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Martina Schröder. Human DEAD-box protein 3 has multiple functions in gene regulation and cell cycle control and is a prime target for viral manipulation. *Biochemical Pharmacology*, 2009, 79 (3), pp.297. <10.1016/j.bcp.2009.08.032>. <hal-00538095>

HAL Id: hal-00538095

<https://hal.science/hal-00538095v1>

Submitted on 21 Nov 2010

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Accepted Manuscript

Title: Human DEAD-box protein 3 has multiple functions in gene regulation and cell cycle control and is a prime target for viral manipulation

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PII: S0006-2952(09)00797-7
DOI: doi:10.1016/j.bcp.2009.08.032
Reference: BCP 10334

To appear in: *BCP*

Received date: 24-6-2009
Revised date: 21-8-2009
Accepted date: 24-8-2009

Please cite this article as: Schröder M, Human DEAD-box protein 3 has multiple functions in gene regulation and cell cycle control and is a prime target for viral manipulation, *Biochemical Pharmacology* (2008), doi:10.1016/j.bcp.2009.08.032

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Running title: Manipulation of DDX3 function by viruses

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Word count: 7685

Abbreviations:

4eBP, eIF4e binding protein; aa, amino acid; Cdc, cell division cycle; CRM-1, chromosome maintenance region-1; DDX3, DEAD-box protein 3; eIF, eukaryotic initiation factor; EJC, Exon junction complex; GSK, glycogen synthase kinase; HBV, Hepatitis B virus; HCC, hepatocellular carcinoma; HCV, Hepatitis C virus; HIV, Human Immunodeficiency virus; HLA, human leukocyte antigen; IAP, inhibitor of apoptosis IFN, interferon; IκB, inhibitor of nuclear factor-κB; IKK, IκB-kinase; IRES, internal ribosome entry site; IRF, interferon regulatory factor, MAVS, mitochondrial antiviral signalling; mRNA, messenger RNA; NES, nuclear export signal; NPC, nuclear pore complex; NS3/4a, non structural 3/4a; PABP, polyA-binding protein; PRR, pattern recognition receptor; RIG, retinoic-acid inducible gene; RLH, RIG-like helicase; RNP, ribonucleoprotein; rRNA, ribosomal RNA; siRNA, small interfering RNA; snRNA, small nuclear RNA; Sp1, specificity protein 1; TAP, tip-associated protein; TBK, TANK-binding kinase; TLR, Toll-like receptor; TRAIL-R, TNF-related apoptosis-inducing ligand- receptor; TRIF, TIR-domain containing adaptor inducing IFNβ; UTR, untranslated region; VACV, vaccinia virus

Abstract

The human DEAD-box RNA helicase DDX3 has been implicated to play a role in the whole repertoire of processes regulating gene expression, including transcription, splicing, mRNA export and translation. It has also been suggested to be involved in cell cycle control and the regulation of apoptosis. In addition, DDX3 was recently shown to be part of innate immune signalling pathways and to contribute to the induction of anti-viral mediators, such as type I interferon. Interestingly, DDX3 appears to be a prime target for viral manipulation: at least four different viruses, namely Hepatitis C virus (HCV), Hepatitis B virus (HBV), Human immunodeficiency virus (HIV) and poxviruses, encode proteins that interact with DDX3 and modulate its function. HIV and HCV seem to co-opt DDX3 and require it for their replication. It has therefore been suggested that DDX3 could be a novel target for the development of drugs against these two viruses, both of which still pose major global health threats. However, in the light of the apparent multifunctionality of DDX3 in the cell, drug development strategies targeting DDX3 will have to be carefully evaluated. This review summarises the available data on the cellular functions of DDX3 and discusses their manipulation by the different viruses known to target DDX3. Understanding the viral strategies for manipulating or co-opting DDX3 in functional and molecular detail can provide valuable insights for the development of strategies to therapeutically target DDX3.

Keywords: DEAD-box helicase, gene expression regulation, hepatitis virus, viral immune evasion, cell growth control, type I interferon

1. Introduction

DDX3 (or DBX) is a member of the DEAD-box family of putative RNA helicases. It was first identified in 1997 as one of five X-chromosomal genes that have homologues in the non-recombining region of the Y-chromosome (DBY, DDX3Y) [1]. The DDX3 (or more correctly DDX3X) gene escapes X-inactivation [1] and is ubiquitously expressed in a wide range of tissues [2]. DEAD-box helicases are involved in a large variety of cellular processes involving RNA, such as splicing, mRNA export, transcriptional and translational regulation, RNA decay and ribosome biogenesis [3]. In recent years, DDX3 has received a lot of interest because of several studies showing its manipulation by viruses that pose major global health threats, such as Human Immunodeficiency Virus (HIV) [4], Hepatitis C Virus (HCV) [5-8] and poxviruses [9]. Due to the finding that HIV and HCV seem to require DDX3 for their replication [4, 8], the inhibition of DDX3 has been suggested as a novel therapeutic strategy for the development of drugs against these viruses [10]. However, it was recently demonstrated that DDX3 is also involved in the induction of anti-viral mediators [9, 11] and it appears to function in protein translation, cell cycle control and apoptosis. Therefore, it seems crucial to understand how exactly DDX3 contributes to these processes in order to design informed strategies for its inhibition or manipulation. This review focuses in particular on the viral manipulation of DDX3 and discusses which cellular functions of DDX3 are targeted by the virus. In many cases, studying a viral protein and its host targets has provided novel insights about the function of the host target. For example, this was also the case with vaccinia virus protein (VACV) K7 which revealed a novel role for DDX3 in anti-viral immunity [9]. Therefore, it has been and will be a valuable approach to deduce information about DDX3 function from the way viruses are targeting it. This could lead to the

development of therapeutic tools that are mimicking or preventing the viral strategy to manipulate DDX3 function.

2. Cellular localisation of DDX3

Several studies have described DDX3 as a protein that constantly shuttles between the cytoplasm and the nucleus, with its export from the nucleus being mediated by the export shuttle protein CRM1 [4, 9, 12]. CRM1 exports proteins containing a leucine-rich nuclear export signal (NES), and indeed DDX3 contains an NES within its N-terminal 22 amino acids (aa). However, the association between CRM1 and DDX3 does not seem to depend on this described NES [4] (discussed further in section 3.3). More recently, it has been demonstrated that DDX3 can also be exported via the TAP-dependent export pathway, which normally mediates the nuclear export of mRNAs [13]. It remains to be demonstrated whether these two pathways are equally involved in nuclear export of DDX3, or whether one pathway is favoured over the other, possibly depending on the cell type or additional co-factors that associate with DDX3. Presumably due to the high rate of nuclear export, most studies investigating DDX3 localisation detected it mainly or exclusively in the cytoplasm [4, 6, 9, 12-14]. DDX3 partially accumulated in the nucleus after inhibition of CRM1 [4, 9, 12, 13] or TAP [13], suggesting that it can indeed be exported from the nucleus through both pathways.

In contrast to these results, two other studies found that endogenous DDX3 was mainly localised in the nuclei of untreated HeLa cells [5, 7]. The reason for this discrepancy is unclear. However, Chao et al. also demonstrated that DDX3 is mainly nuclear in healthy primary epidermis cells, but largely cytoplasmic in skin tissue from cutaneous squamous cell carcinomas [15], suggesting

a difference between transformed and non-transformed cells. It is conceivable that nuclear import and export of DDX3 are regulated; however the mechanisms for this have yet to be uncovered. For example, post-translational modifications could modulate either process, or DDX3 could be retained in the nucleus through the association with nuclear proteins. As the various described cellular functions of DDX3 include both cytoplasmic and nuclear processes (for example translational and transcriptional regulation respectively), nuclear-cytoplasmic shuttling of DDX3 might be closely linked to its participation in these processes. In particular, DDX3 might be regulating or mediating nuclear export of mRNAs through its association with the nuclear export receptors (discussed in more detail in section 3.3).

