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From unwinding to clamping — the DEAD box RNA helicase family

Patrick Linder and Eckhard Jankowsky‡*

Abstract | RNA helicases of the DEAD box family are present in all eukaryotic cells and in many bacteria and Archaea. These highly conserved enzymes are required for RNA metabolism from transcription to degradation and are therefore important players in gene expression. DEAD box proteins use ATP to unwind short duplex RNA in an unusual fashion and remodel RNA–protein complexes, but they can also function as ATP-dependent RNA clamps to provide nucleation centres that establish larger RNA–protein complexes. Structural, mechanistic and molecular biological studies have started to reveal how these conserved proteins can perform such diverse functions and how accessory proteins have a central role in their regulation.

Spliceosome
A large, dynamic complex that is composed of RNA and proteins and is involved in excising introns and joining the exons of pre-mRNA.

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Nearly all aspects of RNA metabolism, from transcription and translation to mRNA decay, involve RNA helicases, which are enzymes that use ATP to bind or remodel RNA and RNA–protein complexes (ribonucleoprotein (RNP) complexes)[1]. RNA helicases are found in all three domains of life, and many viruses also encode one or more of these proteins[2,3]. Together with the structurally related DNA helicases that function in replication, recombination and repair, the RNA helicases are classified into superfamilies and families, based on sequence and structural features[3,4]. DEAD box proteins form the largest helicase family, with 37 members in humans and 26 in *Saccharomyces cerevisiae*[3], and are characterized by the presence of an Asp-Glu-Ala-Asp (DEAD) motif.

DEAD box helicases have central and, in many cases, essential physiological roles in cellular RNA metabolism[5] (FIG. 1). The proteins generally function as part of larger multicomponent assemblies, such as the spliceosome or the eukaryotic translation initiation machinery[6]. Mutations and deregulation of several DEAD box proteins have been linked to disease states, including cancer[7]. Although all DEAD box proteins contain a structurally highly conserved core with conserved ATP-binding and RNA-binding sites, different proteins have been associated with diverse and seemingly unrelated functions, including the disassembly of RNPs, chaperoning during RNA folding and even stabilization of protein complexes on RNA[5,6]. How these highly conserved proteins fulfil such an array of different functions has been a long-standing question.

Research has now started to illuminate the molecular basis for this functional diversity. In this Review, we summarize how structural data, together with biochemical and biophysical studies, have revealed unexpected modes by which these proteins function. We also discuss how novel molecular and cell biological approaches have better defined the cellular roles of DEAD box helicases. We outline our current view on common structural and mechanistic themes that have emerged, and on physical models of how these 'unusual' RNA helicases work.

Structures of DEAD box RNA helicases

A number of structural studies have universally shown that members of the DEAD box family contain a highly conserved helicase core that harbours the binding sites for ATP and RNA[3,4,8]. The core is surrounded by variable auxiliary domains, which are thought to be critical for the diverse functions of these enzymes.

Structure of the helicase core. DEAD box proteins belong to helicase superfamily 2 (SF2)[2]. Similarly to all SF2 helicases, DEAD box proteins are built around a highly conserved helicase core of two virtually identical domains that resemble the bacterial recombination protein recombinase A (RecA)[3,4,8] (FIG. 2a,b). Within this helicase core, at least 12 characteristic sequence motifs are located at conserved positions (FIG. 2a,b). Some of these motifs are conserved across the entire SF2 family, whereas others are found only in the DEAD box family[3]. Motif II, which contains the Asp-Glu-Ala-Asp motif, inspired the name DEAD box for this family[9]. Although the helicase motifs are highly characteristic for DEAD box proteins, some are slightly altered in individual proteins[3]. To determine whether a given protein belongs to the DEAD box family, one has to consider all of the motifs in their entirety[10].

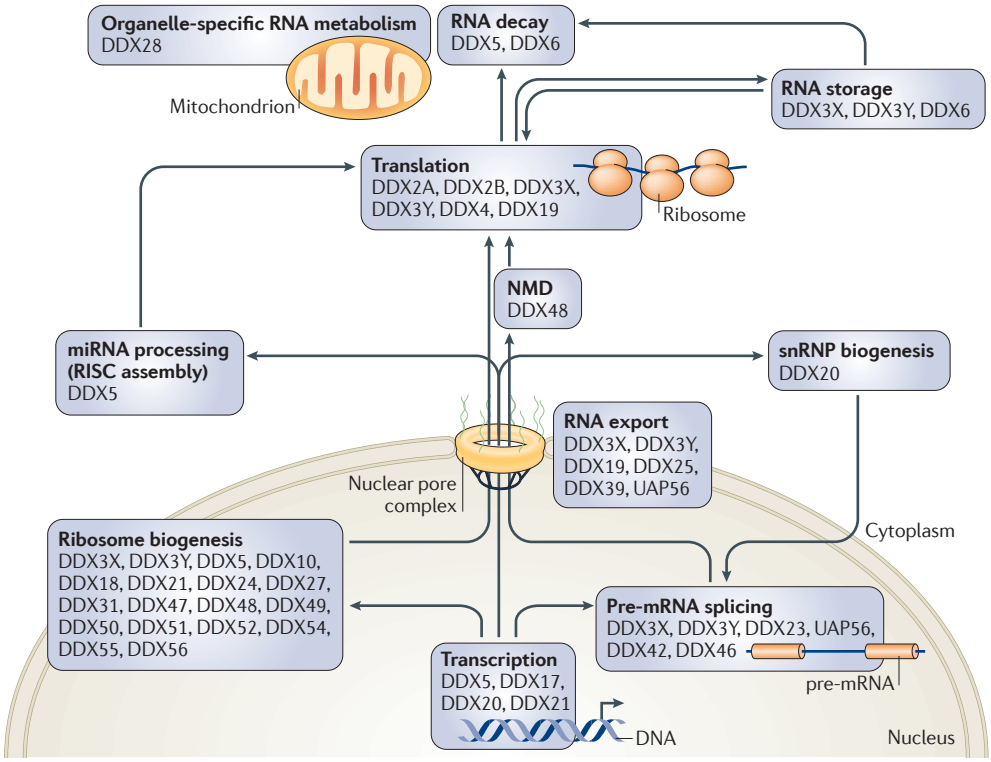


Figure 1 | **Cellular processes involving DEAD box proteins.** RNA helicases of the DEAD box family are involved in various different steps in RNA metabolism. In the nucleus, these include ribosome biogenesis, transcription and pre-mRNA splicing. In the cytoplasm, these include processes like microRNA (miRNA) processing, nonsense-mediated decay (NMD) and protein translation, as well as organelle-specific RNA metabolism. At the interface between the nucleus and the cytoplasm, these enzymes are required for the directional transport of mRNA molecules. Human DEAD box proteins that are involved in these processes are listed; many have been shown, by interaction studies or genetic screens, to be involved in several processes. DDX, DEAD box; RISC, RNA-induced silencing complex; snRNP, small nuclear ribonucleoprotein.

The two helicase domains form a cleft that harbours the ATP-binding site, which is located between the two domains. The associated RNA binds opposite the ATP-binding site, across both domains (FIG. 2b). A host of structural and biochemical work has defined the residues and motifs involved in ATP and RNA binding (FIG. 2c,d). All of these studies show that the cleft between the two domains must be closed to productively bind and hydrolyse ATP[11]. In addition, the studies show how the functional groups that are involved in ATP binding and hydrolysis must be arranged in a highly defined manner (FIG. 2a,b). This arrangement explains the strong conservation of the amino acids involved in ATP binding and hydrolysis (FIG. 2). Many residues of the ATP-binding site form a complex network of interactions[12,13], which makes it a challenge to generate DEAD box protein mutants that are deficient in ATP hydrolysis but intact in ATP binding.

