

# Duplex RNA activated ATPases (DRAs)

## Platforms for RNA sensing, signaling and processing

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Double-stranded RNAs are an important class of functional macromolecules in living systems. They are usually found as part of highly specialized intracellular machines that control diverse cellular events, ranging from virus replication, antiviral defense, RNA interference, to regulation of gene activities and genomic integrity. Within different intracellular machines, the RNA duplex is often found in association with specific RNA-dependent ATPases, including Dicer, RIG-I and DRH-3 proteins. These duplex RNA-activated ATPases represent an emerging group of motor proteins within the large and diverse superfamily 2 nucleic acid-dependent ATPases (which are historically defined as SF2 helicases). The duplex RNA-activated ATPases share characteristic molecular features for duplex RNA recognition, including motifs (e.g., motifs IIa and Vc) and an insertion domain (HEL2i), and they require double-strand RNA binding for their enzymatic activities. Proteins in this family undergo large conformational changes concomitant with RNA binding, ATP binding and ATP hydrolysis in order to achieve their functions, which include the release of signaling domains and the recruitment of partner proteins. The duplex RNA-activated ATPases represent a distinct and fascinating group of nanomechanical molecular motors that are essential for duplex RNA sensing and processing in diverse cellular pathways.

### Background

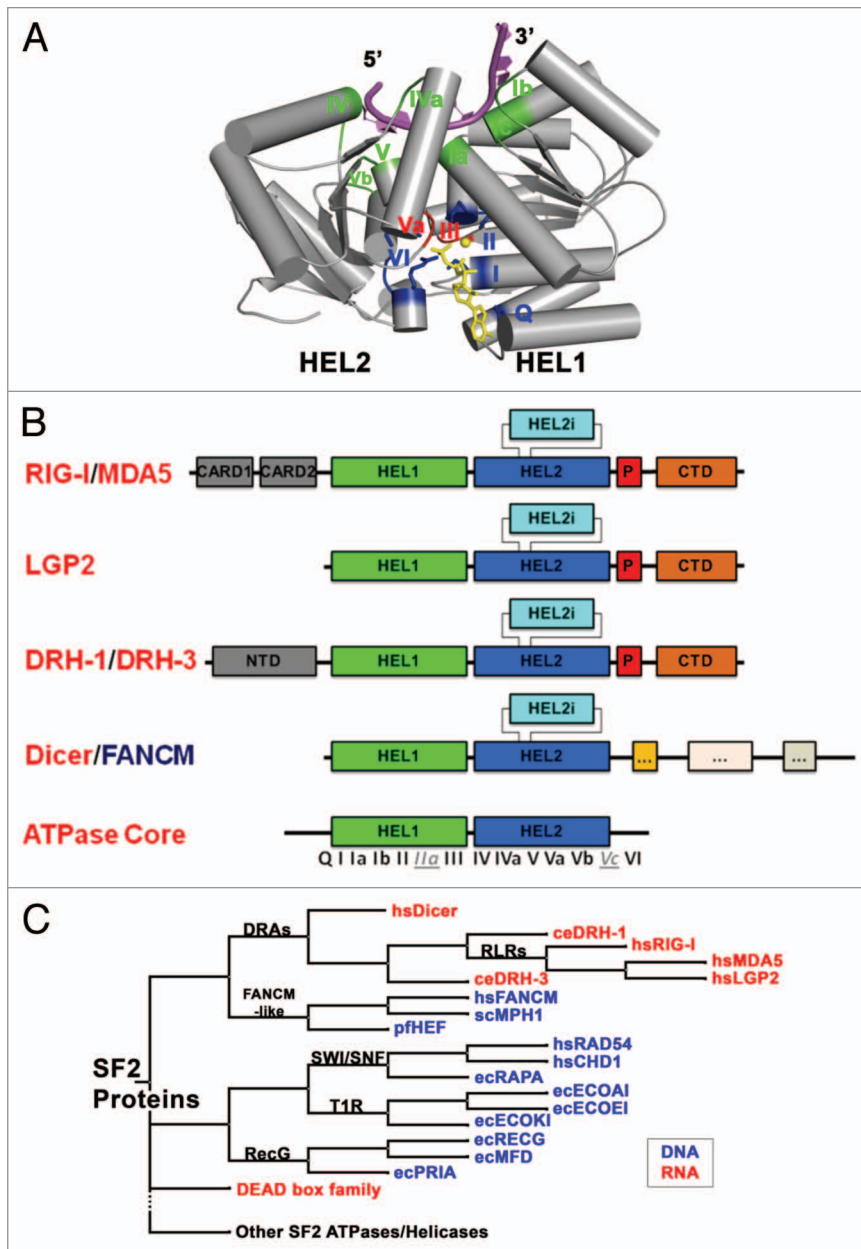
Many cellular processes result in the production of double-stranded RNA molecules, including transcription of convergent cellular genes or mobile genetic elements, self-annealing of cellular transcripts and the replication of common RNA viruses. Duplex RNAs are important for numerous cellular functions, including gene regulation, chromatin remodeling, antiviral defense and maintenance of genomic integrity.<sup>1,2</sup> Most of these processes involve the interaction of double-stranded RNAs with conserved and highly specialized intracellular machines. Well-characterized examples include Dicer and the RIG-I like receptors, as well as Dicer-like RNA helicases 1 and 3 (DRH-1 and -3), which are two mechanical proteins involved in the RNA interference (RNAi)

pathway in worms.<sup>1,3</sup> Although there are fundamental differences between these proteins, they share a similar, highly conserved motor domain that is essential for duplex RNA sensing, signaling and processing. This domain is similar in sequence and form, if not function, to the helicase domain that is found in many DNA and RNA remodeling proteins.<sup>4,5</sup>

