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DDX3X structural analysis: Implications in the pharmacology and innate immunity

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ABSTRACT

The human DEAD-Box Helicase 3 X-Linked (DDX3X) is an ATP-dependent RNA helicase involved in virtually every step of RNA metabolism, ranging from transcription regulation in the nucleus to translation initiation and stress granule (SG) formation, and plays crucial roles in innate immunity, as well as tumorigenesis and viral infections.

This review discusses latest advances in DDX3X biology and structure-function relationship, including the implications of the recent DDX3X crystal structure in complex with double stranded RNA for RNA metabolism, DDX3X involvement in the cross-talk between innate immune responses and cell stress adaptation, and the roles of DDX3X in controlling cell fate.

1. DDX3X roles

DDX3X is a member of the DEAD (Asp-Glu-Ala-Asp)-box helicase family and it was first mapped in 1997 to chromosome X (Xp11.3→p11.23) (Park et al., 1998). Sex chromosome-linked DDX3X/DDX3Y homologs in primates are believed to have arisen through a series of translocation and gene duplication events of an autosomal gene (Wilson and Makova, 2009). Human DDX3X and DDX3Y perform different functions and show specific distribution pattern in different tissues (Ferrandina et al., 2008; Kwong et al., 2005; Soulat et al., 2008). Despite 91% amino acid similarity between human DDX3X and DDX3Y, DDX3X cannot rescue a DDX3Y loss of function mutation (Rosner and Rinkevich, 2007). DDX3Y is specifically expressed in testis (Ditton et al., 2004; Foresta et al., 2000; Rauschendorf et al., 2011), and its mutations are associated with male infertility (Ferlin et al., 2003). The protein is thought to be critical for spermatogenesis in both, human and mouse (Lardone et al., 2007). Deregulated expression of DDX3Y has also been linked to testicular tumors (Gueler et al., 2012). However, most published work on DDX3-like proteins from higher eukaryotes has concentrated on human DDX3X (Sharma and Jankowsky, 2014), the subject of this review. Moreover, DDX3X participates in the WNT/β-catenin signalling pathway that plays an important role in embryonic development (Pene et al., 2015). DDX3X binds to casein kinase 1 ε (CK1ε) triggering a series of phosphorylations leading to the formation of the WNT/β-catenin signalosome (Maga et al., 2008). Lack of DDX3X expression in Xenopus embryogenesis leads to abnormal embryonic development marked by enlarged heads and eyes, shortened tails, and defective melanocyte and eye pigmentation (Maga et al., 2008). In mouse embryos, DDX3X is crucial for both extraembryonic and embryonic development (Gringhuis et al., 2017). Deficient expression of DDX3X leads to higher levels of genome damage and cell cycle arrest during embryogenesis (Gringhuis et al., 2017). Furthermore, DDX3 promotes stem cell maintenance. Undifferentiated embryonic stem cells and embryonal carcinoma cells express high levels of DDX3 compared to differentiated cells. Notably, when DDX3 activities were perturbed, a drastic decrease in the proliferation of undifferentiated stem cells along with an increase in cellular differentiation was observed.

DDX3X has been implicated in avariety of cellular processes involving RNA, such as splicing, mRNA export, transcriptional and translational regulation, RNA decay, ribosome biogenesis, and viral infections (Rocak and Linder, 2004). During the latter, DDX3X initiates antiviral immunity, and at the same time it is hijacked by viruses to aid the translation of viral mRNAs with complex secondary structure on the 5′end of the transcript - thus being involved on both sides of host: pathogen warfare. Here, we review recent biochemical, structural and pharmacological data to understand the role of DDX3X at the crossroad

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of cell stress responses and antiviral immunity. Furthermore, we discuss how the recently discovered crystal structure of DDX3X in complex with double stranded RNA sheds light on possible implications of the protein in RNA metabolism and innate immunity.

2. Lessons from DDX3X structures

DDX3X domains organization. DDX3X plays a role in many cellular and viral processes. Elucidating how the different DDX3X domains function in complex formation with RNA provides insight in the DDX3X structure and how this affects its function.

DEAD box proteins are built around a highly conserved helicase core, formed by two virtually identical domains structurally homologous to RecA-like domains (Caruthers and McKay, 2002) (DDX3X domains D1 and D2, Fig. 1a). Twelve or more characteristic sequence motifs are located at conserved positions within the D1 and D2 (Fig. 1a) (Linder et al., 1989; Linder and Jankowsky, 2011). Motifs I, II, and VI are broadly conserved among RNA helicases of different types, whereas the Q motif is present in only the DEAD-box family. In the three-dimensional structure, the characteristic sequence motifs are arranged to form an RNA binding site as well as a catalytic binding pocket containing an ATP molecule, a divalent ion (e.g., Mg2+), and a water molecule.

The Q motif acts as a nucleotide sensor and regulates nucleotide hydrolysis; forms hydrogen-bond interactions with the Watson– Crick interface of the adenine base, rendering the activation of Q-motif-containing helicases ATP, rather than NTP, dependent. The motif I is a Walker A motif typified by a glycine-lysine-threonine (GKT) motif; contributes to ATP binding and hydrolysis as the lysine coordinates the b-phosphate of ATP and the threonine coordinates the Mg2+ ion. The motifs Ia, Ib, and Ic contribute to RNA substrate binding via interactions with the sugar–phosphate backbone.

The motif II is a Walker B motif containing the aspartate-glutamate-alanine-aspartate (DEAD) sequence motif; participates in ATP binding and hydrolysis as the glutamate coordinates the catalytic water molecule and the aspartate coordinates the Mg2+ ion. The motif III is characteristic serine-alanine-threonine (SAT) amino acid signature interacts with the γ-phosphate of ATP and mediates communication between the ATP and RNA-binding sites. The motifs IV and IVa contain an