3. The role of DDX3 in RNA metabolism

DEAD-box helicases are involved in all aspects of RNA metabolism. Their role is thought to be the unwinding of RNA, i.e. the removal of secondary structure motifs, the unwinding of short RNA-RNA interactions and also the removal of RNA-bound proteins. All DEAD-box helicases contain nine conserved helicase motifs, including the eponymous Asp-Glu-Ala-Asp (D-E-A-D) motif, within a structurally conserved core element forming two recA-like domains. The conserved helicase motifs are involved in ATP binding, ATPase activity, RNA substrate binding and unwinding. However, the N- and C-termini of DEAD-box helicases are much more divergent and thought to confer functional specificity to individual DEAD-box helicases [3].

3.1 Unwinding of RNA

Ded1p, the yeast homologue of DDX3, appears to unwind RNA duplexes (and interestingly DNA-RNA duplexes) in a mode different from canonical translocating helicases [16]. Translocating helicases, e.g. DNA helicases, move along one strand of RNA or DNA directionally and in an energy-dependent manner. In the process, they displace complementary nucleic acid strands and/or interacting proteins.

However, Ded1p unwinds substrates without strict polarity. Based on this and other data, the authors proposed a mechanism by which the separation of the duplex is based on local destabilization of RNA helical regions, meaning that the helicase sitting on the duplex RNA ‘switches’ the two strands apart [16]. This would be a suitable mechanism for DEAD-box helicases, since they mainly appear to be involved in local structural changes of RNA and ribonucleoprotein (RNP) complexes, involving only a small number of base pairs [16, 17]. It is possible that this mechanism distinguishes DEAD-box helicases from the related RNA helicases containing DExD- or DEAH-box motifs, some of which have been shown to be processive RNA helicases [17, 18].

3.2. Splicing

Soon after its discovery the yeast Ded1p was linked to splicing of pre-mRNAs. However, it is still unclear whether Ded1p or mammalian DDX3 actually contribute to splicing [19]. It has been suggested that the C-terminus of DDX3 contains a region which resembles RS-domains found in splicing factors [5]. Ded1p and DDX3 appear to interact with the spliceosome and mRNPs [20, 21] (Figure 1, A). However, DDX3 tightly associated only with spliced mRNAs in an Exon junction complex (EJC)-dependent manner [20]. This would suggest that DDX3 does not have an

active role in splicing, but associates with RNPs after splicing, similar to proteins of the RNA export machinery.

3.3. Nuclear export of RNA

Several different nuclear transport receptors specifically mediate the export of proteins and the various classes of RNAs through the nuclear pore complex. As described in section 1, DDX3 interacts with two of these export shuttle proteins: CRM1, the receptor that exports proteins containing a leucine-rich NES, and TAP, the main mRNA exporter (Figure 1, B). Ribosomal RNAs and small nuclear RNAs (snRNAs) are also exported in a CRM1-dependent manner [22]. The mRNA export shuttle protein TAP gets recruited to spliced mRNAs via the EJC (similar to what was shown for DDX3 [20]). The interaction between DDX3 and TAP involved the C-terminus of DDX3 (aa 536-661) [13]. The authors also demonstrated that DDX3 could be cross-linked to poly(A)-mRNAs in nuclear and cytoplasmic fractions. Binding of DDX3 to mRNAs was not disrupted by siRNA-mediated knock-down of TAP, nor did RNase treatment disrupt the interaction between DDX3 and TAP [13]. Despite the finding that DDX3 binds to both, mRNAs and TAP, the authors were unable to show a contribution of DDX3 to polyA-mRNA export. It therefore seems unlikely that DDX3 is required for general mRNA export. However, it is still possible that DDX3 participates in the nuclear export of a specific subset of mRNAs via TAP. The CRM1-DDX3 interaction is exploited by HIV which seems to 'tag' its incompletely spliced RNAs to the CRM1/DDX3 complex for export out of the nucleus. This is mediated via an interaction between the viral RNA-binding protein rev and DDX3 (Figure 1, B). Hence, DDX3 was shown to be required for the export of HIV RNAs from the nucleus [4] (further discussed in section 5.5). However, the functional relevance of the CRM1-DDX3 interaction in uninfected

cells remains unclear. Yedavalli et al. found that DDX3 was not required for CRM1-dependent export of proteins such as I κ B α [4], therefore DDX3 does not seem to be a general cofactor of CRM1. Despite this, the authors postulated that DDX3 is not merely CRM1 cargo, but an effector molecule of the pathway. This was based on their finding that DDX3 binding to CRM1 was Ran-GTP independent and did not require the NES of DDX3. Rather, the CRM1 interaction was mediated by aa 260-517 of DDX3 [4]. One possibility is that DDX3 mediates the export of specific RNAs through the CRM1 pathway. If DDX3 was needed for the export of snRNA via CRM1 it could indirectly have an effect on splicing by affecting the maturation of snRNAs, which are exported from and re-imported into the nucleus before they assemble with other splicing factors into a functional spliceosome [22]. Also, a small subset of inducible mRNAs, including IFN α 1 mRNA and IFN γ -induced HLA-A mRNA [23, 24] is exported by CRM1 rather than TAP. Given its recently identified role in anti-viral gene expression [9] (further discussed in sections 3.5 and 5.1), it is tempting to speculate that DDX3 might have a role in mediating the export of these and other immuno-relevant mRNAs.

3.4. Protein Translation

The *S. cerevisiae* homologues of DDX3, Ded1p and Dbp1p, function in translation initiation [19]. There is evidence that Ded1p is required (in addition to the DEAD-box containing translation initiation factor eIF4a) for the unwinding of 5' untranslated regions (UTRs), with Ded1p being faster and more efficient in the scanning of long 5'UTRs than eIF4a [25, 26]. It was shown that human DDX3 can substitute for Ded1p in genetic complementation studies, suggesting that the human protein also facilitates translation initiation. It was demonstrated that

DDX3 interacts with several translation initiation factors, namely eIF4e [27], eIF4a, eIF2 α , PABP [13] and eIF3 [14] (Figure 1, C). However, in one of the initial studies, human DDX3 was described to be a repressor rather than a facilitator of cap-dependent protein translation while it enhanced IRES-dependent viral protein translation: DDX3 was shown to interact with eIF4e and to prevent the recruitment of eIF4G to eIF4e, which is required for the initiation of cap-dependent translation [27]. The authors suggested that DDX3 acts like cellular eIF4e-binding proteins (4eBPs) which also prevent translation initiation [27]. Nevertheless, there is now more data supporting a positive role of human DDX3 in translation initiation: One study demonstrated an interaction with eIF3 and a positive role for DDX3 in the translation of a β -globin mRNA [14]. Another study did not reveal an effect of DDX3 shRNA knock-down on global protein translation levels in pulse-labelling experiments, but showed that DDX3 knock-down negatively affects the translation of mRNAs containing complex secondary structures in their 5'UTRs [13], which is reminiscent of the data discussed above for the yeast Ded1p. A DDX3 mutant lacking helicase activity (S382L) [4] was unable to facilitate translation of highly structured mRNAs, suggesting that DDX3 is required to unwind the complex secondary structures of these mRNAs to allow for efficient translation [13]. Taken together, there appears to be more evidence for a positive role of DDX3 in protein translation. However, DDX3 was also found to associate with cytoplasmic stress granules, which are formed in cells after exposure to environmental stress and contain stalled translation pre-initiation complexes [13]. It is possible that the level of DDX3 expression influences whether it has a positive or negative effect on translation, given that the study characterising DDX3 as a translational repressor was based on overexpressed DDX3 [27]. This finding could be explained if the role of DDX3 was to bridge interactions between two or more proteins required for translation initiation. In this case, an excess of DDX3 would saturate

the binding sites and prevent formation of the multi-protein complex, leading to inhibition rather than enhancement of translation. It is also possible that DDX3 affects the translation of specific mRNAs differentially. In the context of DDX3 being a target for viral manipulation, it would be interesting to clarify whether there are indeed differences between cap-dependent (cellular) and IRES-dependent (viral) translation, and whether mRNAs with more complex 5'UTRs are in fact more dependent on DDX3. It has recently been shown that the mRNA of a transcription factor essential for type I interferon production, IRF7, contains a highly structured 5'UTR and that its translation is repressed by 4eBP1/2, thereby putting type I IFN production under translational control [28]. Like in the case of mRNA export, it is intriguing to speculate that DDX3 specifically enhances translation of IRF7 and other immuno-relevant genes.

