.D.T. and B.L.B. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1707063114/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1707063114/-DCSupplemental).



**Fig. 1.** Design and purification of Loquacious-PD and its truncations. (A) Schematic of Loqs-PD and N- and C-terminal truncations. dsRBMs are shown as boxes, and the isoform-specific C terminus is colored red. (B) Coomassie-stained SDS/PAGE gel of purified Loqs-PD and truncations. Molecular mass markers were run in first and last lanes with sizes indicated (kDa). (C) Primary sequence of Loqs-PD. dsRBMs are underlined, and the 22 C-terminal, isoform-specific amino acids are colored in red.

(L), the second dsRBM (R<sub>2</sub>), and the C-terminal tail (C), while the N-terminal region (N) and the first dsRBM (R<sub>1</sub>) were deleted. NR<sub>1</sub>LR<sub>2</sub>C<sub>Δ22</sub> lacked the C-terminal 22 amino acids, which are the only amino acids unique to the PD isoform (Fig. 1C). While many dsRBPs form homodimers in solution (25–27), Loqs-PD and all of its truncations were found to be monomers by sedimentation equilibrium experiments (Fig. S1).

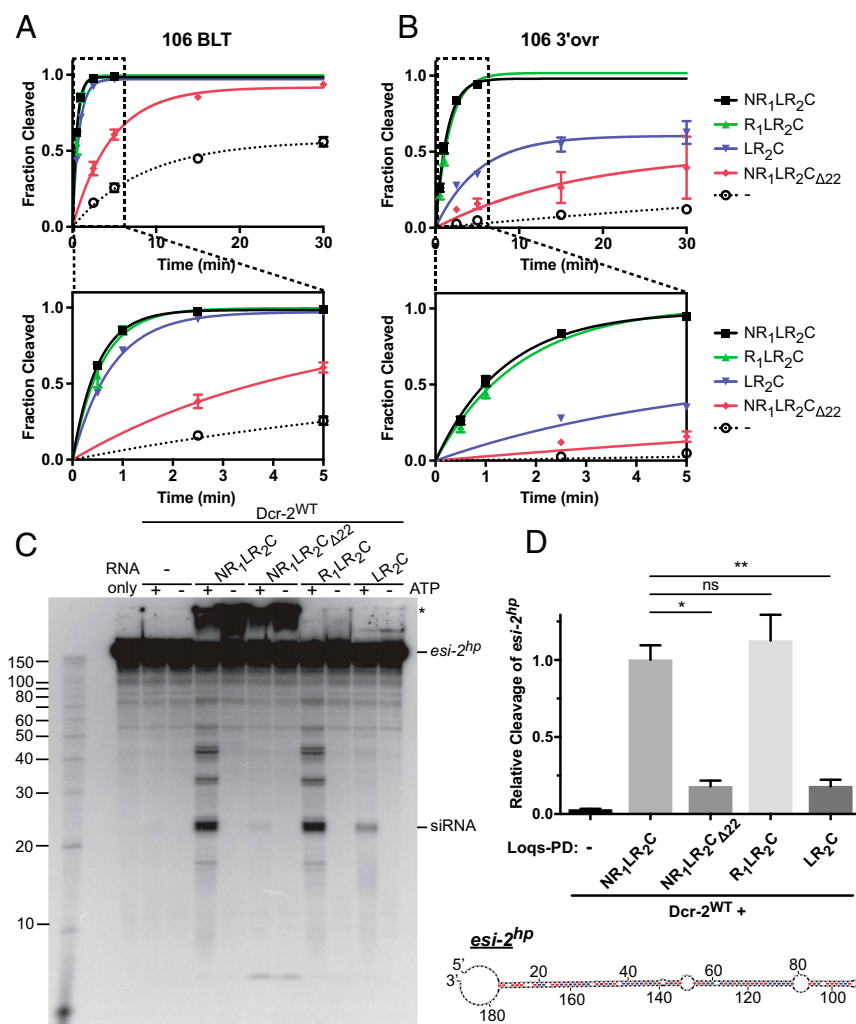
**Loqs-PD Requires the C-Terminal 22 Residues to Fully Enhance Dcr-2 Cleavage.** To determine which domains of Loqs-PD were required to affect Dcr-2 cleavage activity in vitro, we performed single-turnover cleavage assays using dsRNAs with BLT or 3'ovr termini (created by annealing 106-nt sense and antisense RNAs), with Dcr-2<sup>WT</sup> alone or supplemented with Loqs-PD or its truncations (Fig. 2A and B, Table 1, and Fig. S2). As in our prior studies (6), Dcr-2 alone (–, dotted) cleaved 106 BLT dsRNA at a faster rate ( $k_{\text{obs}}$ ,  $0.12 \pm 0.02 \text{ min}^{-1}$ ) than 106 3'ovr dsRNA ( $k_{\text{obs}}$ ,  $0.01 \pm 0.02 \text{ min}^{-1}$ ), emphasizing that dsRNA with BLT termini is an optimal substrate compared with dsRNA with 3'ovr, or other non-BLT termini (suboptimal substrates). Inclusion of full-length Loqs-PD (NR<sub>1</sub>LR<sub>2</sub>C, black lines) dramatically increased the rates of cleavage for both BLT ( $k_{\text{obs}}$ ,  $1.98 \pm 0.05 \text{ min}^{-1}$ ) and 3'ovr ( $k_{\text{obs}}$ ,  $0.73 \pm 0.06 \text{ min}^{-1}$ ) 106 dsRNA, and a similar rate enhancement was observed when the N-terminal 135 residues were removed (R<sub>1</sub>LR<sub>2</sub>C, green lines). Conversely, removal of the C-terminal 22 amino acids from Loqs-PD (NR<sub>1</sub>LR<sub>2</sub>C<sub>Δ22</sub>, red lines) severely compromised the ability of Loqs-PD to stimulate Dcr-2 cleavage activity, for both BLT and 3'ovr dsRNA. Thus, the N terminus of Loqs-PD is dispensable for Loqs-PD effects on Dcr-2 cleavage, while the C-terminal 22 amino acids are essential.

**Loqs-PD Requires both dsRBMs to Enhance Dcr-2 Cleavage of Suboptimal Substrates.** Unexpectedly, additional truncation to remove the first dsRBM (LR<sub>2</sub>C, blue lines) revealed substrate-dependent effects. LR<sub>2</sub>C increased the Dcr-2 cleavage rate for 106 BLT dsRNA to levels approaching that observed after addition of full-length Loqs-PD ( $k_{\text{obs}}$ ,  $1.28 \pm 0.07 \text{ min}^{-1}$  vs.  $k_{\text{obs}}$ ,  $1.98 \pm 0.05 \text{ min}^{-1}$ ). However, while LR<sub>2</sub>C slightly increased the Dcr-2 cleavage rate for 106 3'ovr dsRNA, the rate was ~fourfold slower than that observed in the presence of NR<sub>1</sub>LR<sub>2</sub>C ( $k_{\text{obs}}$ ,  $0.19 \pm 0.04 \text{ min}^{-1}$  vs.  $k_{\text{obs}}$ ,  $0.73 \pm 0.06 \text{ min}^{-1}$ ). To extend these results to a natural, endogenous substrate, we tested a dsRNA derived from *esi-2*, a *Drosophila* endo-siRNA precursor that gives rise to abundant endo-siRNAs in vivo

(13–15). *esi-2* contains 20 inverted repeats capable of forming multiple stem-loop, or hairpin, structures, and we used a substrate with a single inverted repeat flanked by noncomplementary sequences. This substrate, referred to as *esi-2<sup>hairpin</sup>* (*esi-2<sup>hp</sup>*) [previously referred to as *pre-sl* by Miyoshi et al. (22)], is predicted to form a single hairpin with single-stranded overhangs at each terminus (Fig. 2D, Bottom). While the endogenous termini of *esi-2* have not been defined, we previously showed that, in vitro, *esi-2<sup>hp</sup>* recapitulates the Loqs-PD-dependent siRNA production (6) observed in vivo (9, 10). We performed single-turnover cleavage assays of *esi-2<sup>hp</sup>* with Dcr-2<sup>WT</sup> alone, or supplemented with Loqs-PD or its truncations, in the presence (+) or absence (–) of ATP (Fig. 2C and D). Dcr-2 alone was unable to appreciably cleave *esi-2<sup>hp</sup>*, while addition of NR<sub>1</sub>LR<sub>2</sub>C resulted in cleavage and siRNA-sized cleavage products. As with the 3'ovr 106 dsRNA substrate, R<sub>1</sub>LR<sub>2</sub>C enhanced cleavage of *esi-2<sup>hp</sup>* to the same extent as NR<sub>1</sub>LR<sub>2</sub>C, while NR<sub>1</sub>LR<sub>2</sub>C<sub>Δ22</sub> and LR<sub>2</sub>C showed a significantly decreased ability to promote cleavage. All cleavage events were dependent on ATP (Fig. 2C). Thus, while *esi-2<sup>hp</sup>* differs from 3'ovr 106 dsRNA in that its cleavage is completely dependent on Loqs-PD, it is a suboptimal substrate and, like 3'ovr dsRNA, requires both dsRBMs for cleavage.