All of the described structures of DEAD box proteins in the presence of RNA have revealed a highly conserved mode of RNA binding[13–18]. The bound RNA is characteristically bent into a conformation that differs from that seen for nucleic acids bound to most other SF2 and SF1 helicases[14]. The helicase core establishes contacts to the RNA that cover five nucleotides and involve exclusively the sugar phosphate backbone of the RNA (FIG. 2d). Several RNA contacts are made by the protein backbone, explaining why some RNA-binding motifs show low conservation (FIG. 2d). The RNA-binding modules in both helicase domains are highly similar to each other and are suggested to have evolved from an anion-binding module[19]. How the RNA-binding and ATP-binding sites communicate is not clear, but several studies have started to focus on this question. In addition to motifs III and IVa, other conserved residues may be important for this, including the characteristic Phe in motif IV[20].

Structure and orientation of auxiliary domains. In contrast to other helicase families, DEAD box proteins generally do not have other domains inserted into the helicase core[3]. Mammalian DEAD box 24 (DDX24) and DDX1 (and its orthologues) are exceptions to this rule and have insertions in the amino-terminal helicase domain[3]. Flanking the helicase core, all DEAD box proteins contain carboxy-terminal and N-terminal domains, ranging from a few to several hundred amino acids in length[3]. In general, these domains are thought to allow interaction with other proteins or with RNA targets. As most structural studies have been performed on truncated proteins that often encompass little more than the helicase core, structural data for terminal domains are limited. However, the structures of some terminal domains have been determined separately, including the C-terminal domain of YxiN, an orthologue of *Escherichia coli* DbpA, which forms an RNA recognition motif[21] (RRM), and the

terminal domains of bacterial HerA, which fold into an oligomerization module[22] and an RRM[23].

How terminal domains are arranged in relation to the helicase core has only been studied for a limited number of proteins. The helical C-terminal domain of Mss116 extends the RNA-binding site and introduces a second bend in the bound RNA[13]. The arrangement of the oligomerization domain in bacterial HerA has been mapped[22], and fluorescence studies on bacterial YxiN have determined the location of the terminal RRM relative to the helicase core[24]. Each of the terminal domains analysed so far is oriented differently with respect to the helicase core[11].

A range of biochemical activities

Despite the high structural conservation of the helicase core, DEAD box proteins have been associated with an intriguing array of cellular functions, from disassembling RNPs to serving as immobile RNA clamps[1,25]. Similarly to most proteins involved in RNA metabolism, DEAD box proteins usually function within complexes containing dozens or even hundreds of components, such as the spliceosome or the nascent ribosome. It has not been possible to reconstitute either of these complexes, and mechanistic studies are therefore restricted to DEAD box proteins working in isolation or in the presence of very few cofactors. Nevertheless, a remarkable range of ATP-dependent and ATP-independent activities has been reported for DEAD box proteins. Although the extent to which the biochemical features measured in isolation translate into physiological function is not well understood, knowledge of biochemical activities has been essential for devising physical models of how DEAD box proteins function.

Local duplex unwinding. RNA unwinding measured *in vitro* with defined model substrates is considered an excellent proxy for the ATP-dependent binding and remodelling of more complex RNA and RNP structures that DEAD box proteins are thought to perform in the cell during processes such as ribosome biogenesis or pre-mRNA splicing[1,26]. DEAD box proteins are bona fide helicases that use ATP to bind and unwind RNA duplexes[27]. However, appreciable unwinding is restricted to duplexes with fewer than two helical turns, and many DEAD box helicases unwind only duplexes with fewer than 10 or 12 base pairs[6]. Unwinding efficiency also decreases with increasing duplex stability[28,29]. Single-stranded extensions to duplexes, at either the 3′ or 5′ end, stimulate unwinding by many DEAD box proteins[1,6,30,31] but in a few instances extensions are not required — at least *in vitro* — for optimal activity[31].

DEAD box proteins differ from other DNA and RNA helicases in that they show no strict unwinding polarity[32]. It has become clear that this is because DEAD box proteins do not unwind duplexes based on translocation on the RNA[1,6,28,30,31,33]. Instead, DEAD box proteins load directly onto the duplex region and then pry the strands apart in an ATP-dependent fashion[30,34]. This distinct unwinding mode is termed local strand separation[34] (BOX 1). Single-stranded tails aid the loading of the DEAD box protein onto the duplex[30,34]. Notably, these single-stranded regions do not need to be covalently attached to the duplex but must be held in close proximity[30]. For some DEAD box proteins, loading seems to involve multiple protomers of these proteins[34]. Loading of certain DEAD box proteins (for example, CYT19 from *Neurospora crassa* and bacterial DbpA) can also be facilitated by structured RNA[6,33].

Despite quantitative distinctions between different helicases, which may affect their specific biological functions, unwinding by local strand separation is exceptionally well suited for the RNA or RNP remodelling that many DEAD box proteins are thought to conduct in the cell[26]. Their highly localized unwinding mode prevents large-scale unravelling of carefully assembled RNA or RNP structures, and the efficient unwinding of short duplexes is adapted to the separation of duplexes in physiological RNAs and RNPs, which rarely exceed one helical turn. Typical examples of local unwinding are thought to occur during processes that involve guide RNAs (gRNAs), such as the unwinding of small nuclear RNA (snRNA) in pre-mRNA splicing, small nucleolar RNA (snoRNA) in ribosome biogenesis or gRNA in mitochondrial RNA editing[35–38].

ATP utilization in duplex unwinding. Each RNA unwinding event involves only a single ATP molecule, regardless of duplex length[39,40]. However, not every cycle of ATP binding and hydrolysis leads to a strand separation event[39,40] (BOX 1). In addition, most of the DEAD box proteins tested to date require only ATP binding for strand separation, and not ATP hydrolysis[39,41,42]. Instead, ATP hydrolysis is necessary for the fast release of DEAD box proteins from the RNA and thus for substrate turnover[40,42,43]. However, strand separation by bacterial DbpA was reported to be more strongly coupled to ATP hydrolysis than to ATP binding[40]. How can strand separation be coupled to ATP binding for some DEAD box proteins but to ATP hydrolysis for others? To reconcile these observations, it is important to note that the chemical ATP hydrolysis step is fast and reversible, whereas the release of phosphate or ADP is rate limiting for the overall ATPase cycle in the presence of RNA[43]. Therefore, each ATP bound in the catalytic centre can undergo multiple cycles of hydrolysis and reformation before release of the hydrolysis products[43,44]. Strand separation thus appears to be primarily connected to the presence of ATP in the catalytic core, regardless of whether or not it is hydrolysed[45]. As coordination among ATP binding, strand separation, ATP hydrolysis and phosphate or ADP release probably varies for different DEAD box proteins, strand separation may be coupled to ATP hydrolysis in some cases and to ATP binding in others[40,42].

Structural changes during the ATP cycle. Several structural changes accompany different stages of the ATP binding and hydrolysis cycle of DEAD box proteins. It is well established that the two RecA-like domains of the helicase core move relatively freely with respect to each other in the absence of RNA and ATP[26]. Kinetic data suggest that ATP binding in the presence of RNA

Small nuclear RNA
(snRNA). RNA molecules that serve as guides during pre-mRNA processing.

Small nucleolar RNA
(snoRNA). RNA molecules that serve as guides during pre-ribosomal RNA modification.

