Review Article

GCN2 in Viral Defence and the Subversive Tactics Employed by Viruses

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Abstract

The recent SARS-CoV-2 pandemic and associated COVID19 disease illustrates the important role of viral defence mechanisms in ensuring survival and recovery of the host or patient. Viruses absolutely depend on the host's protein synthesis machinery to replicate, meaning that impeding translation is a powerful way to counteract viruses. One major approach used by cells to obstruct protein synthesis is to phosphorylate the alpha subunit of eukaryotic translation initiation factor 2 (eIF2α). Mammals possess four different eIF2α-kinases: PKR, HRI, PEK/PERK, and GCN2. While PKR is currently considered the principal eIF2α-kinase involved in viral defence, the other eIF2α-kinases have also been found to play significant roles. Unsurprisingly, viruses have developed mechanisms to counteract the actions of eIF2α-kinases, or even to exploit them to their benefit. While some of these virulence factors are specific to one eIF2α-kinase, such as GCN2, others target all eIF2α-kinases. This review critically evaluates the current knowledge of viral mechanisms targeting the eIF2α-kinase GCN2. A detailed and in-depth understanding of the molecular mechanisms by which viruses evade host defence mechanisms will help to inform the development of powerful anti-viral measures.

Introduction

All viruses completely rely on the host cell's translation machinery to produce viral proteins and replicate. Hence, shutting down – or drastically reducing – protein synthesis is an effective mechanism for combating viral infection. In extreme cases where the infected cell cannot resume synthesis of even its most fundamental proteins, the cell might initiate a controlled cell death; this would be a form of altruistic cellular suicide for the greater good of the organism. Eliminating the viral threat before the cell succumbs to the stress is key to preventing such drastic measures.

Host cells have developed various strategies to alter the rate of protein synthesis. One powerful mechanism involves a family of protein kinases that phosphorylate the α subunit of the eukaryotic translation Initiation Factor 2 (eIF2α)[1–3]. Mammalian cells harbour four eIF2α-kinases.[1,4] These are General Control Non-derepressible 2 (GCN2, found in virtually all eukaryotes), encoded by the gene *Eukaryotic translation Initiation Factor 2 Alpha*

Kinase 4 (*EIF2AK4* in mammals; Protein Kinase R (PKR, *EIF2AK2*, found in vertebrates); Haem-Regulated Inhibitor of translation (HRI, *EIF2AK1*, found in some fungi and animals); and PKR-like Endoplasmic Reticulum Kinase (PEK/PERK, *EIF2AK3*, found in animals).[5] While each member of this family responds to a specific set of cues – which in large part are stress factors – they all have in common the eIF2α Protein Kinase (PK)-domain. This domain becomes enzymatically active upon detecting a stimulating cue, leading to the phosphorylation of a specific amino acid in eIF2α (Ser-51 in the yeast *Saccharomyces cerevisiae* and mammals). This triggers a series of responses that ultimately enables the cell to counteract the initial insult. Apart from the PK-domain, each member of the eIF2α-kinase family harbours their own unique domains. These distinct domains allow each eIF2α-kinase to detect their specific activating cues. Despite responding to different stimuli, these eIF2α-kinases are all part of a signal transduction pathway that converges at eIF2α phosphorylation; therefore, this response system has been called the Integrated Stress Response (ISR) (Figure 1).

Each of the eIF2α-kinases are best known for responding to specific stresses. GCN2, the focus of this review, was first discovered to help cells cope with starvation for nutrients such as amino acids, and to this point this remains its best studied function. HRI, on the other hand, is named after its role in adjusting globin synthesis to the availability of haem in red blood cells. The characteristic role of PERK is to adjust the rate of protein synthesis to that of protein folding in the endoplasmic reticulum. Whilst all three of these kinases have been linked to combatting viral infection, it is PKR that is mainly recognised for this role.