3.5 Transcriptional regulation

There is increasing evidence that DExD/H RNA helicases can also function in transcriptional regulation of gene promoters, complementing their repertoire of regulatory functions in gene expression. Interestingly, this function often does not require ATPase or helicase activity, making DExD/H proteins truly multifunctional [29]. For DDX3 in particular, there is evidence that it can regulate transcription. It has been shown by chromatin-immunoprecipitation experiments to associate with the E-Cadherin and the IFN β promoter (Figure 1, D). Promoter activation was upregulated by DDX3 in the case of the IFN β promoter [9, 11] and downregulated in the case of the E-Cadherin promoter [30]. In addition, it has been demonstrated that DDX3 can bind to the transcription factor Sp1 and enhance the p21^{waf} promoter in an Sp1-dependent manner [15] (Figure 1, D). It will have to be determined in more detail how DDX3 gets recruited to specific

promoters. Even though DDX3 was initially suggested to contain a leucine-zipper [2], there is little substantiation for the existence of this motif in DDX3 and an ability to bind to specific DNA sequences. It is more likely that DDX3 gets recruited to specific promoters by interacting with promoter-specific transcription factors or other co-activators, as seems to be the case for the p21waf promoter [15]. The recruitment of DDX3 to the IFN β promoter appeared to be independent of IRF3 [11], but it is possible that DDX3 recruitment is mediated by a transcription factor other than IRF3 in this case. Other DExD/H family members, such as DHX9, DDX5 and DDX17, have been shown to bridge interactions between transcription factors and co-activators and/or RNA polymerase II [29] and it will be interesting to see whether DDX3 functions in a similar manner. Another interesting question is whether the role of DDX3 in gene promoter regulation is dependent on its helicase activity. The effect of DDX3 on the IFN β promoter was independent of its ATPase or unwinding activity, since the K230E mutant of DDX3, which has been shown to lose both functions [4], behaved like wild-type DDX3 [9, 11]. On the other hand, the effect of DDX3 on the p21waf promoter appeared to require ATPase but not unwinding activity [15]. It is therefore possible that DDX3 regulates individual promoters in slightly different ways and future studies should reveal the exact mechanism(s) by which DDX3 regulates gene promoter activation and/or suppression. Likewise, it is unlikely that the full range of promoters that are affected by DDX3 has been discovered, and it will be interesting to see how specific or wide-spread the role of DDX3 in transcriptional regulation is. In particular, if DDX3 is taken forward as a therapeutic target, it will be important to determine the extent of its involvement in gene regulation.

3.6 Conclusion: Role of DDX3 in the regulation of gene expression

As discussed in sections 3.2 to 3.5, there is evidence for a role of DDX3 in every step of gene expression: transcription, mRNA maturation, mRNA export and translation (Figure 1). These processes are tightly coordinated, and it is an attractive thought that DDX3 might be involved in integrating these processes to ensure efficient progression of an mRNA through the route from transcription to translation. Since several studies failed to show a role for DDX3 in bulk mRNA export or general protein synthesis, this function of DDX3 might be specific for a subset of mRNAs.

4. DDX3 in cell cycle control, apoptosis and tumorigenesis

There is mounting evidence of a role for DDX3 in cell cycle and cell growth control. The data, however, is ambiguous in that DDX3 has been suggested to be both a tumour suppressor [15, 31] and an oncogene [30]. This conflicting data might point to cell-type specific differences or could be linked to the slightly ambiguous role of DDX3 in protein translation, where an excess of DDX3 appeared to inhibit rather than enhance translation [13, 27].

4.1. Tumour suppressor function of DDX3

It has been shown that DDX3 expression is downregulated in human hepatocarcinoma (HCC) samples, in particular those of male patients and Hepatitis B virus (HBV)-positive patients. It was suggested that lower DDX3 protein levels could contribute to the three times higher prevalence of HCC in men and HBV-induced cellular transformation. Hence, it was postulated that DDX3 acts as a tumour suppressor [15, 31]. The authors also showed that siRNA-mediated knockdown

in the non-transformed murine fibroblast cell line NIH3T3 led to a premature entry into S phase and an increase in cell proliferation [31]. This correlated with lower levels of p21^{waf} and elevated levels of CyclinD1 in the DDX3 knock-down cells [31]. Interestingly, the authors showed that the loss of DDX3 was insufficient for cellular transformation, but the combined loss of DDX3 and overexpression of the oncogene v-ras led to enhanced transformation of NIH3T3 cells. In a follow-up study, it was demonstrated that DDX3 upregulates the p21^{waf} promoter in an Sp-1 dependent manner [15] (also discussed in section 3.5). The authors showed evidence that ectopic expression of DDX3 can inhibit proliferation of tumour cell lines (HCT116, HeLa, HuH-7) and non-transformed cell lines (NIH3T3). This effect of DDX3 was drastically reduced in HCT116 cells lacking p21 expression, suggesting that DDX3 indeed exerts its effect on cell proliferation in a p21-dependent manner [15]. In contrast to these two studies, an earlier study suggested that DDX3 mRNA expression is elevated in 64% of all HCC tissue samples investigated [32]. The reason for this discrepancy is unclear, however it was suggested that it might relate to differential regulation at mRNA and protein level [31]. It will be interesting to determine the exact relationship between DDX3 and p21 and its influence on cell cycle control. Further evidence for a link between DDX3 and cell cycle control is provided by studies on hamster DDX3 [12, 33]: DDX3 appears to influence the levels of the cell cycle regulator Cyclin A and to be phosphorylated by Cyclin B/cdc2 during mitosis [12, 33]. In addition, Hamster tsET24 cells, which are temperature sensitive cells with a point mutation in DDX3 (P267S), exhibit G1 arrest at the non-permissive temperature [34]. The residues phosphorylated by cdc2, most importantly Thr204, sit within conserved helicase motifs of DDX3 and are therefore likely to disrupt substrate binding and helicase activity [33, 35]. It is possible that this is linked to reduced protein translation during mitosis, provided that a role for DDX3 in general protein translation can be substantiated. Further clarification is needed as to how DDX3

function is affected by the cdc2-dependent phosphorylation. It is also unclear how DDX3 contributes to the decrease in Cyclin A levels.

Some of the described studies have initially been inspired by the finding that Hepatitis C Virus core protein interacts with DDX3 (discussed in section 5.3) and future studies should clarify whether and how the core protein of HCV (which has been shown to contribute to cellular transformation) affects the role of DDX3 in cell cycle control.

4.2 DDX3 as an oncogene

It has been suggested that DDX3 has an oncogenic role in breast cancer biogenesis [30]. The study showed that Benzo[a]pyrene diol epoxide (BPDE), a cancer-causing compound found in tobacco smoke, increases levels of DDX3 expression in an immortalized non-tumorigenic human breast epithelial cell line (MCF 10A cells). The authors also demonstrate that DDX3 protein and mRNA levels positively correlate with tumorigenicity of breast epithelial cell lines, suggesting that aggressive breast cancer cell lines have higher levels of DDX3 protein [30]. MCF 10A cells stably overexpressing DDX3 showed an increase in anchorage-independent growth, motility and invasion, which is indicative of cellular transformation [30]. Loss of E-cadherin, which is an adhesion molecule mediating cell-cell contact, has been shown to induce epithelial–mesenchymal transition in several cancers [36]. Hence, the authors tested whether DDX3 overexpression or knockdown affected E-Cadherin expression and demonstrated that DDX3 suppresses the E-Cadherin promoter [30] (see also section 2.5). In contrast to Yan Wu Lee’s laboratory [15, 31], the authors did not find a correlation between DDX3 and p21 protein levels in breast epithelial cells and were unable to see upregulation of a p21 promoter reporter construct by DDX3 [30].