Helicases have been classically defined as enzymes that couple ATP hydrolysis to the unwinding of nucleic acid duplexes, and they were originally phylogenetically grouped into families based on sequence conservation rather than function.<sup>6</sup> However, these family members were subsequently shown to have diverse mechanical functions, of which duplex unwinding is only one type of activity. Therefore, these enzymes are now commonly referred to as nucleic acid remodeling proteins or, perhaps more correctly, as nucleic acid-dependent ATPases.<sup>5,7</sup> Other classifications have grouped these proteins by their nucleic acid target (RNA or DNA), the nucleic acid strandness ( $\alpha$  for single stranded NA or  $\beta$  for double stranded NA) and the translocation polarity on the nucleic acid (A for 3' to 5' or B for 5' to 3') as defined by Wigley et al.<sup>8</sup> Sequence and structure analysis have revealed a common arrangement of conserved motifs for the Superfamily 1 and 2 (SF1 and 2) nucleic acid dependent ATPases.<sup>5</sup> In these proteins, two conserved RecA-like domains lie against each other, forming a cleft that binds and hydrolyzes ATP, thereby serving as the catalytic core. This ATPase core includes conserved motifs Q, I, II and VI (Fig. 1A), which are aligned and rigidified through binding of RNA along the surface of the RecA folds. Conserved motifs Ia, Ib, Ic, IV, IVa, V and Vb mediate RNA binding, while motifs III and Va help to couple nucleic acid binding with ATP hydrolysis. Despite the high degree of conservation in both RNA binding and ATPase motifs, SF1 and SF2 proteins have unique functions and are usually not interchangeable. Specialization in mechanical function and the presence of accessory domains makes each nucleic acid-dependent ATPase unique.<sup>4</sup> These enzymes are involved in every aspect of nucleic acid metabolism in all living organisms and viruses.<sup>5,9-11</sup> Because of their conserved molecular functions, they are also heavily involved in genetic, autoimmune and infectious diseases, and they are potential targets for drug discovery.

Recently, significant progress has been made in our understanding of RNA-dependent ATPases, including the identification and characterization of new examples like DRH-1 and DRH-3<sup>12,13</sup> from nematodes and new structural and functional

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**Figure 1.** DRAs and related nucleic acid-dependent ATPases. **(A)** The conserved SF2 ATPase/helicase core. The figure is prepared from eIF4aIII, which is a component of the exon junction complex (PDB: 2J0S). Positions of the characteristic motifs are highlighted. **(B)** Domain organization of DRAs. Domains are not to the scale. C-terminal regions that resemble the ATPase domains of Dicer and FANCM-like proteins are simplified and are not labeled. **(C)** Schematic cladogram showing DRAs within the SF2 family of proteins, specifically the DEAD box family and double stranded nucleic acid binding ATPases. The alignment and family trees were determined with the UGENE software package.<sup>118</sup> The multiple sequence alignment was run with T-Coffee<sup>119</sup> on the core ATPase/helicase domains listed in **Table 1** and the family tree was determined using the PHYLIP Neighbor Joining method with the Jones-Taylor-Thornton distance matrix. Pair-wise sequence identity for the ATPase core regions of DRAs range from the highest 42% (hsMDA5: hsLGP2) and 36% (hsRIG-I: hsMDA5), to 26% (hsRIG-I: ceDRH-1) and 21% (ceDRH-1: ceDRH-3), with the lowest 14% between hsDicer1 and ceDRH-3.

insights of known cases like Dicer<sup>14</sup> and RIG-I.<sup>15</sup> In this review, we focus on this emerging group of specialized RNA-dependent ATPases that include Dicer, a ribonuclease that plays an essential role in miRNA and siRNA biogenesis;<sup>14</sup> RIG-I, an intracellular

surveillance protein for detecting pathogenic RNA;<sup>15</sup> and DRH-3, Dicer-related-helicase-3, a component of the siRNA pathway from *Caenorhabditis elegans*.<sup>12,13</sup> The mechanistic feature shared by all these proteins [hereafter named Duplex RNA-activated ATPases (DRAs)] is that dsRNA is required to stimulate their ATPase activity and thereby activate all subsequent functions, which is in sharp contrast to other SF2 proteins that are specifically activated by single-stranded RNA. Further, unlike the bona fide RNA helicases, DRAs are unlikely to display RNA unwinding activity.<sup>16,17</sup> Rather, the conformational changes that occur upon binding to RNA and ATP are coupled to other processes, such as the release of signaling domains and binding to partner proteins. Here we review the discovery of the DRAs, highlight recent advances in understanding of their function and discuss how this is related to their structural features.

### Comparison of DRAs with Related Mechanical Proteins

DRA proteins are phylogenetically classified as a subgroup within Helicase Superfamily 2 (known as SF2 proteins, **Fig. 1C**),<sup>4</sup> and present a core ATPase domain that is very similar in both sequence and structure to the DEAD box family ATPases/Helicases. Unlike DEAD box proteins, DRAs contain a unique  $\alpha$ -helical insertion domain (HEL2i) within the second RecA fold of the core ATPase/Helicase domain. Structural studies have shown that this adaptation is important for duplex RNA binding (**Fig. 1B and C**).<sup>17-19</sup> As the closest phylogenetic relatives of RIG-I, innate immune sensors MDA5 and LGP2 are more similar to each other than to RIG-I, although there are conflicting reports on which of these proteins should be considered the evolutionary antecedent of the others.<sup>20,21</sup> A constructed family tree of SF2 proteins from several subgroups suggests that DRH-1 is more closely related to RIG-I than to Dicer (**Fig. 1C**), underscoring the difficulties in naming these proteins based on functional associations.

Perhaps most significant given their function, DRAs are most closely related to proteins that act not on RNA, but on double-stranded DNA (**Fig. 1C**). The DRAs are relatives of the FANCM family of proteins that function during DNA repair, and these include Hef, FANCM, Mph1 and Fml1 ([www.rna-helicase.org/rig.htm](http://www.rna-helicase.org/rig.htm) database) (**Fig. 1 and Table 1**). Members of the FANCM family bind dsDNA and contain a similar  $\alpha$ -helical