Mitochondrial RNA editing
Guide RNA-assisted insertion and modification of the sequence of mitochondrial mRNA in trypanosomes.

occurs in two distinct kinetic steps[43]. Whether these two steps correspond to discrete structural changes is not yet clear. Structures of ATP analogues and RNA bound together captured highly similar conformations of different DEAD box proteins[13–16,18,44]. It is thought that these structures correspond to the conformation that is acquired after the second step of ATP binding and represent the state following the strand separation event[13]. In this conformation, the two RecA-like domains are in a closed conformation[13–16,18,44] and thus complete and organize the ATP-binding and RNA-binding sites (FIG. 2). Structures of DEAD box proteins with RNA bound in the presence of a transition state analogue show virtually no changes in the RNA-binding site, compared with structures in which RNA is bound in the presence of ATP ground state analogues, and only the expected small alteration of the ATP-binding site is seen[13,44]. This is consistent with the extraordinarily tight RNA binding of DEAD box proteins with both ATP ground and transition state analogues[42]. Similar structures with ATP ground and transition state analogues are also consistent with the notion that these conformations represent states after the strand separation event[13]. ADP reduces the affinity of DEAD box proteins for RNA, and thus promotes dissociation of the protein from the substrate[46].

The available structural and mechanistic data provide a coherent picture of the conformations that DEAD box proteins have at major stages of the ATP binding and hydrolysis cycle, although the structural transition accompanying duplex opening by DEAD box proteins is still elusive. Importantly, tight, ATP-dependent RNA binding and duplex unwinding are readily accomplished by the same cycle of conformational changes[13,44]. Arrest of the cycle before the hydrolysis products dissociate can create an immobile RNA clamp[44], whereas progression of the cycle promotes repeated RNA unwinding events.

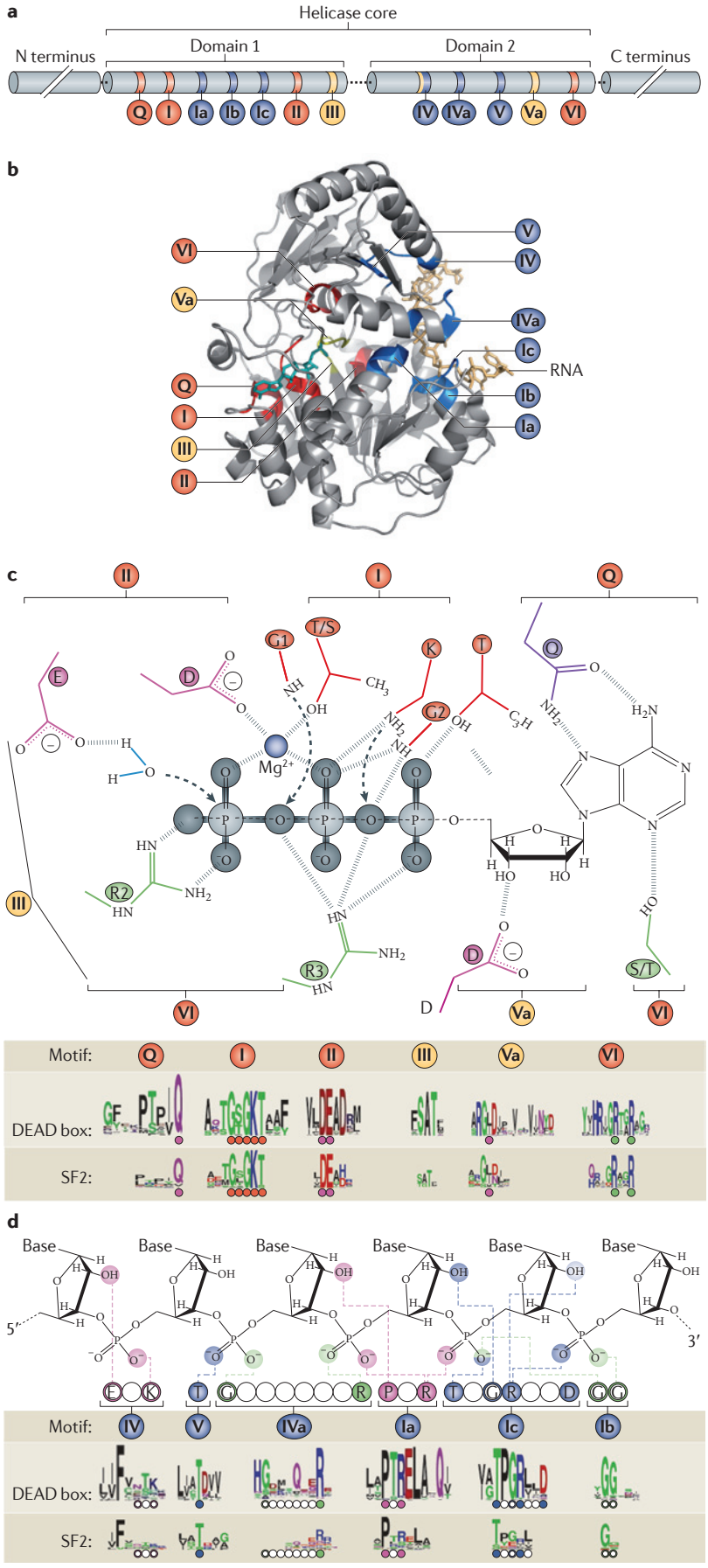
Protein displacement from RNA. In addition to ATP-dependent RNA unwinding and clamping, DEAD box proteins can remove proteins from RNA in an ATP-driven reaction, as has been suggested for the removal of yeast Mud2 by the DEAD box protein Sub2 during pre-mRNA splicing and for the removal of mRNA export factor 67 (Mex67) during mRNA export by DEAD box protein 5 (Dbp5)[47–52]. Moreover, proteins can be displaced from structured and from unstructured RNA, suggesting that duplex unwinding is not required for release[49]. In accordance with a non-processive activity of DEAD box proteins, protein displacement has been directly observed for proteins with a footprint of fewer than eight nucleotides[49–52]. Proteins with larger footprints cannot be displaced by DEAD box proteins, whereas other helicases readily remove such proteins[49,53]. Although it is not known whether protein removal and duplex unwinding by DEAD box proteins rely on identical mechanisms, observations are consistent with a displacement mode that is not based on translocation of the DEAD box protein[50]. The inability to displace a certain set of proteins from RNA might be important for limiting RNA remodelling activity of DEAD box helicases in the cell to avoid complete dissociation of RNP complexes[52].