In addition to the siRNA-sized cleavage products of *esi-2<sup>hp</sup>*, larger products of ~33 and ~43 nts accumulated in a Loqs-PD-dependent manner (Fig. 2C). In vivo, *esi-2* is processed into two adjacent endo-siRNAs, *esi-2.1* and *esi-2.2*, leaving the ~42 nt loop region as a byproduct (14) (Fig. S3A). To get information about the identity of bands observed in our in vitro cleavage assays, we performed Northern blots in which we probed for a region that encompasses *esi-2.1* (nucleotides 30–60, red), the predominant endo-siRNA observed from *esi-2* (13–15), the loop region (nucleotides 79–109, green), or the 3' end of *esi-2<sup>hp</sup>* (nucleotides 158–190, blue) (Fig. S3B). The “red” probe primarily detected siRNA-sized products, suggesting that siRNA-sized products in Fig. 2C include *esi-2.1*. The loop probe primarily detected ~43 nt-sized products, suggesting the ~43-nt band in Fig. 2C corresponds to the loop region of *esi-2<sup>hp</sup>*. Finally, the 3'-end probe detected multiple bands, including likely intermediates, and an ~33 nt product, suggesting the ~33-nt band in Fig. 2C corresponds to the hairpin base. These data are consistent with cleavage of *esi-2<sup>hp</sup>* to produce two siRNAs and byproducts that include the hairpin loop and base and agree with prior analyses of *esi-2* processing in vivo (14).

**Loqs-PD and Its Truncations Bind dsRNA with High Affinity.** To gain insight into the differential ability of the Loqs-PD truncations to enhance Dcr-2 cleavage activity, we measured their dsRNA binding affinity. We performed gel mobility shift assays with each Loqs-PD variant and 106 BLT dsRNA (Fig. 3). NR<sub>1</sub>LR<sub>2</sub>C bound dsRNA with high affinity, exhibiting a  $K_d$  of ~9 nM (Table 1). We observed two faint bands of slower mobility, but the majority of bound or shifted dsRNA appeared as a diffuse smear (Fig. 3A, Top Left), suggesting a subset of complexes dissociate during electrophoresis (28). NR<sub>1</sub>LR<sub>2</sub>C<sub>Δ22</sub> bound dsRNA with the same affinity as NR<sub>1</sub>LR<sub>2</sub>C ( $K_d$  of ~9 nM), and the pattern of shifted dsRNA was also similar (Fig. 3A, Top Right), indicating deletion of the 22 C-terminal amino acids does not compromise dsRNA binding. R<sub>1</sub>LR<sub>2</sub>C bound dsRNA with a slightly higher affinity ( $K_d$  of ~1.4 nM) than NR<sub>1</sub>LR<sub>2</sub>C, suggesting the N-terminal region is inhibitory to dsRNA binding. Further, there was a dramatic change in the pattern of shifted dsRNA. For R<sub>1</sub>LR<sub>2</sub>C, we observed the sequential appearance of ~6 distinct complexes (Fig. 3A, Bottom Left), consistent with a maximal protein to dsRNA stoichiometry of 6:1. In structures with dsRNA, dsRBMs bind ~16 bp along one face of the dsRNA helix such that another dsRBM can bind opposite the first (29, 30). To accommodate six molecules of R<sub>1</sub>LR<sub>2</sub>C (~32 bp) on a 106-bp substrate, we predict binding occurs



**Fig. 2.** Loqs-PD truncations affect Dcr-2 cleavage rates in a substrate-dependent manner. Graphs plot single-turnover cleavage over time for 106 BLT (A) and 3'ovr (B) dsRNA (1 nM) with 30 nM Dcr-2 at 25 °C, in the absence or presence of an equimolar amount of Loqs-PD or its truncations. Portions of *Top* graphs are enlarged below. Data were fit to the pseudofirst order rate equation,  $y = y_0 + A \times (1 - e^{-kt})$ , where  $y$  is fraction cleaved [(cleaved)/(cleaved + uncleaved)],  $A$  is amplitude of rate curve ( $>0.5$ ),  $y_0$  is baseline ( $=0$ ),  $k$  is pseudofirst order rate constant, and  $t$  is time. Data points are mean  $\pm$  SEM ( $n = 3$ ). (C) PhosphorImage shows single-turnover cleavage of  $^{32}$ P-internally labeled *esi-2<sup>hp</sup>* (1 nM) with Dcr-2 (30 nM),  $\pm$ Loqs-PD or its truncations (120 nM),  $\pm$ 5 mM ATP. Cleavage products were separated by 12% denaturing PAGE, and 10-nt RNA ladder is on the left. \*, RNA trapped in well, possibly due to disordered N terminus of Loqs-PD. (D, *Top*) Single-turnover cleavage of *esi-2<sup>hp</sup>*, with 5 mM ATP and Loqs-PD or truncations, was quantified from data as in C (cleavage products divided by total radioactivity in lane) and plotted relative to cleavage with full-length Loqs-PD. Data points are mean  $\pm$  SEM ( $n = 3$ ). Paired  $t$  test—ns,  $P > 0.05$ ; \* $P < 0.02$ ; \*\* $P < 0.005$ . (D, *Bottom*) Predicted secondary structure of *esi-2<sup>hp</sup>* colored according to mFold (58).

along opposite faces of the dsRNA. LR<sub>2</sub>C bound dsRNA with slightly lower affinity ( $K_d$  of  $\sim 29$  nM), consistent with the loss of one of the two dsRBMs. We observed one prominent shift along with a faint second shift. LR<sub>2</sub>C also exhibits a Hill coefficient  $>1$ , suggesting some form of cooperativity may be operative. As summarized in Fig. 3B, removal of the C-terminal 22 amino acids does not alter binding affinity from that of the full-length protein, while removal of the N terminus increases affinity and deletion of both the N terminus and first dsRBM decreases affinity.