**Table 1.** SF2 nucleic acid-dependent ATPases listed in this study

| Family     | NA         | SF2          | Organism <sup>a</sup> | Function                 | Sequence ID <sup>b</sup> | PDB Code <sup>c</sup>                          |
|------------|------------|--------------|-----------------------|--------------------------|--------------------------|--|
| DEAD       | RNA        | Eif4AIII     | hs                    | Exon junction complex    | P38919                   | 2J0Q, 2J0S, 2J0U, 2HYI, 2HXY                   |
| DEAD       | RNA        | VASA         | dm                    | Germ cell development    | P09052                   | 2DB3   |
| DEAD       | RNA        | Mss116       | sc                    | Group II intron splicing | P15424                   | 3I5X, 3I5Y, 3I61, 3I62                         |
| DEAD       | RNA        | Ddx19        | hs                    | Nuclear mRNA export      | Q9NUU7                   | 3EWS, 3G0H                                     |
| Swi/Snf    | DNA        | Rad54        | hs                    | DNA repair               | Q92698                   | 1Z3I, 1Z63, 1Z6A                               |
| Swi/Snf    | DNA        | RapA         | ec                    | Transcription regulation | P60240                   | 3DMQ   |
| Swi/Snf    | DNA        | Chd1         | hs                    | Chromatin remodeling     | O14646                   | 3MWY, 3TED, 2XB0                               |
| RecG       | DNA        | RecG         | ec                    | DNA repair               | P24230                   | 1GM5   |
| RecG       | DNA        | PriA         | ec                    | DNA replication          | P17888                   | None   |
| RecG       | DNA        | MFD          | ec                    | DNA repair               | P30958                   | None   |
| T1R        | DNA        | EcorA1       | ec                    | DNA restriction          | Q07736                   | 2W00 (Ecor124I)                                |
| T1R        | DNA        | EcorE1       | ec                    | DNA restriction          | Q47281                   | 2W00 (Ecor124I)                                |
| T1R        | DNA        | EcorK1       | ec                    | DNA restriction          | P08956                   | 2W00 (Ecor124I)                                |
| <b>DRA</b> | <b>RNA</b> | <b>RIG-I</b> | <b>hs</b>             | <b>Innate immunity</b>   | <b>Q95786</b>            | 2AY2, 4A2P, 4A2Q, 4A2W, 4A36, 3TBK, 2YKG, 3TMI |
| <b>DRA</b> | <b>RNA</b> | <b>MDA5</b>  | <b>hs</b>             | <b>Innate immunity</b>   | <b>Q9BYX4</b>            | None   |
| <b>DRA</b> | <b>RNA</b> | <b>LGP2</b>  | <b>hs</b>             | <b>Innate immunity</b>   | <b>Q96C10</b>            | None   |
| <b>DRA</b> | <b>RNA</b> | <b>Dicer</b> | <b>hs</b>             | <b>Gene silencing</b>    | <b>A9UPY3</b>            | None   |
| <b>DRA</b> | <b>RNA</b> | <b>DRH-1</b> | <b>ce</b>             | <b>Gene silencing</b>    | <b>G5EDI8</b>            | None   |
| <b>DRA</b> | <b>RNA</b> | <b>DRH-3</b> | <b>ce</b>             | <b>Gene silencing</b>    | <b>Q93413</b>            | None   |
| FANCM      | DNA        | FANCM        | hs                    | DNA repair               | Q8IYD8                   | None   |
| FANCM      | DNA        | MPH1         | sc                    | Genome stability         | P40562                   | None   |
| FANCM      | DNA        | Hef          | pf                    | DNA repair               | Q8TZH8                   | 1WP9   |

Notes: Members of DRA family are boxed and bolded. <sup>a</sup> Abbreviations: ce, *Caenorhabditis elegans*; dm, *Drosophila melanogaster*; ec, *Escherichia coli*; hs, *Homo sapiens*; sc, *Saccharomyces cerevisiae*; pf, *Pyrococcus furiosus*. <sup>b</sup> Sequence ID refers to the protein sequence taken from the UniProt Knowledgebase ([www.uniprot.org](http://www.uniprot.org)). <sup>c</sup> PDB Code refers to the structures available from Protein Data Bank ([www.rcsb.org](http://www.rcsb.org)).

HEL2i insertion as the DRAs.<sup>22</sup> Other closely related dsDNA binding proteins include members of the Swi/Snf, RecG and the T1R families (Fig. 1C and Table 1).<sup>4</sup> Swi/Snf proteins have a six  $\alpha$  helix motif that is inserted within two parts of the HEL2 domain. This motif interacts with the 5' strand of dsDNA and occupies the same position relative to HEL1 and HEL2 as the Hef and the RIG-I HEL2i domain.<sup>23-25</sup> Interestingly, RecG proteins have a TRG motif (translocation in RecG) located immediately after motif VI that forms a helical hairpin proposed to be a transmission system for driving double-stranded translocation.<sup>26</sup> This hairpin appears to be analogous to the pincer domain of RIG-I and other DRAs, although the pincer domain is significantly longer and more complex.<sup>27</sup> Similarly, members of the prokaryotic T1R family have an  $\alpha$ -helical domain at their C terminus that plays a role in recognizing foreign dsDNA, either from transmissible plasmids or from phages.<sup>28</sup>