Figure 2 | **Structure of the DEAD box helicase core and substrate interactions. a** | The helicase core region of DEAD box helicases consists of two recombinase A (RecA)-like helicase domains (domains 1 and 2). The conserved sequence motifs within these domains are shown, with colour coding that corresponds to the primary function of the domain (red, ATP binding and hydrolysis; blue, RNA binding; yellow, communication between ATP-binding and RNA-binding sites). The distance between the conserved domains is not drawn to scale. **b** | Structure of the helicase core domains of the DEAD box protein Vasa from *Drosophila melanogaster*. The conserved sequence domains are coloured as in part **a**. The RNA is shown in beige. **c** | Schematic representation of the key residues in the helicase core domain that mediate ATP binding and hydrolysis, based on the structure of Vasa[14]. Brackets indicate the approximate binding surface for the conserved sequence domains represented by the numbers. Functionalities that coordinate the catalytic water (E, motif II), stabilize the transition state (R2, motif VI) and coordinate the Mg^{2+} (D, motif II; T/S, motif I) and the β-phosphate of ATP (K, motif I) are largely conserved among other helicase families[3]. Sequence logos of the conserved domains involved in ATP binding and hydrolysis are shown at the bottom. These logos are constructed from sequence alignments of all of the DEAD box and superfamily 2 (SF2) helicases from *Escherichia coli*, *Bacillus subtilis*, *Saccharomyces cerevisiae* and *Homo sapiens*[3]. The coloured dots below the sequence logos correspond to the residues shown in the schematic and the colours of the dots and bonds emphasize the different residues. **d** | Schematic representation of key residues involved in RNA binding, based on the structure of Vasa[14]. Letters indicate the residues of the sequence motifs, which are also indicated in the sequence logos shown at the bottom. The motifs are not in numerical order but in the order by which they are arranged in the structure. Contacts to residues on the RNA are shown as dotted lines, and colour coding emphasizes potential functional similarities between motifs located in domains 1 and 2 (for example, between motif IV in domain 2 and motif Ia in domain 1). The dots under the key residues correspond to the number of amino acids in the respective sequence motifs. Filled dots represent a side-chain contact; dots with a coloured rim show a backbone contact to the RNA; and white dots represent intervening residues without a function in RNA binding. Sequence logos were constructed as in part **c**. Figure is modified, with permission, from REF. 141 © (2010) Royal Society of Chemistry.

Strand annealing activity. Many helicases, including several DEAD box proteins, not only catalyse the unwinding of duplexes but also show strand annealing activity, at least *in vitro*[26,54–58]. For example, nucleolar RNA helicase 2 (RH2; also known as Gu and DDX21) displays intrinsic ATP-independent RNA folding activity, the intramolecular version of strand annealing[59]. Although not all DEAD box proteins show pronounced strand annealing activity *in vitro*, many may have such activity *in vivo*[56]. The DEAD box proteins Ded1 and Mss116, which are important for cytoplasmic translation and mitochondrial RNA metabolism, respectively, are among the most potent strand annealers[56,57]. Their annealing activity does not require ATP[56,57], although strand exchange by cyanobacterial CrhR is ATP-dependent[55] and ADP and ATP modulate annealing by Ded1 (REF. 56).

Transition state analogue
A compound that mimics the structure of the transition state, which is the state with the highest energy along the reaction coordinate.

ATP ground state
In helicases, this typically corresponds to the reaction state when ATP is bound by the enzyme but is not yet hydrolysed.



The strand annealing reaction by DEAD box proteins is not the reverse of the unwinding reaction[60]. As a result, DEAD box proteins that catalyse both ATP-dependent duplex unwinding and ATP-independent strand annealing can promote RNA rearrangements that are much more complex than simple duplex unwinding or helix formation[60,61]. This ability is thought to be pivotal for the function of DEAD box proteins in RNA remodelling reactions, especially those that promote RNA chaperoning[61].

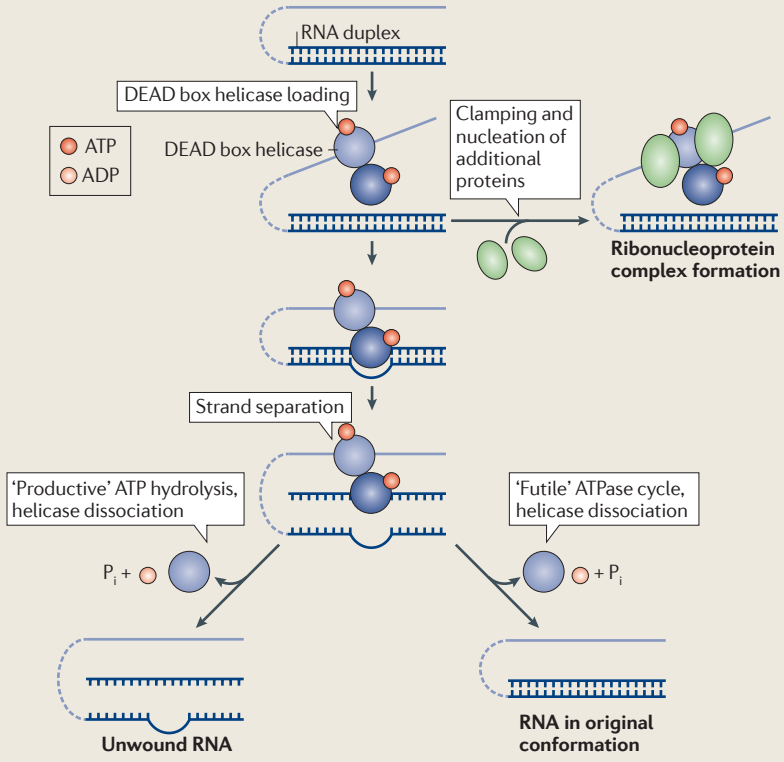
Emerging themes for physiological functions

Most eukaryotic and bacterial DEAD box proteins have now been assigned functions in one or more biological processes, including ribosome biogenesis, pre-mRNA splicing, mRNA export, translation and RNA decay[6]. However, understanding the molecular basis for how DEAD box proteins function in these biological processes has proven challenging[9]. To devise physical models for how DEAD box proteins act, it is important to define where a given protein binds its RNA target, whether and how the RNA structure is changed, and in which manner ATP is utilized for this reaction. In addition, it is critical to elucidate how biochemical functions of the DEAD box protein are modulated by other factors, which invariably surround the helicase in large multicomponent complexes. Answering these questions will require cell and molecular biological approaches, as well as biochemical and structural studies conducted in physiological contexts. Significant progress has been made in several cases, and one can begin to sketch physical models for the biological function of several DEAD box proteins. Some highly interesting models are emerging; here, we focus on illustrative examples provided by exon junction complex (EJC) protein eIF4AIII (also known as DDX48), eukaryotic translation initiation factor 4A (eIF4A; also known as DDX2A and DDX2B) and mRNA export factor Dbp5.

eIF4AIII as an ATP-dependent RNA clamp. The DEAD box protein eIF4AIII is an essential part of the EJC[62], a multiprotein assembly that is deposited 20–24 nucleotides upstream of exon–exon junctions during pre-mRNA splicing in higher eukaryotes[63]. The EJC consists of four core components, eIF4AIII, MAGOH, MLN51 and Y14 (REFS 15,16,62), and a larger number of associated factors[64] (FIG. 3a,b). Although it is not clear exactly how the EJC is deposited on RNA during splicing, it is well established that the core EJC remains stably bound to the spliced RNA after export into the cytoplasm[62]. Here, the EJC affects several steps of RNA metabolism, including nonsense-mediated RNA decay (NMD), translation and RNA localization to various destinations in the cell[65]. Small interfering RNA (siRNA)-mediated depletion of eIF4AIII leads to a defect in NMD[66–68]. Consistent with EJC deposition during splicing, eIF4AIII colocalizes with the splicing factor SC35 (also known as SRSF2) in nuclear speckles[66,67], and EJC components do not associate with the mRNA of genes that lack introns[69]. Recently, differential deposition of EJCs on specific mRNAs was reported, suggesting that this

