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Review

DExD/H-box RNA helicases as mediators of anti-viral innate immunity and essential host factors for viral replication ☆

Anthony Fullam, Martina Schröder *

Institute of Immunology, National University of Ireland Maynooth, Maynooth, Co. Kildare, Ireland

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ABSTRACT

Traditional functions of DExD/H-box helicases are concerned with RNA metabolism; they have been shown to play a part in nearly every cellular process that involves RNA. On the other hand, it is accepted that DexD/H-box helicases also engage in activities that do not require helicase activity. A number of DExD/H-box helicases have been shown to be involved in anti-viral immunity. The RIG-like helicases, RIG-I, mda5 and lgp2, act as important cytosolic pattern recognition receptors for viral RNA. Detection of viral nucleic acids by the RIG-like helicases or other anti-viral pattern recognition receptors leads to the induction of type I interferons and pro-inflammatory cytokines. More recently, additional DExD/H-box helicases have also been implicated to act as cytosolic sensors of viral nucleic acids, including DDX3, DDX41, DHX9, DDX60, DDX1 and DHX36. However, there is evidence that at least some of these helicases might have more downstream functions in pattern recognition receptor signalling pathways, as signalling adaptors or transcriptional regulators. In an interesting twist, a lot of DExD/H-box helicases have also been identified as essential host factors for the replication of different viruses, suggesting that viruses 'hijack' their RNA helicase activities for their benefit. Interestingly, DDX3, DDX1 and DHX9 are among the helicases that are required for the replication of a diverse range of viruses. This might suggest that these helicases are highly contested targets in the ongoing 'arms race' between viruses and the host immune system. This article is part of a Special Issue entitled: The Biology of RNA helicases — Modulation for life.

1. Introduction

DExD/H-box helicases are part of the large SF2 helicase superfamily. Within the DExD/H-box family, helicases are further distinguished based on the amino acid sequence of the eponymous conserved helicase motif II (DEAD, DEAH, DExH and DExD helicases). DExD/H-box helicases share (at least) eight conserved motifs that are involved in ATP binding, ATP hydrolysis, nucleic acid binding, and RNA unwinding activity. These conserved motifs are contained within a conserved structural fold that comprises two RecA-like globular domains connected by a flexible linker region [1,2]. This structural fold facilitates the motor protein function associated with helicase activity. In general, SF2 helicases display an 'open conformation' when they are not bound to a nucleic acid substrate, where the two domains can quite flexibly rotate around the linker. The ATP binding site is located in the cleft between the two domains and the nucleic acid binding sites stretch across both domains. When ATP and a nucleic acid substrate are bound, the cleft between the two domains

Abbreviations: 5′ ppp, 5′ triphosphate; AIM2, Absent In Melanoma 2; ARE, AU-rich element; CARD, Caspase Activation and Recruitment Domains; CDN, cyclic dinucleotide; cGAMP, cyclic-GMP-AMP; cGAS, cGAMP Synthase; CRM1, Chromosome Maintenance Region 1; CstF, Cleavage Stimulation Factor; CTD, C-terminal domain; CTE, Constitutive Transport Element; DAI, DNA-Dependent Activator of IFN-Regulatory Factors; ds, Double Stranded; EMCV, Encephalomyocarditis Virus; ER, Endoplasmic Reticulum; EXOSC1, Exosome Component 1; FMCV, Foot-And-Mouth-Disease Virus; HBV, Hepatitis B Virus; HCC, Human Hepatocellular Carcinoma; HCMV, Human Cytomegalovirus; HCV, Hepatitis C Virus; HIV, Human Immunodeficiency Virus; HSV, Herpes Simplex Virus; IBV, Infectious Bronchitis Virus; IFI16, Interferon-Inducible Myeloid Differentiation Transcriptional Activator; IFN, Interferon; IKK, IκB Kinase; IRF, Interferon Regulatory Factor; ISG, Interferon Stimulated Gene; JCV, John Cunningham Virus; LTR, Long Terminal Repeat; MAMs, Mitochondria-Associated Membranes; MAVS, Mitochondrial Antiviral-Signalling Protein; mDCs, Myeloid Dendritic Cells; MyD88, Myeloid Differentiation Primary Response Gene (88); NES, Nuclear Export Signal; NF/NFAR, nuclear factor/nuclear factor associated with dsRNA; NF- B, Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells; NIK, Nuclear Factor KappaB-Inducing Kinase; nsp14, Non-Structural Protein 14; PBMC, Peripheral Blood Mononuclear Cell; PCE, Posttranscriptional Control Element; pDC, Plasmacytoid Dendritic Cells; PML, Progressive Multifocal Leukoencephalopathy; PRR, Pattern Recognition Receptor; RHA, RNA Helicase A; RIG, Retinoic Acid-Inducible Gene 1; RLH, RIG-I-like Helicase; RRE, Rev-Responsive Element; SARS, Severe Acute Respiratory Syndrome; SF2, Splicing Factor 2; SNX2, Sorting Nexin-2; STING, Stimulator of IFN Genes; TAP, Tip Associated Protein; TAR, Trans-Activation Response; TBK1, TANK Binding Kinase; TCR, Transcriptional Control Region; TIR, Toll-IL-1 receptor; TLR, Toll-Like Receptor; TRAF3, TNF Receptor-Associated Factor 3; TRIF, TIR-domain-containing adapter-inducing interferon-β; UTR, Untranslated Region

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* Corresponding author. Tel.: +353 1 708 6853; fax: +353 1 708 6227.
E-mail addresses: fullama@tcd.ie (A. Fullam), martina.schroeder@nuim.ie (M. Schröder).

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closes. This 'closed conformation' allows for productive ATP binding and hydrolysis. ATP hydrolysis is likely associated with the release of the nucleic acid substrate [1–4].

Apart from the conserved functional core, most DExD/H-box helicases contain additional variable N- and C-terminal regions that confer functional specificity to individual helicases. For example, these extended regions have been shown to modulate enzymatic activity, to contain additional RNA-binding domains or to provide docking sites for protein–protein interactions [5].

In recent years it has emerged that several DExD/H-box helicases contribute to anti-viral immunity, either by acting as sensors for viral nucleic acids or by facilitating downstream signalling events. In general, pathogens are detected by innate immune cells with the help of germline-encoded pattern recognition receptors (PRRs) that recognise conserved structures present in certain pathogen classes, known as pathogen-associated molecular patterns (PAMPs) [6]. Viral PAMPs are mainly nucleic acids, including viral genomic RNA and DNA, as well as replication intermediates [7]. Double-stranded (ds)RNA, in particular, has long been recognised as a viral PAMP and is detected by several cellular dsRNA-binding proteins [8]. The main groups of PRRs sensing viral nucleic acids are endosomal Toll-like receptors (TLRs), the RIG-like helicases (RLHs) which are part of the DExH family of RNA helicases, and cytoplasmic DNA receptors [8]. In addition, several DExD/H-box helicases that are not part of the RIG-I family have recently been implicated in the sensing of viral nucleic acids and/or downstream signalling pathways [9–14]. A general characteristic of anti-viral PRR signalling is the induction of type I interferons (IFN-α and IFN-β), cytokines with potent anti-viral activity. Their anti-viral activity is largely mediated by interferon-stimulated genes (ISGs) that encode proteins with direct anti-viral effector functions [15]. Activation of the transcription factors IRF3 or IRF7 is required for induction of type I interferons [16], and signalling pathways downstream of antiviral PRRs engage kinases that phosphorylate and activate IRF3/7. TLR3, the RLHs and most DNA receptors discovered to date utilise the IKK-related kinases TBK1 and IKKε for phosphorylation of IRF3/7 [17–21]. Their upstream signalling pathways are quite divergent: TLR3 depends on the TIR-domain containing adaptor molecule TRIF [22], the RLHs utilise a CARD-domain containing mitochondrial adaptor called MAVS (also called IPS-1, Cardif or VISA) [18,23–25], and most cytosolic DNA receptors depend on an ER-resident adaptor molecule called STING [26].