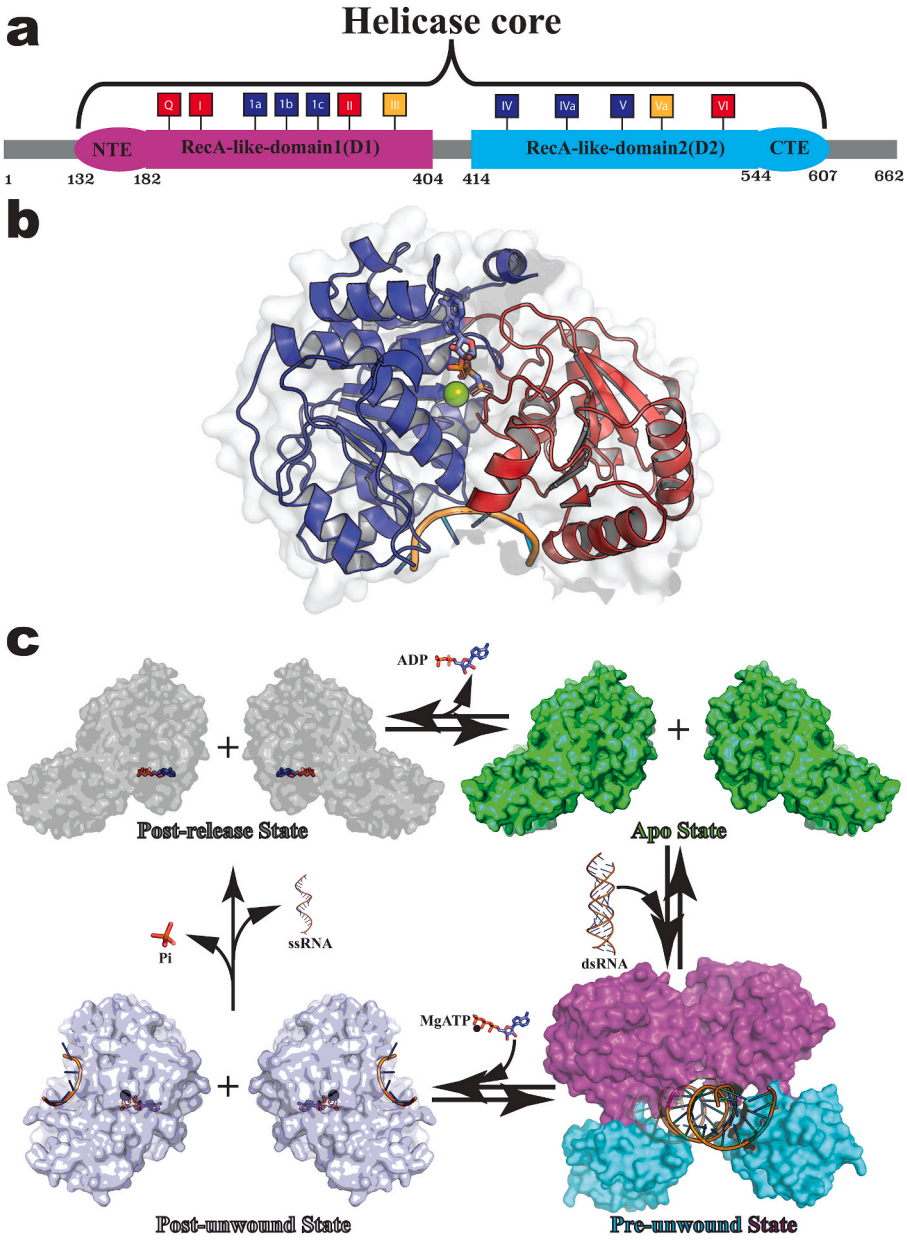

Fig. 1. Protein domains organization and conserved motifs of DDX3X RNA helicase. (a) The catalytic helicase core is composed of two RecA-like domains, domain 1(encompassing helicase motifs Q, I, II, III) and domain2 (encompassing helicase motifs IV, V and VI). These domains are essential for RNA binding and ATPase activity.
Structure of VASA protein and DEAD-box helicase functional cycle. (b) Crystal structure of VASA (PDBid:2db3) in complex with single strand RNA and AMPPNP bound to a Mg^{+2} ion. The N-terminus domain is in the blue cartoon representation, whereas the C-terminus domain is in red overlapped with a transparent surface representation of the entire molecule. The RNA molecule in cartoon representation is in orange, whereas the AMPPNP in a ball-and-stick representation and coloured according to the atoms colour codes. The Mg^{+2} is shown in a space-filling representation and coloured in yellow. (c) The functional cycle illustrating the four-step mechanism of RNA duplex unwinding by DEAD-box helicase. All the proteins are in surface representation; the apo-DDX3X is in green (PDBid:5E7I), the two domains binding of DDX3X in complex with double stranded RNA (PDBid:6O5F) are in cyan and purple, whereas the RNA in ribbon representation is coloured orange with the bases in violet, VASA (PDBid:2DB3) in light grey bound to the RNA in orange, the ligand AMPPNP in a ball-and-stick representation and coloured according to the atoms colour codes and the Mg^{+2} in a space-filling representation coloured in black. The post-release state (PDB:4PXA) in dark grey with the ATP in a ball-and-stick representation and coloured according to the atoms colour codes. The phosphate atom is coloured according to the colour code atoms. The figure was prepared with PyMOL (http://www.pymol.org/). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

evolutionarily conserved phenylalanine residue and contribute to substrate binding through interactions with the RNA. The domains IV and IVa contain an evolutionarily conserved phenylalanine residue and contribute to substrate binding through interactions with the RNA. The domain V participates in RNA substrate binding. The domain Va mediates communication between the ATP and RNA-binding sites.

The domain VI is involved in ATP binding and hydrolysis, and contains two highly conserved arginine residues, which play important roles in stabilising the transition state. More recently the functional core of DDX3X was redefined to encompass not only D1 and D2 domains, but also short N- and C-terminal extensions beyond the two RecA-like domains (Floor et al., 2016) (NTE and CTE, respectively) (Fig. 1a).

The two helicase domains D1 and D2 form a cleft harbouring the enzyme ATP-binding site (Fig. 1b). Key residues mediating ATP binding and hydrolysis form a complex network of interactions, making the generation of DEAD box protein mutants deficient in ATP hydrolysis but competent for ATP binding a challenging task (Linder and Jankowsky, 2011; Strohmeier et al., 2011; Del Campo and Lambowitz, 2009). The DDX3X RNA binding residues also span both domains and face the ATP-binding site at the opposite end of the interdomain cleft (Fig. 1b).

Understanding DDX3X functional cycle through the structures. After complex formation with double stranded RNA (DDX3X-dsRNA), ATP binding leads to unwinding of the dsRNA duplex, resulting in a complex of DDX3X, a single-stranded RNA (ssRNA), and ATP (DDX3X-ssRNA-ATP). Then, ATP hydrolysis facilitates the release of the ssRNA (Putnam and Jankowsky, 2013; Liu et al., 2008; Chen et al., 2008). Structures of DDX3X and its orthologues capture snapshots of the four distinct states of the enzyme's cycle: i) the apo-DDX3X dimer; ii) the pre-unwound DDX3X-dsRNA-DDX3X ternary complex; iii) the post-unwound DDX3X-ssRNA-ATP ternary complex; and iv) the post-release DDX3X-ADP complex (Fig. 1c). The crystal structure of apo-DDX3X (e.g. PDB id:5E7I) (Floor et al., 2016) first revealed that the DDX3X D1D2 core is flanked by largely unstructured N- and C-terminal tails, and yet harbouring important functional motifs (Sharma and Jankowsky, 2014).