Again, it is quite unclear what causes this discrepancy, unless one assumes that the effect of DDX3 on the p21 promoter is cell-type specific. It is certain that DDX3 is only one of many regulatory factors influencing cell proliferation and cellular transformation, so the overall outcome of enhanced or reduced DDX3 levels might depend on the presence/absence of other co-factors and the activation of signalling pathways.

4.3. DDX3 and apoptosis

The development of cancer can be linked to enhanced cell proliferation and/or reduced levels of programmed cell death (apoptosis). So far, there is only little data regarding a role for DDX3 in apoptosis and further studies will be needed to clarify this. Chang et al. reported preliminary data that Caspase 6 and -9 cannot be activated in the absence of DDX3, hence protecting DDX3 knock-down cells from apoptosis [31]. Their data therefore suggest that loss of DDX3 leads to enhanced cellular proliferation and reduced apoptosis, both features that promote tumorigenesis [31]. In contrast, the only study dedicated to a role for DDX3 in apoptosis describes an anti-apoptotic role for DDX3 [37]. The authors identified an antiapoptotic complex consisting of GSK3, DDX3 and c-IAP1, which binds to death receptors and prevents signalling. Death receptor stimulation led to inactivation of GSK3 and caspase-dependent cleavage of c-IAP1 and DDX3 [37]. The exact role of DDX3 in this complex is unclear. However, knockdown of DDX3 enhanced signalling through the death receptor TRAIL-R2 and activation of Caspase-3 [37]. In the light of the limited amount of data on the role of DDX3 in apoptosis, there is scope for further studies to clarify this potentially important function of DDX3. The possibility that DDX3 differentially affects intrinsic and extrinsic apoptotic signalling pathways should be considered. Signalling by death receptors requires assembly of the death inducing signalling complex (DISC)

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4 comprising the initiator caspase 8. The intrinsic or mitochondrial pathway is regulated e.g. by
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6 Bcl-2 family proteins and involves the initiator caspase 9. The data summarised above seems to
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8 suggest that DDX3 is required for activation of the intrinsic pathway [31], but downregulates
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10 activation of the extrinsic pathway [37]. However, this appears to make limited physiological
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12 sense, so clarification of the role of DDX3 in apoptosis is needed and should be addressed in
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14 further studies.
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21 **4.4. Conclusion: DDX3 in tumorigenesis**

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26 The data available on the role of DDX3 in cell cycle regulation, cellular transformation and
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28 apoptosis is still rather limited, so further studies should bring clarification and additional
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30 mechanistic insight into the involvement of DDX3 into these processes. Due to its
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32 multifunctionality, it is possible that DDX3 can have both positive and negative effects on cell
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34 proliferation depending on the cell type and environmental conditions. It will be important to
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36 understand and reconcile these effects of DDX3 on cellular events contributing to tumorigenesis,
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38 before DDX3 can be explored as a therapeutic target for viral infections and auto-immune
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40 diseases (further discussed in sections 5.5 and 6).
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48 **5. DDX3 as a target of viral manipulation**

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52 One of the most intriguing features of DDX3 is its manipulation by viruses. Because viruses have
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54 limited genome sizes and are highly optimized through evolution, they tend to target key
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56 regulators within the host cell machinery to help them replicate. On the one hand, this comprises
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58 recruitment of host factors, such as transcription factors or polymerases, that directly participate
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in replication of the virus. On the other hand, a lot of viruses encode immunoregulatory proteins that inhibit or modulate the immune response, so that the virus can either avoid detection by the immune system altogether or interfere with the ensuing signalling pathways leading to cytokine expression. In particular, a lot of viruses have evolved mechanisms to inhibit the induction or function of type I interferons (IFN), potent anti-viral mediators that were named after their 'interfering' effect on viral replication [38]. DDX3 has recently been shown to contribute to IFN β production downstream of pattern recognition receptor activation [9, 11]. This would make it a potential target for viral evasion strategies aimed at the IFN pathway, and indeed, we have shown that the poxvirus protein K7 targets DDX3 and interferes with IFN β promoter activation [9]. On the other hand, HIV and HCV depend on DDX3 for their replication, exploiting its nuclear export and helicase function. Hence, they seem to co-opt rather than inhibit DDX3. However, it is possible that some viruses recruit DDX3 to help with replication of the viral genome while simultaneously sequestering and preventing DDX3 from inducing type I IFNs, meaning that these viruses would get dual benefit from their interaction with DDX3.

5.1 DDX3 participates in innate immune signalling and enhances the induction of anti-viral mediators.

We and others have recently described a positive role for DDX3 in the induction of IFN β , revealing an unexpected function of DDX3 in anti-viral gene expression [9, 11].

Viral recognition by the innate immune system is mediated by different classes of pattern recognition receptors (PRRs): The subset of endosomal Toll-like receptors (TLRs) that comprises TLR3, TLR7, TLR8 and TLR9 recognises the different classes of viral nucleic acids within the

lumen of the endosome. On the other hand, RIG-like helicases (RLH) (RNA helicases belonging to the DExH family) recognise viral RNAs in the cytoplasm of cells [39]. Both sets of anti-viral PRRs lead to the activation of the transcription factors NF- κ B and Interferon regulatory factors (IRF)3 and IRF7, which are required for IFN β promoter induction. IKK ϵ and the related kinase TBK1 are required for the phosphorylation-induced activation of IRF3 and IRF7 downstream of most anti-viral PRRs (TLR3, TLR4 and the RLHs). We have recently shown that DDX3 interacts with IKK ϵ after Sendai virus infection, and contributes to IRF3 activation and IFN β and promoter activation (Figure 2) [9]. This novel role of DDX3 in anti-viral innate immune signalling was simultaneously described in another study identifying DDX3 as a TBK1-interacting protein and phosphorylation target [11]. In accordance with our data, the authors also demonstrated that knock-down of endogenous DDX3 by siRNAs reduced IFN β promoter induction downstream of different PRRs [11]. Both studies agree that ATPase and helicase function of DDX3 are dispensable for the effect of DDX3 on the IFN β promoter, showing that a mutation in the Walker A motif (K230E) of DDX3 does not affect this function of DDX3 [9, 11] (see section 3.5).

Using a series of truncation mutants, we showed that the N-terminus of DDX3 is required for its effect on the IFN β promoter, since truncations lacking the first 139 aa were unable to mediate promoter induction [9]. Soulat et al. identified several serine/threonine residues within the two recA-like domains of DDX3 (between aa 181 and aa 543) as TBK1 phosphorylation sites, and showed that S/T \rightarrow A mutation of putative TBK1 phosphorylation motifs contained within either recA-like domain of DDX3 led to a decrease in IFN β promoter induction [11]. They also demonstrated by chromatin immunoprecipitation that DDX3 bound to the IFN β promoter

enhancer region (with no requirement for IRF3) [11]. Their data suggest that DDX3 acts as a transcriptional co-factor that is activated by TBK1-phosphorylation, much in the same way as IRF3 and IRF7 themselves. Apart from the IFN β promoter, there is further evidence that DDX3 can bind to and regulate gene promoters, as discussed in section 3.5.

The exact molecular detail of how DDX3 contributes to IRF3 activation and IFN β promoter activation remains to be elucidated, e.g. the functional relevance of the DDX3 phosphorylation by TBK1/IKK ϵ is currently unclear.

5.2 Poxvirus protein K7 targets DDX3 to inhibit anti-viral gene expression

We have recently identified the vaccinia virus (VACV) protein K7 as a viral protein that interacts with DDX3 and inhibits its function in the IFN induction pathway. [9]. The potent inhibitory effect of K7 on TLR-and RLH-dependent IRF3/7 activation and IFN β promoter induction led us to investigate a potential role of its host target DDX3 in this innate immune signalling pathway [9]. Interestingly, K7 binds to the N-terminal region of DDX3 (aa 1-139) that we found to be required for the effect on the IFN β promoter [9]. It is therefore conceivable that binding of K7 to this region of DDX3 prevents downstream signalling (Figure 2). In a more recent study, we have narrowed down the K7-DDX3 binding site to the region between aa 61-90 of DDX3 [40].

The N-terminus of DDX3 contains several other interesting motifs, including the NES (see section 2) [4] and the potential eIF4e interaction site (aa 38-43) (see section 3.4) [27].