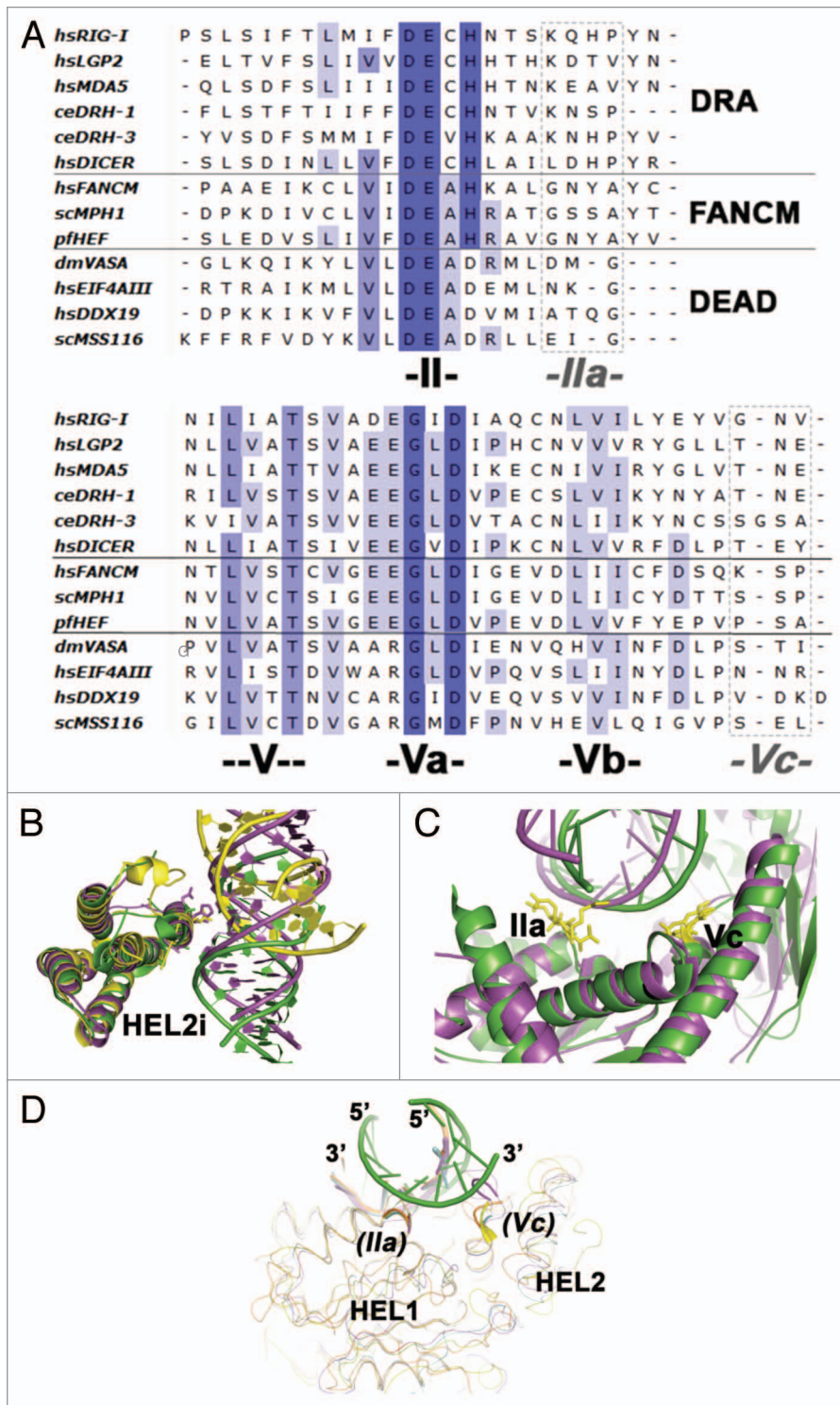
In addition to the conserved ATPase/Helicase core and the specialized RNA recognition and transduction domains, DRAs contain two semi-conserved motifs that contribute to binding of double-stranded nucleic acid (Fig. 2). In these proteins, motifs IIa and Vc form contacts with the 5'-3' "second strand," which contrasts with the other nucleic acid binding motifs that contact only the 3'-5' "tracking strand" that is bound by all translocative helicases. Motif IIa in particular was initially noted in the *Sulfolobus solfataricus* Rad54 structure and later within the motor subunit of the T1R restriction modification enzyme, EcoR124, from *Escherichia coli*.<sup>23,29</sup> Not surprisingly, motif IIa also appears to be present in members of the DEAD box family, and its functional role in duplex RNA binding is supported by recent structural studies of DEAD-box protein Mss116 bound to duplex RNA.<sup>30</sup> Motifs IIa and Vc are primarily conserved structurally

rather than in primary sequence (Fig. 2).<sup>17,23,29</sup> Although there are semi-conserved lysines and asparagines in motif IIa and a semi-conserved asparagine in motif Vc, the majority of the contacts made with nucleic acids involve the peptide backbone.

By contrast, RNA-dependent ATPases that function as helicase enzymes preferentially bind single stranded nucleic acid before unwinding adjacent duplex regions. Two distinct mechanisms of unwinding have been proposed, and these include melting of the RNA backbone through local distortions of the A-form duplex RNA, as hypothesized for the DEAD box family of helicases including Ded1p and Mss116p,<sup>31</sup> and displacement of adjacent duplex strands during translocation, as shown for the viral SF2 DExH helicases including NS3<sup>32</sup> and NPH-II.<sup>32,33</sup> In contrast to these bona fide helicases, DRAs preferentially bind RNA duplex instead of single stranded regions,<sup>16,34,35</sup> and in all existing structures of RNA-RIG-I complexes, the duplex RNA maintains an undistorted A-form conformation.<sup>17-19,36</sup> Furthermore, the crucial  $\beta$ -hairpin motif that participates in strand separation by DExH helicases is missing in RIG-I and other DRAs.<sup>17,37,38</sup> Therefore, because DRAs have no structural features designed to disrupt duplex RNA, and no structural motifs for coupling translocation with strand separation, it is not surprising that DRAs have not yet been shown to function as unwindases.

### The Molecular and Structural Biology of DRAs

Structural studies on nucleic acid-dependent ATPases are hampered by the intrinsic flexibility that arises from their function as molecular motors. In these proteins, the two conserved Rec-A like domains are loosely connected in the absence of nucleic acid and ATP. This is particularly true for DEAD box proteins, which tend



**Figure 2.** Sequence and structural features of RIG-I that contribute to duplex RNA recognition. (A) Sequence alignment of DRAs and other SF2 proteins. Notice motifs IIa and Vc (boxed in dotted lines) are not very conserved in amino acid sequence. (B) HEL2i domain juxtaposes with the duplex RNA backbone (PDB codes: 3TMI in green; 2YKG in yellow; 4A36, in magenta). (C) Specialized motifs IIa and Vc recognize the top strand of the duplex RNA (Bottom strand or tracking strand is the strand nucleic acid that binds to the SF2 $\alpha$  proteins; Top strand is the complementary strand). (D) Possible structural conservation of motif IIa and motif Vc found in DEAD-box RNA family members. Figure shows the aligned structures of DEAD-box protein:ssRNA complexes with duplex RNA (PDB codes: 2J05, 3I5X, 3G0H and 2DB3). The possible presence of motif IIa and Vc in DEAD-box proteins are labeled in parenthesis.

to be captured crystallographically only in the presence of both ssRNA and ATP analogs,<sup>39-43</sup> thereby limiting our understanding of their functional cycles. Structural studies of DRAs face the same challenges as those focused on DEAD box proteins. Adding to this difficulty, DRAs are large multidomain proteins with several moving parts that usually function within even larger protein complexes. Nevertheless, recent cryo-electron microscopy studies of Dicer<sup>44-46</sup> and crystallographic studies on RIG-I have advanced our understanding of the biological function and mechanical properties of DRA proteins.<sup>15,17-19</sup>