PKR has been found to be required for combating a large array of viruses.[6] PKR can be activated in many ways in response to viral infection, such as by viral double-stranded RNA (dsRNA, its canonical activator molecule). Activation can also occur via conditions likely to result from viral infection, such as ER stress and cytokines resulting from an immune response (for a review, see [7]). Therefore, it comes as no surprise that PKR has been considered the main eIF2α-kinase involved in viral defence. Thus far, research on eIF2α-kinases and their role in viral defence primarily centres around PKR, and several reviews on this topic have been published, e.g.[6,8,9] However, PKR does not con-

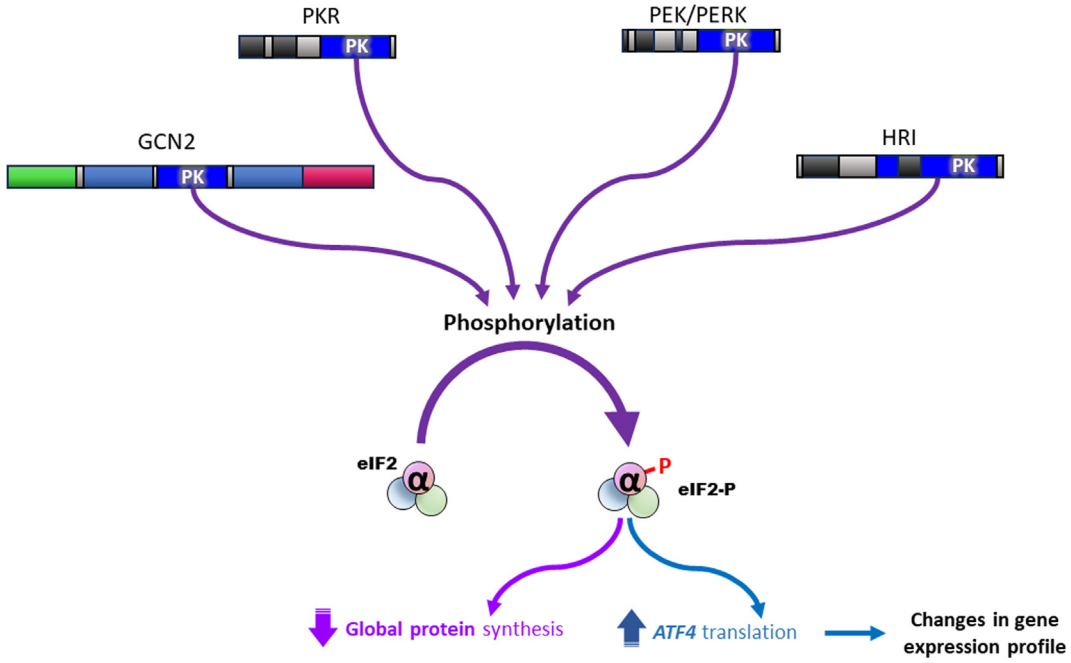

Figure 1. Schematic diagrams of the four mammalian eIF2α-kinases and the ISR signalling pathway. Mammals harbour four distinct eIF2α-kinases, namely GCN2, PKR, PEK/PERK, and HRI. All eIF2α-kinases share a conserved eIF2α Protein Kinase (PK)-domain, but also contain unique domains. For more detail on these domains see.[181] The unique domains are required for the detection of distinctive activating cues by each eIF2α-kinase, leading to autophosphorylation of the eIF2α protein kinase (not shown in Figure), followed by the phosphorylation of eIF2 at its α subunit. eIF2α phosphorylation elicits a dual response. On one hand global protein synthesis is decreased. On the other hand, translation of specific mRNAs, notably that of ATF4, is increased. ATF4 is a transcription factor, and increased ATF4 protein levels lead to a shift in the cell's gene expression profile to help cells combat the initial insult. Despite responding to different stimuli, eIF2α-kinases are part of a signal transduction pathway that converges on eIF2α; therefore, this response system has been called the Integrated Stress Response (ISR).

tribute to the defence against all viruses. For example, the anti-viral response to influenza and Vaccinia Virus (VV) is not observably affected in mice lacking PKR.[10] Instead, findings suggest that another yet to be identified eIF2α-kinase triggers the response against these viruses.[10] Critically, a specific virus does not necessarily stimulate all eIF2α-kinases. Instead, the type of infecting virus determines which eIF2α-kinase(s) will become activated and mount an anti-viral response. Hence, it appears that eIF2α-kinases collectively contribute to viral defence.

This underscores the need to gain a full picture of the roles each eIF2α-kinase plays in viral defence. For this reason, this review focusses solely on GCN2, the molecular processes by which it becomes activated during viral infection, and the mechanisms employed by viruses to counteract GCN2. The review commences by presenting a few examples of the importance of GCN2 in viral defence (see I), followed by an overview of the signalling pathways governing GCN2 (see II). This will then allow the reader to delve into the mechanisms by which GCN2 recognises and antagonises viral infections (see III), and to fully appreciate the ways by which viruses counteract GCN2 (see IV). A selection of experiments is presented to showcase how specific findings led to the discovery of the underlying molecular mechanisms. While this review intends to give a comprehensive evaluation of the knowledge acquired to date on this topic, it does not intend to be exhaustive, and we apologise to the authors whose work is not cited.