TLR7 and TLR9 engage a different signalling pathway which depends on the TIR-adaptor molecule MyD88 and the kinases NIK and IKKα for phosphorylation and activation of IRF7 [27,28]. This pathway is mainly active in plasmacytoid dendritic cells (pDCs), which express TLR7 and TLR9 and constitutively high levels of IRF7, allowing them to produce high levels of IFN-α in an early response to viral infections [29].

Several DExD/H-box helicases that are not part of the RIG-I family, including DDX3, DDX60, DDX41, DDX1, DHX9 and DHX36, have recently been implicated as additional receptors for viral nucleic acids, and to induce IFN induction via TIR adaptor — or MAVS-dependent signalling [9–14].

Because type I interferons are such potent anti-viral cytokines, many viruses have evolved strategies for interfering with their induction [30,31]. In fact, we know numerous examples of viral immune evasion proteins that either mask viral RNA ligands or directly inhibit the RLHs or downstream signalling molecules involved in type I IFN induction [32,33]. In many cases, the study of such viral immune evasion proteins has led to new insights into components of the host anti-viral immune response [33].

Intriguingly, apart from their roles in viral recognition and anti-viral immunity, RNA helicases are also actively recruited by viruses to facilitate their replication cycles. Interestingly, while all pro- and eukaryotic genomes contain RNA helicase genes, a lot of viruses do not seem to encode their own RNA helicases. This suggests that viruses rely heavily on host RNA helicases to mediate RNA remodelling events that are part of their replication cycle or required for viral gene expression. As such, several DExD/H-box helicases have been identified as essential host factors for the replication of different viruses, including those that pose major global health threats, such as HIV and Hepatitis C Virus (HCV).

It is fascinating that in many cases the very same RNA helicases that are 'hijacked' by viruses as essential host factors are also the ones implicated in anti-viral innate immune responses. This might indicate that RNA helicases are in fact at the very centre of an ongoing evolutionary 'arms race' between viruses and the host immune system.

2. RNA helicases in innate anti-viral immunity

2.1. The RIG-like helicases

The RIG-like helicases (RLHs), RIG-I, mda5 and lgp2, are part of the DExH RNA helicase family, and are recognised as one of the most important groups of anti-viral PRRs [34]. In contrast to other DExD/H-box helicases that have recently been implicated in anti-viral immunity (discussed below), RIG-I and mda5 contain CARD signalling domains [35] that allow them to interact with the mitochondrial CARD-domain containing adaptor molecule MAVS [24]. In addition to the N-terminal tandem CARD domains and the central helicase core region comprising the conserved two RecA-like domains, RLHs also contain a C-terminal regulatory domain (CTD or RD) which mediates specific recognition of viral RNAs [36,37]. Intensive research over the last few years has defined the RNA ligands for RIG-I. It is now widely accepted that RIG-I binds with high affinity to blunt-ended RNAs containing a free 5′ triphosphate end (5′ppp), followed by a short double stranded region of at least 19 bp [38]. Studies in Influenza and Sendai virus-infected cells have confirmed these requirements and demonstrated that RIG-I stimulatory activity resides primarily in progeny viral genomes, which contain a 5′ppp and adopt so-called 'panhandle' structures through intramolecular base-pairing [39,40]. Baum et al. identified SeV defective interfering (DI) copy-back genomes as particularly strong RIG-I ligands in SeV infected cells. These DI genomes consist of a 546-nt RNA molecule with a 5′ppp and a 92nt long perfect dsRNA portion [39]. Recent structural studies have also provided a lot of insights into ligand binding and activation of the RLHs. Specificity of 5′ppp binding is mostly conferred by the CTD, while the helicase core region binds to the double-stranded part of the RNA ligand. This binding does not confer sequence specificity, as contacts are made exclusively with the phosphoribose backbone of the RNA [41]. Initial binding of the 5′ppp end to the CTD appears to allow for subsequent cooperative binding of the double-stranded region of the RNA and ATP to the helicase core domain [42].

This binding results in a conformational change to the typical 'closed form' of DExD/H-box helicases that allows for efficient ATP hydrolysis [42,43]. It has been shown that RIG-I exists in an 'auto-inhibited' conformation in the absence of RNA [37,44], where the CARD domains are not accessible due to an internal interaction between the second CARD domain and the second RecA-like helicase domain [42,45]. The conformational change that follows ATP and RNA binding disrupts this internal interaction and releases the tandem CARD domains [42,43]. The free CARD domains of RIG-I can then initiate signalling by interacting with the CARD-domains of the MAVS adaptor molecule [42,46].

RIG-I has also been described to bind to, and be activated by, longer dsRNA ligands (~100–400 bp) lacking a 5′ppp end. In this case, a number of RIG-I molecules seemed to cooperatively assemble side by side on the dsRNA strand [47]. It is possible that a high concentration of dsRNA can overcome the initial requirement for 5′ppp binding to the CTD, as this is believed to facilitate binding of the RNA ligand to the helicase core region by increasing the local concentration of dsRNA. In addition, RIG-I can also be activated by RNase L cleavage products of viral and cellular RNAs, which do not contain a 5′ppp end [48]. It is unclear how RIG-I can recognise these cleavage products, and it will be interesting to determine whether a co-receptor, such as lgp2 (see below) is required for this activation.

It is also unclear whether RIG-I actually acts as an RNA helicase in the sense of unwinding its double-stranded RNA ligands. It was initially reported that recombinant RIG-I can unwind short duplexes containing a 3′overhang [49]. However, RNAs containing a 3′overhang were not good IFN-inducers in cells [49], and it is now believed that RIG-I does not efficiently bind to RNAs with single-stranded overhangs [50], suggesting that these are not physiological ligands for RIG-I. It has also been suggested that while RIG-I can translocate on RNA [51], it might lack a phenylalanine loop motif required for RNA unwinding [52]. It is therefore possible that RIG-I acts more like a motor protein by converting the energy from ATP hydrolysis into a mechanical force that drives the CARD and helicase domains apart and thereby releases the auto-inhibited state.

The free CARD domains then appear to bind to K63-linked free polyubiquitin chains and thereby induce tetramerisation of RIG-I. These higher order complexes of RIG-I and ubiquitin were shown to be strong inducers of type I IFN [53]. The RIG-I CARD domains can also be directly ubiquitinated by Trim25 [54], which should sterically prevent them from interacting with the helicase domain to re-instate the autoinhibited state [42]. Recently, it has been shown that the 14-3-3ε chaperone protein is involved in facilitating a stable TRIM25-RIG-I interaction and shuttling of the RIG-I complex to the mitochondrial membrane where it can interact with MAVS [55]. It has become clear in recent years that organelle membrane reorganisation events are closely linked with the induction of innate immune signalling pathways. Mitochondrial fusion events [56,57], as well as interactions between mitochondria, mitochondria-associated membranes (MAMs) and peroxisomes [58] seem to be necessary to achieve signalling through MAVS. Also, a recent study showed that the signalling molecule TRAF3 normally resides in ER-associated compartments and the cis-Golgi, while smaller TRAF3-containing speckles appeared following virus infection [59]. Translocation of TRAF3 to mitochondria was mediated by Sec16a and p115, proteins that function in ER-to-Golgi vesicle transport. Knockdown of these proteins inhibited dsRNA-induced IFN induction, showing that this translocation of TRAF3 is crucial for assembly of MAVS-signalling complexes [59]. TRAF3 is required for IRF3/7 activation downstream of anti-viral PRRs and consequently for the induction of type I interferons [60].