Box 1 | **Formation of RNP nucleation centres and RNA unwinding**

DEAD box helicases load directly onto the duplex region of an RNA, aided by single-stranded or structured nucleic acid regions (see the figure). These regions do not have to be covalently connected to the helix, although they need to be proximal. Loading can involve multiple protomers of a helicase, as shown, but can also be mediated by a single helicase that has accessory protein domains. The exact mechanisms by which the loading process takes place are not clear. However, loading onto an RNA helix can occur at either end of an RNA, or internally, and on either strand[34]. Once loaded, a DEAD box helicase can serve as a nucleation centre to recruit additional proteins and establish a larger ribonucleoprotein complex. Alternatively, the helicase can catalyse strand separation. Binding of a DEAD box protein to double-stranded RNA can locally open the duplex strands; this step requires ATP but not necessarily ATP hydrolysis[39,42]. The localized helix opening reduces the number of base pairs in the duplex and the remaining base pairs dissociate without further action from the enzyme. As a consequence, the unwinding rate constant decreases with RNA duplex length and stability. The actual hydrolysis of the ATP γ-phosphate may have little effect on strand separation, which can occur before and after the reversible chemical cleavage step. Strand separation probably occurs as long as ATP is bound in the active site, regardless of whether or not it is hydrolysed. However, ATP hydrolysis is important for generating ADP, the release of which triggers further reaction steps. Dissociation of inorganic phosphate (P_i) or ADP promotes the release of the DEAD box protein from the RNA. This step is critical for the turnover of multiple RNA substrates, which requires enzyme recycling. Depending on the substrate and probably on the particular physiological conditions, the ATP hydrolysis can be 'productive', resulting in unwinding of the RNA, or 'futile', resulting in the substrate returning to its original conformation upon helicase dissociation. Figure is modified, with permission, from REF. 42 © (2008) National Academy of Sciences, USA.



is tightly controlled[69]. How this regulation occurs, and whether eIF4AIII is required for this discrimination, is not known. Removal of the EJC from mRNA is thought to require additional factors and ongoing translation[70].

Crystal structure analysis shows that eIF4AIII binds RNA in an ATP-dependent fashion and serves as a platform for the assembly of the EJC core factors[15,16] (FIG. 3c). Although RNA stimulates ATP hydrolysis by eIF4AIII, the protein retains a very high affinity for RNA in the presence of the MAGOH–Y14 dimer, which inhibits ATP hydrolysis by eIF4AIII[62,71]. The MAGOH–Y14 dimer primes eIF4AIII for binding of MLN51 (REF. 62). The ability of eIF4AIII to form a long-lived, highly stable RNP is referred to as RNA clamping[62].

Mutations in motifs I and II of eIF4AIII do not seem to affect clamp formation during establishment of the EJC and/or during NMD[72]. However, structural studies indicate that ATP is bound in the EJC[15,16]. Biochemical data, in conjunction with a structure containing an ATP transition state analogue (ADP–aluminium fluoride (ADP–AlF_x)), have shown that hydrolysis can occur within the complex without loss of the high affinity for RNA[44]. The EJC structure with the ATP transition state analogue reveals that Y14 inhibits the release of the ATP hydrolysis products[44]. As a result, ATP remains trapped in the active site, although both ATP hydrolysis and reformation occur continuously[44].

The idea that a DEAD box helicase could function as an ATP-dependent, immobile RNA clamp, the antithesis to a canonical translocating helicase, was quite unexpected. However, studies have shown that the same cycle of ATP-driven changes in RNA affinity can give rise to both a high-affinity RNA clamp and duplex unwinding by local strand separation[6,39,42]. In fact, DEAD box proteins might traverse a clamp-like state of high RNA affinity during unwinding, before the products of ATP hydrolysis dissociate and RNA is released (BOX 1). Consistent with this notion, eIF4AIII unwinds RNA duplexes in an ATP-dependent fashion *in vitro*[71]. Whether RNA unwinding is involved in EJC deposition is not known. Interestingly, the inherent lack of sequence specificity for eIF4AIII seems to be essential for its binding to a wide variety of mRNAs at defined positions. Pronounced sequence specificity would probably be detrimental because it would limit deposition of EJCs strictly to particular positions on the RNA.

The conceptual elegance of eIF4AIII serving as a sequence-independent RNP assembly platform inspired suggestions that other DEAD box proteins might also function as immobile RNA clamps. This is particularly attractive for DEAD box proteins that affect several consecutive steps of mRNA processing. But, so far, evidence for this is limited to a putative clamping activity of a DDX3 and Ded1 orthologue in *Chironomus tentans*, which shows continuous mRNP binding *in vivo*[73], and perhaps to yeast Dbp5, which has been reported to interact with the transcription machinery and is also required downstream for mRNA export[74,75].

eIF4A as a multifunctional target. Another paradigm of DEAD box protein function is exemplified by eIF4A[76,77]. Despite high similarity to eIF4AIII[78], eIF4A seems to be targeted by a wide range of factors that regulate translation initiation, a pivotal step in gene expression[10,76,79]. The translation initiation factor eIF4A is the prototypical DEAD box protein[77]. ATP-dependent RNA helicase activity and RNA remodelling by any RNA helicase were first demonstrated with eIF4A[80], and key features of the

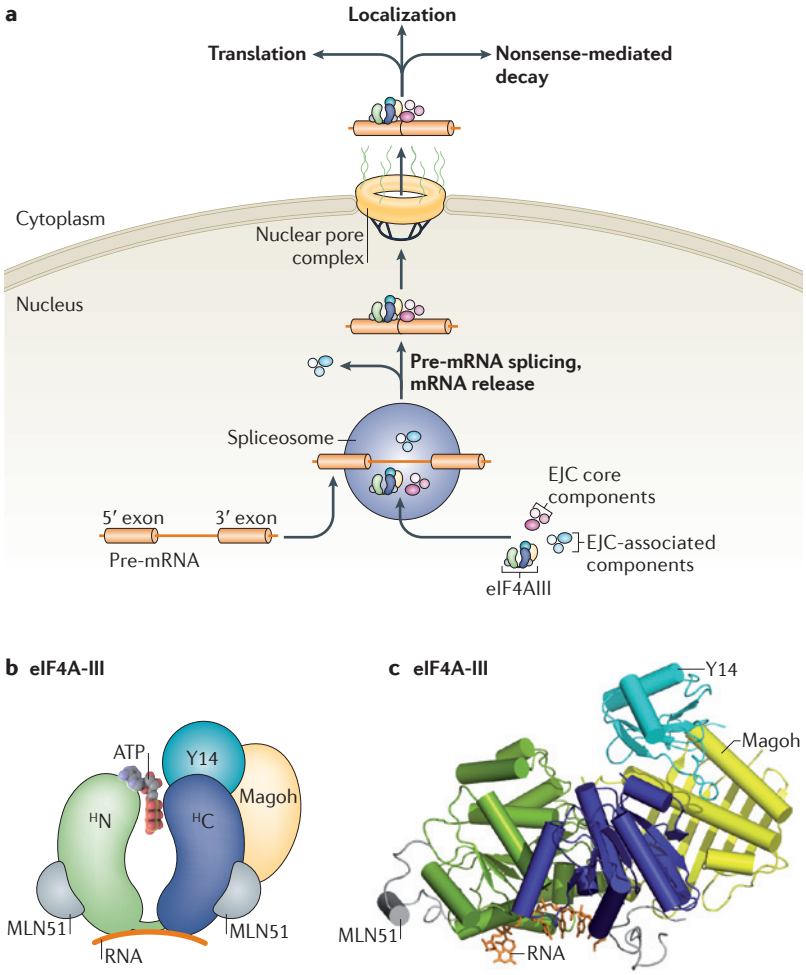


Figure 3 | **eIF4AIII clamps the EJC to the mRNA. a** | Other core components and associated components of the exon junction complex (EJC) associate with eukaryotic initiation factor 4AIII (eIF4AIII) and pre-mRNA as part of the spliceosome in the nucleus. Core components of the EJC, including eIF4AIII, remain associated with the mRNA after splicing, during mRNA export through the nuclear pore complex and during different steps of mRNA metabolism in the cytosol. The number of associated components shown is not representative. **b** | Schematic structural representation of the EJC core factors, MLN51, MAGOH and Y14, and ATP bound to the two helicase core domains of eIF4AIII[15]. ^{H}N represents the amino-terminal helicase core domain and ^{H}C represents the carboxy-terminal helicase core domain. **c** | Crystal structure of the core EJC bound to RNA. Image in part **c** is reproduced, with permission, from REF. 15 © (2006) The American Association for the Advancement of Science.