I) Relevance of GCN2 in Viral Defence

Studies continue to demonstrate the importance of GCN2 in the defence against viruses. For instance, GCN2 promotes host cell survival following infection with certain viruses. When infected with Sindbis Virus (SV), immortalised Mouse Embryonic Fibroblasts (MEFs) lacking GCN2 ($GCN2^{-/-}$) display a higher mortality rate than MEFs containing GCN2 ($GCN2^{+/+}$).[11] Similarly, mice infected with Mouse *Cytomegalovirus* (MCMV) show a 20% lower survival rate when they lack functional GCN2, as compared to mice containing functional GCN2.[12] This suggests that GCN2 is relevant for the host cells to overcome viral infection.

In line with this, published studies suggest that GCN2 can hamper the replication of certain viruses. In one such example, in Henrietta Lacks (HeLa) P4 cells, the knockdown of GCN2 via small inhibitory RNA silencing (siRNA) leads to an almost 2-fold increase in infectivity to Human Immunodeficiency Virus 1 (HIV-1).[13] Furthermore, as one would expect, overexpression of functional GCN2 in NIH 3T3 cells (a MEF cell line) severely reduces the replication of SV, whereas this is not observed in cells overexpressing catalytically inactive GCN2.[11] Although these observations were derived from cultured cell lines, they seem to translate to an entire organism as well. For example, in studies using SV and living mice, it was found that GCN2 is important for viral defence during early stages of infection. In particular, it was found that three to four days post-nasal infection, $GCN2^{-/-}$ mice have significantly higher SV titres in their brains as compared to wildtype animals, while at 5 days post-infection the viral titre in $GCN2^{-/-}$ and wildtype brain are similar.[11] Together this shows that GCN2 can dampen viral replication, although its relevance may be influenced by the stage of infection.

In line with the fact that GCN2 controls protein synthesis, one would expect that GCN2 blunts viral replication by severely reducing the rate of global protein synthesis. Accordingly, total protein synthesis in HeLa P4 cells is markedly reduced in response to early HIV-1 infection, and this is not observed in cells with reduced GCN2 abundance. This is in line with the idea that GCN2 is required for dampening protein synthesis in response to viral infection.[13]

Given that viruses depend on the host's translation machinery, one would expect that GCN2-mediated suppression of translation also impedes translation of viral proteins. If this is the case, then cells lacking functional GCN2 should show increased synthesis of viral proteins. This has in fact been observed. For instance, SV-infected $GCN2^{-/-}$ MEFs produce significantly more viral proteins as compared to wildtype MEFs, and this correlates with higher mortality of $GCN2^{-/-}$ MEFs.[11] Similar findings were obtained for Vesicular Stomatitis Virus (VSV).[11] In NIH 3T3 cells, overexpression of GCN2 reduces the amount of SV or VSV proteins produced, but overexpression of catalytically inactive GCN2 does not elicit this effect, demonstrating that GCN2 activity is required to dampen translation of viral proteins.[11]

Finally, since GCN2 needs to be activated to suppress protein synthesis, it is reasonable to expect that viral infection would trigger the activation of GCN2. GCN2 activation can be easily scored by assessing its auto-phosphorylation (a key step in GCN2 activation), and subsequent phosphorylation of its substrate, eIF2α. For example, HeLa P4 cells infected with HIV-1 show increased GCN2 auto-phosphorylation as compared to uninfected cells,[13] indicating that GCN2 senses HIV-1 infection. Furthermore, infection of MEFs with Murine *Norovirus* (MNV) leads to increased eIF2α phosphorylation levels, but this is not the case in $GCN2^{-/-}$ MEFs.[14] The fact that the presence of GCN2 in MEFs is required to

increase eIF2α phosphorylation demonstrates that GCN2 is the sole eIF2α-kinase relevant for detecting MNV infection.