The other two members of the RLH family, Mda5 and lgp2, are not as well characterised as RIG-I. Mda5 has been implicated in the response to long synthetic dsRNA (poly(I:C)), and appears to mediate responses to distinct sets of viruses compared with RIG-I. Mda5 has mainly been implicated in the response to picornaviruses, vaccinia virus and reovirus [61–65]. It has been suggested that mda5 recognises particularly large and branched RNA structures termed 'RNA webs' that are produced during the replication of certain viruses, such as EMCV (a picornavirus) and vaccinia virus [66].

The CTDs of mda5 and lgp2 are structurally very similar to the CTD of RIG-I. However, while they also mediate binding to blunt-ended dsRNA molecules, the CTDs of mda5 and lgp2 have no specificity for 5′ppp ends [67,68]. The lgp2 CTD, but not the mda5 CTD, also appears to mediate the same autoinhibited state that has been described for RIG-I [69]. From the structure of the mda5 CTD it is initially unclear why mda5 is preferentially activated by long dsRNA [67]. In this context, it is interesting to note that the CTD of mda5 has a much lower affinity for dsRNA molecules than the CTDs of RIG-I and lgp2 [67]. Thus, it is interesting that mda5 appears to cooperatively assemble on dsRNA filaments, similar to what had been described for RIG-I activation by longer dsRNAs without a 5′ppp. This cooperative assembly of mda5 might overcome the initial low affinity of its CTD for dsRNA, although it has been questioned whether the CTD is required for RNA binding at all [70]. The CTD appears to be required for cooperative assembly of mda5 filaments on dsRNA, possibly through mediating an intermolecular interaction with another mda5 molecule [70,71]. In any case, the ensuing mda5-coated RNA filaments appear to be well suited to induce oligomerisation of MAVS via CARD-CARD interactions [70–72].

Lgp2 displays a similar helicase core and CTD as RIG-I and mda5, but lacks the CARD signalling domains [35]. Due to its lack of signalling domains, it was initially believed to be an inhibitory RLH and overexpression of lgp2 indeed suppressed RIG-I signalling [73]. Studies on lgp2 knockout mice however offered a more differentiated view of the role of lgp2 [74–77]. They provide evidence that lgp2 enhances mda5-dependent IFN induction in response to picornavirus infection. As the affinity of the mda5 CTD for dsRNA is relatively low, lgp2 might serve as a co-receptor that facilitates RNA binding to mda5, e.g. by increasing the local concentration of suitable RNA ligands [74,75]. It has recently been shown that the basal ATP hydrolysis activity displayed by lgp2, which distinguishes it from mda5 and RIG-I, is required for enhanced binding of lgp2 to dsRNA, in particular to imperfect dsRNA substrates containing noncomplementary bulges [78]. The authors showed that this basal ATP hydrolysis activity of lgp2 was required for its enhancement of mda5-mediated IFN induction, suggesting that lgp2 might bind to dsRNA first before sensitizing mda5 to these RNA ligands [78]. The extent of lgp2 involvement in mda5 signalling might depend on the nature of the dsRNA ligand, e.g. its overall length and the presence of secondary structures, such as bulges and hairpins.

The effects of lgp2 on RIG-I signalling appear to be even more complex and might also depend on the exact nature of the RNA ligand. As mentioned above, lgp2 was initially described to inhibit RIG-I signalling through an interaction between its CTD and the helicase domain of RIG-I [69,73]. In keeping with this, Lgp2 knockout mice were shown to display enhanced IFN production to VSV infection [74], and endogenous lgp2 limited the responses to seasonal influenza virus strains that activate IRF3 and induce IFN [77]. However, another study suggested that loss of lgp2 leads to defects in RIG-I-mediated VSV and Sendai virus recognition [75]. Lgp2 also appears to be required for responses to cytoplasmic poly(dA:dT) DNA, *Listeria monocytogenes* and vaccinia virus, which are likely mediated by RIG-I ligands generated through RNA polymerase III activity [76]. This controversial data on the relationship between RIG-I and lgp2 might suggest that lgp2 can act as both an inhibitor and activator of RIG-I, possibly depending on the nature of the RNA ligand and the cellular concentration of lgp2. It will require further investigations to understand whether and how lgp2 positively and negatively modulates RIG-I-dependent responses to different dsRNA ligands, and also how exactly it enhances mda5 recognition of dsRNA.

2.2. DDX3

DDX3 has two homologues, DDX3X and DDX3Y, that were first described in 1997 in a study of the non-recombining region of the Y chromosome [79]. DDX3X is ubiquitously expressed in most tissues, while expression of the DDX3Y protein appears to be restricted to the male germline [80]. Like many DEAD-box helicases, DDX3(X) has been implicated in various different aspects of RNA metabolism, including transcriptional regulation, splicing, mRNA export, ribosome biogenesis and translational regulation [81]. It has been suggested to regulate transcription of the E-Cadherin, p21/waf and *ifnb* promoters [82–84], and recent studies appear to manifest a role for DDX3 in translational regulation, in particular for mRNAs with complex 5′UTRs [85,86].

Two simultaneous reports in 2008 provided the first evidence that DDX3 is a component of anti-viral innate immune signalling pathways [84,87]. The study we were involved in identified DDX3 as a molecular host target of the vaccinia virus protein K7. K7 inhibited *ifnb* induction at the level of IKKε and TBK1, the two key kinases that phosphorylate IRF3/7 downstream of TLR3, TLR4, the RLHs, and most known cytoplasmic DNA receptors. We showed that DDX3 interacted with IKKε after Sendai virus stimulation and that it enhanced induction of the *ifnb* promoter [87]. The intrinsically unstructured N-terminal region of DDX3 was required for this activity, but its ATPase and RNA unwinding activities were not [87]. The second

report concurrently revealed that DDX interacts with, and is a phosphorylation target of, TBK1. TBK1-mediated phosphorylation of DDX3 was shown to be required for the effect of DDX3 on the *ifnb* promoter [84]. This study therefore also supported a role for DDX3 in *ifnb* promoter induction downstream of TBK1/IKKε. However, the authors also demonstrated that DDX3 was recruited to the *ifnb* promoter enhancer region, suggesting that DDX3 acts as a transcriptional regulator after being activated by TBK1 [84].

Another study also supported a role for DDX3 in the RLH pathway leading to type I IFN induction, but placed DDX3 upstream of TBK1/IKKε [13]. Oshiumi et al. demonstrated an interaction between DDX3 and MAVS and suggested that DDX3 acts as a sensor for viral RNA in conjunction with RIG-I and mda5. The authors proposed that DDX3 can sensitize the RLH system for dsRNA ligands at early stages of infection when levels of the IFN-inducible RIG-I are still low [13]. The same group later demonstrated that the HCV Core protein, which had previously been shown to interact with DDX3, can disrupt the MAVS-DDX3 interaction and thus act as a viral immune evasion protein preventing IFN induction [88]. Contrary to this, another report suggested that HCV Core protein actually triggers IFN induction through DDX3 [89]. Culture-adapted strains of HCV had acquired mutations in the N-terminal DDX3-binding region of the Core protein, which lowered their binding affinity for DDX3. This resulted in reduced TBK1-dependent IFN induction and enhanced replication of the culture adapted strains [89]. As DDX3 is required for HCV replication [90,91], the DDX3-HCV Core protein interaction will also be discussed in this context in Section 3.1.