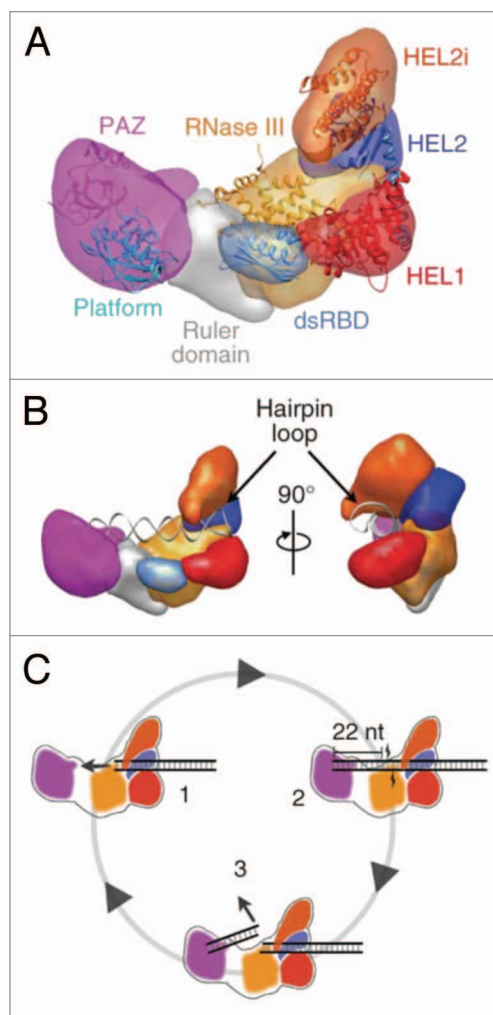
**Dicer: the small RNA processing machine.** Ever since Fire et al., published the groundbreaking paper on RNA interference (RNAi),<sup>47</sup> great strides have been made in understanding the biogenesis and functional mechanisms of the small RNAs that facilitate dsRNA-mediated gene regulation.<sup>1,48-53</sup> There are two major types of small RNAs: microRNA (miRNA) and small interfering RNA (siRNA). While these two RNAs differ in their pathway of biogenesis, they share similarities in function. Of central importance to the RNAi pathway is the formation of an RNA-induced silencing complex (RISC),<sup>54,55</sup> which binds to the target mRNA and results in downregulation of gene expression by either RNA degradation or translational arrest.<sup>1,52</sup> RISC is assembled from the RISC-loading complex (RLC), which includes dsRNA, Ago, Dicer and other additional dsRNA binding and accessory proteins.<sup>56,57</sup>

Dicer plays a major role in gene regulation by processing dsRNA precursors into short fragments that are used to target the silencing of specific genes.<sup>58-61</sup> Dicer cleaves long duplex precursor RNAs (pre-miRNA or pre-siRNA) into short miRNA and siRNA fragments and then loads the correct “guide” strand into RISC. Dicer received its name because of its dsRNA cleavage, or “dicing” activity.<sup>58</sup> Phylogenetically, Dicer is a class III RNase, members of which are conserved among eukaryotic species.<sup>62</sup> In humans, there is only one Dicer protein, *hsDicer1*, however *Drosophila* and plants contain two and four Dicer proteins respectively. Human Dicer1 mutations have been found in various cancer syndromes,<sup>63-65</sup> emphasizing its fundamental roles in gene regulation. In general, all Dicer and Dicer

like proteins (DCL) from eukaryotic species share a similar domain architecture, containing a SF2 RNA-dependent ATPase domain at the N terminus, a DUF283 domain (Domain of Unknown Function), a PAZ domain, two tandem RNase III domains and a dsRNA-binding domain (dsRBD) at the C terminus<sup>14,44</sup> (Fig. 1B). The first structural insights into Dicer components came from a crystal structure of a Dicer homolog obtained from the unicellular eukaryote *Giardia intestinalis*. This structure revealed a specific spatial arrangement of the PAZ domain relative to the two RNaseIII domains,<sup>66</sup> suggesting that Dicer contains a molecular ruler that enables it to generate dsRNA fragments of specific length. Unfortunately, unlike Dicer genes from other organisms, *Giardia intestinalis* Dicer does not contain an ATPase motor domain.

The ATPase motor domain of Dicer is highly conserved across species and it is phylogenetically distinguishable as a DRA protein (Fig. 1C). The precise biochemical function of the motor domain is still unclear, and it is not yet known whether the RNA-dependent ATPase activity is actually linked to duplex unwinding, and whether the motor domain behaves like a helicase. Recent studies have indicated that it plays a role in helping to select the “guide strand” from the two duplex strands that are initially bound within the RISC-loading complex. This is accomplished by sensing thermodynamic features of the RNA duplex, and determining which terminus is more easily opened.<sup>67</sup> The selected siRNA guide strand will then be loaded into the Ago protein, resulting in formation of a functional RISC complex.<sup>67</sup>

The overall three-dimensional architecture and domain organization of Dicer is well conserved among orthologs.<sup>14,44</sup> Dicer adopts an L-shape as determined by negative-stain electron microscopy (EM).<sup>44</sup> Using a streptavidin tagging method and domain deletion constructs, Lau et al. accurately located the position of the motor domain at the base of the L shaped structure (Fig. 3). Furthermore, when the motor domain of the RIG-I was docked into the EM structure of Dicer, the RNA binding interfaces of the motor domain and the RNase III domain creates an adjacent central RNA binding groove.<sup>44</sup> A complex between Dicer and its TRBP (TAR RNA Binding Protein, an accessory protein of Dicer and a dsRBD) forms a similar L shape with a long edge of 150 Å and a 100 Å extension at the bottom end.<sup>45</sup> Because of the small size and intrinsic flexibility of TRBP, it is difficult to accurately assign its location, particularly in the absence of a siRNA or miRNA substrate. A low resolution EM structure of the human RISC-loading complex (containing Dicer, AGO2 and TRBP in a 1:1:1 stoichiometric ratio) was obtained by cross-linking the complex. In the resulting model, AGO2 was proposed to interact with the C-terminal region of Dicer.<sup>68</sup> The RNA binding site of AGO2 is located in close proximity to the C-terminal region of Dicer, pointing away from the N-terminal motor domain. This model is consistent with biochemical data suggesting that the motor domain of Dicer may not be required for loading mature siRNA into the AGO2.<sup>69</sup> Two discrete conformations of the Dicer motor domain have been identified, suggesting that it may adopt multiple conformations on dsRNA.<sup>44</sup> This structural flexibility contributes to specific dsRNA recognition and may support a processive dicing mechanism (Fig. 3).<sup>35,70,71</sup> Not surprisingly, it