typical DEAD box unwinding mechanism were also first elucidated with eIF4A[28]. Similarly to eIF4AIII, eIF4A essentially contains only the helicase core, with only very short N-terminal and C-terminal extensions[77]. Compared with many other DEAD box proteins, eIF4A has low affinity for RNA *in vitro*[28,29]. Accordingly, the RNA-stimulated ATPase and unwinding activities shown by eIF4A alone are low.

eIF4A is one of the most abundant proteins in many cell types[81,82]. Together with the large scaffolding protein eIF4G and the cap-binding protein eIF4E, eIF4A forms the eIF4F complex[83] (FIG. 4). However, an appreciable fraction of eIF4A is not bound in eIF4F[77]. eIF4A also associates with the initiation factors eIF4B and eIF4H, which are closely related RNA-binding proteins that enhance eIF4A association with RNA[84] and thereby increase the ability of eIF4A to unwind RNA and hydrolyse ATP in an RNA-stimulated fashion[85,86,87]. Together, these factors all promote formation of a translation initiation complex that allows binding of the small 40S ribosomal unit and other initiation factors to mRNA[88].

The interaction between eIF4G and eIF4A has been studied by structural, biochemical and biophysical approaches. HEAT (huntingtin, EF3, PP2A and TOR1) domain 1 of eIF4G binds to both RecA-like domains in the helicase core of eIF4A, thereby closing the core domains[89,90] (FIG. 4). However, the two core domains do not close completely, as observed for eIF4AIII in the EJC[89,91]. The eIF4G-induced, half-open form of eIF4A promotes RNA binding and also accelerates phosphate release, which is presumably the rate-limiting step in the ATPase cycle for eIF4A[91]. These findings are consistent with the well-documented stimulation of the RNA-dependent ATPase activity of eIF4A by eIF4G[85]. In addition, full-length eIF4G might also increase binding of eIF4A to RNA by providing additional RNA-binding sites[85]. A further study indicated that eIF4G promotes the formation of a complex of eIF4A, eIF4B and RNA[92]. Moreover, eIF4G accelerates phosphate release and thereby RNA release. The scaffolding protein eIF4G may therefore act in a catalytic fashion, inducing multiple rounds of formation of eIF4A–eIF4B–RNA[92], which would also be consistent with the structural and biochemical evidence (FIG. 4). Other studies suggest that eIF4G, together with eIF4A and eIF4H, promotes ribosome scanning[88]. These scenarios are not mutually exclusive and it is possible that several distinct complexes are formed by eIF4A and eIF4G in response to different signals or on different RNAs.

Despite the progress made in understanding how eIF4A interacts with initiation factors and regulators, the exact function of eIF4A in translation initiation is still elusive. Extrapolating from its unwinding activity[80], and the correlation of an increased need for eIF4A during translation of mRNAs with structured 5′ untranslated regions (UTRs), eIF4A is often suggested to unwind RNA secondary structures in the 5′ UTR that would otherwise inhibit ribosome scanning[93,94]. But the unwinding of such structures by eIF4A during translation initiation has not been shown directly, and eIF4A-independent scanning has been seen[95]. Moreover, the DEAD box protein Ded1 and the non-DEAD box protein DEAH box 29 (DHX29) affect translation of mRNAs with long and structured 5′ UTRs, respectively[96,97]. One alternative, and not mutually exclusive, possibility is that eIF4A induces conformational changes in the translation initiation complex, or that ATP-modulated, transient binding to RNA may be necessary for ordered initiation to occur[92]. Defining the mRNA sites to which eIF4A binds might provide insights into its many functions.

The eIF4F complex is essential for the translation of most, if not all, cellular mRNAs[83] and is an important target for regulation[98,99]. Abolishing this can lead to neoplastic transformation and tumour formation[100,101]. For example, programmed cell death 4 (PDCD4), which is a natural

Nonsense-mediated RNA decay
(NMD). A process by which mRNA molecules that contain a stop codon within the open reading frame are subjected to rapid degradation to avoid synthesis of deleterious truncated proteins.

Nuclear speckles
Subnuclear structures that are enriched with pre-mRNA and many different proteins that are involved in splicing.

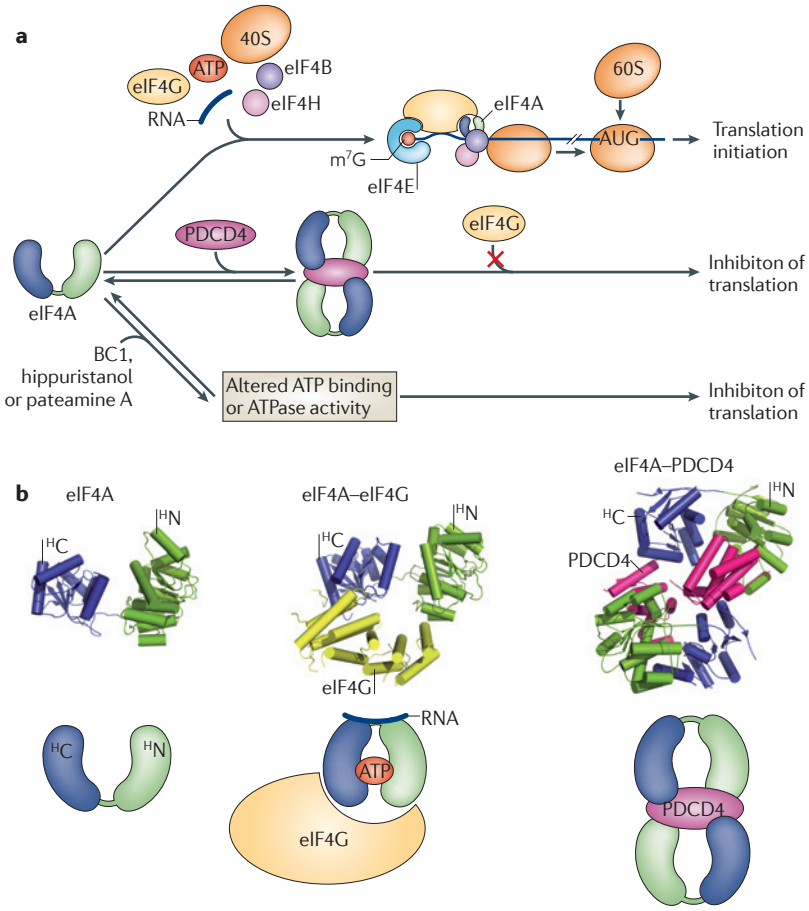


Figure 4 | **Regulation of eIF4A during translation initiation. a** | During cap-dependent initiation of translation, eukaryotic initiation factor 4A (eIF4A) binds ATP, eIF4G and RNA (top). The cap-binding complex eIF4F is composed of the cap-binding protein eIF4E, the scaffolding protein eIF4G and the translation initiation factor eIF4A. The 40S initiation complex then scans through the 5′ untranslated region for the first AUG initiator codon to begin translation[76,88,92]. Association of programmed cell death 4 (PDCD4) with two molecules of eIF4A (middle) triggers a conformational change that prevents association with eIF4G and thereby inhibits the initiation of translation. Association of eIF4A with the non-coding RNA BC1 inhibits translation by stimulating the ATPase activity of eIF4A (bottom)[109]. eIF4A binding to the small molecules hippuristanol or pateamine A also prevent translation by altering the ATP binding or ATPase activity of eIF4A[105,106]. **b** | Structures of eIF4A bound to regulating proteins. The schematic structural representation shown at the bottom emphasizes the orientation of the two helicase core domains in the free enzyme and when bound to eIF4G and RNA, or when bound to PDCD4. ^{H}C, carboxy-terminal helicase core domain; ^{H}N, amino-terminal helicase core domain; m^{7}G, 7-methylguanosine. The structure of the free eIF4A enzyme is reproduced, with permission, from REF. 142 © (2000) National Academy of Sciences, USA. The structure of eIF4A bound to a fragment of eIF4G is reproduced, with permission, from REF. 89 © (2006) National Academy of Sciences, USA. The structure of the eIF4A–PDCD4 complex is reproduced, with permission, from REF. 103 © (2009) Macmillan Publishers Ltd. All rights reserved.