Unfortunately, the N-terminus of DDX3 also appears to be highly flexible and is missing in the

available crystal structure of DDX3 [35], so that we have no structural information about this functionally important region of DDX3.

We will continue to explore the molecular detail and nature of the interaction between K7 and DDX3 with the aim of understanding how poxviruses manipulate DDX3, and how the region of DDX3 targeted by K7 contributes to the cellular functions of DDX3.

5.3. DDX3 interacts with HCV Core protein and is required for HCV replication

HCV core protein was the first viral protein to be described as a DDX3-interacting protein. HCV core protein is a structural protein, which forms the viral nucleocapsid. However, there is evidence that the core protein has other functions, e.g. it has been described to modulate viral and cellular gene expression. Three independent publications described the interaction between DDX3 and HCV core protein; however their studies were hampered by limited data being available on the cellular function of DDX3 at the time of discovery. Owsianka et al. mapped the interaction to the C-terminal serine-rich region of DDX3 (aa 553-622), which they suggested to resemble RS-domains found in splicing factors. They also found DDX3 associated with nuclear speckles, and therefore suggested that DDX3 plays a role in splicing [5]. Expression of HCV core protein, however, did not affect the localisation of DDX3 to nuclear speckles. In contrast, it affected the distribution of the cytoplasmic fraction of DDX3 and led to strong co-localisation of DDX3 and core protein in distinct spots in the cytoplasm, suggesting that the core protein targets a cytoplasmic function of DDX3 [5]. Yan-Hwa Wu Lee's group also identified the interaction between HCV Core protein and DDX3, which they initially called Cap-Rf (Core-associated protein-RNA helicase full-length) and believed to be distinct from, but closely related to, DDX3.

They also mapped the interaction to the C-terminal region of DDX3 (aa 473-611). In addition, they showed that addition of recombinant core protein enhanced the ATPase activity of DDX3 [7]. In their study, DDX3 expression also induced expression of a luciferase reporter construct, pCMV-luc, and co-expression of HCV core protein further enhanced the induction. This was a first indication that DDX3 might be involved in transcriptional regulation of gene promoters (as discussed in section 3.5), and furthermore suggested that HCV core protein activates or enhances DDX3 function [7]. Finally, the third publication describing the DDX3-Core protein interaction showed that human DDX3 can substitute for homologous yeast Ded1p [6]. HCV core protein negatively affected the growth of DDX3-substituted *ded1*-deletion yeast and inhibited the translation of capped, but not un-capped, RNA in an in vitro translation assay. The authors concluded that DDX3 functions as an RNA helicase and is involved in translation initiation of capped RNAs (as discussed in section 3.4). Their data suggests indirectly that HCV core protein interferes with this function of DDX3 [6].

As the evidence is mounting for a role for DDX3 in both transcriptional and post-transcriptional regulation of gene expression (see section 3), cell cycle regulation (see section 4) and innate immune signalling (section 5.1), it appears that DDX3 is a highly multifunctional cellular protein. It is not completely inconceivable that HCV core protein could enhance a particular function of DDX3 while simultaneously inhibiting one of its other functions, possibly explaining some of the diverse findings described above. However, to date it is not clear what the functional relevance of the DDX3-Core protein interaction is. It has recently been demonstrated that DDX3 is required for HCV RNA replication, with genome-length HCV RNA strongly and subgenomic replicon RNA partially suppressed in DDX3 knock-down cells [8]. This suggests that HCV needs DDX3 to aid in the replication of its RNA (possibly by unwinding double-stranded RNA species),

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4 however the exact contribution of DDX3 remains to be determined. It also does not explain the
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6 core protein-DDX3 interaction, given that the core protein is not directly involved in viral
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8 genome replication.
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14 There is strong epidemiological evidence that chronic HCV infection is linked to the
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16 development of hepatocellular carcinomas (HCC), even though the molecular mechanisms
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18 underlying the progression to cancer are not very well defined [41]. It is likely that
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20 immunopathological mechanisms linked to chronic inflammation play a role, but there is
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22 evidence that HCV proteins, including the core protein, are intrinsically oncogenic [41]. As
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24 discussed in section 4.2, publications by Wu Lee's group suggest that DDX3 expression is
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26 downregulated in certain hepatocellular carcinomas (HCC), in particular those occurring in HBV-
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28 positive and male patients [15, 27, 31]. They also demonstrated that knock-down of DDX3 with
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30 siRNA enhances cell proliferation and downregulates the expression of the cell cycle regulator
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32 p21waf, which appeared to depend on cooperative action between DDX3 and Sp1 (see sections
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34 3.5 and 4.1). This suggested that DDX3 acts as a tumour suppressor gene by upregulating p21waf
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36 levels [15]. The authors argue that lower levels of DDX3 or functional inactivation of DDX3
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38 might contribute to the development of HCC, potentially explaining the higher rate of HCC in
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40 males and the link between HBV and HCV-infection and HCC [15]. HCV infection does not
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42 appear to change DDX3 protein levels, however it is possible (though remains to be
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44 demonstrated) that the HCV core protein functionally interferes with the effect of DDX3 on the
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58 As discussed above, some viruses might benefit from DDX3 sequestration in two different ways,
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60 one being the disruption of signalling pathways leading to IFN β promoter activation. Upon
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introduction of HCV core protein into cells, a striking re-distribution of DDX3 occurs from a diffuse cytoplasmic localization into distinct cytoplasmic spots co-localised with HCV core protein [5]. This may suggest that the HCV core protein sequesters DDX3 from some of its cellular functions, such as IFN β promoter activation, by pulling it into these cytoplasmic complexes. On the other hand, HCV has other extremely potent mechanisms for inhibiting IRF3 activation and IFN β induction, e.g. the cleaving of the signalling adaptors TRIF and MAVS by the viral protease NS3/4a [42]; hence inhibition of DDX3 might be unnecessary or redundant for HCV.

For lack of experimental data, the functional consequences of the HCV core protein-DDX3 interaction remain mysterious and further research is required to investigate whether and how the core protein affects the diverse cellular functions described for DDX3. It is important to determine whether this interaction contributes to viral replication, persistence and/or the development of virus-induced carcinomas.

5.4 DDX3 inhibits HBV replication

Hepatitis B virus is the prototypic member of the hepadnavirus family. Replication of the genome occurs by reverse transcription of the pregenomic RNA template, is mediated by the HBV polymerase which binds to an RNA stem loop and occurs entirely within nucleocapsids. It was recently shown that DDX3 binds to HBV polymerase, in an interaction that did not appear to be mediated by RNA [43]. DDX3 was incorporated into nucleocapsids together with HBV polymerase and inhibited the initial step of reverse transcription in a manner that seemed to

depend on the ATPase-activity of DDX3 [43]. A lot of questions remain unanswered, such as the exact mechanism of inhibition and the physiological relevance of the finding. It is interesting that DDX3 is targeted by two hepatitis viruses, raising the question as to whether targeting DDX3 might contribute to liver cell tropism [43]. However, HCV and HBV belong to different virus families and have different replication mechanisms, making a common strategy for targeting DDX3 unlikely. In addition, while DDX3 is required for HCV replication, it blocks HBV replication. Interestingly, it has been shown that DDX3 levels are decreased in a fraction of HBV-induced HCC cases [31]. This could be a viral mechanism to relieve the inhibitory effect of DDX3 on its replication and might contribute to the development of HBV-induced HCC (see section 4.1).