**Figure 3.** (A) Segmented map of human Dicer with crystal structures of homologous domains docked. (B) Model for pre-miRNA recognition. A pre-miRNA hairpin is modeled into the proposed binding channel of Dicer, with the stem-loop fit into the RNA-binding cleft of the protein. (C) Schematic for processive dicing in which dsRNA is translocated into the nuclease core (1). The PAZ domain (purple) recognizes the dsRNA end, positioning RNase III (orange) for cleavage (2). The siRNA product is released while the dsRNA substrate remains bound to the protein (3). Reprinted with permission from Lau et al.<sup>44</sup>

is similar to structural rearrangements observed when the RIG-I motor domain binds to RNA duplex.<sup>18,19</sup>

**RIG-I: The innate immune sensor for viral RNA detection and defense.** A diverse group of cytoplasmic surveillance proteins sensitively detect the presence of viral genomes and gene products and then initiate inflammatory responses that enable vertebrates to fight viral infections.<sup>72,73</sup> These proteins form the foundation of our innate immune response. The RIG-I-like receptors (RLRs) are a specialized subclass of DRA proteins that detect double stranded viral RNAs in the cytoplasm and initiate a series of signaling events to elicit an antiviral response. The RLR motor proteins include RIG-I,<sup>74</sup> MDA5 (Melanoma Differentiation Associated gene 5)<sup>34</sup> and LGP2 (Laboratory of Genetics and Physiology 2).<sup>75</sup> They were initially identified in

different biological contexts and were later re-discovered to be key members in antiviral innate immunity.<sup>72,73,76</sup> RIG-I is the most extensively studied member of the RLRs and has been demonstrated to be the major antiviral RLR. RIG-I recognizes a broad range of viruses, including negative stranded viruses, e.g., vesicular stomatitis virus, Sendai virus, influenza virus and rabies virus; positive stranded viruses such as dengue virus, Japanese encephalitis virus, West Nile virus and Hepatitis C virus; dsRNA virus (reovirus) and DNA virus (Epstein-Barr virus).<sup>3,77</sup> MDA5 is both structurally and functionally similar to RIG-I and complements RIG-I by recognizing a distinct set of virus RNAs although there might be some overlap.<sup>3</sup> LGP2 is thought to serve as a feedback regulator but its exact function is still not clearly defined.<sup>78,79</sup>

RIG-I contains two tandem caspase activation and recruitment domains (CARDs; CARD1 and CARD2) at its N-terminus, which mediate a downstream signaling relay; a central DRA motor domain, and a C-terminal domain (CTD) that facilitates viral RNA recognition (Fig. 1B).<sup>15,17-19,80</sup> It is commonly believed that RIG-I is inactive in resting cells and it is activated upon detection and binding of viral RNA. The activated RIG-I is believed to hydrolyze ATP and initiate a signaling cascade and type I interferon (IFN) response via the adaptor protein MAVS, also known as IPS-1, VISA or CARDIF.<sup>3</sup> MAVS in turn activates several transcription factors including IRF3, IRF7 and NF- $\kappa$ B, and leads to the production of IFN and inflammatory cytokines.<sup>77,80,81</sup> Moreover, RIG-I displays apoptosis-inducing properties in tumor cells.<sup>82,83</sup> Effective therapeutic RIG-I antagonists and agonists may provide new tools for the treatment of viral infections and cancer.<sup>84</sup>

Recent research has focused on characterizing the molecular determinants for RNA-RIG-I recognition, the mechanisms of activation and signaling, and regulatory pathways that help control RIG-I signaling. Structural and biochemical studies on RIG-I have revealed that 5' tri-phosphorylated blunt-ended duplex RNAs are the optimal substrate for RIG-I binding and activation.<sup>17-19,36,73,85,86,120</sup> The exact length of the duplex is unclear although it is generally accepted that RIG-I recognizes RNA that is ten to hundreds of base pairs in length, while MDA5 forms filaments on longer RNA in the thousands of base pairs.<sup>85,87-90</sup> The CTD is primarily responsible for 5' tri-phosphate recognition and both the CTD and helicase domain form critical contacts with the RNA duplex.<sup>15,18,19,85,91-93</sup> The latest structural studies indicate RNA binding induces a dramatic conformational change in RIG-I (Fig. 4A and B). The role of ATP binding and hydrolysis has not been determined, although mutations in the ATPase domain are clearly deleterious to function.<sup>15,18,19</sup> Post translational modifications of RIG-I, including ubiquitination, phosphorylation and SUMOylation, have been reported to be important for its function.<sup>94-96</sup> Non-covalent polyubiquitin binding to the CARDs is likely to be essential for full activation of RIG-I and possibly oligomerization.<sup>97,98</sup>

In the resting state, RIG-I adopts an autoinhibited conformation in which the motor domain is sterically blocked.<sup>15,18</sup> The CARDs are trapped in a fixed conformation relative to the HEL domains (synonymous with Rec-A folds 1 and 2) through an interaction between the second CARD and the insertion domain (HEL2i) (Fig. 4A).<sup>18</sup> This conformation was speculated to inhibit

both CARD1 ubiquitination by the ubiquitin E3 ligase TRIM25 and non-covalent binding of polyubiquitin to the CARDs, both of which are required for RIG-I activation. In the apoenzyme state the RNA binding surfaces of RIG-I (and particularly the CTD) are largely exposed, allowing RIG-I to search for viral RNAs. The CTD, which is connected to the HEL domain through a long and flexible pincer domain, enhances the specificity of RIG-I for tri-phosphorylated RNA.<sup>18</sup>