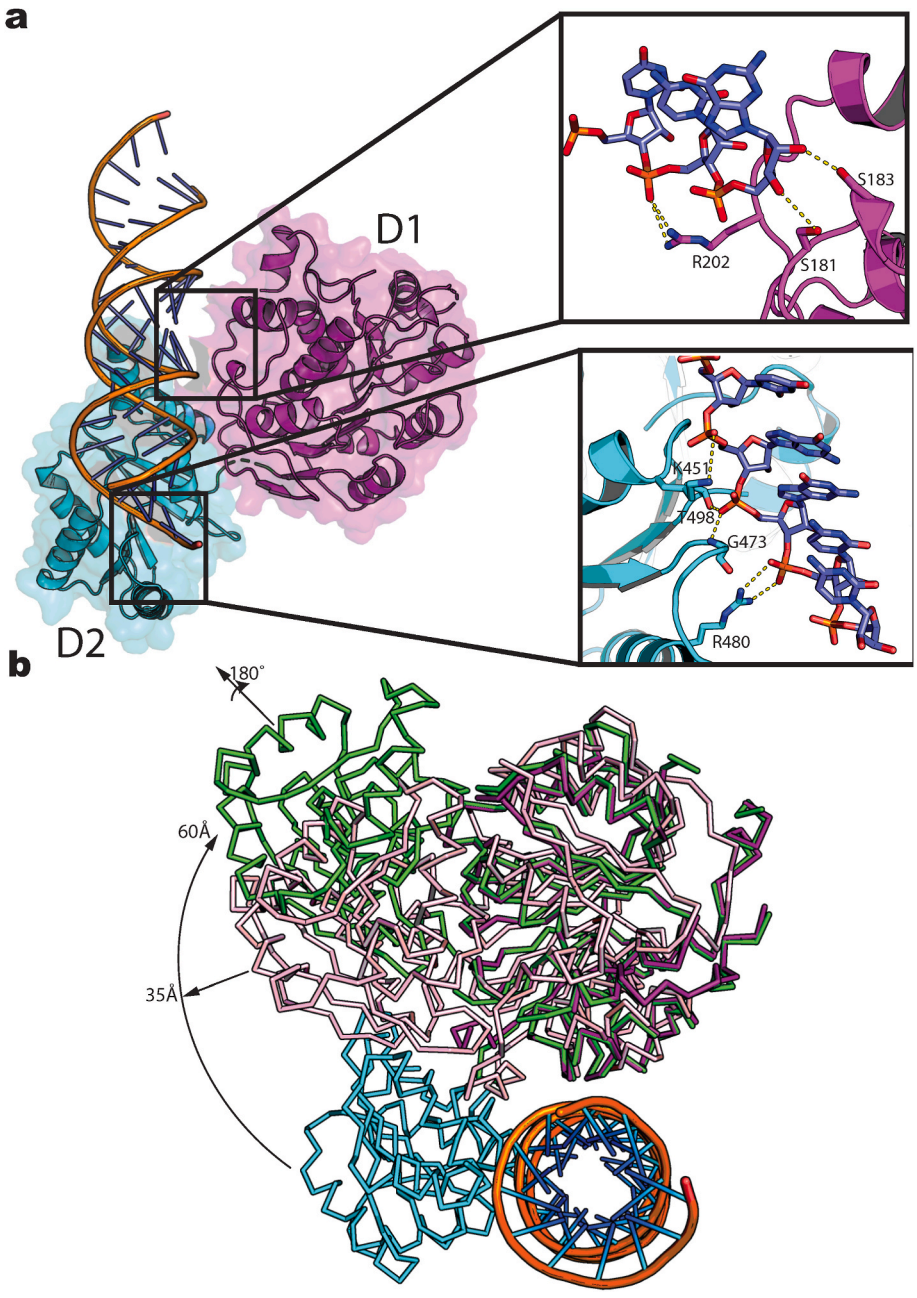

Fig. 2. The structure of DDX3X bound to duplex RNA. (a) The two RNA binding domains (D1 in purple and D2 in cyan) are in ribbon representation, overlapped with their surface representation, whereas the RNA in ribbon representation is in orange with the bases coloured in purple. The insets show the hydrogen bonds network between both the phosphates backbone and the oxygen atoms of the nucleotides of the RNA and the side chains of the protein. (b) Conformational changes between the apo-DDX3X (PDBid:5E7I), DDX3X in complex with the double stranded RNA (PDBid:5E7I) and VASA (PDBid:2DB3) structures. The structural alignment was performed by C-α superposition. Structures superposition was performed with Coot (Emsley et al., 2010). The figure was prepared with PyMOL (http://www.pymol.org/). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

In particular, the N-terminus of DDX3X contains a nuclear export sequence (Yedavalli et al., 2004), whereas the C-terminal tail contains a low complexity region essential for DDX3X oligomerization (Sharma and Jankowsky, 2014; Putnam et al., 2015) and binding to DDX3X cellular partners (Oshiumi et al., 2010).

Like the vast majority of DEAD box proteins, the RNA binding sites in the helicase core contact only the RNA backbone (Mallam et al., 2012; Sengoku et al., 2006; Song and Ji, 2019), so that DDX3X lacks inherent RNA sequence-specificity and as such can bind a wide variety of mRNAs. The same sequence-independent binding mode enables DDX3X association with duplex RNAs (Song and Ji, 2019). A crystal structure of DDX3X domains D1D2 in complex with a synthetic 23-bp dsRNA with a 1-nt 3' overhang on one terminal end of the duplex (strand 1: 3′-CCGGUGAGAACGCUUACUGGAAC-5′, strand2: 5′-GGUCAUUCG CAAGAGUGGCCUUGC-3′), in the pre-unwound state has only recently been determined (PDB id:6O5F) (Song and Ji, 2019) (Fig. 2a). It was obtained in absence of ATP and AMPPNP (a non-hydrolyzable ATP analogue). The structure of this dimer reveals that each DDX3X monomer mostly recognizes one strand of a 2-turn A-form RNA duplex, which requires an unpaired 3' region for effective unwinding. The D1 and D2 domains interact respectively with the phosphate backbone and the RNA 2′-OH groups (Song and Ji, 2019) (insets in Fig. 2a), the latter interactions underpinning RNA over DNA specificity. Binding of dsRNA to apo-DDX3X generates a total of 26 hydrogen bonds between the two D1D2 cores and dsRNA to stabilize the pre-unwound complex (Song and Ji, 2019). Contacts between the two D1s in the dimer (with a buried surface area (bsa) of ~350 $Å^2$), between the D1 pair and dsRNA (bsa~710 $Å^2$), and between each D2 and the RNA strand associated with it (bsa~560 $Å^2$) further stabilize the pre-unwound assembly (Song and Ji, 2019). The same complex was also studied in solution by small angle X-ray scattering (SAXS), confirming that the crystal structure captures a physiologically relevant quaternary state (Song and Ji, 2019). Previous efforts to generate DDX3X inhibitors have targeted the ATP binding site (Yang et al., 2020) or the RNA binding site, as done for the VASA protein - the *Drosophila melanogaster* DDX3X ortholog (*Dm*DDX3X) (Brai et al., 2016).

ATP binding to the DDX3X-dsRNA-DDX3X complex triggers large conformational changes, which cause dsRNA unwinding, each DDX3X monomer in the dimer pulling its associated RNA strand away from the complementary one, resulting in two post-unwound DDX3X-ssRNA-ATP complexes. The conformational changes induced by ATP binding unwind the RNA duplex in a cooperative manner, as demonstrated by Hill cooperativity analysis of dsRNA unwinding upon ATP-hydrolysis activities (Song and Ji, 2019). However, some data suggest that full-length DDX3X exhibits a three-molecule cooperativity, where a third DDX3X monomer could play a RNA-loading role and thereby optimise the RNA-unwinding efficiency (Sharma et al., 2017). Like its ortholog Ded1p, DDX3X unwinds RNA duplexes most efficiently as oligomers. (Sharma et al., 2017). Removing the 69 C-terminal amino acids from Ded1p C-terminus, a region of low complexity (Sharma and Jankowsky, 2014), diminished oligomerization both in the cell and in vitro. The deletion of the entire Ded1p C-terminus still allows dimer formation with low efficiency, heavily affecting both the ATPase RNA stimulated and unwinding activities. This data suggests that the unstructured region present at Ded1p C-terminus, as well as at DDX3X C-terminus, is part of the protein-protein interface in the trimer, playing a crucial part in the enzymatic activities.

In the post-unwound state, ATP hydrolysis triggers a third wave of conformational changes, promoting release of ssRNA from DDX3X (Fig. 1c). An atomic model of the post-unwounded state is provided by the structure of VASA (PDBid: 2DB3) (Sengoku et al., 2006) in complex with an ATP analogue and a single stranded RNA (Fig. 2a). The relative orientation of the D1 and D2 domains undergoes a shift of ~60 Å and a rotation of ~180° from apo to post-unwounded state (Song and Ji, 2019) (Fig. 2b).

Following ATP hydrolysis and ssRNA release, a fourth step in the enzymatic cycle, the post release state (Fig. 1c), was captured by the structure of a truncated form of DDX3X in complex with ADP (PDB id: 4PXA) (Epling et al., 2015). This structure shares the same overall conformation of the apo form, suggesting a free nucleotide release from the binding site to reach the initial state of the enzymatic cycle.