tumour suppressor protein, inhibits translation by binding to eIF4A[102]. One PDCD4 molecule binds two eIF4A protomers, inhibiting enzymatic activity by trapping the DEAD box protomers in an inactive conformation and preventing their binding to eIF4G[103,104] (FIG. 4).

Two natural products, hippuristanol and pateamine A, inhibit translation initiation directly by modulating the enzymatic activity of eIF4A[105–107]. Hippuristanol is highly specific: it inhibits ATP binding to eIF4A but does not affect the eIF4AIII homologue. By contrast, pateamine A stimulates the RNA-stimulated ATPase activity of eIF4A but also seems to be active on eIF4AIII[108]. Similarly to the function of pateamine A, the non-coding RNA BC1 binds specifically to eIF4A and stimulates its ATPase activity but blocks its unwinding activity[109] to prevent translation initiation in neurons.

Although much remains to be learned about the function of eIF4A in translation initiation, studies show that it can be specifically targeted by many regulating factors, despite the structural conservation of its helicase core in other DEAD box proteins. This may be because, in several cases, cofactors modulate the orientation of the two helicase domains in eIF4A and thereby influence enzymatic activities as well as the binding of other cofactors, occasionally in a mutually exclusive fashion.

Local activation of Dbp5 during RNA export. Dbp5 (DDX19 and DDX25 in vertebrates) represents a further emerging paradigm of how DEAD box proteins are regulated: through local activation. It interacts with the transcription machinery and with nascent RNPs[74,75] to mediate mRNA export from the nucleus[110,111], and it has also been implicated in the termination of translation[112,113]. Although it is not known whether Dbp5 remains bound to mRNAs from transcription to export, as eIF4AIII does, elegant studies have demonstrated that Dbp5 activity must be highly localized during mRNA export[114,115].

Dbp5 predominantly localizes to the cytoplasm and is particularly concentrated at the rim of nuclear pores, where it associates with cytoplasmic fibrils of the nuclear pore complex[116]. This localization requires the nucleoporin protein Nup159 (NUP214 in mammals)[117,118]. Genetic analyses suggest that Dbp5 affects the release of the mRNA export factor Mex67 from RNA, which is a critical step for mRNA export[48]. In addition, Dbp5 has been implicated in the release of nuclear polyadenylated RNA-binding 2 (Nab2) from mRNA[51]. How exactly Dbp5 affects the release of these two proteins is not clear.

The structure and the biochemical analysis of the mammalian homologue of Dbp5, DDX19, reveal an interesting potential mode of self-regulation[119]. In this analysis, it was shown that the sequence that lies just to the N-terminal side of the Q-motif of DDX19 inhibits ATP hydrolysis, by competing with the Arg finger (motif VI), unless this extension becomes displaced by RNA.

Dbp5 also interacts with nuclear pore protein Gle1 (REFS 117,120), and the C-terminal region of Gle1 stimulates RNA-dependent ATPase activity of Dbp5 *in vitro*[114,121]. Gle1 mutants that fail to activate Dbp5 also affect mRNA export[114]. These findings suggest that Gle1 is a specific cofactor of Dbp5 (REF. 114). Several observations suggest that Gle1 activates Dbp5 in a manner that requires the small metabolite inositol hexakisphosphate ($InsP_6$)[114,115]. Mutations in the $InsP_6$ pathway genetically interact with *gle1* mutants[122] and, *in vitro*, $InsP_6$ further increases the Gle1-dependent stimulation of

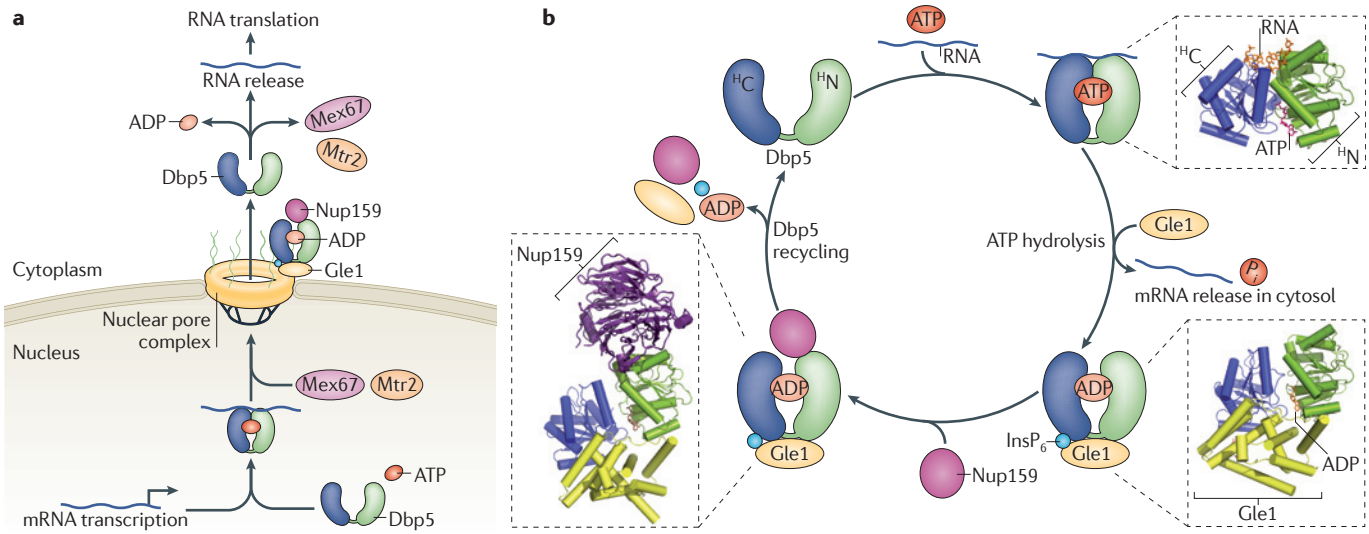


Figure 5 | **Dbp5 control of mRNA export. a** | In yeast, DEAD box protein 5 (Dbp5) mediates export of mRNAs to the cytosol through the nuclear pore complex. ATP-associated Dbp5 associates with mRNA in the nucleus and is recruited together with mRNA export factor 67 (Mex67) and mRNA transport regulator 2 (Mtr2) to the nuclear pore complex through association with the nucleoporin Nup159, which localizes to the cytoplasmic fibrils of the pore. Here, Gle1 association and ATP hydrolysis triggers release of mRNA in the cytosol and dissociation of Gle1, inositol hexakisphosphate ($InsP_6$), Mex67, Mtr2 and Nup159 to allow recycling of the export factors. **b** | During the mechanochemical cycle of Dbp5, ATP and RNA association trigger a conformational change in Dbp5. ATP hydrolysis allows release of mRNA into the cytosol. The association of Gle1, $InsP_6$ and Nup159 with Dbp5 further modulates its conformation to allow Dbp5 recycling. Structures of Dbp5 and its interaction proteins at different stages are also shown ($InsP_6$ is not visible). Binding of Gel1 and $InsP_6$ induce RNA dissociation, and release of inorganic phosphate (P_i) presumably accompanies this step[121]. Nup159 blocks the RNA binding site[17]. The crystal structure of Dbp5 bound to RNA and ATP is based on Fan *et al.*[18]. ^{H}C, carboxy-terminal helicase core domain; ^{H}N, amino-terminal helicase core domain. Figure is modified, with permission, from REF. 121 © (2011) Macmillan Publishers Ltd. All rights reserved.