Compounds inhibiting the catalytic activity of GCN2 have been used to test whether GCN2 is required for viral defence. For example, the GCN2 inhibitor A-92 hampers the increase in eIF2α phosphorylation in MNV-infected MEFs in a dose-dependent manner.[14] Furthermore, the GCN2-inhibiting compound SP600125 dampens nutrient deprivation-induced HIV-1 transcription in J-Lat A1 cells (a Jurkat cell-based model of latent HIV infection), and HIV-1 reactivation in U1 cells (a subclone of HIV-1-infected U937 promonocytic cells).[15] These findings imply the relevance of GCN2 in viral defence, though additional experiments may be required to validate that these observations were not due to these compounds also inhibiting other kinases, in particular other eIF2α-kinases.

Compounds activating GCN2 have also been used to test the antiviral potential of GCN2 activation. For instance, the compound MG132 leads to enhanced eIF2α phosphorylation by inhibiting the proteasome – a cellular complex that degrades proteins that are damaged or no longer needed.[16] Enhanced eIF2α phosphorylation not only correlates with a reduction in total protein synthesis, but also a reduction in VSV protein synthesis in infected MEFs. This phenomenon is not observed in GCN2$^{-/-}$ MEFs, where a significant reduction in eIF2α phosphorylation was also observed.[16] This indicates that proteasomal inhibition by MG132 stimulates GCN2-mediated eIF2α phosphorylation, and that GCN2 activation is required for the antiviral effect of MG132. This raises the intriguing possibility that pharmacological activation of GCN2 could be used as a means to reduce viral protein synthesis in the case of VSV. It will be interesting to investigate whether this mechanism of GCN2 activation is an effective measure in whole organisms, and whether such measures could be used to treat other viral infections.

Together, these few examples clearly demonstrate the significant role that GCN2 plays in the cellular defence against viruses. Upon sensing viral infection, GCN2 effectively dampens mRNA translation, thereby hampering the production of viral proteins. Since protein production is crucial for viruses to replicate and infect other cells, this reduces the severity of infection and promotes survival of host cells. GCN2 could therefore act as an early responder to viral infection to mitigate its impact on the organism while other components of the cellular anti-viral response act to remove the threat.

II) The GCN2 Protein

Before discussing the links between GCN2 and viruses in molecular detail, it is necessary to first introduce GCN2 and some of the additional components of the ISR.

IIa) The domain structure of GCN2

GCN2 is a highly conserved protein found in virtually all eukaryotes.[1] So far five domains have been identified (Figure 2). The N-terminal RWD domain (named for its presence in RING finger containing proteins, WD-repeat containing proteins, and yeast DEAD (DExD)-like helicases) is essential for direct binding to the effector protein GCN1. The RWD domain is followed by a charged region, and then a pseudokinase domain (YPK). The YPK domain bears homology to Protein Kinase (PK)-domains but it lacks some of the amino acids critical for enzymatic activity. Adjacent to the YPK domain is the PK-domain which phosphorylates eIF2α. C-terminal to the PK-domain is a domain with homology to Histidyl-tRNA Synthetases (HisRS-like domain) which is not enzymatically functional as an aminoacyl-tRNA synthetase. Together with the C-terminal Domain (CTD), the HisRS-like domain specifically binds uncharged tRNAs.[17] The HisRS-like domain harbours conserved residues corresponding to those required for tRNA binding in aminoacyl-tRNA synthetases, such as the amino acids Tyr-Arg (position 1050–1051 in yeast Gcn2) within the m2 motif.[18] Gel shift assays have shown that these amino acids are critical for binding to uncharged tRNAs.[17] Finally, the CTD serves as a ribosomal binding site, and also constitutes the major site for GCN2 dimerisation.[19–21]

Since several viral proteins target the PK-domain of eIF2α-kinases (see IVa-b), it is useful to understand the architecture of this domain in more detail. As a typical member of Ser/Thr kinases, the PK-domain contains 12 subdomains (I-XII). Each of these are recognisable by containing distinctive patterns of conserved residues that are not interrupted by large amino acid insertions.[22,23] Structurally, the PK-domain consists of the N (N-terminal) and C (C-terminal) lobe, with the catalytic cleft positioned between these.[22–27] While the N-lobe (encompassing subdomains I-IV) mediates dimerisation of the PK-domain and plays a critical role in anchoring and orienting the nucleotide ATP, the C-lobe (VI-XI) is involved in both binding the substrate eIF2α and in catalysing the transfer of the ATP terminal phosphate group to eIF2α. Subdomain V spans the two lobes, and functions as a hinge that can allow inter-lobe mobility. The C-lobe also contains the activation loop which encompasses auto-phosphorylation sites critical for the PK-domain to adopt a catalytically active conformation.[28] Two separate features in the C-lobe facilitate binding of the substrate eIF2α, which are the αG helix and the P + 1 loop within the activation segment.[25,29]