3. Targeting the DDX3X ATPase and helicase domains

DDX3X structures are instrumental for structure-based drug design to control or inhibit DDX3X function in health and disease. In particular, DDX3X is hijacked for replication by various RNA viruses such as human immunodeficiency virus 1 (HIV-1), Cytomegalovirus (CMV), Dengue virus (DENV), West-Nile virus (WNV), Vaccinia Virus, Hepatitis B virus (HBV), Hepatitis C virus (HCV), Influenza virus, Japanese Encephalitis virus (JEV) and Norovirus (NV). DDX3X is therefore an interesting target for antiviral therapy (Ariumi et al., 2007; Benfield et al., 2013; Chahar et al., 2013; Kalverda et al., 2009; Lenarcic et al., 2015; Li et al., 2013; Noble et al., 2010; Vashist et al., 2012). According to the DepMap portal (Tsherniak et al., 2017), DDX3X is a druggable structure and the druggability is ligand based. Initial attempts to design DDX3X inhibitors were focusing on its helicase and ATPase activities. A DDX3X crystal structure in complex with AMP (Hogbom et al., 2007) allowed pharmacophore modeling and molecular docking and enabled the design of RING-expanded nucleoside (REN) analogues inhibiting DDX3X helicase activity (Table 1) (Maga et al., 2008). These REN analogues successfully attenuate HIV-1 replication (Yedavalli et al., 2008) and are active against both HIV-1 and HCV (Zhang et al., 2014). Another REN analogue, the small molecule RK-33 (Kondaskar et al., 2010; Kumar et al., 2008), targets DDX3X to suppress its ATPase activity in lung cancer models (Bol et al., 2015). RK-33 showed high specificity in targeting cancer cells with mild side effects. Its chemical structure was docked in the ATP binding site of DDX3X structure (Hogbom et al., 2007) to identify the key interactions involved in the binding, and estimate the free energy of binding between RK-33 and DDX3X (ΔG ~ -8 kcal/mol) (Bol et al., 2015). RK-33 went on to show antiviral activity against human parainfluenza virus type 3 (hPIV-3), respiratory syncytial virus (RSV), DENV-2, Zika virus (ZIKV) and WNV (Yang et al., 2020).

In a separate line of investigation, before the structure of DDX3X in complex with double strand RNA was solved, attempts at targeting the DDX3X RNA binding site used an homology model based on Vasa helicase in complex with single strand RNA and ATP analogues, as template, resulting in the discovery of small molecule 16d, successfully hampering HCV, WNV, DENV and HIV-1 replication, which further demonstrated the relevance of DDX3X as an antiviral target (Brai et al., 2016; Riva et al., 2020). Interestingly, 16d inhibited the replication levels of HIV-1 treatment resistant strains, in absence of cellular toxicity. (Brai et al., 2016). The same inhibitor was chemically joined to small molecules targeting DDX3X helicase activity (Fazi et al., 2015), which resulted in hybrid compounds with strong activity against WNV and enhanced aqueous solubility (Brai et al., 2019). The recently elucidated DDX3X structure in complex with dsRNA (Song and Ji, 2019) now offers new routes to target the dsRNA binding site and improve on the existing compounds.

4. DDX3X in stress granules

Besides viral replication inhibition, RK-33 and 16d affect stress granule (SG) assembly (Cui et al., 2020). SGs are large cytoplasmic RNP (ribonucleoprotein) foci, induced by environmental stressors (ranging from oxidative stress to viral infections) via silencing of mRNA translation. SGs harbour abortive translation initiation complexes containing polyadenylated mRNA bound to early initiation factors and small ribosomal subunits, as well as RNA binding proteins, transcription factors, translation regulators, signalling molecules and RNA helicases, including DDX3X (Ivanov et al., 2019; Shih et al., 2012; Soto-Rifo et al., 2012) (Fig. 3). An initial investigation of the role of DDX3X in SG

Table 1
Small molecules and genetic studies targeting DDX3X.

Development of RNA helicase inhibitors	Role of DDX3X gene on viral replication cycle
Discovery of the first small molecule inhibitor of human DDX3 specifically designed to target the RNA binding site: towards the next generation HIV-1 inhibitors. Radi M, Falchi F, Garbelli A, Samuele A, Bernardo V, Paolucci S, Baldanti F, Schenone S, Manetti F, Maga G, Botta M. Bioorg Med Chem Lett. 2012 Mar 1; 22 (5):2094–8.	Knockdown of cellular RNA helicase DDX3 by short hairpin RNAs suppresses HIV-1 viral replication without inducing apoptosis. Ishaq M, Hu J, Wu X, Fu Q, Yang Y, Liu Q, Guo D. Mol Biotechnol. 2008 Jul; 39(3):231–8.
Dual inhibition of HCV and HIV by ring-expanded nucleosides containing the 5:7-fused imidazo diazepine ring system. In vitro results and implications. Zhang N, Zhang P, Baier A, Cova L, Hosmane RS. Bioorg Med Chem Lett. 2014 Feb 15; 24 (4):1154–7.	DDX3 Interacts with Influenza A Virus NS1 and NP Proteins and Exerts Antiviral Function through Regulation of Stress Granule Formation. Thulasi Raman SN, Liu G, Pyo HM, Cui YC, Xu F, Ayalew LE, Tikoo SK, Zhou Y. J Virol. 2016 Jan 20; 90(7):3661–75.
Homology Model-Based Virtual Screening for the Identification of Human Helicase DDX3 Inhibitors. Fazi R, Tintori C, Brai A, Botta L, Selvaraj M, Garbelli A, Maga G, Botta M. J Chem Inf Model. 2015 Nov 23; 55 (11):2443–54.	DDX3 suppresses type I interferons and favors viral replication during Arenavirus infection. Loureiro ME, Zorzetto-Fernandes AL, Radoshitzky S, Chi X, Dallari S, Marooki N, Lèger P, Foscaldi S, Harjono V, Sharma S, Zid BM, López N, de la Torre JC, Bavari S, Zúñiga E. PLoS Pathog. 2018 Jul 12; 14(7): e1007125.
Human DDX3 protein is a valuable target to develop broad spectrum antiviral agents. Brai A, Fazi R, Tintori C, Zamperini C, Bugli F, Sanguinetti M, Stigliano E, Esté J, Badia R, Franco S, Martinez MA, Martinez JP, Meyerhans A, Saladini F, Zazzi M, Garbelli A, Maga G, Botta M. Proc Natl Acad Sci U S A. 2016 May 10; 113(19):5388–93.	Requirement of DDX3 DEAD box RNA helicase for HIV-1 Rev-RRE export function. Yedavalli VS, Neuveut C, Chi YH, Kleiman L, Jeang KT. Cell. 2004 Oct 29; 119(3):381–92.
DDX3X Helicase Inhibitors as a New Strategy To Fight the West Nile Virus Infection. Brai A, Martelli F, Riva V, Garbelli A, Fazi R, Zamperini C, Pollutri A, Falsitta L, Ronzini S, Maccari L, Maga G, Giannecchini S, Botta M. J Med Chem. 2019 Mar 14; 62(5):2333–2347.	A DEAD box protein facilitates HIV-1 replication as a cellular co-factor of Rev. Fang J, Kubota S, Yang B, Zhou N, Zhang H, Godbout R, Pomerantz RJ. Virology. 2004 Dec 20; 330(2):471–80.
Unique Domain for a Unique Target: Selective Inhibitors of Host Cell DDX3X to Fight Emerging Viruses. Riva V, Garbelli A, Brai A, Casiraghi F, Fazi R, Trivisani CI, Boccuto A, Saladini F, Vicenti I, Martelli F, Zazzi M, Giannecchini S, Dreassi E, Botta M, Maga G. J Med Chem. 2020 Sep 10; 63 (17):9876–9887.	DDX3 DEAD-Box RNA helicase inhibits hepatitis B virus reverse transcription by incorporation into nucleocapsids. Wang H, Kim S, Ryu WS. Virol. 2009 Jun; 83(11):5815–24.
Exploring the Implication of DDX3X in DENV Infection: Discovery of the First-in-Class DDX3X Fluorescent Inhibitor. Brai A, Boccuto A, Monti M, Marchi S, Vicenti I, Saladini F, Trivisani CI, Pollutri A, Trombetta CM, Montomoli E, Riva V, Garbelli A, Nola EM, Zazzi M, Maga G, Dreassi E, Botta M. ACS Med Chem Lett. 2020 Apr 9; 11 (5):956–962.	Hepatitis B virus polymerase blocks pattern recognition receptor signaling via interaction with DDX3: implications for immune evasion. Wang H, Ryu WS. PLoS Pathog. 2010 Jul 15; 6(7): e1000986.
DDX3X inhibitors, an effective way to overcome HIV-1 resistance targeting host proteins. Brai A, Riva V, Saladini F, Zamperini C, Trivisani CI, Garbelli A, Pennisi C, Giannini A, Boccuto A, Bugli F, Martini M, Sanguinetti M, Zazzi M, Dreassi E, Botta M, Maga G. Eur J Med Chem. 2020 Aug 15; 200:112319.	From promoting to inhibiting: diverse roles of helicases in HIV-1 Replication. Lorgeoux RP, Guo F, Liang C. Retrovirology. 2012 Sep 28; 9:79.
Synthesis and Antiviral Activity of Novel 1,3,4-Thiadiazole Inhibitors of DDX3X. Brai A, Ronzini S, Riva V, Botta L, Zamperini C, Borgini M, Trivisani CI, Garbelli A, Pennisi C, Boccuto A, Saladini F, Zazzi M, Maga G, Botta M. Molecules. 2019 Nov 4; 24(21):3988.	A motif unique to the human DEAD-box protein DDX3 is important for nucleic acid binding, ATP hydrolysis, RNA/DNA unwinding and HIV-1 replication. Garbelli A, Beermann S, Di Cicco G, Dietrich U, Maga G PLoS One 2011, 6:e19810.