Several other studies also support a role for DDX3 in *ifnb* induction. While it had first been shown that DDX3 interacts with the HBV polymerase and inhibits HBV reverse transcription [92], two subsequent studies showed that the HBV polymerase acts as a viral immune evasion protein by disrupting the interaction between DDX3 and IKKε/TBK1 [93,94]. This is strongly reminiscent of the way vaccinia virus protein K7 targets DDX3 to prevent induction of *ifnb* [87]. DDX3 also appears to be involved in IFN-β induction downstream of the cytoplasmic DNA receptor DAI. DAI-dependent IFN-β induction in response to Human Cytomegalovirus (HCMV) was greatly diminished upon knockdown of DDX3 [95].

In summary, these reports clearly implicate DDX3 in anti-viral innate immune signalling pathways leading to type I IFN induction. However, despite the strength of this evidence, the exact nature of the involvement of DDX3 in these pathways is not yet clear. Most of the evidence points to a function as a signalling intermediate downstream of TBK1/IKKε [84,87,93–95], however DDX3 also interacts with MAVS and has been suggested to act as a PRR that senses viral RNA [13]. Finally, it has also been shown to bind to the *ifnb* promoter directly, suggesting it acts as a transcriptional regulator [84].

Our research group has recently confirmed direct interactions between DDX3 and IKKε, and between DDX3 and the transcription factor IRF3 [96]. Phosphorylation of DDX3 at serine 102 by IKKε was required for the recruitment of IRF3 into the complex, and mutation of serine 102 in DDX3 led to reduced IRF3 activation and *ifnb* promoter induction [96]. We therefore propose that DDX3 acts as a scaffolding adaptor that coordinates the activation of IKKε and its substrate IRF3 and thereby contributes to *ifnb* induction.

Future research will have to clarify whether DDX3 can also act as a direct viral RNA sensor and/or a transcriptional regulator, as these are also compatible with the described functions of DEAD-box helicases and are not necessarily mutually exclusive with its role as a signalling adaptor.

2.3. DDX41

In order to comprehensively address the potential involvement of other DExD/H-box helicases in innate immunity, Yong-Jun Liu's group carried out an siRNA screen with 59 members of the DExD/H-box helicase family. They found that IFN and pro-inflammatory cytokine production in response to cytosolic DNA was impaired in murine myeloid (m)DCs lacking DDX41 [10]. The authors showed that DDX41 bound specifically to DNA rather than RNA, and that this binding required the Walker A and B motifs of DDX41 [10]. As Walker motifs usually mediate ATP binding and hydrolysis, this might indicate that ATP binding is required for DNA binding by DDX41.

Several other receptors for cytoplasmic DNA have been identified, including IFI16, DAI, and RNA Polymerase III [97]. IFI16, in particular, has also been shown to sense viral DNA in human myeloid cells [19]. In fact, in the study by Yong-Jun Liu's group, DDX41 and IFI16 were both required for the response to cytosolic DNA and DNA viruses in the human monocytic THP1 cell line [10]. The authors suggested that the constitutively expressed DDX41 acts as an early sensor of viral DNA, while IFI16 is upregulated at later stages of viral infections in a DDX41-dependent manner. Indeed, IFI16 expression was nearly completely absent in cells lacking DDX41 [10].

The molecule STING has been shown to act as a crucial adaptor molecule for cytoplasmic DNA receptors [98]. Here, STING was also required for the response to cytosolic DNA, as well as to HSV-1, L. monocytogenes and adenovirus infection [10]. DDX41 interacted with STING and TBK1 (but not p204, the murine IFI16 orthologue), in keeping with its proposed role as a separate DNA receptor [10]. Another recent publication confirmed a role for DDX41 in the response to adenovirus infection in the murine macrophage cell line Raw264.7. In this study, TBK1, STING and Aim2 were required to induce IRF3 activation in addition to DDX41, but not DAI or p204 [99]. Aim2 is a cytosolic DNA receptor that is closely related to IFI16, but has mainly been linked to inflammasome and Caspase-1 activation, rather than IFN induction [100].

Recently, DDX41 has also been proposed to bind directly to cyclic dinucleotides (CDNs), namely cyclic-di-GMP and cyclic-di-AMP [101]. These bacterial second messenger molecules act as PAMPs that signal the presence of an intracellular bacterial infection and induce a type I IFN response. DDX41 has been shown to induce a STING-TBK1-IRF3-dependent type I IFN response, following its binding to CDNs [101]. However, STING itself can also directly bind CDNs, and it was previously proposed that STING can directly sense the presence of CDNs without the need for an upstream receptor [102]. More recently, two studies from the Chen lab described cyclic-GMP-AMP (cGAMP) as a novel second messenger that is generated in response to cytoplasmic DNA in mammalian cells [103,104]. They identified a nucleotidyltransferase, cGAMP synthase (cGAS), which generates cGAMP in response to cytosolic DNA [104]. They implicated cGAS as a universal DNA sensor that triggers the activation of downstream molecules, such as STING, in a cGAMP-dependent manner [104]. The respective roles of DDX41 and STING in CDN sensing remain to be clarified, and also whether direct binding of cytosolic DNA to these two proteins is required.

There are very few previous reports on cellular functions for DDX41, but in one such study DDX41 (or ABS, Abstrakt) was suggested to interact with Sorting Nexin-2 (SNX2) [105]. The function of SNX2 is also largely unclear, but it is part of the retromer complex that shuttles receptors from endosomes to the trans-Golgi network [106]. It is possible that SNX2 is also involved in other intracellular trafficking events [107]. As mentioned above, it is becoming increasingly clear that innate immune responses are intricately linked with membrane reorganisation and vesicle transport events [59,108]. Reminiscent of what was observed for TRAF3, STING appears to traffic from the ER to the Golgi in response to cytoplasmic DNA sensing. It eventually assembles with TBK1 in cytoplasmic punctuate structures [108]. Therefore, it is an intriguing idea that DDX41 might be involved in STING trafficking through its interaction with a sorting nexin; a potential role of DDX41 that might warrant further investigation.

2.4. DHX9

Yong-Jun Liu's group also identified DHX9 (also known as RNA Helicase A (RHA) or Nuclear DNA Helicase II (NDHII)) as a sensor

for double-stranded RNA in myeloid cells [9], and as a sensor for CpG DNA in plasmacytoid dendritic cells (pDCs) [14]. In myeloid DCs, type I IFN and pro-inflammatory cytokine production in response to poly(I:C) was attenuated after knockdown of DHX9. Binding of DHX9 to poly(I:C) required its two N-terminal double-stranded RNA-binding domains rather than the helicase core region. The C-terminus of DHX9 was shown to interact with the CARD domain of the mitochondrial adaptor MAVS, suggesting that DHX9 links into the RLH signalling pathway. In fact, either RIG-I or mda5 were also required for the response to poly(I:C) in addition to DHX9, depending on the exact nature of the RNA ligand used [9]. In pDCs, DHX9 was shown to be required for the induction of IFN-α and TNF-α by CpG-B DNA, in a MyD88-dependent manner (see also Section 2.6) [14]. There is also additional evidence for a role of DHX9 in innate immunity. It is an interferon-inducible gene and has been shown to get phosphorylated by the dsRNA-binding kinase PKR [109], providing a link between DHX9 and responses to viral dsRNA in a different context. DHX9 phosphorylation by PKR was investigated in the context of its requirement for HIV replication and decreased its affinity for RNA [109] (see also Section 3.3).