GCN2 resides in the cell as a latent dimer, facilitated by autoinhibitory intramolecular interactions.[19,24,30–32] Contributing to the latency

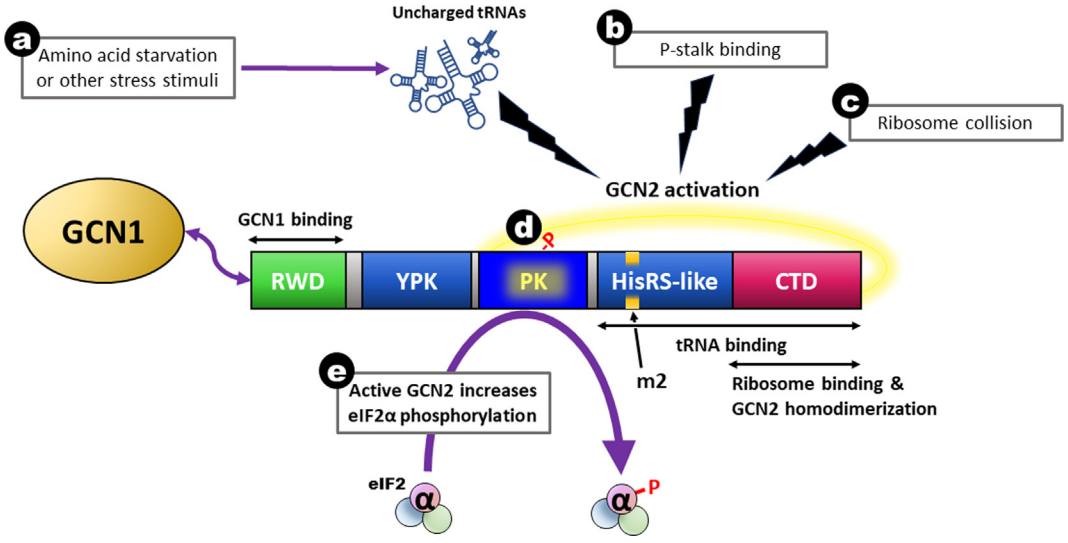

Figure 2. Domain composition of GCN2 and the mechanisms proposed to activate GCN2. GCN2 is composed of several domains (from the N- to the C-terminus, image not to scale): The N-terminal RWD domain essential for direct interaction with the effector protein GCN1**;** the enzymatically inactive pseudokinase domain (YPK); the Protein Kinase (PK)-domain responsible for phosphorylating eIF2α; the domain with homology to Histidyl tRNA Synthetases (HisRS-like domain) containing the m2 motif responsible for binding uncharged tRNA; and the C-terminal Domain (CTD) harbouring the major dimerisation domain as well as the major site for ribosome binding. The HisRS-like domain, together with the CTD, has affinity to uncharged tRNAs. **a-c. Several mechanisms have been proposed to stimulate GCN2**: **a.** Amino acid starvation leads to the accumulation of uncharged tRNAs, which then bind to the HisRS-like domain**; b.** Under certain conditions, the ribosomal P-stalk can directly bind to and stimulate GCN2; **c.** Ribosome collision was proposed to promote GCN2 activation. **d.** GCN2 stimulation and concomitant GCN2 auto-phosphorylation **e.** leads to GCN2-mediated phosphorylation of eIF2α.

of GCN2, the rigidity of the hinge leads to a closed bi-lobal conformation, with the active site cleft being partially closed, and the ATP-binding pocket occluded. Furthermore, in the N-lobe the unfavourable orientation of the αC helix prevents the proper positioning of ATP in the catalytic site.[24,31–35] After recognising a stimulating signal, GCN2 undergoes conformational changes, relieving autoinhibitory intramolecular interactions and eliciting GCN2 auto-phosphorylation, allowing GCN2 to finally adopt a fully active conformation.[19,24,27,28,30–35] GCN2 is then capable of phosphorylating its substrate eIF2α. It is for this reason that GCN2 auto-phosphorylation is considered a reliable measure of GCN2 activation.