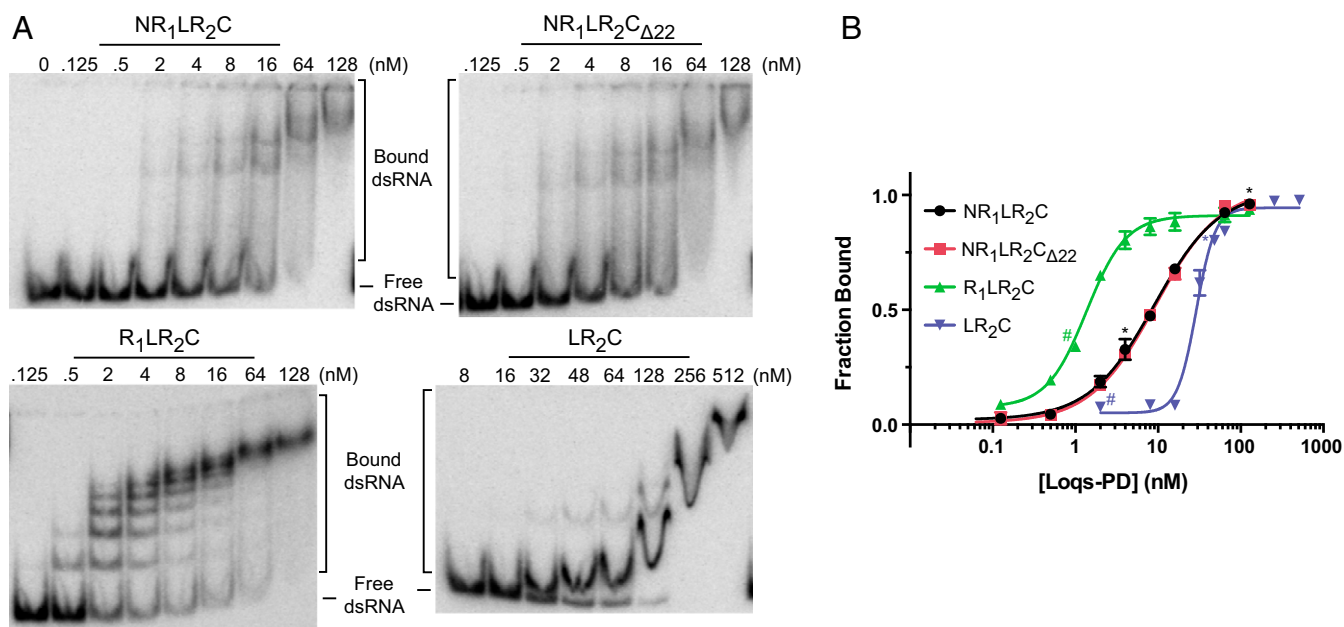
**The C-Terminal 22 Residues of Loqs-PD Are Necessary for Interaction with Dcr-2.** Previous studies suggest the C-terminal 22 residues of Loqs-PD are required to interact with Dcr-2 (21, 22). However, the interaction has not been monitored with purified proteins. Whether Loqs-PD and Dcr-2 interact in the absence of RNA also is untested. To address these questions, we used purified proteins in pull-down experiments with His-tagged Loqs-PD variants and untagged Dcr-2. To facilitate formation of a stable complex, we used a Dcr-2 variant in which both RNaseIII and helicase activity were disrupted by point mutations (Dcr-2<sup>RIII,K34A</sup>). We

**Table 1. Summary of  $k_{obs}$ ,  $t_{1/2}$ , and  $K_d$  values**

Loqs-PD	Cleavage of 106 BLT		Cleavage of 106 3'ovr		Binding of 106 BLT	
	$k_{obs}$ , min <sup>-1</sup>	$t_{1/2}$ , min	$k_{obs}$ , min <sup>-1</sup>	$t_{1/2}$ , min	$K_d$ , nM	$h$
—	0.12 $\pm$ 0.02	5.98	0.01 $\pm$ 0.02	66.3	n/a	n/a
NR <sub>1</sub> LR <sub>2</sub> C	1.98 $\pm$ 0.05	0.35	0.73 $\pm$ 0.06	0.95	8.97 $\pm$ 0.66	1.1 $\pm$ 0.1
R <sub>1</sub> LR <sub>2</sub> C	1.73 $\pm$ 0.18	0.40	0.60 $\pm$ 0.07	1.16	1.36 $\pm$ 0.12	1.8 $\pm$ 0.2
NR <sub>1</sub> LR <sub>2</sub> C $\Delta$ 22	0.21 $\pm$ 0.02	3.25	0.06 $\pm$ 0.07	12.0	9.34 $\pm$ 0.75	1.1 $\pm$ 0.1
LR <sub>2</sub> C	1.28 $\pm$ 0.07	0.54	0.19 $\pm$ 0.04	3.57	29.01 $\pm$ 1.3	3.8 $\pm$ 0.6
His-LR <sub>2</sub> C	1.53 $\pm$ 0.06	0.45	n.d.	n.d.	29.93 $\pm$ 1.2	3.3 $\pm$ 0.4
His-LR <sub>2</sub> C <sup>K,A</sup>	0.14 $\pm$ 0.01	4.81	n.d.	n.d.	1,631 $\pm$ 43	5.0 $\pm$ 0.5
His-LR <sub>2</sub> C <sup>KKK,EAA</sup>	0.12 $\pm$ 0.01	5.62	n.d.	n.d.	1,318 $\pm$ 69	3.5 $\pm$ 0.5
His-NR <sub>1</sub> LR <sub>2</sub> C <sup>FF,AA</sup>	n.d.	n.d.	n.d.	n.d.	9.47 $\pm$ 1.1	1.0 $\pm$ 0.1

Values shown are mean  $\pm$  SEM ( $n = 3$ ). n/a, not applicable; n.d., not determined.





**Fig. 3.** Loqs-PD and its truncations bind dsRNA with high affinity. (A) Representative PhosphorImages for gel shift experiments with 106 BLT dsRNA (10 pM),  $^{32}$ P-end-labeled on the sense strand and incubated with indicated concentrations of Loqs-PD or its truncations. Free dsRNA was separated from bound dsRNA by native PAGE on a 4% 19:1 polyacrylamide gel. (B) Radioactivity in gels, as in A, was quantified to generate binding isotherms.  $\text{dsRNA}_{\text{total}}$  and  $\text{dsRNA}_{\text{free}}$  were quantified to determine fraction bound ( $1 - (\text{dsRNA}_{\text{free}}/\text{dsRNA}_{\text{total}})$ ), and data were fit using the Hill formalism,  $\text{fraction bound} = 1/(1 + (K_d^n/[P]^n))$ , where  $K_d$  is the dissociation constant,  $n$  is the Hill coefficient, and  $[P]$  is the protein concentration. Data points are mean  $\pm$  SEM ( $n = 3$  unless marked otherwise;  $*n = 2$ ;  $\#n = 1$ ).

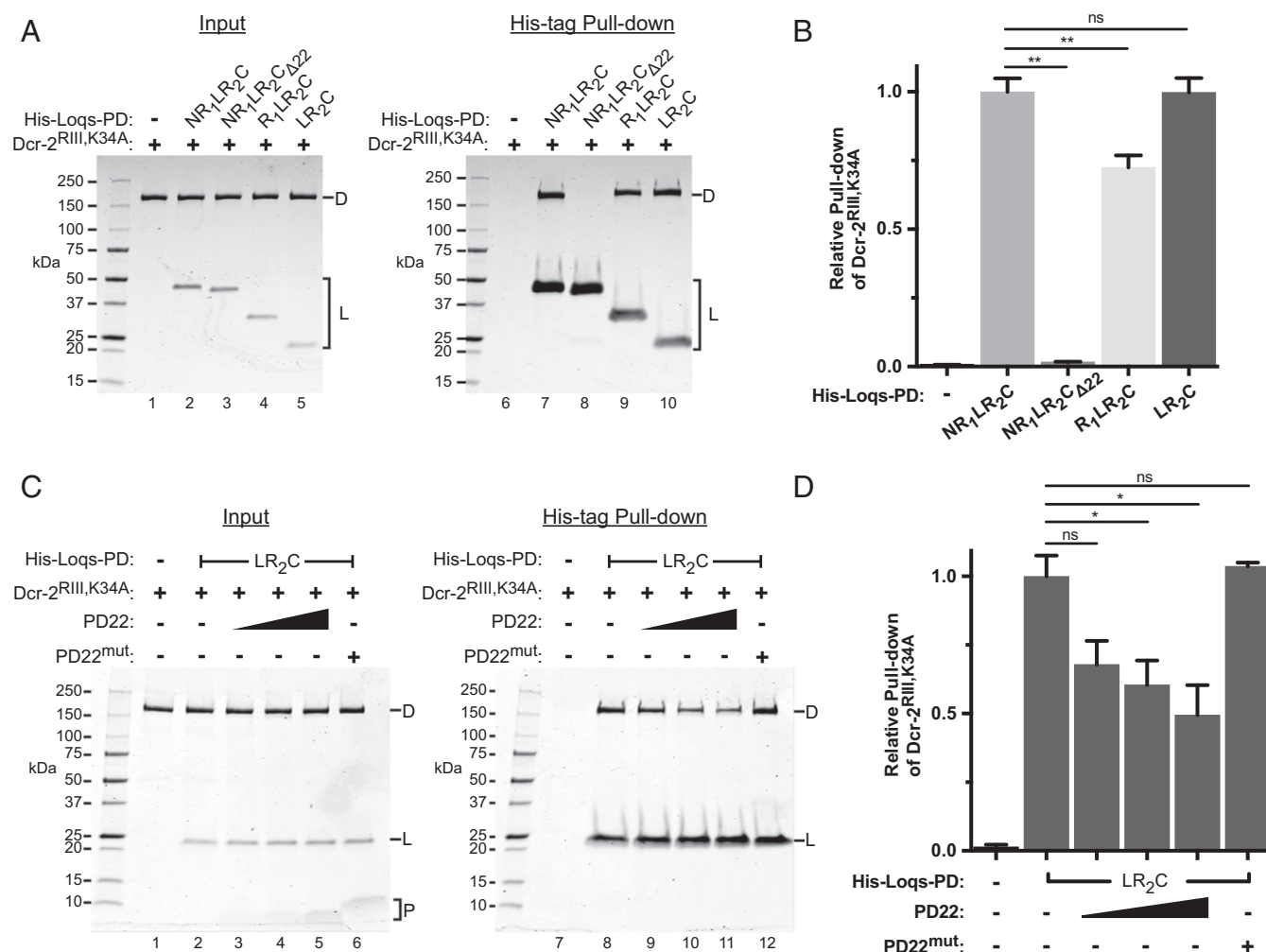
found that  $\text{NR}_1\text{LR}_2\text{C}$  was able to pull down Dcr-2 (Fig. 4A, lane 7), confirming a direct interaction. Additionally, our recombinant proteins were free of RNA as measured by  $A_{260/280}$ , suggesting this interaction is RNA-independent. By contrast,  $\text{NR}_1\text{LR}_2\text{C}_{\Delta 22}$  was unable to pull down Dcr-2 (Fig. 4A, lane 8), confirming that the C terminus of Loqs-PD is required for a direct, RNA-independent interaction with Dcr-2.  $\text{R}_1\text{LR}_2\text{C}$  and  $\text{LR}_2\text{C}$  were both able to pull down Dcr-2 (Fig. 4A, lanes 9 and 10), although  $\text{R}_1\text{LR}_2\text{C}$  pulled down slightly less Dcr-2 than  $\text{NR}_1\text{LR}_2\text{C}$  or  $\text{LR}_2\text{C}$  (Fig. 4B). Thus, Loqs-PD directly binds Dcr-2, and its C-terminal 22 amino acids are required for this interaction.