DHX9 has been extensively characterised as a transcriptional regulator, consistent with its mostly nuclear localisation [5]. Interestingly, it was shown that DHX9 is recruited into PML nuclear bodies after IFN-α-stimulation where it associated with nascent RNA polymerase II (mRNA) transcripts [110]. This observation suggests that DHX9 might have a role in regulating transcription of IFN-stimulated genes (ISGs) or subsequent processing of ISG mRNAs [110]. This potential function of DHX9 also clearly implicates DHX9 in anti-viral immune responses, as the anti-viral effects of IFNs are largely executed by IFN-stimulated genes. In fact, many proteins involved in the detection of viral infections are themselves interferon-inducible, including RIG-I, mda5 and IRF7.

In the context of this evidence for one or more functions of DHX9 in antiviral immunity, it is very interesting to note that DHX9 is actively recruited by many different viruses and required for their replication (discussed in Section 3.3). This suggests that DHX9 might be a highly contested protein in the battle between viruses and the innate immune system.

2.5. DDX1/DDX21/DHX36 complex

In yet another publication from Yong-Jun Liu's group, DDX1 was identified as a dsRNA-sensing receptor [11]. In a pulldown with poly(I:C) followed by mass spectrometry analysis, DDX1 as well as DDX21 and DHX36 were identified. The authors then showed that only DDX1 directly bound to poly(I:C), a slightly unexpected finding as DHX36 and DDX21 would be expected to bind dsRNA via their conserved helicase motifs. Instead, DDX21 and DHX36 were placed downstream of DDX1. DDX21 bound to both DDX1 and DHX36, suggesting that it bridges the interaction between these two helicases [11]. DDX21 also interacted with itself, suggesting that the complex might be a DDX1-DDX21-DDX21-DHX36 complex. Both DDX21 and DHX36 interacted with TRIF, the TIR-adaptor molecule that mediates signalling downstream of TLR3 and TLR4 [11]. This suggested that DDX1 senses dsRNA and then triggers signalling via DDX21 and DHX36 to TRIF. Knockdown of DDX1, DDX21 or DHX36 inhibited IFN induction in response to long or short poly(I:C), Influenza Virus and Reovirus [11]. Depending on the nature of the RNA ligand, knockdown of RIG-I, mda5 or TLR3 also strongly inhibited IFN induction [11].

Previous reports on DDX1 function appeared to point towards a role for DDX1 in 3′ processing of mRNAs [111,112]. It was shown to interact with nuclear ribonucleoprotein K, poly(A)-RNA [112], and Cleavage stimulatory factor (CstF) 64, and to localise to nuclear cleavage bodies [111]. In addition, DDX1 was shown to bind to the p65 subunit of the major pro-inflammatory transcription factor NF-κB, thereby enhancing NF-κB dependent transcription [113].

DDX21 was previously identified as nucleolar protein regulating ribosomal (r)RNA processing. Knockdown of DDX21 inhibited 18S and 28S rRNA production and slowed down cell proliferation [114], possibly by inducing cell cycle arrest at the G1/S-Phase [115]. DDX21 (originally named RH-II/GuA) has also been shown to interact directly with the transcription factor c-jun and to participate in c-jun mediated transcriptional activation of target genes [116].

DHX36 (also called RHAU or G4R1) is a predominantly nuclear protein, but appears to shuttle out of the nucleus as part of RNPs. It can be recruited into stress granules via its N-terminal RNA-binding domain [117]. It has also been shown to be involved in the degradation of ARE-containing RNAs [118]. Furthermore, DHX36 has been described to recognise and remove so-called quadruplex knots in G-rich RNA and DNA (G4-loops) [119,120]. Its specificity for G4-loops is mediated by the N-terminal RNA-binding domain of DHX36 rather than its helicase core region [119]. G4 loops are found in telomeric repeats, but they also seem to be present in gene promoter regions where they block transcription [121]. Indeed, DHX36 has been suggested to resolve G4-loops present in the promoter of the transcriptional regulator YY1 and to thus facilitate its transcription [122]. YY1 has recently been shown to negatively regulate the *ifnb* promoter, providing another potential link between DHX36 and anti-viral immunity [123].

As discussed further in Section 2.6, DHX36 has also been suggested to sense CpG-A oligonucleotides in plasmacytoid DCs [14]. As CpG-A oligonucleotides contain polyG sequences at their 5′ and/or 3′ ends, they might form G4-loops or similar structures that can specifically be detected by DHX36.

2.6. DHX36 and DHX9 as CpG sensors

As mentioned above, DHX36 was also characterised in a separate study from Liu's group as a CpG-A binding protein, whereas DHX9 was identified as a CpG-B binding protein [14]. CpG-A and CpG-B oligonucleotides are known to induce IFN-α and proinflammatory cytokine production through TLR9, with CpG-A inducing stronger IFN-α production [124,125]. Knockdown of DHX9 or DHX36 reduced IFN-α induction in response to CpG-A and CpG-B respectively [14]. Knockdown of Myd88 also completely abolished responses to CpG-A and CpG-B, suggesting that these helicases signal through a MyD88-dependent pathway. Indeed, the authors showed that both helicases bound to the TIR domain of MyD88 [14]. However, knockdown of TLR9 also nearly completely abolished activation of IRF7 and NF-κB downstream of CpG-A and CpG-B [14], suggesting that both TLR9 and DHX9/DHX36 are required for CpG sensing in pDCs.

Both DHX9 and DHX36 have previously been described as DNA-binding proteins, supporting their potential roles as DNA sensors. As mentioned in Section 2.5, DHX36 was shown to resolve G4 loops in both DNA and RNA [120]. DHX9 was characterised as a helicase that could unwind both DNA and RNA [126]. Recently, it has been shown that DHX9 might in fact also be involved in unwinding unusual DNA structures, such as intramolecular triplexes [127]. As CpG oligonucleotides contain repetitive regions of GC nucleotides and poly(G) stretches in the case of CpG-A, they might be prone to forming unusual DNA structures, such as G4-loops or triplex structures, possibly explaining the observed binding of DHX9 and DHX36 to these CpG-DNA oligonucleotides.

The characterisation of DHX9 as a dsRNA (Section 2.4) and CpG-A sensing protein is intriguing, yet it is also puzzling how the same protein can detect different nucleic acid ligands and then induce signalling through either MAVS (Section 2.4) or MyD88 depending on the nature of the ligand and the cell type [9,14]. Similarly, DHX36 is suggested to facilitate signalling through TRIF downstream of DDX1, as well as acting as a receptor for CpG-B DNA in a MyD88-dependent manner. Clearly, future studies will have to further ascertain the exact mechanisms by which both DHX36 and DHX9 contribute to these innate immune signalling pathways.

2.7. DDX60

Seya's laboratory recently identified a role for DDX60 in RLH-mediated signalling. Their study showed that DDX60 acts in conjunction with RIG-I or mda5 to mediate responses to viral dsRNA [12]. Production of IFN-β and the IFN-inducible chemokine IP10 in response to transfected poly(I:C), Sendai Virus, VSV, Poliovirus or HSV-1 infection was reduced in cells after DDX60 knockdown. VSV and poliovirus replication were also reduced following knockdown of DDX60 [12]. While DDX60 has sequence similarity with the RNA exosome cofactor SKIV2L and interacted with two exosome components, EXOSC1 and EXOSC4, the antiviral activity of DDX60 appeared to be independent of the RNA exosome [12]. Instead, the authors showed that DDX60 interacted with the RIG-I, mda5 and lgp2 and enhanced their signalling.

In a large screen for the anti-viral activities of ISGs, DDX60 was also recently shown to have anti-viral activity against HCV [128]. As HCV genomic RNA is recognised by RIG-I, this report appears to confirm a role for DDX60 in the RLH pathway.

Thus, DDX60 was proposed to act as a cofactor for RLH-dependent detection of dsRNA [12], potentially sensitizing the RLHs for dsRNA. It will be interesting to address the question whether all RLH-mediated responses require DDX60 or whether it selectively facilitates the detection of specific RNA (or even DNA) ligands.