5.5 DDX3 is required for nuclear export of HIV RNA and viral replication

In a paper that drew much attention to DDX3, Yedavalli et al. described a role for DDX3 in HIV replication and suggested it as a potential therapeutic target for HIV infection [4]. DDX3 was upregulated in HIV-infected cells in a tat-dependent manner [4]. The authors demonstrated that DDX3 interacts with the HIV rev protein and the cellular nuclear export protein CRM1. Through the rev-DDX3 interaction, HIV seems to target its incompletely spliced mRNAs to CRM1 for export from the nucleus (see section 3.3). Downregulation of DDX3 levels in the cell using antisense technology inhibited the export of partially spliced HIV RNAs from the nucleus and consequently HIV replication [4]. This function of DDX3 was dependent on its helicase activity [4], so DDX3's role in this process might be the unwinding of secondary structures within the HIV RNA or the removal of RNA-bound proteins to facilitate export. In this context, the authors also found DDX3 to interact with nucleoporins and to localize to the cytoplasmic side of nuclear

pores, which is reminiscent of the Yeast Dpb5p helicase that functions in nuclear-cytoplasmic export of mRNAs [4]. Most importantly, it was possible to inhibit HIV replication by reducing DDX3 protein levels in the cell [4]. The possibility to exploit the identification of DDX3 as an essential host factor for HIV replication for the development of novel anti-HIV therapeutics is exciting, and will be discussed in more detail in section 6.1.

5.6 Conclusion: Viral manipulation of DDX3

It is highly intriguing that DDX3 is the target of manipulation by at least four very different viruses. Each of the viruses seems to co-opt or inhibit a different function of the astonishingly multifunctional DDX3 protein; however one has to wonder whether there is some common denominator that makes DDX3 such an attractive target for viral manipulation. Its newly discovered role in IFN β promoter induction would lead us to assume that other viruses, apart from poxviruses, have evolved mechanisms for inhibiting this particular function of DDX3. As mentioned before, it is possible that viruses that appear to co-opt DDX3 also simultaneously inhibit its function in IFN β induction. Studying these virus-DDX3 interactions in more detail will provide us with novel insights into the biology of DDX3 and potentially lead to the development of anti-viral drugs and the therapeutic targeting of DDX3.

6. Therapeutic potential

6.1. DDX3 as a target for HIV and HCV therapy

Both, human and viral helicases have been successfully targeted therapeutically in the past [10]. Given that DDX3 seems to be required for replication of HCV and HIV, it appears, and was suggested early on, that DDX3 could be a promising target for drug development against these two viruses that pose major global health threats [10]. Targeting a host factor rather than a viral protein might make it more difficult for a virus to develop resistance against the drug. On the other hand, DDX3 obviously has not evolved for the purpose of the virus, and hence one needs to be careful with the inhibition of a host protein which appears to have a multitude of cellular functions, none of which has been elucidated in much detail yet. As described above, tampering with DDX3 has the potential to affect different levels of gene expression, cellular proliferation and transformation, and long-term suppression of DDX3 function could therefore lead to serious complications, including the development of cancer. However, it has been shown that knock-down of DDX3 in cells can inhibit HIV replication without affecting cell viability [44]. In addition, two studies have been published in which small molecule inhibitors were used to inhibit the ATPase activity of DDX3 (which is required for mediating HIV mRNA export), again without affecting cell viability or showing toxicity in mouse models [45, 46]. However, it is likely that none of these studies would have revealed effects of DDX3 inhibition on cellular transformation events. Not all of the cellular functions of DDX3 require ATPase activity. Hence inhibiting this enzymatic activity of DDX3 should leave the ATPase-independent cellular functions of DDX3 intact, such as the induction of type I IFNs [9, 11]. However, it was suggested that upregulation of the p21^{waf} promoter requires the ATPase (but not unwinding) activity of DDX3 [15]. This cellular function of DDX3 is linked to cell cycle control; hence inhibiting this function carries a risk of contributing to tumorigenesis.

Since DDX3 is also required by HCV for its replication, its inhibition could also be a valid strategy for the development of novel HCV drugs. However, even less is known about the exact molecular mechanisms of the HCV-DDX3 interaction, and there is evidence linking DDX3 inactivation during virus infection to the development of liver carcinomas.

The safest strategy would be to develop drugs that very specifically target the virus-DDX3 interaction. This requires a detailed understanding of the molecular and functional details of the interaction and further studies should aim to achieve this.

6.2. Cancer

As discussed in section 4, current data on the role of DDX3 in cell cycle control, cellular transformation events and apoptosis is ambiguous; hence it seems difficult to imagine targeting DDX3 for cancer therapy at the moment. However, should it emerge in the future that hyperactivation or inactivation of DDX3 are clearly linked to certain cancer types, this could lead to the development of cancer chemotherapies targeting DDX3.

6.3. Autoimmune diseases and viral infections

It has recently been shown that DDX3 seems to have a role in anti-viral immunity by contributing to type I IFN production [9, 11]. Vaccinia virus K7 protein was shown to target this function of DDX3 in order to inhibit type I IFN production [9]. Since type I IFN production is a key target of many viral evasion strategies, we will most likely find that other viruses have evolved similar mechanisms to inhibit this particular function of DDX3. Studying these DDX3-virus interactions

could lead to the development of anti-viral therapeutics, which disrupt the viral strategy and restore DDX3 function. On the other hand, hyperactivation of the IFN system, e.g. in autoimmune diseases, could call for therapeutic inhibition of DDX3 function in this pathway. This could be achieved by mimicking the viral strategies for interfering with DDX3 with small molecule inhibitors.

7. Finishing remarks

Even though research on DDX3 has accelerated in recent years and consequently our knowledge about the cellular functions of DDX3 has grown, the overall picture still remains fragmentary. It will be important to resolve the seemingly contradictory data on the role of DDX3 in cell growth control and the regulation of protein translation. It should also be attempted to establish whether the various effects of DDX3 on cellular events are indeed mediated by independent functions of DDX3, or whether some of them could be interconnected. Another interesting area of research will be the regulation of DDX3 activity by post-translational modification, as there is evidence for DDX3 being phosphorylated by cdc2, IKK ϵ and TBK1 [11, 33]. Most likely, we will also discover other promoters that are regulated by DDX3 and learn about additional viral strategies to inhibit or co-opt DDX3. Studying the manipulation of DDX3 by viruses in detail can provide us with novel mechanistic insights about the function(s) of DDX3 and might lead to the development of therapeutics mimicking or inhibiting the viral strategies.

Acknowledgements

Work in the author's laboratory is supported by Science Foundation Ireland (09/RFP/BIC2188) and the Irish Health Research Board (HRA/2009/171). M.S. is also supported by a Career Development Fellowship from the Irish Health Research Board (PD2007/35).

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Figure legends

Figure 1: DDX3 regulates gene expression at different levels.

A. DDX3 and its yeast homologue have been described to interact with splicing factors and RNPs (see section 3.1). **B.** DDX3 can be exported from the nucleus by the CRM1 and the TAP export shuttles, both of which it has been shown to interact with. The TAP exporter is mainly responsible for mRNA export from the nucleus and binds to mature RNPs after splicing. DDX3 associates with both TAP and RNPs, but didn't seem to be required for bulk mRNA export. DDX3 also binds to CRM1, which is exploited by HIV. The viral RNA-binding protein rev binds to DDX3 and thereby targets its partially spliced RNAs to the CRM1 export shuttle for export from the nucleus. Knock-down of DDX3 was shown to inhibit HIV replication. The physiological role of the CRM1-DDX3 interaction is unknown (see section 3.2). **C.** DDX3 has been implicated in regulating the initiation of translation. It binds to several translation initiation factors, namely eIF4e, eIF4a, eIF2 α , eIF3 and PABP. There is slightly conflicting data on whether DDX3 has a positive or negative role on translation initiation, as discussed in section 3.3. **D.** DDX3 plays a role in the transcriptional regulation of gene promoters. It has been shown to downregulate the E-Cadherin promoter, while it enhances IFN β and p21^{waf} promoter induction. It is likely to be recruited to gene promoters by other transcription factors or co-activators (discussed in section 3.5). The different steps in the gene expression process are tightly co-ordinated and it is possible that DDX3 serves as an integrating factor, accompanying an mRNA along the way from transcription to translation.

In the online colour version of this figure, proteins that have been shown to interact with DDX3 are depicted in blue.

Figure 2: The role of DDX3 in innate immune signalling

DDX3 was shown to interact with and be phosphorylated by TBK1 and IKK ϵ . The interaction between DDX3 and IKK ϵ was transiently induced during Sendai virus infection, linking DDX3 into the signalling pathway from RIG-I (the pattern recognition receptor that mediates Sendai virus recognition) to IRF3 activation and IFN β promoter activation (section 5.1). IKK ϵ and TBK1 also mediate IRF3 activation downstream of other pattern recognition receptors, such as TLR3 and TLR4, and consequently DDX3 also enhanced IFN β promoter induction downstream of these receptors. DDX3 was found to associate with the IFN β promoter enhancer, suggesting that it acts as a transcriptional co-activator (section 3.5 and 5.1).