It is believed that viral activation of RIG-I signaling occurs in a carefully choreographed sequence of events. Binding of viral RNA is the initial trigger for RIG-I activation, whereupon the motor domain (comprised of HEL1, HEL2 and HEL2i) of RIG-I forms a ring-shaped clamp around the sugar-phosphate backbone of the duplex and the CTD caps the helical terminus, even in the absence of a 5' triphosphate. The tight and specific interaction of the CTD with the duplex terminus may prevent RIG-I from binding with high affinity to internal sites on the duplex<sup>17-19,36,120</sup> (Fig. 4B). Structural analysis suggests that binding of RIG-I to RNA alone may not be sufficient to disrupt the autoinhibitory interaction between the CARD2 and HEL2i domains,<sup>19</sup> hinting that an additional trigger might be needed to activate signaling. In the crystal structures of RIG-I:dsRNA with AlFx and BeFx, ATP binding appears to bring the RIG-I helicase into a more closed and compact conformation relative to RIG-I structures that contain only dsRNA (Fig. 4C).<sup>17,18,36</sup> This ATP-induced conformational change shifts the CTD and HEL2i toward each other, resulting in a clash between the CARDs and CTD (Fig. 4C). Consequently the structure is likely to reorganize, reorienting the relative positions of the CARDs and HEL2i, and potentially releasing the CARDs which makes them available for interaction with MAVS and activates the innate immune response (Fig. 4D).<sup>97,98</sup>

In agreement with this idea, ATP is required in vitro reconstitution of the RIG-I signaling pathway,<sup>97,99</sup> although ATP hydrolysis and turnover is not essential.<sup>100</sup> Activation of RIG-I is therefore a tightly-regulated, multi-checkpoint process, starting with recognition of the correct RNA substrate, followed by ATP binding, and then subsequent coupled structural rearrangements that release auto-inhibition and switch RIG-I into a signaling-competent state (Fig. 4).<sup>15,101</sup>

**DRH-3: Attenuating the siRNA pathway in *Caenorhabditis elegans*.** A group of endogenous siRNAs, named 22G endo-siRNA, from *Caenorhabditis elegans* are linked to a variety of biological processes that are vital to maintaining genetic stability, including transposon silencing and chromosome segregation in germline cells.<sup>102-104</sup> Defects in the endo-RNAi pathway can result in many forms of genetic instability such as loss of chromosomes during mitosis, abnormal gene expression and increased sensitivity to X-ray irradiation.<sup>102-105</sup> These siRNAs are classified as secondary siRNA molecules because they are produced directly by RNA-dependent RNA polymerase (RdRP) transcription, without a double-stranded RNA intermediate or cleavage.<sup>106,107</sup>

Dicer-related helicase 3 (DRH-3) is a large multi-domain, multi-functional protein that is essential for the biogenesis of these endogenous secondary siRNAs.<sup>12,13,103,104</sup> DRH-3 interacts with members of the *C. elegans* RNAi machinery, including Dicer (DCR-1) and the RdRP, RRF-1.<sup>12,13</sup> A large protein (1119 amino

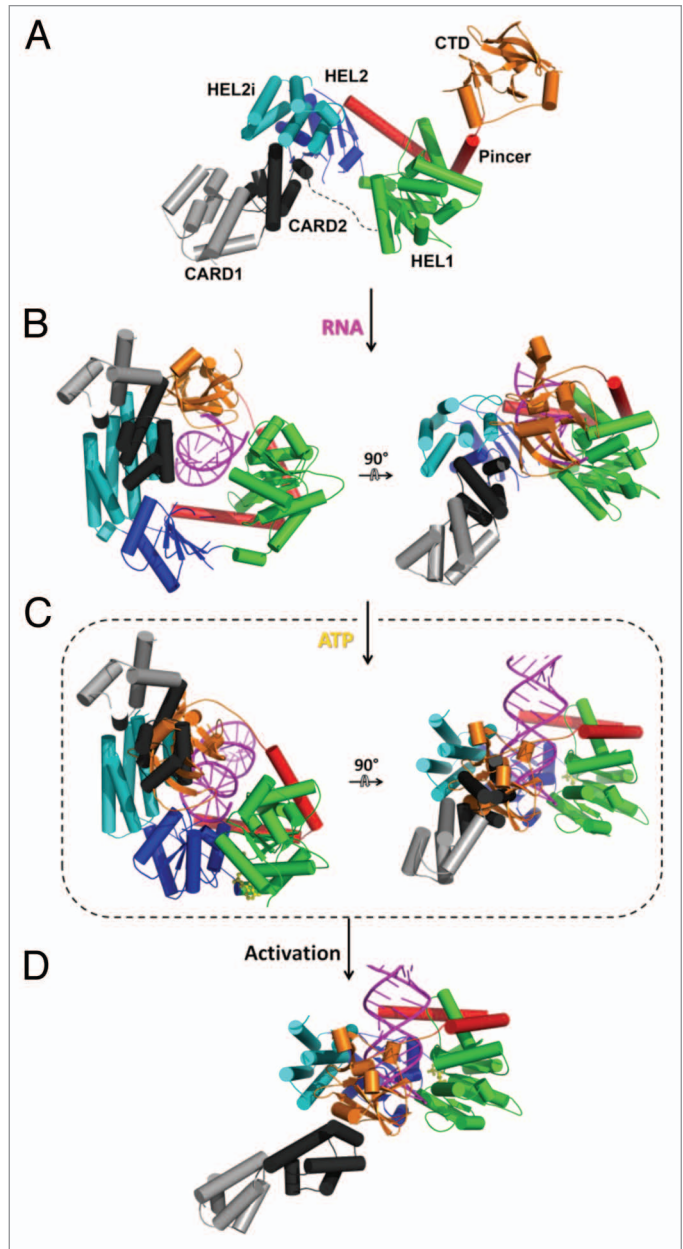
acids, ca. 130 kDa), DRH-3 contains 3 sub-domains (Fig. 1B). These include an N-terminal domain of novel sequence that lacks a known homolog, and the central motor domain that is common to DRA proteins.<sup>5,10,108</sup> The C-terminal domain of DRH-3 shares sequence similarity with the CTD that plays an important role in RNA and triphosphate recognition by RIG-I. Although there are no structural studies of DRH-3, a preliminary biochemical characterization has been reported. Several key features of DRH-3 are now apparent: the protein binds more strongly to dsRNA than to ssRNA; potent ATP hydrolysis by DRH-3 is only stimulated by dsRNA; DRH-3 does not have unwinding activity.<sup>16</sup> DRH-1, a homolog of DRH-3, is implicated in both germline and somatic RNA interference (RNAi) pathways as well as virus sensing and viral siRNA formation<sup>109,110</sup> and may be an equally important target for future biochemical and structural studies.