assembly was assessed by treatment of a human osteosarcoma cell line with arsenite, an inducer of SGs, in the presence of RK-33 or 16d. The presence of DDX3X inhibitors RK-33 and 16d resulted in decreased SG assembly (with RK-33 the more potent compound), confirming involvement of DDX3X in the process (Cui et al., 2020). Based on its proposed DDX3X binding mode and inhibition kinetics (Brai et al., 2016) 16d is a competitive inhibitor, which together with the high RNA content of SGs, explains its relatively weak activity. Conversely, K-33 targets the ATP binding site and locks DDX3X in an inactive conformation unable to bind dsRNA, making the inhibitor less sensitive to high RNA concentration in SGs. Moreover, in influenza A virus infections, it has been shown that DDX3X can interact with viral NS1 and NP proteins (Thulasi Raman et al., 2016). Furthermore, these viral proteins co-localize with DDX3X in virus-induced SGs, an observation paired with the reduction of viral replication, suggesting that DDX3X functions as an antiviral protein via SGs (Thulasi Raman et al., 2016).

In contrast, the SG core protein G3BP1 exerts a pro-viral role in viral translation initiation, indicating that viruses can use SG components for their propagation (Hosmillo et al., 2019). However, whether G3BP1 and DDX3X in SGs interact or regulate each other remains unknown. Further investigations will be required to fully understand the role of DDX3X in SG assembly.

In addition to its presence in SGs, DDX3X also shuttles between nuclear and cytoplasmic compartments: identification of its cellular trajectories, although difficult to assess, would be of paramount importance to dissect the roles of the protein during viral infections. As a first attempt to locate DDX3X in DENV infection, a fluorescent inhibitor targeting the RNA-binding domain of DDX3X was developed (Brai et al., 2020). Treatment with the DDX3X inhibitor *in cellula* increased the ratio of viable cells count over infected cells; DDX3X migrated to perinuclear spots 6–24 h after infection with DENV serotype 2 (DENV-2), while the DENV-2 non-structural protein NS5 migrated to the nucleus 12 h after infection (Brai et al., 2020). These data suggest that DENV-2 recruits DDX3X to specific cytoplasmic locations, possibly a viral propagation strategy. However, it is also possible that the host cell is responsible for recruitment of DDX3X to these cellular compartments, in order to halt viral message translation and limit viral replication – much in the same way DDX3X works in SGs.

Notably, DDX3X seems to be involved in live-or-die cell-fate decisions under stress conditions (Samir et al., 2019). DDX3X drives inflammasome activation by interacting with leucine rich containing (NLR) and pyrin domain containing receptor 3 (NLRP3), a multimeric protein complex responsible for induction of pro-inflammatory interleukin-1 beta (IL-1β) and interleukin-18 (IL-18) responses as well as pyroptosis. Interestingly, stress granule assembly sequesters DDX3X and thereby inhibits NLRP3 inflammasome activation (Samir et al., 2019). The authors suggest that macrophages use the availability of DDX3X to interpret stress signals and choose between pro-survival stress granules and pyroptosis.

The interaction of DDX3X with NLRP3 is therefore important in determining cell-fate. DDX3X interacts with full-length NLRP3 and its

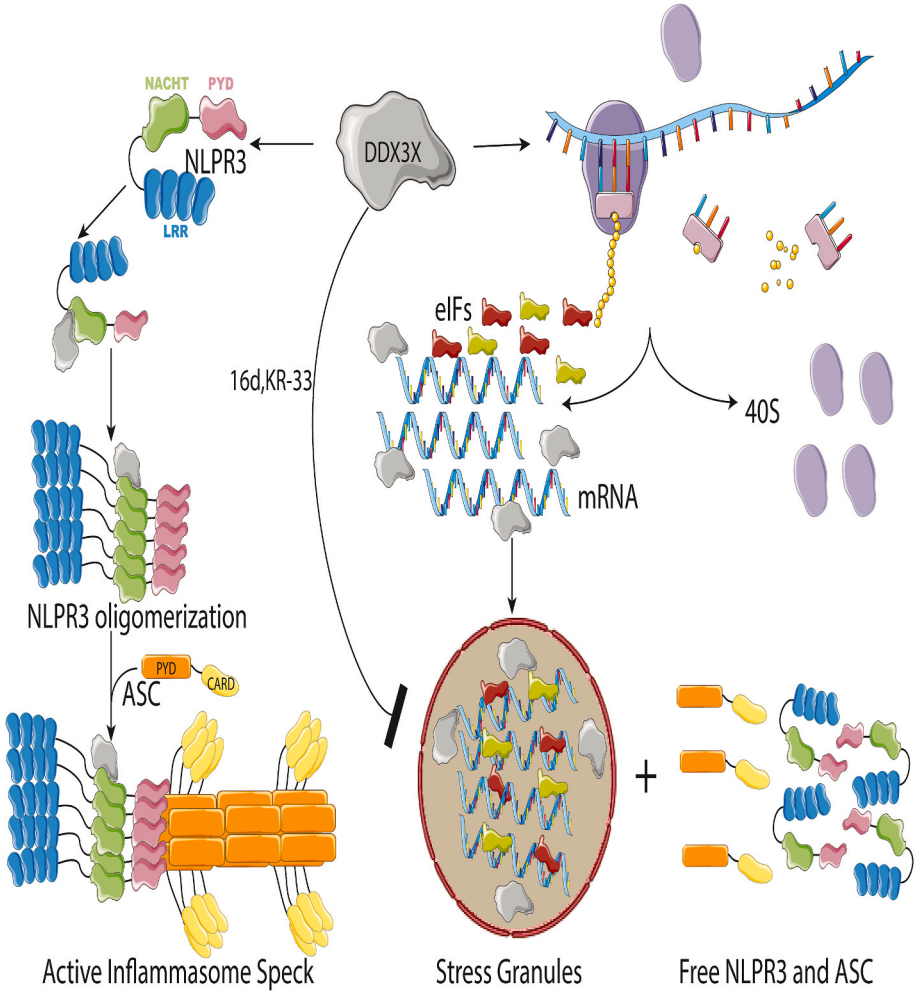

Fig. 3. Role of DDX3X in the inflammasome activation. Interplay between the inflammasome and stress granules triggering the cell-fate decisions. DDX3X promotes NLRP3 inflammasome activation and the pro-death cell-fate decision probably by interacting with the NLRP3 NACHT domain through its helicase domain. Induction of stress granules causes the sequestration of DDX3X (along with 40S ribosomal subunits and translation initiation factors (eIFs)), thus making it unavailable for NLRP3 inflammasome activation and thereby allowing the cells to make a pro-survival cell-fate choice.
The presence of the DDX3X inhibitors 16d and RK-33 interfere with the stress granules formation. The figure was prepared with Servier Medical ART (https://smart.servier.com/category/cellular-biology/cell-membrane/).