IIb) Mechanism of GCN2 activation in uninfected cells

Studies so far suggest that GCN2 protects host cells against viruses exclusively in its activated state. Therefore, it comes as no surprise that many viral mechanisms have arisen that target the process of GCN2 activation. For this reason, this chapter provides an overview of the current knowledge on how GCN2 is stimulated in 'uninfected' cells (Figure 2). GCN2 activation plays many roles in the cell aside from defending against viruses, such as its well-established role in responding to amino acid starvation. Whilst much research has been done to better understand this activation pathway, the molecular mechanisms underlying the activation of GCN2 still remain elusive. Currently, studies suggest more than one mechanism of GCN2 activation. Since this topic is not the main focus of this review, the current working models for GCN2 activation are only briefly described, without outlining all scientific discoveries that have led to these models.

Currently, two models have been proposed for GCN2 activation. The initially proposed – and still current – working model is based on the fact that under amino acid starvation the respective tRNAs cannot be aminoacylated, leading to the accumulation of uncharged tRNAs (Figure 2a). These are recognised by GCN2 as a direct starvation signal.[17,36,37] The exact mechanism by which GCN2 detects these uncharged tRNAs is still unknown. It has been proposed that, when there is a shortage of an aminoacylated tRNA required for translation, a cognate but *uncharged* tRNA can enter the ribosomal Aminoacyl acceptor site (A-site) in its place. This uncharged tRNA is then detected by the HisRS-like domain of GCN2, leading to GCN2 auto-phosphorylation (Figure 2d). As GCN2 auto-phosphorylation allows the PK-domain to adopt an active conformation (and thus is a mark of GCN2 activation), GCN2 may then phosphory-

late its substrate, eIF2α (Figure 2e). The protein GCN1 is required for GCN2 activation *in vivo*.[38] GCN1 directly binds GCN2,[39,40] and both proteins need to bind to ribosomes to allow for the activation of GCN2.[20,21,39,41,42] The role of GCN1 may be to promote delivery of uncharged tRNAs to the A-site, to promote delivery of the uncharged tRNA to GCN2, and/or to position GCN2 on the ribosome in such a way that it can access the uncharged tRNA within the A-site.[39]

Further supporting the idea that uncharged tRNAs are an activating ligand for GCN2 was obtained by an independent study.[43] Treatment of Human Embryonic Kidney (HEK)-293 T cells with halofuginone leads to the specific accumulation of uncharged $tRNA^{Pro}$, and this is associated with an increase in GCN2 auto-phosphorylation and eIF2α phosphorylation.[43] Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR) conducted on GCN2-precipitates show a 3-fold enrichment of $tRNA^{Pro}$, as compared to cells not treated with halofuginone. Together with the fact that halofuginone treatment leads to a dramatic reduction in the abundance of aminoacylated $tRNA^{Pro}$, this supports the idea that the uncharged form of $tRNA^{Pro}$ is detected by and bound to GCN2. The HisRS-like domain is involved in detecting the uncharged $tRNA^{Pro}$, as is suggested by the fact that the co-precipitation of $tRNA^{Pro}$ with GCN2 is abolished by the m2 mutation in the HisRS-like domain.[43]

Research findings have led to the proposal of a second working model that suggests a more indirect involvement of tRNAs in the activation of GCN2. Instead, the ribosomal P-stalk is implicated in mediating GCN2 activation (Figure 2b).[44–46] The P-stalk is involved in the cyclic recruitment of elongation factors during each round of translation elongation.[47–49] Given that the absence of a cognate aminoacylated tRNA causes a pause in translation, this working model proposes that the resulting stalled ribosome does not recruit translation factors to its ribosomal P-stalk. Consequently, the P-stalk is able to instead contact and activate GCN2.

The uncharged tRNA and P-stalk activation models for GCN2 are not mutually exclusive. There is evidence that they come into play differentially, depending on whether the stress signal is starvation-dependent or starvation-independent.[50] Nevertheless, a recent study in yeast has shown that Gcn2 activation requires Gcn1, Gcn2-ribosome association, and the Gcn2 HisRS-like domain for detecting either type of activating cue.[50] A model was proposed in which the mechanism of GCN2 activation depends on the activating cue. Under starvation conditions, uncharged tRNA binds to the HisRS-like domain to stimulate GCN2. In contrast, during starvation-independent ribosome stalling, P-stalk proteins instead interact with the HisRS-like domain to stimulate GCN2.[50] An independent study in mammalian cells led to similar conclusions, in that GCN2 is activated by at least two mechanisms, some of which necessitate binding of uncharged tRNA to GCN2, while others require ribosome stalling/collisions.[43] This study also found that the GCN2 HisRS-like domain is required for both mechanisms of GCN2 activation.[43]