To determine whether the C-terminal 22 residues of Loqs-PD alone were able to bind Dcr-2, we synthesized the 22-residue peptide (PD22) and performed a competition experiment in which we pulled down Dcr-2 with  $\text{LR}_2\text{C}$  in the presence of increasing amounts of PD22 (Fig. 4C and D). PD22 effectively competed for binding with  $\text{LR}_2\text{C}$  as seen by the dose-dependent decrease in the amount of Dcr-2 pulled down by  $\text{LR}_2\text{C}$  (Fig. 4C, lanes 9–11 compared with lane 8). To control for nonspecific effects from the high concentration of peptide used, a mutated version of PD22 ( $\text{PD22}^{\text{mut}}$ , described below) was tested at the highest concentration of peptide assayed.  $\text{PD22}^{\text{mut}}$  did not compete for the interaction between  $\text{LR}_2\text{C}$  and Dcr-2 (Fig. 4C, lane 12), confirming the specificity of the PD22 interaction. We attempted direct binding studies by fluorescence polarization using a fluorescein-labeled version of PD22 but were unable to saturate binding without using prohibitively high concentrations of Dcr-2. Without quantitative binding studies, we cannot rule out that other portions of Loqs-PD contribute to binding, but our analyses indicate the C-terminal 22 amino acids of Loqs-PD directly interact with Dcr-2.

**dsRNA Binding Is Required by Loqs-PD to Affect Dcr-2 Cleavage Activity.** Our cleavage assays with  $\text{NR}_1\text{LR}_2\text{C}_{\Delta 22}$  (Fig. 2) confirmed that the C-terminal 22 residues of Loqs-PD were essential for enhancing Dcr-2 cleavage activity. Our pull-downs (Fig. 4) provided an explanation in that those residues were required for

interaction with Dcr-2. An outstanding question was whether dsRNA-binding by Loqs-PD was also required to enhance Dcr-2 cleavage activity. To test this, we disrupted dsRNA-binding activity of Loqs-PD in a construct capable of enhancing Dcr-2 activity. We selected  $\text{LR}_2\text{C}$  because it contained a single dsRBM yet was able to enhance Dcr-2 activity toward a 106 BLT dsRNA to a similar extent as full-length Loqs-PD (Fig. 2A). dsRBMs contain a highly conserved KKxxK motif, which mediates direct interaction with the phosphate backbone of dsRNA (29–31). To disrupt the dsRNA-binding activity of  $\text{LR}_2\text{C}$ , we mutated lysine 301, present in the KKxxK motif of dsRBM2 (Fig. 5A and B). We performed gel shift assays of 106 BLT dsRNA with His- $\text{LR}_2\text{C}$  or His- $\text{LR}_2\text{C}^{\text{K},\text{A}}$  (Fig. 5C). Indeed, mutation of lysine 301 to alanine resulted in a  $\sim 55$ -fold reduction in binding affinity by His- $\text{LR}_2\text{C}^{\text{K},\text{A}}$  compared with His- $\text{LR}_2\text{C}$  (Fig. 5D and Table 1). The presence of the 6xHis tag had no effect on dsRNA binding ( $K_d \sim 29$  nM vs.  $K_d \sim 30$  nM, respectively) (Table 1). To ensure that the decrease in dsRNA-binding affinity was due to the mutation and not a secondary affect of protein misfolding, we compared His- $\text{LR}_2\text{C}$  and His- $\text{LR}_2\text{C}^{\text{K},\text{A}}$  by circular dichroism (CD) spectroscopy (Fig. S4A). CD spectra reflect the secondary structure composition of a protein (32), and there was no significant difference between the His- $\text{LR}_2\text{C}$  and His- $\text{LR}_2\text{C}^{\text{K},\text{A}}$  spectra, suggesting the K301A mutation did not grossly affect protein folding. Thus,  $\text{LR}_2\text{C}^{\text{K},\text{A}}$  was properly folded but had greatly reduced affinity for dsRNA.

We performed pull-downs with His- $\text{LR}_2\text{C}^{\text{K},\text{A}}$  and found that the dsRBM mutation had no effect on Dcr-2 binding (Fig. 5E and F). This result emphasized that the interaction between Dcr-2 and Loqs-PD is independent of dsRNA. After determining that His- $\text{LR}_2\text{C}^{\text{K},\text{A}}$  had greatly reduced dsRNA-binding affinity but was still capable of interacting with Dcr-2, we tested whether His- $\text{LR}_2\text{C}^{\text{K},\text{A}}$  could affect Dcr-2 cleavage activity. We performed single-turnover cleavage assays of 106 BLT dsRNA by Dcr-2<sup>WT</sup> alone (–) or supplemented with His- $\text{LR}_2\text{C}$  or His- $\text{LR}_2\text{C}^{\text{K},\text{A}}$  (Fig. 5G). His- $\text{LR}_2\text{C}^{\text{K},\text{A}}$  was unable to increase the rate of Dcr-2 cleavage (Table 1), indicating dsRNA binding is required under