The vaccinia virus protein K7 binds to the N-terminus of DDX3, which was found to be required for IFN β promoter induction. K7 is a potent inhibitor of IRF3 activation and IFN β promoter induction. This suggests that K7 binding to DDX3 prevents it from exerting its effect on IFN induction (section 5.2).

In the online colour version of this figure, proteins that have been shown to interact with DDX3 are depicted in blue.

Interaction of DDX3 with viruses						
Virus	Effect of DDX3 on viral replication	Viral interacting protein	Cellular function of DDX3 targeted	Helicase activity required	Therapeutic potential	References
HCV	+	Core	Translation? p21 promoter activation? Helicase function?	?	Inhibit DDX3 function in HCV replication.	[5-8]
HBV	–	HBV Pol	?	ATPase: Yes Helicase: No		[43]
HIV	+	rev	Nuclear-cytoplasmic shuttling	Yes	Inhibit helicase activity, Inhibit nuclear-cytoplasmic shuttling?	[4], [44-46]
VACV	(-)*	K7	IFN β promoter induction	No	Use K7 mimetics to inhibit DDX3 in IFN-dependent autoimmune disorders.	[9], [40]
* has not been determined. K7 inhibits role of DDX3 in IFN β induction, hence role of DDX3 appears to be antiviral.						