In addition to adjusting protein synthesis in response to external stresses such as starvation, it has been proposed that GCN2-activation also serves as a mechanism to decrease the likelihood of ribosome collisions (Figure 2c).[51,52] Supporting this idea, GCN2 can become activated by ribosomal stalling in a manner that seems unrelated to the presence of uncharged tRNA molecules.[13,39,41,45,46,48–51] An elongating ribosome that experiences stalling can form a roadblock, into which a succeeding elongating ribosome collides, resulting in a disome.[52] GCN2 activation, which involves GCN2 auto-phosphorylation (Figure 2d), and subsequent eIF2α phosphorylation (Figure 2e) leads to a reduction in global protein synthesis, resulting in a 'lighter' load of mRNAs with elongating ribosomes and thereby reducing the probability of further ribosome collisions.

GCN1 has been found to associate with disomes, raising the possibility that disome-bound GCN1 elicits GCN2 activation.[51,53,54] Supporting this idea, studies suggest that an increase in transient collisions leads to GCN2 activation (likely in conjunction with GCN1).[55,56] Hence, the formation of disomes may be implicated in GCN2 activation, though it remains to be validated experimentally whether GCN2 also binds to disomes for its activation.

Ribosome stalling and the resulting collisions are considered to be the key cellular indicators of aberrant translation, which also triggers the activation of the Ribosome Quality Control (RQC) system. The RQC mechanism ensures that stalled ribosomes are resolved and recycled to re-enter translation, a process that involves ubiquitination of ribosomal proteins.[56] Research suggests that the GCN2/ISR and the RQC pathways are linked,[54,57] and studies in mammals have led to a model where the persistence of disomes determines the type of cellular response.[51,56] Under unstressed conditions, short-lived disomes are resolved via dedicated pathways such as RQC. Severe collisions would stimulate the ribotoxic stress response pathway, leading to cell cycle arrest or apoptosis. Under 'intermediate' stress on the other hand, GCN2-mediated eIF2α phosphorylation decreases the rate of translation initiation to reduce the ribosomal load on mRNAs and thus the likelihood of further ribosome collisions.[51] The evidence so far strongly points to a coordinated interplay between the GCN2/ISR and RQC regulatory pathways in sensing unresolved ribosome stalling/collision in eukaryotes.[51,56] This interplay is of significance to viral replication as well, given

that viruses place a heavy burden on the host's translation machinery to produce large quantities of viral proteins.

Due to the remarkable conservation of GCN2 and its signalling pathway, the budding yeast *Saccharomyces cerevisiae* was instrumental in uncovering the basic biological function of Gcn2, how Gcn2 becomes activated and how it is regulated. The knowledge gained in this most amenable eukaryotic model organism led to a surge in research focused on GCN2 in higher eukaryotes, particularly in relation to health and disease in humans and mammalian models. Some recent studies suggest that the human activation pathway may contain additional subtleties, but this area warrants further investigation. Therefore, the exact mechanisms for GCN2 activation under different stress conditions remain to be further investigated in both yeast and human systems.

IIc) The function of eIF2α

eIF2α is one of the three subunits of eIF2, which is essential for facilitating the initiation of protein synthesis.[58] eIF2 forms a ternary complex with GTP and initiator methionyl tRNA (Met-tRNA$_i^{Met}$) to aid the ribosome in detecting the translation start codon. After subsequent GTP hydrolysis, eIF2 is released in its GDP-bound state. The GDP then needs to be replaced by GTP, which is mediated by the guanine nucleotide exchange factor called eukaryotic translation Initiation Factor 2B (eIF2B). eIF2-GTP can then bind Met-tRNA$_i^{Met}$ again to form a ternary complex capable of promoting the next round of translation initiation. Hence, a perpetual rate of GDP-GTP exchange ensures plentiful abundance of ternary complexes and thus supports a rapid rate of protein synthesis. Phosphorylation of Ser-51 in the alpha subunit of eIF2 by the eIF2α-kinases (Figure 1) turns eIF2 from a substrate into an inhibitor of eIF2B. The concomitant decrease in the rate of GDP-GTP exchange leads to reduced levels of ternary complex. This can affect translation in two ways.