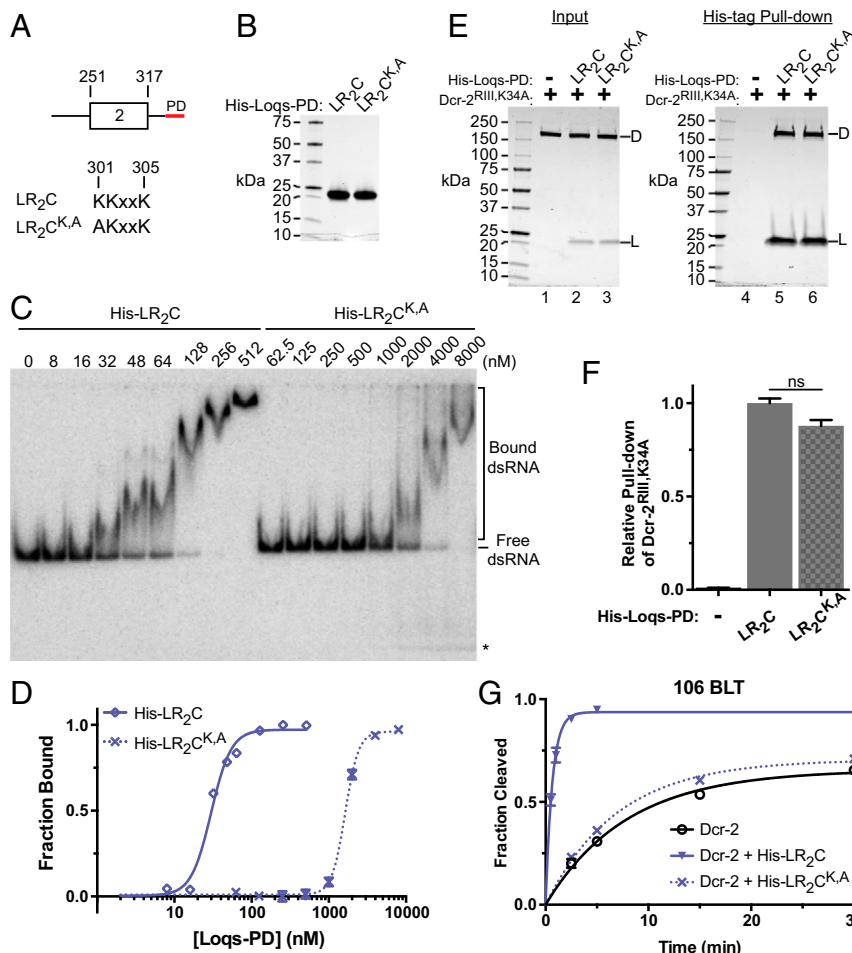
**Fig. 4.** C-terminal 22 amino acids of Loqs-PD mediate direct interaction with Dcr-2. (A) Coomassie-stained SDS/PAGE gels show input (Left, 5% of total) and pull-down (Right, 100%) of Dcr-2<sup>R111,K34A</sup> (D, 2  $\mu$ M) in the absence (–) or presence of His-tagged Loqs-PD or truncations (L, 4  $\mu$ M). Molecular mass markers (kDa) are on Left. (B) Data as in A were quantified to determine amount of Dcr-2 pulled down by each Loqs-PD variant. Values were normalized to Dcr-2 in the input and plotted relative to amount pulled down by His-Loqs-PD (His-NR<sub>1</sub>LR<sub>2</sub>C). Data points are mean  $\pm$  SEM ( $n = 3$ ); paired  $t$  test—ns,  $P > 0.05$ ; \*\* $P < 0.003$ . (C) Pull-down performed as in A with increasing amounts of PD22 peptide (P, 10, 20, 40  $\mu$ M) as a binding competitor. (D) Data were quantified as in B for pull-downs as in C. Data points are mean  $\pm$  SEM ( $n = 3$ ). Paired  $t$  test—ns,  $P > 0.05$ ; \* $P < 0.04$ .

the conditions tested. In addition to the single point mutant, we made a more severe mutant in which all three lysines of the KKxxK motif were mutated to EAxXA (Fig. S4 B and C). We obtained similar results for K,A and KKK,EAA mutants in all of the above experiments (Table 1 and Fig. S4). Thus, Loqs-PD must bind dsRNA as well as Dcr-2 to enhance Dcr-2 cleavage activity.

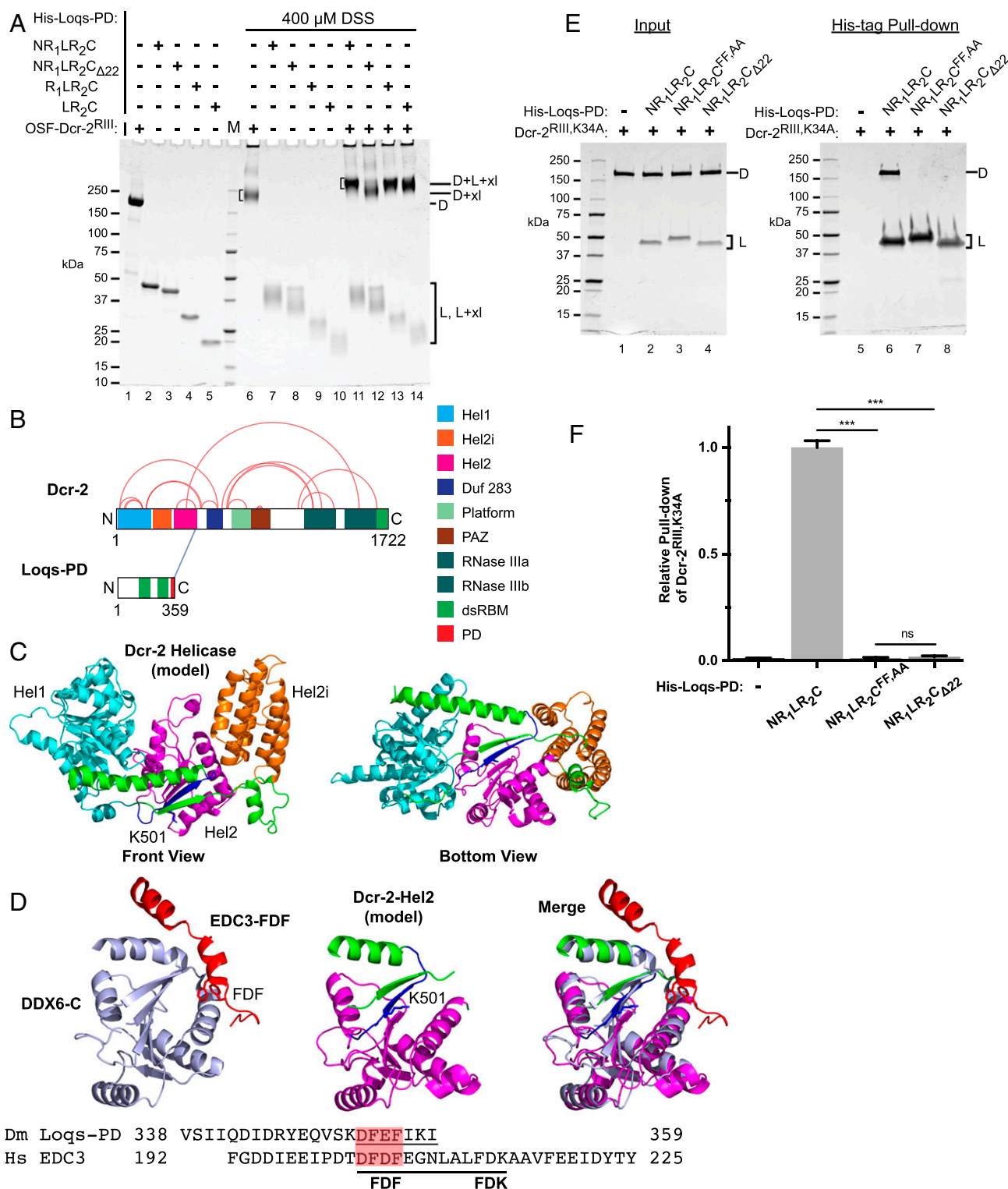
**Loqs-PD Binds the Hel2 Subdomain of Dcr-2's Helicase.** Previous studies indicate Loqs-PD interacts with the helicase domain of Dcr-2 (21), but the exact binding interface is unknown. The helicase domain of Dcr-2 contains two RecA-like domains (Hel1 and Hel2) separated by a Hef-like insertion domain (Hel2i). To identify the region of Dcr-2 that binds Loqs-PD, we coupled protein cross-linking with mass spectrometry (XL-MS), in which hybrid peptides, resulting from intra- or interprotein cross-links, are identified and sequenced by liquid chromatography and tandem MS (LC-MS/MS) (33–35). We performed chemical cross-linking with disuccinimidyl suberate (DSS), a homo-bifunctional NHS-ester cross-linker that primarily reacts with primary amines of lysine side chains or the N terminus (36).