NACHT([NAIP (neuronal apoptosis inhibitory protein), CIITA (MHC class II transcription activator), HET-E (incompatibility locus protein from Podospora anserina) and TP1 (telomerase-associated protein)]) domain (also known as the nucleotide-binding domain; NBD) when it is co-expressed with the same protein constructs in HEK293T cells (Samir et al., 2019). This suggests a binding site for DDX3X in the NACHT (NBD) domain of NLRP3 (Fig. 3), which in turn implies a role for DDX3X in NLRP3 oligomerization (Samir et al., 2019). In the same study, a series of N- and C-terminal truncations and helicase domains deletion mutants have been generated (Samir et al., 2019): the mutants were able to interact with NLRP3, and the authors suggest that the DDX3X-NLRP3 interaction might be mediated by intrinsically disordered regions (Banani et al., 2017) present in the N and C termini and the helicase domain of DDX3X, and the disordered regions in the NACHT domain. These regions are indeed predicted disordered in the DDX3X structure and are involved in interactions with several cellular partners (Sharma and Jankowsky, 2014). The conclusions drawn by these deletion mutagenesis experiments should be further validated by biophysical methods: recent advancements in imaging techniques, such as cryo-Electron Microscopy and Tomography, now offer the opportunity to visualize the NLRP3-DDX3X complex and shed light on the molecular details underpinning its formation. Alternatively, X-ray crystallography could be used to elucidate the structure of DDX3X in complex with the NACHT (NBD) domain of NLRP3, as already successfully demonstrated for the structure determination of DDX3X in complex with dsRNA. Sequestering of DDX3X into SGs during assembly leads to depletion of the cytoplasmic DDX3X pool, thereby preventing its interaction with NLRP3. SGs and NLRP3 therefore need to compete for cytoplasmic DDX3X, enabling the cells to make a live-or-die cell-fate decision based on DDX3X availability (Samir et al., 2019). Of note - it is also possible that DDX3X-induced pyroptosis plays a role in limiting the number of infected cells and as such ongoing viral replication. Targeting the DDX3X ATPase and helicase domains reduces SG assembly, so that DDX3X inhibitors have the potential to skew the cell's live-or-die decision and give the cell the upper hand in the response/control of viral replication.

5. Implications of DDX3X structures for antiviral innate immunity

DDX3X senses viruses. The recent crystal structure of DDX3X in complex with double stranded RNA adds another piece to the puzzle of the protein role in the antiviral innate immune response. As it turns out, besides its roles in RNA metabolism, DDX3X also functions as a pattern recognition receptor (PRRs) after sensing viral pathogen-associated molecular patterns (PAMPs). Similar to the PRRs retinoic acid inducible gene I (RIG-I) and melanoma differentiation associated gene 5 (MDA5), activated DDX3X engages with the mitochondrial antiviral-signaling protein (MAVS), ultimately leading to the induction of both IFN-β and interferon-stimulated genes (ISGs) transcription (Seth et al., 2005). However, triggering of the RIG-I-like receptor (RLR) signaling pathway by DDX3X, in contrast to RIG-I and MDA5, is not fully elucidated. While RIG-I and MDA5 both interact with MAVS via N-terminal CARD domains, DDX3X lacks a CARD domain (Kawai et al., 2005) (Figs. 1 and 4). Instead, biochemical studies have suggested that the C-terminal domain of DDX3X could be involved in the interaction with

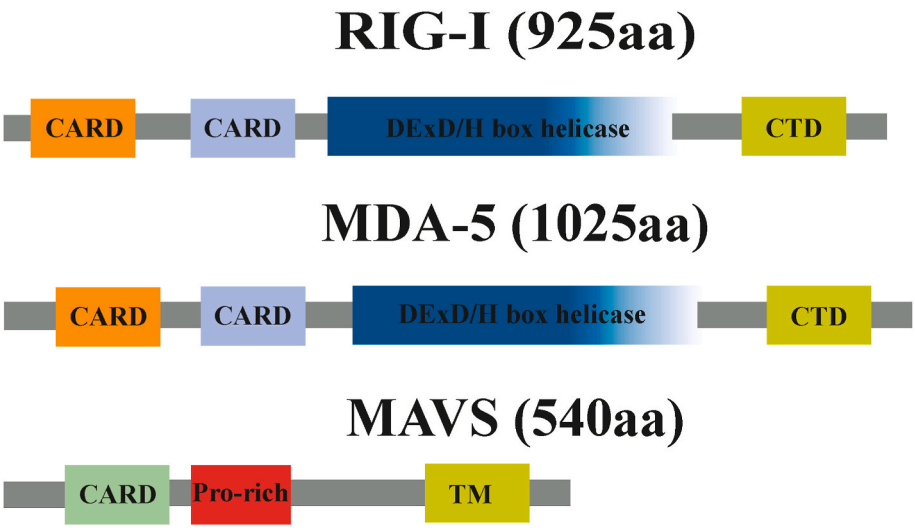

Fig. 4. Protein domains organization of RIG-I, MDA5, MAVS. RIGI and MDA5 contain tandem caspase activation and recruitment domain (CARD) regions at their N-terminal domain. A schematic representation of the MAVS protein showing its CARD domain, the proline reach region and the transmembrane region (TM).

the CARD domain of MAVS (Oshiumi et al., 2010), by an interaction between the unfolded region in the C-terminus of the DDX3X and the highly structured MAVS CARD domain. The HCV virus core protein interacts with the C-terminus of DDX3X, hereby interfering with MAVS-induced IFN-β responses, providing further evidence that the C-terminal domain of DDX3X is involved in binding to MAVS (Oshiumi et al., 2010).

DDX3X interacts with abortive HIV-1 RNA. Engagement of DDX3X with MAVS occurs upon detection of short HIV-1 RNA transcripts (Fig. 5). These abortive HIV-1 RNAs are generated during the early steps