2.8. Concluding remarks on RNA helicases in innate immunity

While the RIG-like helicases (RLHs) have a clearly established role as viral RNA sensors in the cytosol, the evidence that other DExD/H-box helicases can also act as sensors for viral RNA or DNA ligands is still a little bit ambiguous. The RLHs recognise their specific RNA ligands mainly through their C-terminal regulatory domain and not the conserved helicase core region. It generally appears to be the case for DExD/H-box helicases that interactions with specific RNA ligands are mediated by adjacent additional RNA-binding domains or are facilitated through interactions with other RNA-binding proteins [2,4]. In their N-terminal tandem CARD domains, the RLHs also contain a clearly defined signalling domain that allows them to trigger signalling through a homologous interaction with the CARD-domain containing adaptor molecule MAVS.

For most other DexD/H-box helicases that have been implicated in innate immune responses to viral nucleic acids, it is less clear whether they act as *bona fide* receptors that initiate signalling in response to binding viral RNA or DNA. DDX3 has been suggested to act as a receptor for dsRNA [13], yet there is also strong evidence that it functions downstream of TBK1 and IKKε either as a signalling adaptor and/or transcriptional regulator [84,87,93–96]. As DexD/H-box helicases bind to RNA and in some cases also to DNA in a rather indiscriminate manner via the conserved helicase motifs, it is not unexpected to observe them interacting with various synthetic or viral nucleic acids. However, several DExD/H-box helicases, including DDX3 and DHX9, have additional functions that are independent of their RNA unwinding activity and in many cases also their ATPase activity [5,84,87]. Mere *in vitro* binding to RNA or DNA should therefore not be sufficient to characterise a DExD/H-box protein as a nucleic acid sensor.

It might be worth considering that not all of the helicases implicated in RNA or DNA sensing are independent receptors, but could either act as co-receptors for established nucleic-acid binding PRRs, play downstream roles in their signalling pathways or, even more generally, influence the transcriptional or posttranscriptional regulation of immuno-relevant genes, such as interferons or interferon-stimulated genes. Thus, it should be investigated how exactly the newly identified DExD/H-box helicase nucleic-acid sensing pathways intersect or cooperate with the established nucleic-acid receptors, such as the RLHs and TLR9. The current proposed roles for the DExD/H-box helicases described in this section are summarised in Fig. 1.

3. RNA helicases in viral replication

As mentioned above, not all viruses encode their own RNA helicases and many cellular helicases have been identified as essential host factors for viral replication. It appears that viruses rely heavily on host helicases for RNA unwinding functionality. Presumably, akin to their numerous functions in the processes mediating eukaryotic gene expression, RNA helicases are required for equivalent steps during the expression of viral genes. In addition to that, viruses might require additional RNA unwinding activity for remodelling genomic RNA structures, remodelling structures of replication intermediates, or for packaging their genomic RNA into virions.

The role of RNA helicases in the replication cycles of different virus classes has not been systematically addressed. However siRNA screens continue to identify DExD/H-box helicases as essential cofactors for the replication of individual viruses, and proteomics screens with viral proteins as baits often identify interacting DExD/H-box helicases. We will focus here on the DExD/H-box helicases that appear to have an obvious dual function as viral replication cofactors and innate immune mediators, namely DDX3, DDX1 and DHX9. For a more comprehensive overview of the roles of RNA helicases in viral replication, we direct the reader towards a recent review by Ranji and Boris–Lawrie [129]. The role of RNA helicases in HIV infection has also recently been reviewed in detail [130].

3.1. DDX3

In Section 2.2, we have described how viral immune evasion proteins inhibit DDX3′s function in anti-viral innate immunity, with the consequence of suppressing type I IFN production. On the other hand, DDX3 has also been shown to be an essential host factor for the replication of several different viruses, most notably HIV and HCV [90,91,131].

DDX3 was shown to be required for HIV replication and the export of unspliced and partially spliced HIV RNAs from the nucleus [131]. These viral RNAs contain the so-called Rev-Responsive Element (RRE) that is recognised by the HIV regulatory protein Rev. Rev contains a leucine-rich nuclear export signal (NES) and binds to CRM1 (Chromosome Maintenance Region 1), the cellular export shuttle protein that mediates export of NES-containing proteins [132]. Thus, RRE-containing HIV RNAs are exported via the CRM1 pathway rather than the main export pathway for fully spliced cellular mRNAs mediated by the TAP (tip associated protein) export shuttle protein.

Export of RRE-containing HIV RNAs apparently also requires DDX3, which was shown to bind to HIV Rev and CRM1 and to facilitate export of RRE-containing HIV RNAs out of the nucleus [131]. The authors proposed that DDX3 acts as an effector of CRM1-mediated transport rather than mere cargo, as neither RanGTP binding nor the putative NES of DDX3 were required for its binding to CRM1 [131]. On the other hand, the ATPase and RNA unwinding activity of DDX3 were required for HIV replication [131]. It was later confirmed in a separate study that depletion of endogenous DDX3 using shRNA inhibits the export of HIV RNAs and viral replication, while not affecting cell viability [133].

Essential cofactors of viral infections are interesting potential drug targets, as it is thought that viruses will find it harder to evolve resistance to drugs that target cellular host factors rather than viral proteins. This is particularly relevant for HIV, as the emergence of resistance to antiretroviral drugs poses a significant problem with current HIV treatment regimes. Several studies have already attempted to develop inhibitors for DDX3, mainly directed against its ATPase activity: Ring expanded nucleoside analogues inhibiting the ATPase activity of DDX3 suppressed HIV replication without displaying any apparent cellular toxicity [134]. Another group identified small molecule inhibitors of DDX3's ATPase activity using pharmacophore modelling, and showed that these were also able to block HIV replication [135,136]. A recent study developed inhibitors targeting the RNA-binding interface of