When treated with DSS, Dcr-2 migrated slightly slower during SDS/PAGE (D vs. D+xl) (Fig. 6A, compare lanes 1 and 6). Loqs-PD and its truncations migrated slightly faster after DSS treatment, with broader, more diffuse bands (L vs. L+xl; Fig. 6A, compare lanes 2–5 and 7–10). In both cases, the altered SDS/PAGE mobility is likely due to intraprotein cross-linking. When Dcr-2 was incubated with NR<sub>1</sub>LR<sub>2</sub>C and treated with DSS, the main Dcr-2 band (D+xl) shifted to a higher molecular mass species, suggesting formation of a covalent adduct between Loqs-PD and Dcr-2 (D+L+xl) (Fig. 6A, compare lanes 6 and 11). Consistent with the requirement of the C-terminal 22 amino acids for interacting with Dcr-2 in pull-down assays (Fig. 4A and B), the D+L+xl species was greatly reduced when cross-linking was performed with NR<sub>1</sub>LR<sub>2</sub>CΔ22 (Fig. 6A, compare lanes 11 and 12). Cross-linking performed with Dcr-2 and R<sub>1</sub>LR<sub>2</sub>C or LR<sub>2</sub>C also resulted in the D+L+xl species (Fig. 6A, lanes 13 and 14). The agreement between our cross-linking and pull-downs suggests DSS cross-linking captures the native interaction between Loqs-PD and Dcr-2.

To identify the sites of cross-linking between Dcr-2 and Loqs-PD, we analyzed the in-gel tryptic digest of the D+L+xl species by LC-MS/MS. We identified peptides mapping to both Dcr-2







**Fig. 6.** Loqs-PD interacts with the Hel2 domain of Dcr-2 via an FDF-like motif. (A) DSS cross-linking of OSF-Dcr-2<sup>RIII</sup> (2 μM), in the presence or absence, of Loqs-PD or its truncations (4 μM). Representative Coomassie-stained SDS/PAGE gel of individual proteins without (lanes 1–5, D and L) or with DSS (lanes 6–10, D+xl and L+xl) or together with DSS (lanes 11–14, D+L+xl) (n > 3). Brackets mark bands excised for subsequent XL-MS analysis. (B) Schematic [xVis (59)] of Dcr-2 and Loqs-PD with color-coded domains depicting intra- and interprotein cross-links identified by MS/MS analysis using ProteinProspector2 (57) in D+L+xl species (n = 2). (C) Homology model of Dcr-2 helicase domain with Hel1 (cyan), Hel2i (orange), and Hel2 (magenta). The Dcr-2-derived peptide that cross-linked to Loqs-PD is shown (blue), with the site of DSS cross-linking, lysine 501, in stick representation. (D, Top Left) Complex of DDX6-C (gray) and EDC3-FDF (red). FDF motif is shown in stick representation (PDB ID code 2WAX). (D, Top Middle) Hel2 from Dcr-2 homology model, colored as in C. (D, Top Right) Structural superposition of DDX6-C:EDC3-FDF complex and homology model of Dcr-2 Hel2 domain. (D, Bottom) Sequences of *Homo sapiens* EDC3 and C-terminal 22 residues from Loqs-PD. The FDF-motif in EDC3 and the putative FDF-motif in Loqs-PD are shaded in red. (E) Coomassie-stained SDS/PAGE gels of input (Left, 5% of total) and pull-down (Right, 100%) for Dcr-2<sup>RIII,K34A</sup> (2 μM) with His-tagged Loqs-PD constructs (4 μM). Molecular mass markers are to the left. (F) Quantification as in Fig. 4B for data as in E. Data points are mean ± SEM (n = 3). Paired t test—ns, P > 0.05; \*\*\*P < 0.001.





the dsRNA positioned outside of the helicase domain, thus favoring distributive cleavage (Fig. 7C, *Top Right*). Based on the experiments reported here, we predict the substrate-dependent conformations of the dsRNA–Dcr-2 complex dictate which domains of Loqs-PD are required to enhance cleavage.

For an optimal substrate, either R<sub>1</sub>LR<sub>2</sub>C or LR<sub>2</sub>C is sufficient to enhance Dcr-2 cleavage (Figs. 2A and 7C, *Left*). In Fig. 7C, *Bottom*, R<sub>1</sub>LR<sub>2</sub>C/LR<sub>2</sub>C is shown interacting with Dcr-2 and the dsRNA substrate, consistent with our findings that dsRNA binding (Fig. 5) and Dcr-2 binding (Figs. 2 and 4A and B) are both necessary for Loqs-PD to affect Dcr-2 activity. For an optimal substrate, we predict the dsRNA–Dcr-2 complex is in the closed conformation, which based on crystal structures of RNA-bound RIG-I-like helicases (44–48) would position the dsRNA and Hel2 in close proximity such that LR<sub>2</sub>C is sufficient to simultaneously bind both. Thus, we depict LR<sub>2</sub>C (highlighted as solid lines in Fig. 7C, *Bottom*) holding the dsRNA in the correct orientation relative to the helicase domain to stabilize the closed conformation of the dsRNA–Dcr-2 complex.

For suboptimal substrates, in contrast, R<sub>1</sub>LR<sub>2</sub>C is the only variant sufficient to fully enhance Dcr-2 cleavage (Figs. 2B–D and 7C, *Right*). For a suboptimal substrate, we predict the dsRNA–Dcr-2 complex is predominantly in an open conformation, which based on low-resolution cryo-EM reconstructions of human Dicer bound to an siRNA (49) may position the dsRNA and Hel2 farther apart such that LR<sub>2</sub>C is no longer sufficient to simultaneously bind both (Fig. 7C, *Top Right*). This model is consistent with our findings that LR<sub>2</sub>C interacted with Dcr-2 comparable to full-length Loqs-PD (Fig. 4A and B) and bound dsRNA (Fig. 3) yet was not sufficient to fully enhance cleavage of suboptimal substrates (Fig. 2B–D). Given that LR<sub>2</sub>C leads to a partial increase in cleavage of suboptimal substrates compared with NR<sub>1</sub>LR<sub>2</sub>C, we hypothesize that Dcr-2 occasionally transitions into the closed conformation (Fig. 7C, dashed arrow) such that LR<sub>2</sub>C can stabilize it to promote cleavage. In our model, inclusion of the first dsRBM extends the reach of R<sub>1</sub>LR<sub>2</sub>C, allowing it to now simultaneously bind both Dcr-2 and the dsRNA substrate. We predict this allows R<sub>1</sub>LR<sub>2</sub>C to reposition the substrate within the helicase domain of Dcr-2 such that it can now adopt the closed conformation and be stabilized by LR<sub>2</sub>C (Fig. 7C, *Bottom*). Additional studies are needed to fully elucidate the structures and dynamics of the different conformations discussed.

As a general mechanism, we propose Loqs-PD coordinates Dcr-2 binding with dsRNA binding to promote or stabilize a conformational change in the helicase domain of Dcr-2, which correlates with increased cleavage. Consistent with this model, Loqs-PD has no effect in the absence of ATP (6) (Fig. 2C), which we predict is required for the conformational change in Dcr-2. In vivo, under ATP-replete conditions, we expect Loqs-PD directly facilitates endo-siRNA biogenesis by this mechanism, although we cannot rule out the possibility that other factors may further enhance the efficiency.

Many of the annotated Dicer–dsRBP interactions require the helicase domain of Dicer (50, 51), but the exact interface is unknown, with the exception of human TRBP and Dicer. Biochemical and structural studies indicate the third dsRBM of TRBP interacts with Hel2i of Dicer (52, 53). A recent study suggests this interaction is conserved in the fly homologs Dcr-1 and Loqs-PB (20). In contrast, our data indicate that Loqs-PD primarily interacts with Hel2 of Dcr-2's helicase (Fig. 6), identifying an additional Dicer–dsRBP interaction interface. There are conflicting reports as to whether Loqs-PD and R2D2 simultaneously interact with Dcr-2 (22) or whether their binding is mutually exclusive (21, 23). Additional studies are required to determine whether R2D2 binds the same Hel2 interface we have described for Loqs-PD or interacts with Hel2i of Dcr-2 in a manner analogous to TRBP and Dicer. It remains to be seen if

other ATP-dependent Dicers such as *Schizosaccharomyces pombe* Dcr1 and *Caenorhabditis elegans* DCR-1 interact with dsRBPs similarly to Dcr-2 and Loqs-PD. Protein–protein interactions mediated by small motifs located in disordered regions have become a dominant theme among RNP assemblies (40, 54).

## Materials and Methods

**Protein Expression and Purification.** Loqs-PD and Dcr-2 were purified from *Escherichia coli* and Sf9 cells, respectively, as described (6) (*SI Materials and Methods*).