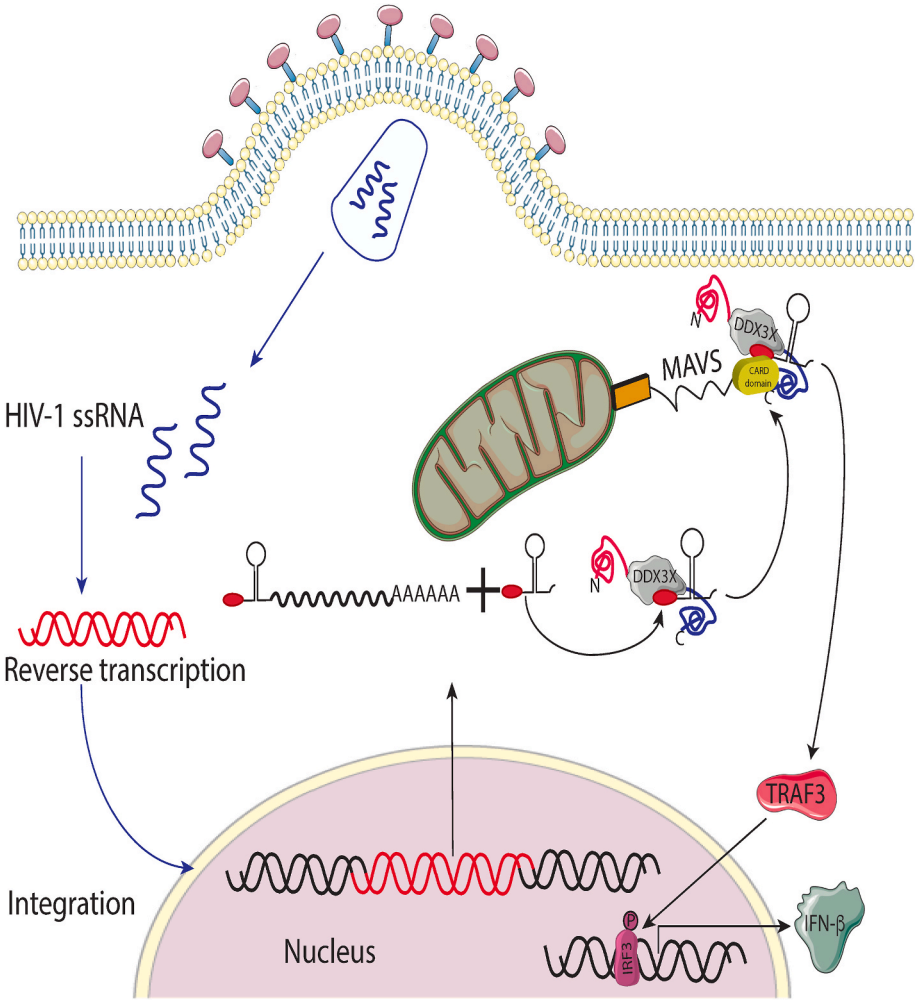

Fig. 5. DDX3X-MAVS signalling during HIV-1 infection. Once HIV-1 virus enters the cell, releases its viral genome. After reverse transcription of the two single-stranded (ss) viral RNA genomes into a linear double stranded DNA within the cytoplasm, the viral DNA is integrated into the host genome within the nucleus. Following the integration, viral DNA is transcribed in the full-length RNA transcripts. Some of them are exported from the nucleus and serve as mRNAs. HIV-1 infection results also in abortive HIV-1 RNA, containing the first 58 viral nucleotides, that translocate to the cytoplasm where they are collected by DDX3X to form a complex that associates with the MAVS. This complex triggers the subsequent activation of TRAF3, which leads to phosphorylation of IRF3 and the synthesis and cellular release of INF-β and ultimately activation of antiviral immune responses. The figure was prepared with Servier Medical ART (https://smart.servier.com/category/cellular-biology/cell-membrane/).

of HIV-1 transcription. Although the precise abortive HIV-1 RNA-DDX3X interaction has not been structurally characterized, it is known that DDX3X recognizes the complex secondary RNA structure (TAR hairpin) in close proximity to the 5′ m7GTP cap moiety. When the TAR structure forms high molecular weight complexes with DDX3X (Soto-Rifo et al., 2013), multiple copies of DDX3X are bound to the TAR region, suggesting a cooperative mechanism of unwinding of the RNA duplex during translation (Song and Ji, 2019; Sharma et al., 2017). The crystal structure of DDX3X in complex with dsRNA (Fig. 2a) offers a model for understanding the binding of DDX3X to the TAR hairpin region during viral translation. The TAR hairpin structure and the m7GTP cap moiety embedded at the basis of the duplex could recruit more copies of DDX3X, anchoring the HIV-1 RNA as it enters translation process. The large conformational changes of DDX3X upon binding the mRNA suggest further rearrangements upon association with the 5′-UTR of HIV-1 RNA, before translation starts. However, structural investigation of full length DDX3X and abortive HIV-1 RNAs TAR region complex assembly needs to be carried out to confirm the complex formation and the following binding to the MAVS CARD domain through the DDX3X C-terminal unstructured region.

DDX3X facilitates antiviral responses. Besides direct involvement in the RIG-I signaling pathway, DDX3X is capable of translational control of molecules involved in the RIG-I signaling pathway. The protein activator of the interferon-induced protein kinase (PACT) is a facilitator of RIG-dependent sensing of viral RNA by functioning as a double-stranded (ds) RNA-binding protein and activator of RIG-I. DDX3X is important for translation of PACT mRNA via its 5′UTR. PACT mRNA contains GC-rich sequence (74.6%GC) at its 5′UTR, predicted to form a stable secondary structure. Dual-luciferase reporter assay was performed with firefly luciferase (Fluc) gene containing the 5′ UTR of PACT and Renilla luciferase (Rluc) with an unstructured 5′ UTR as control. Significant reduction of the relative Fluc/Rluc activity was observed in DDX3-knockdown cells without changing the level of the reporter mRNAs. These data suggest that knockdown of DDX3X decreases PACT translation and subsequent antiviral responses, of which the latter one were partially restored upon rescuing PACT (Lai et al., 2016). HCV uses the DDX3X-PACT axes to circumvent antiviral immunity via binding of the HCV core protein to DDX3X, hereby interfering with its role in the translation of PACT (Lai et al., 2016). As described, the HCV core protein plays a decisive role in the regulation of DDX3X during viral infection, however, its exact role is controversial. When bound to DDX3X it interferes with DDX3X-MAVS or DDX3X-PACT axes, attenuating or abrogating antiviral type I IFN responses. In contrast, the HCV core protein-DDX3X interaction has also been shown to be necessary for INF-β responses (Kang et al., 2012). Mutations in the HCV core protein (K23E and V31A) were associated with weaker interactions with DDX3X and a decrease in IFN-β (Kang et al., 2012). Interestingly, HIV-1 also circumvents antiviral responses at a translational level. However, this immune evasion does not involve DDX3X, but the protein kinase R (PKR), which similarly to DDX3X, is activated by abortive HIV-1 RNA. Activation of PKR leads to phosphorylation of the downstream target eIF2α, resulting in translational inhibition of cellular mRNAs and viral mRNAs during infection (Garcia et al., 2006). HIV-1 escapes PKR-dependent translational inhibition via various ways including competitive substrates such as the HIV-1 Tat protein (McMillan et al., 1995). In addition, the PKR activator PACT that normally enhances phosphorylation of PKR, has been described to inhibit PKR activity during HIV-1 infection, hereby allowing viral mRNA translation and thus viral replication (Clerzius et al., 2013).

DDX3X triggers IRF3 and NF-kB via *distinct signaling.* Alongside MAVS, DDX3X also binds directly to the RIG-I signaling pathway components IKKε and IKKα, leading to IRF3 and NF-κB activation and thus induction of type I IFN and pro-inflammatory cytokines, respectively (Gu et al., 2013) (Gu et al., 2017) (Fig. 6). DDX3X binds IKKε and IKKα