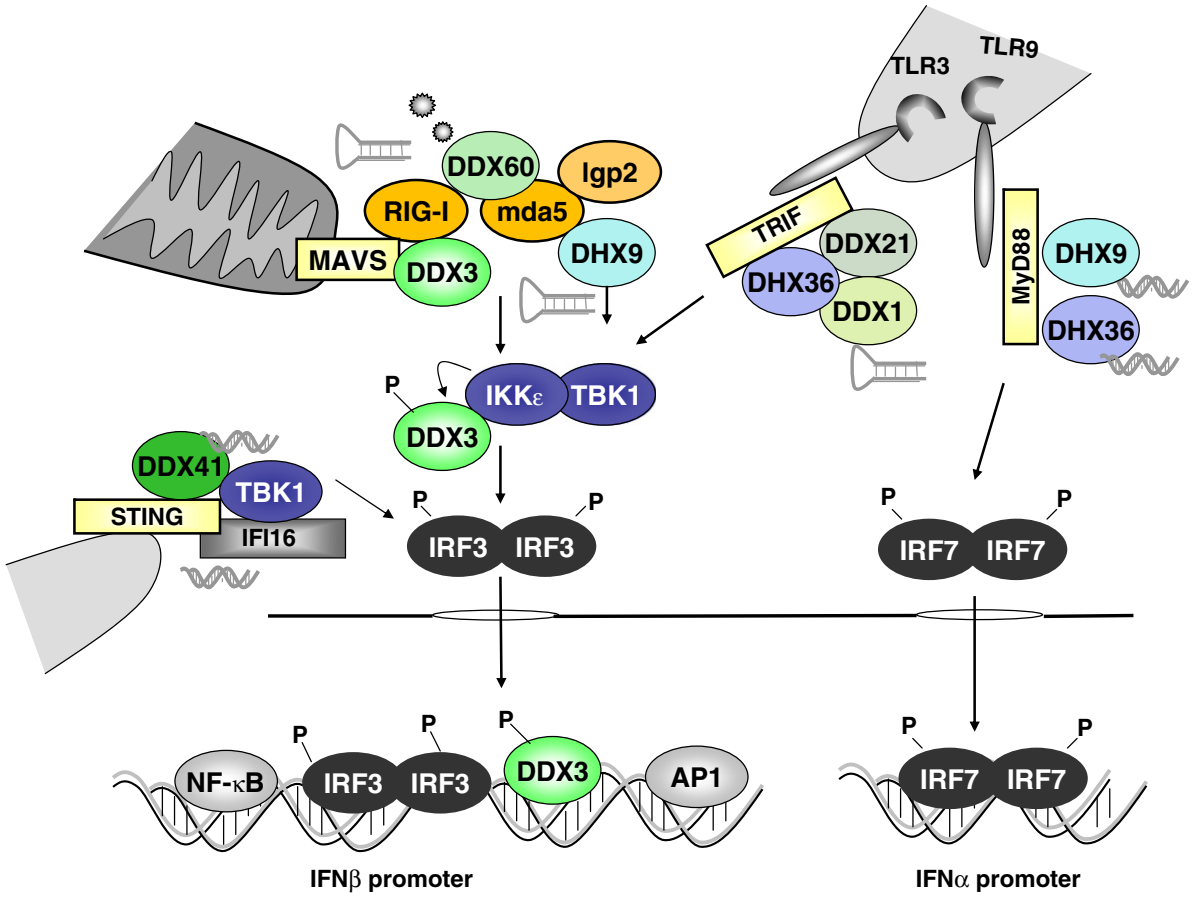

Fig. 1. The proposed roles of DExD/H-box helicases in anti-viral innate immune signalling. While the RIG-like helicases, RIG-I, mda5 and lgp2, have clearly defined roles in the sensing of viral nucleic acids in the cytosol, the exact function of other DExD/H-box helicases is more ambiguous. DDX3 has been suggested to act as a sensor of viral dsRNA in conjunction with the RLHs, as a signalling intermediate downstream of TBK1 and IKKε and as a transcriptional regulator of the *ifnb* promoter. DDX60 has also been proposed to sensitize the RLH to viral dsRNA ligands. DDX41 seems to sense cytoplasmic DNA and signal in a STING-dependent manner. DHX9 has been proposed to sense dsRNA and to signal in a MAVS-dependent manner, and separately it was implicated in the response to CpG-B DNA where it signals through MyD88. A complex of DDX1, DDX21 and DHX36 has been suggested to detect dsRNA and to signal via TRIF. DHX36 has also been shown to trigger IFN-α production in response to CpG-A DNA via a MyD88-dependent pathway.

DDX3. These inhibitors also blocked ATPase and unwinding activity of DDX3 and suppressed HIV replication in human PBMCs [137].

These studies suggest that DDX3 might indeed be a viable target for the development of novel anti-HIV drugs. However, it is vital that we understand more about the various cellular functions of DDX3 and in particular the role of its ATPase activity, in order to develop safe and specific strategies for targeting DDX3 therapeutically.

DDX3 was shown to interact with the Hepatitis C Virus (HCV) Core protein, which forms part of the viral nucleocapsid [138–140]. While the functional consequences of this interaction have still not been fully established, it was more recently shown that DDX3 is required for HCV replication [90,91]. However, it is still unclear whether the interaction between DDX3 and the HCV Core protein is required for the effect of DDX3 on HCV replication [141,142]. Expression of the HCV Core protein induces a striking relocalisation of DDX3 in the cell, as it strongly colocalises with the HCV Core protein [91]. This suggested that sequestering of DDX3 by the HCV Core protein might disrupt other cellular functions of DDX3. As mentioned in Section 2.2., it was suggested that the HCV Core protein can affect the function of DDX3 in IFN induction [88,89].

In contrast to its role as an essential host factor in HCV replication, DDX3 inhibited the replication of another hepatotropic virus, Hepatitis B Virus. A study by Wang et al. showed that DDX3 interacts with HBV polymerase, gets incorporated into nucleocapsids and inhibits viral reverse transcription [93]. As mentioned in Section 2.2., it has since also been demonstrated that the HBV polymerase blocks the function of DDX3 in innate immune signalling pathways leading to IFN induction [93,94]. Interestingly, in an analysis of human hepatocellular carcinoma (HCC) samples, DDX3 levels were found to be reduced in HCC samples from HBV-positive patients, while no reduction was observed in samples from HCV-positive patients [143]. Hepatocellular carcinoma (HCC) is a highly prevalent cancer in the modern world and is strongly linked with hepatitis virus infection [144]. The above-mentioned study linked DDX3 to the progression of cancer by showing that knockdown of DDX3 led to early entry into S-phase and an increased growth rate [143]. It was suggested that while HBV downregulates DDX3 expression (possibly to escape its inhibitory effect on viral reverse transcription), HCV might disable DDX3 function through the Core protein interaction [143]. Future research should clarify the mechanism by which DDX3 contributes to HCV replication and elucidate the functional consequences of the Core protein-DDX3 interaction on viral replication and HCV-mediated pathological effects, such as the development of liver cirrhosis and HCC.

Recent studies suggested that DDX3 is also required for Norovirus [145] and West-Nile Virus replication [146]. These findings might indicate that a requirement of DDX3 for viral replication is a more universal feature of positive-stranded RNA viruses.

3.2. DDX1

Similar to DDX3, DDX1 has been shown to facilitate the nuclear export of Rev-dependent HIV RNAs [147]. Recent biochemical studies showed that DDX1 promotes oligomerisation of Rev on the RRE contained in Rev-dependent HIV RNAs [148]. DDX1 can bind to

Rev-RRE complexes, and its ATPase activity was stimulated to a similar degree by free RNA or Rev-bound RNA [149]. These findings suggest that DDX1 plays a role in the assembly of Rev-RRE-RNA complexes, possibly preparing them for efficient export through the CRM1 pathway. It would be interesting to address whether DDX1 and DDX3 have distinct roles in HIV RNA export, as both of these helicases have been shown to be involved in the export of Rev-dependent RNAs.

DDX1 was also shown to bind to the transcriptional control region (TCR) of the John Cunningham (JC) Virus together with cleavage stimulation factor (CstF). JC virus is a human polyomavirus, a dsDNA virus that replicates in the nucleus, and can cause progressive multifocal leukoencephalopathy (PML) in immunosuppressed patients. DDX1 expression promoted transcription of viral genes, while knockdown of DDX1 led to reduced expression levels of JCV proteins [150,151]. DDX1 levels in cells were shown to correlate with permissiveness to JCV infection, and exogenous expression of DDX1 restored susceptibility to JCV infection.

DDX1 also interacts with Infectious Bronchitis Virus (IBV) non-structural protein 14 (nsp14) and nsp14 of the SARS-Coronavirus [152]. Coronavirus nsp14 proteins contain 3′-5′ exoribonuclease activity and appear to be involved in ensuring a sufficient level of fidelity during the replication of the exceptionally large Coronavirus RNA genome [153]. Upon IBV infection of Vero cells, DDX1 redistributed from the nucleus to the cytoplasm, where it colocalised with nsp14 and newly-synthesized viral RNAs [152]. These findings suggest a role for DDX1 in genomic or subgenomic coronavirus RNA replication or transcription, however only a small effect on IBV replication in a cell culture system was found [152].

DDX1 was also found to associate with the HCV 3′(+)UTR and its reverse complementary 5′(−)UTR. However, a role for DDX1 in HCV replication could not be confirmed [154].