Dbp5 activities[114,115]. Moreover, several dominant mutations in Dbp5 and Gle1 that tighten the interaction between the two proteins can bypass the requirement of $InsP_6$ (REF. 114). Thus, activation of Dbp5 by Gle1 in an $InsP_6$-dependent manner might then enable Dbp5 to release Mex67 and Nab2 from mRNA, thus facilitating mRNA export.

Important insights have been gained from structural and biochemical analysis of the interaction of Dbp5, Gle1, $InsP_6$ and Nup159 (REF. 121) (FIG. 5). $InsP_6$ binds at the interface between Gle1 and Dbp5 (REF. 121). Perhaps most remarkably, the Gle1 domain that binds Dbp5 closely resembles the eIF4A-binding domain in eIF4G (compare FIG. 5b with FIG. 4b). The structural folds of the DEAD box binding domains in Gle1 and eIF4G are virtually identical, despite the absence of sequence similarity, and the binding sites on both RecA-like domains of the helicase core of Dbp5 are essentially identical to the binding sites in eIF4A that contact eIF4G[121]. Gle1 and $InsP_6$ bind the ADP-bound form of Dbp5 (REF. 121) (FIG. 5), and Gle1 stimulates Dbp5 activities by promoting the concurrent release of RNA, again similarly to the findings for eIF4A and eIF4G[121] (FIG. 4). Binding of Nup159 blocks the RNA-binding site of Dbp5 (REFS 17,121) (FIG. 5) and this is required for the release of ADP, adding a new level of DEAD box protein regulation by partner proteins[123,124]. So far, such an ADP-release factor has not been described for any other DEAD box protein, but it may not be unique to Dbp5. These structural studies provide a compelling and instructive picture of how Dbp5 is locally activated during mRNA export, even though it is not clear what nucleotide-dependent changes Dbp5 makes to exporting mRNPs. It is also remarkable that Gle1 and eIF4G, two very different proteins, both activate two distinct DEAD box proteins using a similar structural and mechanistic basis. A part of the sites that these regulators bind seems to be a hotspot for association with DEAD box helicases, as suggested by studies of DDX6 bound to a fragment of its regulator enhancer of mRNA-decapping protein 3 (EDC3)[125], and of eIF4AIII binding to its regulator UPF3B[126].

Expanding the roles for DEAD box proteins. Although we concentrate in this Review on only three instructive examples, it is important to note that there are additional ways in which DEAD box proteins can regulate RNA-dependent processes. For example, significant attention has been focused on several fungal mitochondrial DEAD box helicases, including Mss116 and CYT19, which act as RNA chaperones to promote folding of several mitochondrial RNA introns into their native conformations[45,57,61,127–130]. For this function, it seems critical that the DEAD box proteins bind and affect RNA structure in a non-sequence- or structure-specific fashion, much like the way in which traditional protein chaperones act on proteins[6].

Next-generation sequencing
High-throughput sequencing technologies in which millions of (usually short) pieces of sequence are produced in parallel.

Other emerging themes are exemplified by the proposed functions of the DEAD box helicase Sub2 (UAP56 in vertebrates) in promoting protein–protein interactions in an ATP-dependent manner, a function that is important for the assembly of spliceosomes on mRNAs[131]. The involvement of DEAD box proteins, such as Dbp4, in the release of snoRNAs from the pre-ribosome is another example of an important way in which RNPs can be modulated[132]. Although these different roles for DEAD box helicases are conceptually intriguing, it is not yet well understood how all of these DEAD box proteins function and whether their roles involve bona fide unwinding or rather destabilization of RNP complexes.

Several DEAD box proteins have also been shown to perform tasks that may not directly involve RNA, although those roles are usually in addition to their RNA-associated functions. Roles in transcriptional regulation have been shown for DDX20 (also known as gemin 3), p68 (also known as DDX5), p72 (also known as DDX17) and RH2 (REF. 133). DDX20 represses transcription, probably through the recruitment of histone deacetylases[134]. p68 and p72 are transcriptional co-activators for nuclear oestrogen receptor-α (ERα)[135,136], and p68 also acts as a potent co-activator of the tumour suppressor p53 (REF. 137). Although the interacting region between p68 and p72 and ERα includes part of the conserved helicase core and RNA binding is important for ERα co-activation, p68 helicase activity per se is not required[135,136]. Clearly, these activities are not the general rule and further work is required to elucidate how these roles are fulfilled and whether other DEAD box proteins display similar activities.

Future challenges and perspective

Despite the impressive progress made in our understanding of the structures, functions and biological roles of DEAD box helicases, many important questions remain unanswered. We almost completely lack information about where exactly these proteins bind to their RNA or RNP targets. For DEAD box proteins that function in mRNA metabolism, it is also unclear whether they bind all mRNAs or only a subset of them. Our lack of knowledge about RNA-binding sites has prevented us from establishing complete physical models for how DEAD box proteins function and has precluded further elucidation of whether and how these helicases alter RNA structures. So far, exact RNA-binding sites are known only for eIF4AIII, in the context of the EJC[69], and for bacterial DbpA and its orthologues, which bind with high affinity to a ribosomal RNA hairpin[138]. The vast majority of DEAD box proteins lack inherent substrate specificity, and physiological RNA binding sites need to be explicitly determined.

Novel approaches that combine *in vivo* crosslinking with high-throughput next-generation sequencing (such as crosslinking and immunoprecipitation (CLIP) and crosslinking and analysis of cDNAs (CRAC)) are now making it more feasible to identify RNA targets and RNA-binding sites, even on a genome-wide scale. CRAC was recently used to determine the RNA-binding sites of the non-DEAD box RNA helicase Prp43 on pre-ribosomal RNA[139]. A similar approach was also used to determine RNA sites bound by eIF4AIII in the EJC[69]. In future, CLIP and CRAC techniques should have a key role in illuminating RNA-binding sites for DEAD box proteins.

When it becomes possible to determine physiological RNA-binding sites, it will be important to define whether and how RNA structure is changed by DEAD box proteins, what roles ATP has in these reactions and how other proteins modulate the DEAD box proteins, and vice versa. In addition, the functional impact of post-translational modifications on these events will need to be investigated; DDX3, for example, is phosphorylated at a Thr residue within the Q-motif, which abrogates its activity[140]. Collectively, these are significant challenges, but the impressive progress made over the past few years is reason to be optimistic that we will see a rapidly continuing increase in our understanding of how DEAD box proteins function and of their central roles in cellular RNA metabolism.

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Competing interests statement

The authors declare no competing financial interests.

DATABASES

The RNA Helicase Database: http://www.rnahelicase.org

FURTHER INFORMATION

Patrick Linder's homepage:
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