DRH-3 has a domain organization that is very similar to RIG-I. It is tempting to speculate that DRH-3 might bind to the RNA duplexes generated by the endogenous siRNA pathway and recruit signaling partners through its NTD. This speculation is further supported by the absence of helicase activity and the preference for canonical RNA duplex binding.<sup>16</sup>

### Concluding Remarks and Future Directions for Research on DRA Proteins

DRA proteins share several characteristic features that distinguish them from other groups of RNA-dependent ATPases. First, in addition to the conserved motifs that classify DRAs as SF2 RNA-dependent ATPases, DRAs contain unique motifs (e.g., motifs IIa and Vc) and domains (HEL2i) that specialize in duplex RNA recognition. Second, although the literature on DRAs is somewhat unclear on this point, DRAs do not appear to possess RNA unwinding activity and they may accomplish their biological function by simply binding duplex RNA or by translocating along the duplex without unwinding. Lastly, the DRAs discussed in this review are all part of larger protein complexes that function in duplex RNA sensing and processing.

One of the most intriguing questions about DRAs is whether they, in fact, require ATP hydrolysis for function. At the present time, it is not established that DRAs require ATP binding and/or hydrolysis and, like DEAD-box proteins, they may only utilize ATP for recycling. ATPase activity is unnecessary for pre-miRNA processing by human Dicer,<sup>35,69,111</sup> but in contrast, *Drosophila* Dicer-2 appears to require ATP for siRNA production.<sup>55,112</sup> The ATPase motor domain from *C. elegans* DCR is required for the biogenesis of some but not all siRNAs.<sup>113</sup> Evidently, there is no consensus for the function of ATP hydrolysis by Dicers from different species. RIG-I has shown a clear dependence on ATP for in vitro reconstitution,<sup>97,98</sup> but mutagenesis studies by Bamming and Horvath suggest that signaling by RIG-I and MDA5 can occur independent of ATPase enzymatic activity.<sup>100</sup> To reconcile this, recent structural data suggests that ATP binding but not necessarily hydrolysis induces a conformational change on the RIG-I helicase domain that may eventually lead to RIG-I activation (Fig. 4). Further experiments are needed to verify this structure-driven hypothesis.



**Figure 4.** Structural basis for dsRNA recognition and activation of RLRs. Models were created by aligning and merging known duck and human RIG-I structures and considering our recent solution hydrodynamic studies on RIG-I conformational dynamics upon RNA and ATP binding.<sup>36</sup> (A) Model of full length RIG-I apoenzyme based on structures of duck RIG-I (PDB: 4A2W) and the CTD (PDB: 4A2V). In the autoinhibited conformation, the N-terminal CARDs are sequestered from signaling and maintain RIG-I in an autoinhibited state. (B) RIG-I switches into a semi-closed conformation upon RNA binding. Binding of dsRNA to the CTD brings the HEL domains in contact with dsRNA (PDB: 4A2W and 2YKG). (C) ATP binding (yellow) closes the HEL domains and causes a clash between the CARDs and CTD (PDB: 4A2W, 4A36 and 2YKG). (D) The change in conformation upon dsRNA and ATP binding releases the CARD domains for signaling.

Although the recent crystal structures of RIG-I advance our understanding of DRAs, there are several questions that remain unanswered. One question is whether DRAs recognize specific RNA sequences or structures. Several studies suggest that Dicer

recognizes specific terminal structures of RNA as evidenced by the fact that human dicer is more efficient in processing pre-miRNA than siRNA.<sup>71</sup> Furthermore, *Drosophila* Dicer-1 recognizes the terminal loop structure of pre-miRNAs through its motor domain,<sup>70</sup> and both *Drosophila* Dicer-2 and *C. elegans* DCR-1 differentiate the end structures of long duplex RNAs for endo-siRNA processing.<sup>114</sup> This suggests there might be a special structural feature in the Dicer RNA-dependent ATPase domain that is responsible for recognizing RNA ends. RIG-I specifically recognizes tri-phosphorylated RNA through its accessory CTD domain, and given that RIG-I recognizes a broad but distinct set of RNA viruses, it will be interesting to determine if RIG-I can recognize unique viral RNA sequences or structures, in addition to 5' triphosphate and duplex RNA.

Oligomerization is a variable characteristic that can be important for the function of SF2 proteins, including DRAs. To generalize, the minimum functional unit of SF2 proteins is monomeric, but there is biochemical evidence suggesting that oligomerization can enhance biological activity of certain SF2 proteins.<sup>115,116</sup> As for DRAs, there is no indication that Dicer forms oligomers, however RIG-I has been proposed to dimerize<sup>93</sup> or tetramerize<sup>97</sup> upon activation. The exact molecular basis for RIG-I oligomerization is still unknown; however the downstream target of RIG-I, MAVS, forms filaments upon activation. Distinct from RIG-I, MDA5 cooperatively binds to long duplex RNAs and may form a filament-like structure itself,<sup>115,117</sup> which would be unique not

only among DRAs but also SF2 proteins in general. It is therefore important to establish whether oligomerization is obligatory for RIG-I or MDA5 function.

Given the central role of DRA proteins in diverse cellular pathways that range from epigenetic regulation to the innate immune response, it will be interesting to characterize the regulatory cofactors that help to specify and control the activity of DRAs. It will also be exciting to identify new DRA proteins that have distinct molecular functions. The DRA proteins seem to be markers of interesting biology, and investigation of this protein family will continue to yield major insights into the nanomechanical features of living systems.

An informative review on the molecular mechanism of RIG-I activation was published while this manuscript was under review, which is agreeable to our model (see ref. 121).

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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