In summary, DDX1 appears to be recruited by several viruses with different genomes and replication cycles. The exact function of DDX1 in uninfected cells is unclear, but it appears to be involved in the 3′ maturation of mRNAs [111,112]. It is an interesting observation that coronavirus infection induced translocation of DDX1 from the nucleus to the cytoplasm [152], where it could conceivably form the DDX1-DDX21-DHX36-TRIF complex that appears to be involved in the sensing of dsRNA (see section 2.5) [11].

3.3. DHX9

DHX9 is another DExD/H-box helicase that appears to be an attractive 'prey' for viruses, as there is ample evidence that it is required for the replication of several different viruses. Interestingly, the structure of DHX9 is quite similar to that of the Flavivirus NS3 helicase [155].

DHX9 is required for HIV replication [156] and appears to be involved in several different steps of the HIV replication cycle. It was shown to bind to the double-stranded stem of the HIV TAR leader RNA and to enhance transcription from the HIV-LTR [157]. Phosphorylation by PKR within the N-terminal RNA binding domain reduced the affinity of DHX9 for RNA and binding to the HIV TAR [109]. In a separate study, DHX9 increased the levels of unspliced HIV CTE- and RRE-containing mRNAs and was therefore proposed to release incompletely spliced HIV RNAs from spliceosomes for export out of the nucleus [158]. DHX9 has also been suggested to facilitate export of RRE- and CTE-containing viral RNAs through an alternative nuclear export pathway mediated by the Sam68 cofactor and TAP [159]. There is increasing evidence that DHX9 facilitates translation of HIV RNAs through binding to the 5′UTR [160]. DHX9 has also been shown to be incorporated into HIV virions by interacting with the HIV gag protein and HIV RNA. When DHX9 was knocked down in virus producer cells, the resulting virions were less infective and had a defect at the level of reverse transcription [161]. Another recent study showed that DHX9 facilitates the annealing of $tRNA_3^{Lys}$ to HIV gag mRNA. The viral RNA with its associated $tRNA_3^{Lys}$ is then incorporated into assembling virions [162]. As DHX9 has also been shown to facilitate translation of gag mRNA [160], it appears that DHX9 can regulate two subsequent steps in the viral life cycle. The authors suggested that DHX9 together with the HIV gag protein remodels the viral RNA which switches it from being translated to being incorporated into the newly assembling virions [162]. Thus, as stated above, DHX9 appears to be involved in virtually all steps regulating the expression of HIV genes as well as the assembly of new virions. This is not inconceivable, as other viral and cellular proteins that regulate transcription or export of HIV RNAs, such as HIV Tat, HIV Rev, DDX3 and Sam68, have also been shown to regulate translation of viral RNAs [86,163,164].

It still needs to be clarified whether DHX9 really independently affects all of these different stages of HIV gene expression and replication. However, if this is indeed the case, it suggests that DHX9 might be an attractive target for HIV (and other viruses) due to its multi-functionality.

DHX9 has also been shown to facilitate translation initiation in several other lymphotropic retroviruses. DHX9 interacted with the so-called posttranscriptional control element (PCE), a stem-loop structure located at the 5′end of retroviral (and some cellular) mRNAs. DHX9 is likely to recognise common structural features of the PCE rather than a specific RNA sequence and to promote translation by facilitating ribosome access [165].

DHX9 was shown to be required for replication of Foot-and-mouth-disease Virus (FMDV), a picornavirus. Upon FMDV infection, a striking redistribution of DHX9 from the nucleus to the cytoplasm occurred and DHX9 localised at sites of viral replication, where it associated with the 5′terminus of the FMDV genome and viral proteins [166].

Similarly, DHX9 and other NF/NFAR proteins were shown to associate with the 5′ and 3′ termini of the HCV genomic RNA and to be required for HCV replication [167]. Interestingly, this study also observed a nuclear to cytoplasmic re-localisation of DHX9. Reminiscent of the proposed mechanism of DHX9 in HIV RNA translation and packaging [162], the authors suggested that DHX9 is involved in creating a circular loop structure between the 3′ and 5′ termini of the genome which is required for coordinating translation and replication of the HCV RNA [167].

DHX9 was also shown to be required for influenza virus replication [168]. It interacted with the viral NS1 protein in an RNA-dependent manner and enhanced viral RNA replication and transcription. Interestingly, the study that implicated DHX9 as a sensor for dsRNA in myeloid DCs showed that influenza virus-induced IFN-α production was reduced in cells after DHX9 knockdown (see section 2.4) [9]. This points to a potentially very interesting dual function for DHX9, in that it might be mediating IFN-induction in response to a virus that actively recruits it to facilitate viral replication. On the other hand, the reduced IFN-α production observed by Zhang et al. might also be a result of decreased viral replication in the absence of DHX9, however viral replication was not assessed in this study [9].

3.4. Other RNA helicases involved in viral replication

DDX3, DDX1 and DHX9 are by no means the only DExD/H-box helicases that are required for viral replication. A recent study identified DDX5, DDX17 and DDX21 as HIV Rev-binding proteins and added them to the long list of helicases that are required for HIV replication [130,169]. The P-body and stress granule component DDX6 was shown to be required for HIV-1, Foamy Virus, HCV and West Nile Virus replication [170–173]. In many cases, a single virus appears to require multiple different cellular DExD/H-box helicases to facilitate different steps in its life cycle [130]. This is likely to reflect the fundamental roles that DExD/H-box helicases play in cellular and viral gene expression. Future studies will undoubtedly add even more helicases to the long list of essential host factors for different viruses.

3.5. Summary: RNA helicases as attractive viral targets

Many DExD/H-box helicases have been identified as essential host factors for the replication of different viruses. Well studied viruses,

like HIV and HCV, appear to require a number of different cellular helicases to facilitate their gene expression and replication. As DExD/H-box helicases facilitate and coordinate cellular gene expression, they also appear to be required to mediate the equivalent steps in viral gene expression. In addition, they have also been shown to coordinate the assembly of viral genomic RNA into newly assembling virions.

4. Concluding remarks

As summarised in this review, DExD/H-box helicases appear to be located at the frontline of the evolutionary arms race between viruses and the host immune system. As ubiquitously expressed nucleic acid binding proteins, they should be well-suited to detect invasion of viral nucleic acid species. Due to intensive research over the years and recent structural studies, we now have a good understanding of how the RIG-like helicases selectively bind viral RNA ligands and trigger signalling pathways in response. For other DExD/H-box helicases that have recently been implicated in anti-viral immunity, these fundamental questions remain to be addressed. In particular, it needs to be clarified whether 'sensing' nucleic acids is their main role and if so, how exactly they distinguish between cellular and viral nucleic acids. It will also be interesting to investigate how they co-operate or intersect with other nucleic acid sensing pathways. It might be worth considering whether some of the helicases act as co-receptors or have (additional) downstream roles in anti-viral innate immune pathways.

DDX3, DHX9 and DDX1 have been implicated in anti-viral immunity, yet are also required for the replication of several different viruses with different genome organisation. This poses the question whether their anti-viral signalling function has evolved in response to being 'hijacked' by viruses, or vice versa. In several cases, it has been shown that the subcellular localisation of these helicases changed when they interacted with viral RNA, namely from a primarily nuclear localisation to cytoplasmic viral replication sites. It might be this redistribution that allowed them to come in contact with the adaptors mediating innate immune pathways. Or have viruses evolved to primarily co-opt helicases that are involved in anti-viral immunity, a strategy that would give them the dual benefit of acquiring RNA unwinding activity while simultaneously disrupting anti-viral immune responses?

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