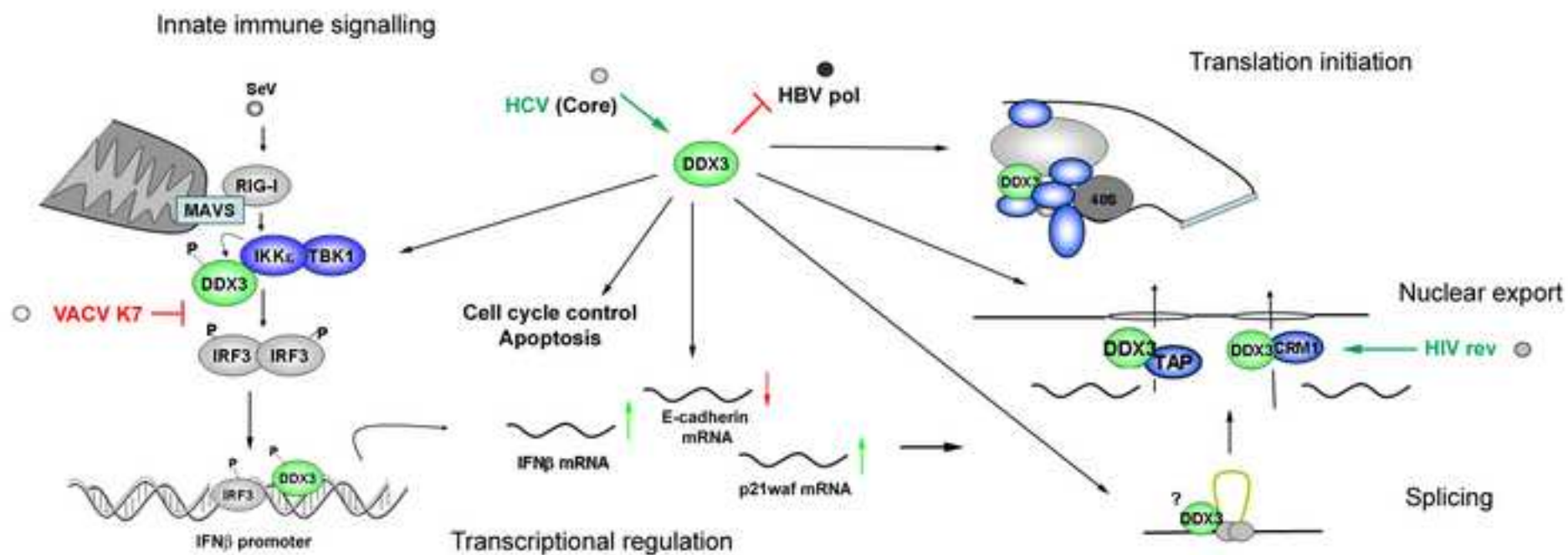


Figure 1

Figure 1

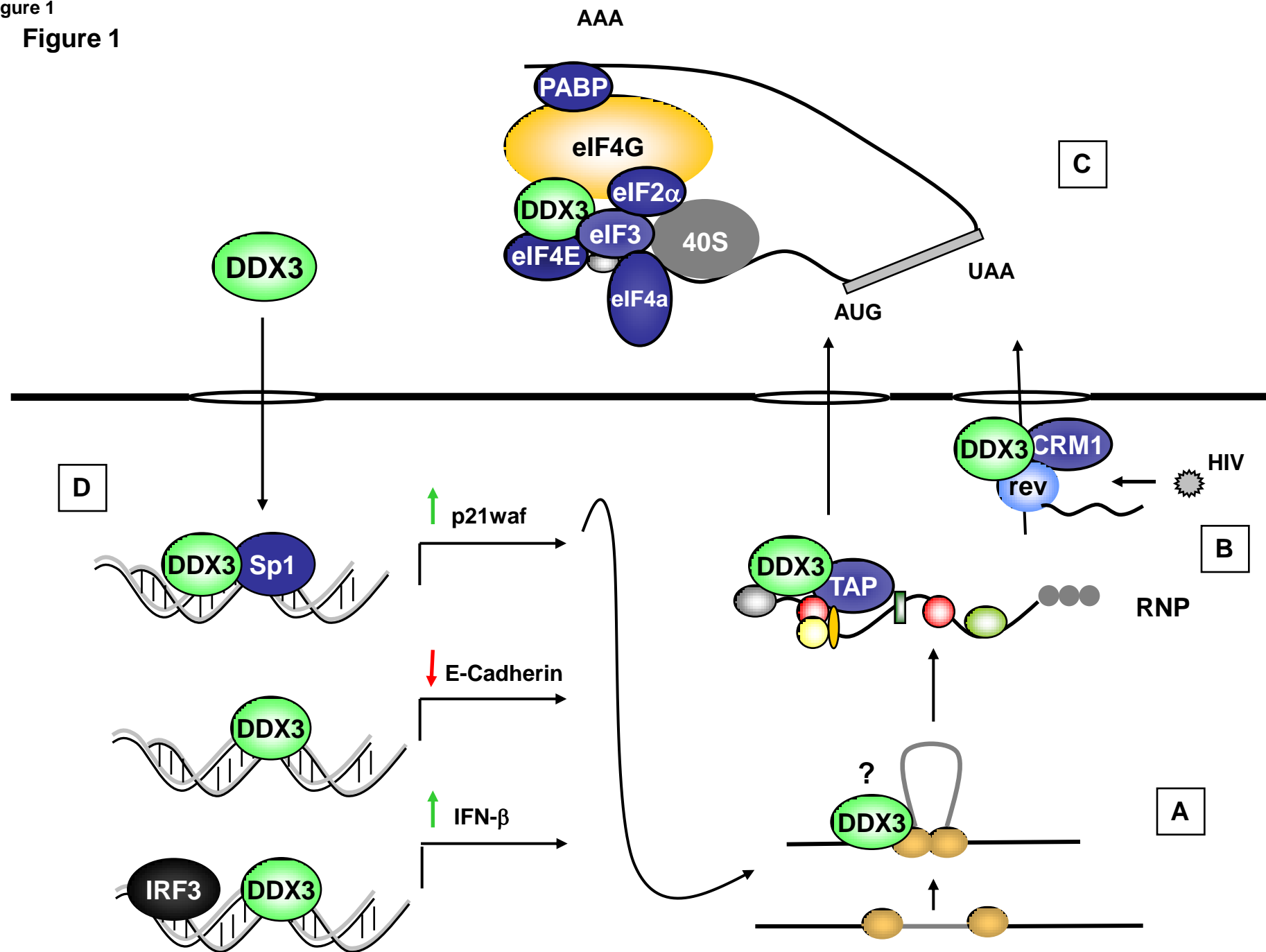


Figure 1-grey
Figure 1

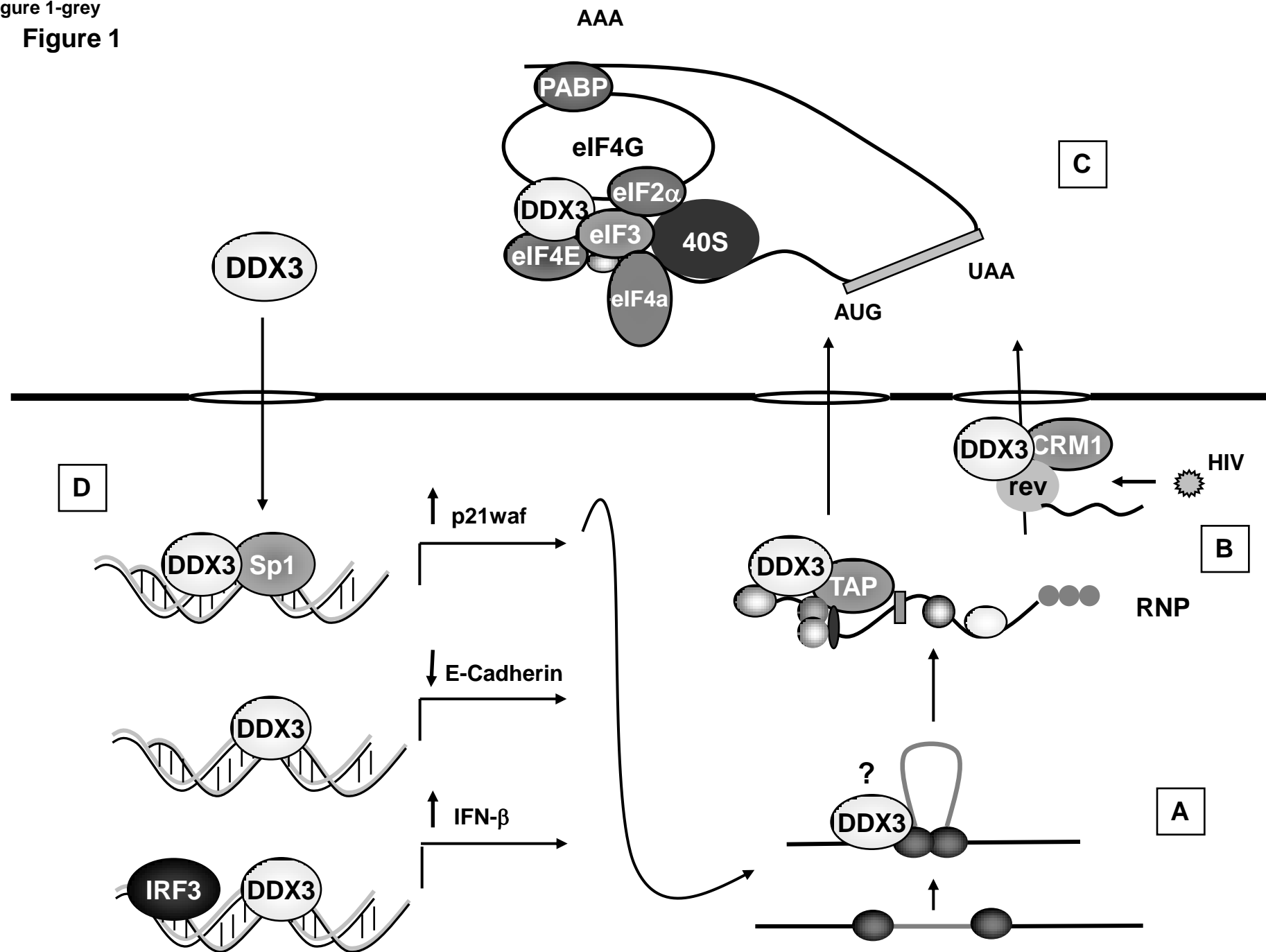


Figure 2

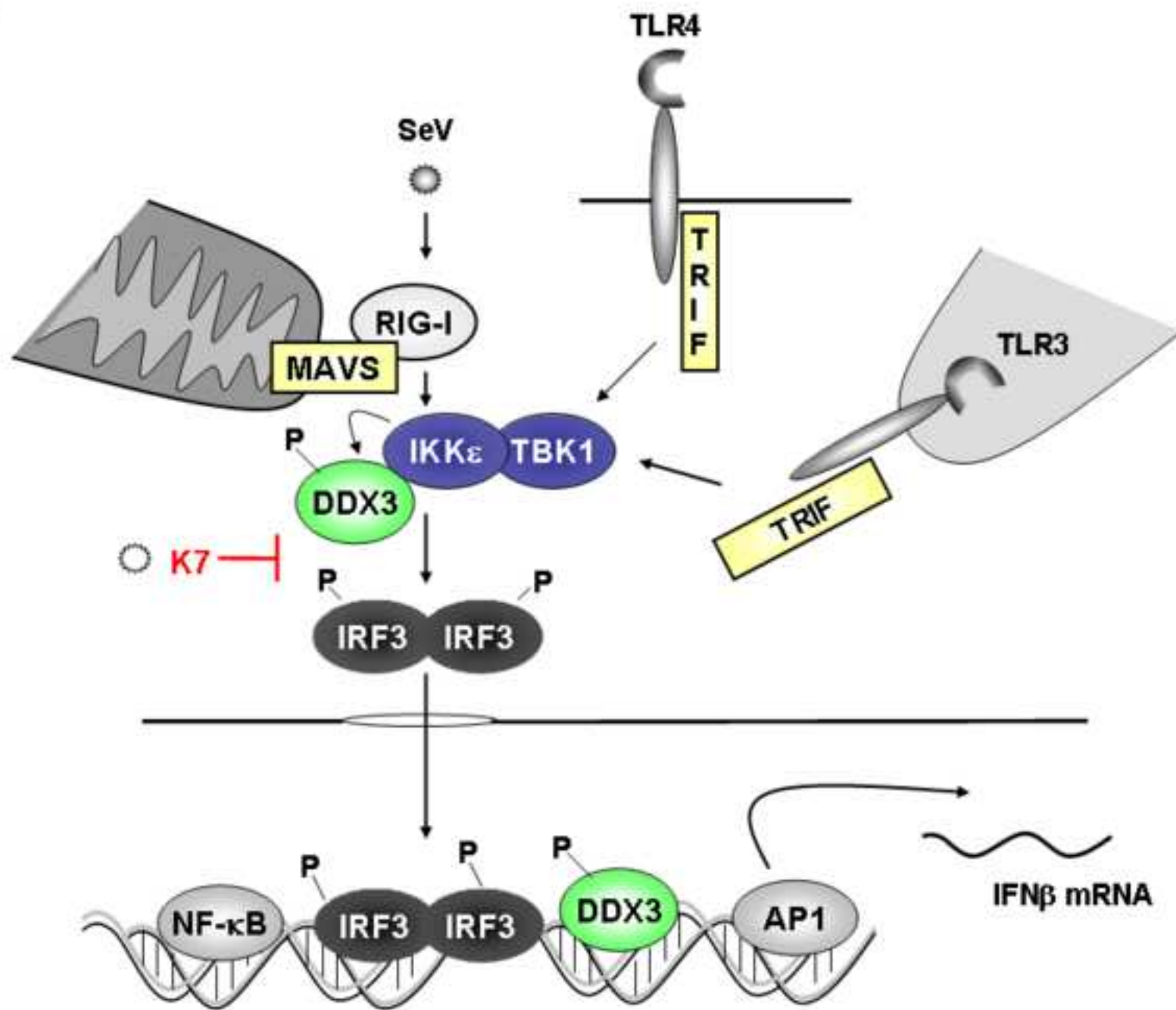


Figure 2-grey
Figure 2