**Synthesis of PD22 Peptide.** PD22 and PD22<sup>mut</sup> peptides were chemically synthesized as described (55) (*SI Materials and Methods*). Peptide sequences were as follows: PD22: VSIQDIDRYEQVSKDFEFIKI; PD22<sup>mut</sup>: VSIQDIDRYEQVSKDAEAIKI.

**In Vitro Transcription of RNA Substrates.** We prepared 106 dsRNA as described (6). In the plasmid, each RNA strand was flanked by a hammerhead (5' side) and HDV (3' side) ribozyme to ensure accurate termini. <sup>32</sup>P-end-labeled 106 sense RNA was annealed with 106 BLT or 3'ovr antisense RNA to generate 106 BLT and 106 3'ovr dsRNA, respectively. *esi-2<sup>hp</sup>* was cloned into the same ribozyme plasmid and prepared as described for 106 dsRNA with minor changes (see *SI Materials and Methods* for details). Sequences of 106 dsRNAs and *esi-2<sup>hp</sup>* are in *SI Materials and Methods*.

**Gel Shift and Cleavage Assays.** Gel shift and single-turnover cleavage assays were performed as described (6) with minor changes (see *SI Materials and Methods* for details).

**Pull-Down Assays.** Dcr-2<sup>RIII,K34A</sup> (2 μM) and His-Loqs-PD (4 μM) were incubated together in pull-down buffer (25 mM Tris, pH 8, 175 mM KCl, 10 mM MgCl<sub>2</sub>, 10 mM imidazole, 1 mM TCEP, 5% glycerol, 0.1% nonidet P-40) for 1 h at 4 °C and added to prewashed His-Select Resin (Sigma-Aldrich) for 2 h at 4 °C. Resin and bound proteins were pelleted by centrifugation, and unbound protein (supernatant) was removed. Resin was washed with chilled pull-down buffer, and bound protein was eluted in pull-down buffer containing 300 mM imidazole. Proteins were resolved on a 4–15% gradient gel and stained with Coomassie Brilliant Blue. Competition pull-down assays were performed as described above with addition of PD22 (10, 20, and 40 μM) or PD22<sup>mut</sup> (40 μM). Bound proteins were resolved on a 4–20% gradient gel by SDS/PAGE.

**Chemical Cross-Linking.** Dcr-2<sup>RIII,K34A</sup> or OSF-Dcr-2<sup>RIII</sup>, and Loqs-PD and its truncations, were dialyzed into cross-linking buffer (20 mM Hepes, pH 7.8, 100 mM KCl, 10 mM MgCl<sub>2</sub>, 1 mM TCEP, 5% glycerol). Cross-linking reactions were assembled with Dcr-2 (2 μM) and/or Loqs-PD (4 μM) and incubated (25 °C, 30 min). DSS (5 mM in DMSO; Sigma-Aldrich) was added to make 100–400 μM final, and cross-linking was quenched after an additional 30 min at 25 °C with 30 mM Tris, pH 8. Cross-linked proteins were resolved by SDS/PAGE (4–15%) and detected with Coomassie Brilliant Blue.

**MS and Identification of Cross-Linked Peptides.** Bands corresponding to D+L+xl and D+xl were excised and subjected to in-gel digestion by trypsin and Lys-C. Peptides were extracted, reduced, treated with iodoacetamide, and analyzed using a nano-LC–MS/MS system equipped with a nano-HPLC pump (2D-ultra; Eksigent) and a maxis II ETD mass spectrometer (Bruker Daltonics). The maxis II ETD mass spectrometer was equipped with a capillary spray ion source.

Cross-linked peptides were identified using the webserver version of ProteinProspector2 (v5.18.0/1) ([prospector.ucsf.edu/prospector/mshome.htm](http://prospector.ucsf.edu/prospector/mshome.htm)). A custom database was made containing amino acid sequences of Dcr-2<sup>RIII,K34A</sup> or OSF-Dcr-2<sup>RIII</sup>, His-Loqs-PD, and 20 decoy proteins. Loqs-PD and Dcr-2 sequences were each randomized 10 times using Decoy Database Builder (56) to generate decoy targets. Up to three missed cleavages were allowed. The MS1 and MS2 mass tolerances were both set to 11 ppm. DSS was specified as the cross-linker. Spectra were annotated as potential cross-linked products if the ProteinProspector total cross-linked product score was >20, and the score difference was >0. To identify high-confidence cross-link products (57), a score difference >8.5 was used, and spectra were manually verified.

**Homology Modeling.** A homology model of the Dcr-2 helicase domain (residues 1–539) was generated using the Robetta webserver ([robeta.bakerlab.org](http://robeta.bakerlab.org); reference parent structure was DDX3X [Protein Data Bank (PDB) ID code 4PXA]).

**ACKNOWLEDGMENTS.** We thank members of the B.L.B. and Cazalla laboratories for discussion and feedback and Patrick W. Erickson for assistance with CD spectroscopy. Oligonucleotides were synthesized by the DNA/Peptide Facility, part of the Health Sciences Center Cores at the University of Utah. MS was performed at the Mass Spectrometry and Proteomics Core Facility at the University of Utah. MS equipment was obtained through NCR Shared

Instrumentation Grant 1 S10 RR020883-01 and 1 S10 RR025532-01A1. Research reported in this publication was supported by funding from National Institute of General Medical Sciences of the National Institutes of Health Grant R01GM121706 and the H.A. and Edna Benning Presidential Endowed Chair (to B.L.B.) and by funding from National Institute of General Medical Sciences of the NIH Grant P50-GM082545 (to D.M.E.).