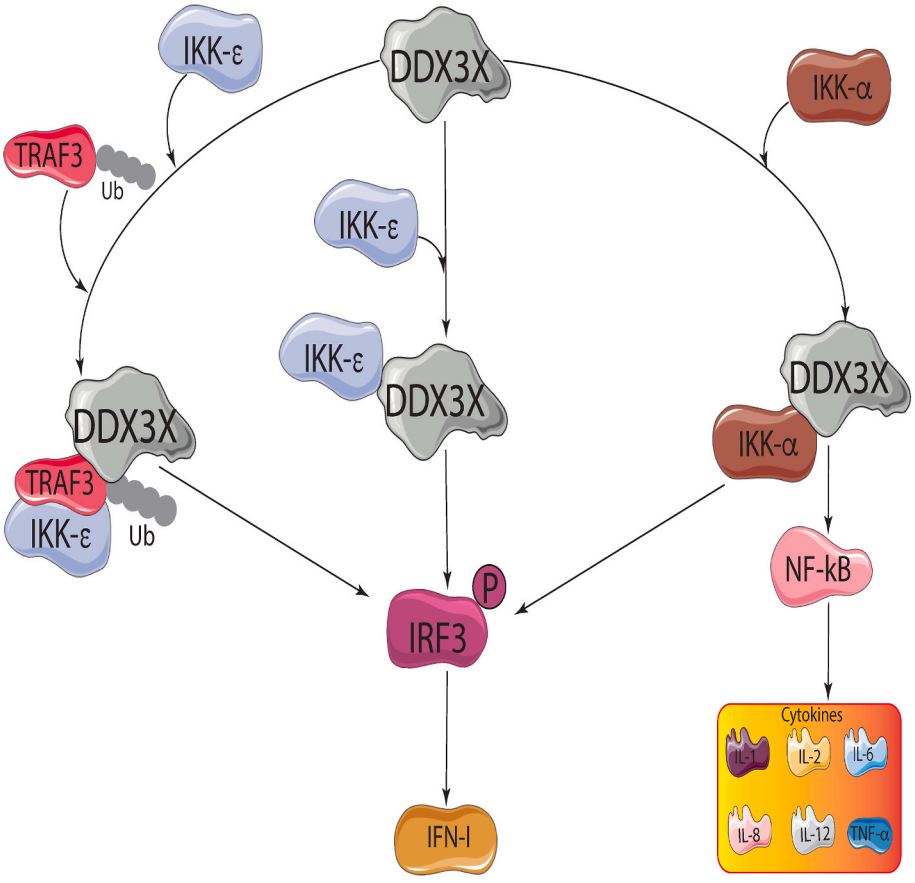

Fig. 6. Role of DDX3X in innate immunity. DDX3X can trigger the phosphorylation and activation of IRF3 in three different ways; by interacting with TRAF3-IKKε complex or with IKKα through residues in its NTE, and alternatively with IKKε, via residues in disordered N-terminus. Moreover, the interaction between DDX3X and IKKα activates the NF-Kb with the following production of cytokines. The figure was prepared with Servier Medical ART (https://smart.servier.com/category/cellular-biology/cell-membrane/).

differently: IKKα interacts with DDX3X NTE domain (Fig. 1) (Fullam et al., 2018). IKKε interacts with the DDX3X disordered N-terminal regions. These observations suggest that the folding state of DDX3X be a determining factor in whether IKKε or IKKα-mediated immunity occurs. During HCV infection, the 3′ UTR of HCV interacts with DDX3X, which can then activate IKKα and result in antiviral type I IFN. At the same time, the DDX3X-IKKα interaction is also exploited by HCV for transcriptional regulation of sterol regulatory element-binding proteins (SREBPs), important for lipid-droplet (LD) formation, and favouring viral replication (Li et al., 2013). The factors determining whether DDX3X functions as a viral sensor leading to antiviral responses or favours viral replication remain elusive and may depend on many factors such as availability of free DDX3X in the cytoplasm, the conformational status of DDX3X and the presence of other host and viral molecules interfering with DDX3X-dependent processes. Pène et al. elegantly showed that the presence of HCV 3' UTR RNA leads to a redistribution of DDX3X and IKKα to SGs and not LDs early in the infection process (Pene et al., 2015). At later time points (24–72 h post infection), the HCV core protein reaches stable expression levels and co-localises with DDX3X and LDs (Pene et al., 2015), suggesting that the DDX3X-IKKα interaction is dynamic and may therefore initially trigger viral sensing and only later facilitate HCV replication.

Besides IKKα, a second protein interacts with DDX3X NTE region is TRAF3, acting downstream of MAVS and upstream of IKKε (Fig. 6). Spatio-sequential analysis following Sendai virus (SeV) infection has revealed that the recruitment of DDX3X and IKKε occurs first, followed by a wave of ubiquitination of TRAF3, hereby strengthening the interaction between MAVS and TRAF3 (Gu et al., 2017). Subsequently, the DDX3X-TRAF3-IKKε complex is released from MAVS and a second wave of TRAF3 ubiquitination occurs, followed by IRF3 recruitment (Gu et al., 2017). Silencing DDX3X by RNAi abrogates the cascade of events blocking IRF3 activation and preventing type I IFN responses (Gringhuis et al., 2017), suggesting a critical role of protein-protein interactions in innate antiviral immunity. Although DDX3X uses a similar aminoacid sequence to bind IKKα and TRAF3, DDX3X-TRAF3 interactions activate IKKε, ultimately inducing type I IFN. These observations suggest that drugs targeting the IKKα and TRAF3 binding regions of DDX3X may enhance antiviral immune activity through both type I IFN as well as cytokine responses. Inhibitors of IKKα-DDX3X and TRAF3-DDX3X interactions would also allow IKKε activation only, without induction of cytokine responses. Alongside its role as a positive regulator of IKKε, DDX3X can modulate NF-κB signaling. NF-κB is sequestered in the cytoplasm by inhibitory subunit IKKβ, which upon phosphorylation is degraded, allowing for nuclear translocation of the NF-κB subunit p65 to the nucleus. Xiang et al. elegantly showed that DDX3X suppresses the transcriptional activity of NF-κB via direct engagement of the functional ATP-dependent RNA helicase domain of DDX3X to the N-terminal Rel Homology domain (RHD) of p65, in 293 T cells (Xiang et al., 2016). In contrast, activation of DDX3X using synthetic mimics of short HIV-1 RNA led to p65 translocation in human dendritic cells, suggesting that DDX3X functions as a positive regulator for NF-κB (Stunnenberg et al., 2020).

Although much is unknown, these studies provide a rationale for further investigating different DDX3X domains, in health and during viral infections, to determine and possibly modulate the final immune response induced by DDX3X.

6. Concluding remarks

DDX3X is a key player, involved in virtually every aspect of RNA metabolism. Its versatility is mediated by its multidomain structure and its ability to multimerise: core domains support ATP-hydrolysis and dsRNA unwinding, while the unstructured N- and C-termini mediate interactions with many cellular and viral partners within a crowded cellular environment. During viral infections, DDX3X exerts a dual role - facilitating the translation of viral mRNA as well as functioning as a PRR that induces antiviral immunity and/or engages with the RIG-I signalling pathway. By offering a hub for so many protein-protein interactions, DDX3X has the capacity to determine cell fate by being sequestered in SGs or by binding to inflammasome components. The location of DDX3X in the cell and the availability of its structural domains (e.g. ATPase, helicase domains) also determine its effect on viral replication and affect innate immunity. All in all, the data available so far prompt further investigation of DDX3X domains as targets for antiviral innate immunity modulation. At the same time, the DDX3X-dependency of many cellular RNA regulation processes is the Achilles' heel in the strategy for generating a new class of DDX3X inhibitors for antiviral and anticancer therapy (Brai et al., 2016; Garbelli et al., 2011; Maga et al., 2011). To date, available DDX3X inhibitors suppress either its ATPase or its helicase activity, exerting a heavy impact on the overall host RNA metabolism. Elucidation of DDX3X key interactions with viral proteins and other host factors will open the way to targeting DDX3X protein-protein interactions (Ivanov et al., 2019) (PPIs) as a means of achieving better selectivity without interfering with its physiological roles within the cell. Our prediction is that structure-based drug discovery of DDX3X-PPIs modulators will play a crucial role towards new weapons to be added to the arsenal currently at our disposal to fight viruses and regulate cellular responses to virally induced stress.

CRediT authorship contribution statement

L.D.C. designed and prepared the manuscript. M.S. participated in the discussion and prepared the manuscript. T.B.H.G. supervised all aspects of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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