- Lee YS, et al. (2004) Distinct roles for Drosophila Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. *Cell* 117:69–81.
- Carthew RW, Sontheimer EJ (2009) Origins and mechanisms of miRNAs and siRNAs. *Cell* 136:642–655.
- Wang X-H, et al. (2006) RNA interference directs innate immunity against viruses in adult Drosophila. *Science* 312:452–454.
- Sabin LR, et al. (2013) Dicer-2 processes diverse viral RNA species. *PLoS One* 8:e55458.
- Welker NC, et al. (2011) Dicer's helicase domain discriminates dsRNA termini to promote an altered reaction mode. *Mol Cell* 41:589–599.
- Sinha NK, Trettin KD, Aruscavage PJ, Bass BL (2015) Drosophila dicer-2 cleavage is mediated by helicase- and dsRNA termini-dependent states that are modulated by loquacious-PD. *Mol Cell* 58:406–417.
- Deddouche S, et al. (2008) The DExD/H-box helicase Dicer-2 mediates the induction of antiviral activity in Drosophila. *Nat Immunol* 9:1425–1432.
- Marques JT, et al. (2013) Functional specialization of the small interfering RNA pathway in response to virus infection. *PLoS Pathog* 9:e1003579.
- Zhou R, et al. (2009) Processing of Drosophila endo-siRNAs depends on a specific loquacious isoform. *RNA* 15:1886–1895.
- Hartig JV, Esslinger S, Böttcher R, Saito K, Förstemann K (2009) Endo-siRNAs depend on a new isoform of loquacious and target artificially introduced, high-copy sequences. *EMBO J* 28:2932–2944.
- Ghildiyal M, et al. (2008) Endogenous siRNAs derived from transposons and mRNAs in Drosophila somatic cells. *Science* 320:1077–1081.
- Watanabe T, et al. (2008) Endogenous siRNAs from naturally formed dsRNAs regulate transcripts in mouse oocytes. *Nature* 453:539–543.
- Czech B, et al. (2008) An endogenous small interfering RNA pathway in Drosophila. *Nature* 453:798–802.
- Okamura K, et al. (2008) The Drosophila hairpin RNA pathway generates endogenous short interfering RNAs. *Nature* 453:803–806.
- Kawamura Y, et al. (2008) Drosophila endogenous small RNAs bind to Argonaute 2 in somatic cells. *Nature* 453:793–797.
- Chung W-J, Okamura K, Martin R, Lai EC (2008) Endogenous RNA interference provides a somatic defense against Drosophila transposons. *Curr Biol* 18:795–802.
- Jiang F, et al. (2005) Dicer-1 and R3D1-L catalyze microRNA maturation in Drosophila. *Genes Dev* 19:1674–1679.
- Saito K, Ishizuka A, Siomi H, Siomi MC (2005) Processing of pre-microRNAs by the Dicer-1-Loquacious complex in Drosophila cells. *PLoS Biol* 3:e235.
- Fukunaga R, et al. (2012) Dicer partner proteins tune the length of mature miRNAs in flies and mammals. *Cell* 151:533–546.
- Lim MYT, et al. (2016) The Drosophila Dicer-1 partner loquacious enhances miRNA processing from hairpins with unstable structures at the dicing site. *Cell Rep* 15:1795–1808.
- Hartig JV, Förstemann K (2011) Loqs-PD and R2D2 define independent pathways for RISC generation in Drosophila. *Nucleic Acids Res* 39:3836–3851.
- Miyoshi K, Miyoshi T, Hartig JV, Siomi H, Siomi MC (2010) Molecular mechanisms that funnel RNA precursors into endogenous small-interfering RNA and microRNA biogenesis pathways in Drosophila. *RNA* 16:506–515.
- Marques JT, et al. (2010) Loqs and R2D2 act sequentially in the siRNA pathway in Drosophila. *Nat Struct Mol Biol* 17:24–30.
- Parker GS, Maity TS, Bass BL (2008) dsRNA binding properties of RDE-4 and TRBP reflect their distinct roles in RNAi. *J Mol Biol* 384:967–979.
- Cosentino GP, et al. (1995) Double-stranded-RNA-dependent protein kinase and TAR RNA-binding protein form homo- and heterodimers in vivo. *Proc Natl Acad Sci USA* 92:9445–9449.
- Hitti EG, Sallacz NB, Schoft VK, Jantsch MF (2004) Oligomerization activity of a double-stranded RNA-binding domain. *FEBS Lett* 574:25–30.
- Parker GS, Eckert DM, Bass BL (2006) RDE-4 preferentially binds long dsRNA and its dimerization is necessary for cleavage of dsRNA to siRNA. *RNA* 12:807–818.
- Carey J (1991) Gel retardation. *Methods Enzymol* 208:103–117.
- Ryter JM, Schultz SC (1998) Molecular basis of double-stranded RNA-protein interactions: Structure of a dsRNA-binding domain complexed with dsRNA. *EMBO J* 17:7505–7513.
- Ramos A, et al. (2000) RNA recognition by a Staufen double-stranded RNA-binding domain. *EMBO J* 19:997–1009.
- Krovat BC, Jantsch MF (1996) Comparative mutational analysis of the double-stranded RNA binding domains of Xenopus laevis RNA-binding protein A. *J Biol Chem* 271:28112–28119.
- Provencher SW, Glöckner J (1981) Estimation of globular protein secondary structure from circular dichroism. *Biochemistry* 20:33–37.
- Holding AN (2015) XL-MS: Protein cross-linking coupled with mass spectrometry. *Methods* 89:54–63.
- Liu F, Heck AJR (2015) Interrogating the architecture of protein assemblies and protein interaction networks by cross-linking mass spectrometry. *Curr Opin Struct Biol* 35:100–108.
- Leitner A, Faini M, Stengel F, Aebersold R (2016) Crosslinking and mass spectrometry: An integrated technology to understand the structure and function of molecular machines. *Trends Biochem Sci* 41:20–32.
- Mädler S, Bich C, Touboul D, Zenobi R (2009) Chemical cross-linking with NHS esters: A systematic study on amino acid reactivities. *J Mass Spectrom* 44:694–706.
- Merkley ED, et al. (2014) Distance restraints from crosslinking mass spectrometry: Mining a molecular dynamics simulation database to evaluate lysine-lysine distances. *Protein Sci* 23:747–759.
- Kim DE, Chivian D, Baker D (2004) Protein structure prediction and analysis using the Robetta server. *Nucleic Acids Res* 32:W526–W531.
- Fairman-Williams ME, Guenther U-P, Jankowsky E (2010) SF1 and SF2 helicases: Family matters. *Curr Opin Struct Biol* 20:313–324.
- Jonas S, Izaurralde E (2013) The role of disordered protein regions in the assembly of decapping complexes and RNP granules. *Genes Dev* 27:2628–2641.
- Tritschler F, et al. (2008) Similar modes of interaction enable Trailer Hitch and EDC3 to associate with DCP1 and Me31B in distinct protein complexes. *Mol Cell Biol* 28:6695–6708.
- Sharif H, et al. (2013) Structural analysis of the yeast Dhh1-Pat1 complex reveals how Dhh1 engages Pat1, Edc3 and RNA in mutually exclusive interactions. *Nucleic Acids Res* 41:8377–8390.
- Tritschler F, et al. (2009) Structural basis for the mutually exclusive anchoring of P body components EDC3 and Tral to the DEAD box protein DDX6/Me31B. *Mol Cell* 33:661–668.
- Kowalinski E, et al. (2011) Structural basis for the activation of innate immune pattern-recognition receptor RIG-I by viral RNA. *Cell* 147:423–435.
- Jiang F, et al. (2011) Structural basis of RNA recognition and activation by innate immune receptor RIG-I. *Nature* 479:423–427.
- Luo D, et al. (2011) Structural insights into RNA recognition by RIG-I. *Cell* 147:409–422.
- Wu B, et al. (2013) Structural basis for dsRNA recognition, filament formation, and antiviral signal activation by MDA5. *Cell* 152:276–289.
- Uchikawa E, et al. (2016) Structural analysis of dsRNA binding to anti-viral pattern recognition receptors LGP2 and MDA5. *Mol Cell* 62:586–602.
- Taylor DW, et al. (2013) Substrate-specific structural rearrangements of human Dicer. *Nat Struct Mol Biol* 20:662–670.
- Ota H, et al. (2013) ADAR1 forms a complex with Dicer to promote microRNA processing and RNA-induced gene silencing. *Cell* 153:575–589.
- Nishida KM, et al. (2013) Roles of R2D2, a cytoplasmic D2 body component, in the endogenous siRNA pathway in Drosophila. *Mol Cell* 49:680–691.
- Daniels SM, et al. (2009) Characterization of the TRBP domain required for dicer interaction and function in RNA interference. *BMC Mol Biol* 10:38.
- Wilson RC, et al. (2015) Dicer-TRBP complex formation ensures accurate mammalian microRNA biogenesis. *Mol Cell* 57:397–407.
- Calabretta S, Richard S (2015) Emerging roles of disordered sequences in RNA-binding proteins. *Trends Biochem Sci* 40:662–672.
- Petersen ME, Jacobsen MT, Kay MS (2016) Synthesis of tumor necrosis factor  $\alpha$  for use as a mirror-image phage display target. *Org Biomol Chem* 14:5298–5303.
- Reidegeld KA, et al. (2008) An easy-to-use Decoy Database Builder software tool, implementing different decoy strategies for false discovery rate calculation in automated MS/MS protein identifications. *Proteomics* 8:1129–1137.
- Trnka MJ, Baker PR, Robinson PJJ, Burlingame AL, Chalkley RJ (2014) Matching cross-linked peptide spectra: Only as good as the worst identification. *Mol Cell Proteomics* 13:420–434.
- Zuker M (2003) Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* 31:3406–3415.
- Grimm M, Zimniak T, Kahraman A, Herzog F (2015) xVis: A web server for the schematic visualization and interpretation of crosslink-derived spatial restraints. *Nucleic Acids Res* 43:W362–W369.
- Morse DP (2000) Identification of messenger RNAs that contain inosine. *Methods Enzymol* 318:48–67.
- Pall GS, Codony-Servat C, Byrne J, Ritchie L, Hamilton A (2007) Carbodiimide-mediated cross-linking of RNA to nylon membranes improves the detection of siRNA, miRNA and piRNA by northern blot. *Nucleic Acids Res* 35:e60.
- Greenfield NJ (2006) Using circular dichroism spectra to estimate protein secondary structure. *Nat Protoc* 1:2876–2890.
- Cole JL (2004) Analysis of heterogeneous interactions. *Methods Enzymol* 384:212–232.
- Laue TM, Shah BD, Ridgeway TM, Pelletier SL (1992) Computer-aided interpretation of analytical sedimentation data for proteins. *Analytical Ultracentrifugation in Biochemistry and Polymer Science*, eds Harding SE, et al. (The Royal Society of Chemistry, Cambridge, UK), pp 90–125.