On one hand, low abundance of ternary complex leads to the increased translation of specific mRNAs (Figure 1). This regulation is mediated by specific unique upstream Open Reading Frames (uORFs) present in these mRNAs that impair translation of the main ORF under non-stressed conditions (reviewed in [3]). However, low availability of ternary complexes allows ribosomes to overcome these inhibitory uORFs, leading to enhanced translation of the main ORF. These ORFs code for transcription factors, such as General control non-derepressable 4 (Gcn4) in yeast or Activating Transcription Factor 4 (ATF4) in mammals.[3] Thus, increased levels of phosphorylated eIF2α (eIF2α-P) essentially lead to an increased abundance of these transcription factors, thereby altering the expression profile of a large array of genes and allowing the cell to adjust to and overcome the initial insult.[1,3] The increase in eIF2α-P levels, or the elevated translation of ATF4, has also been found to lead to enhanced translation of a selection of mRNAs containing an Internal Ribosome Entry Site (IRES).[59,60] IRESs can be found in stress-responsive transcripts, such as for the high affinity cationic amino acid transporter 1 (cat-1),[59] which would enhance amino acid uptake to rectify cellular shortage of amino acids. Although various molecular mechanisms have been proposed for IRES-mediated translation initiation, it seems that all of these utilize translation factors other than the traditional ternary complex.[61] The exact mechanisms by which an increase in eIF2α-P levels enhances IRES-mediated translation initiation are still being deciphered.[61] Non-AUG translation start sites provide an additional layer of translation regulation.[62] Some of these start sites are not affected by the ISR response, or are even upregulated specifically under ISR conditions.

Concurrent with the increased translation of specific mRNAs containing unique uORFs, IRES elements, or non-AUG translation start sites, low availability of ternary complex leads to a reduction in global translation (Figure 1). The degree by which global protein synthesis is dampened depends on the extent of eIF2α phosphorylation. While strong eIF2α phosphorylation can halt translation, weak eIF2α phosphorylation may not affect global protein synthesis but still lead to increased translation of specific mRNAs.[58] At intermediate levels, the amount of eIF2α-P could determine the rate of global protein synthesis. When it comes to viral infection, the impairment of protein synthesis would be desired to prevent viral replication.

III) GCN2 Stimulation by Viral Infections

To be employed in viral defence, GCN2 must first detect the presence of viral infection, which in turn leads to its stimulation. The mechanism(s) of GCN2 activation upon viral infection is currently not fully understood. Nevertheless, it appears that GCN2 can be activated via different mechanisms depending on the type of virus (Table 1, Figure 3). Here, an overview of the current understanding of virally-induced GCN2 activation is provided. Generally, the virally-induced activation of GCN2 and the subsequent increase in eIF2α phosphorylation leads to the attenuation of translation, thereby diminishing the synthesis of viral proteins (Figure 3f). The last section of this chapter (see IIId) introduces alternative mechanisms of GCN2-mediated viral defence that do not rely on eIF2α phosphorylation.

IIIa) Viral infection eliciting or mimicking amino acid starvation

In the host cell, some viruses may indirectly trigger amino acid shortage or elicit the accumulation of uncharged tRNAs, leading to GCN2 activation.

Yellow Fever Virus (YFV) is one such virus that appears to lead to a depletion of amino acids (Figure 3a). For example, infection of human monocyte-derived Dendritic Cells (hmDCs) with live attenuated Yellow Fever strain 17D (YF-17D) vaccine leads to the depletion of free Arg as assessed by Liquid Chromatography-Mass Spectrometry (LC-MS).[63] Exposing YF-17D to heat or UV-irradiation renders it biologically inactive. This inactivated form fails to trigger a decrease in amino acid levels, indicating the need for live virus – and thus a physiological activity by the virus – in this process. Notably, YF-17D-mediated amino acid depletion correlates with an increase in phosphorylation of GCN2 and eIF2α.[63] A similar phenomenon was found in mouse Bone Marrow–derived Dendritic Cells (BMDCs), where YF-17D infection leads to enhanced eIF2α-P levels, but biologically inactive YF17D does not (GCN2 phosphorylation was not investigated). The mechanism causing a decrease in the amino acid pool by YF-17D remains to be uncovered. One possibility is that the increased translation of viral proteins utilises the intracellular pool of amino acids faster than they can be replenished, leading to the observed amino acid starvation.

HIV is also known to cause dysregulation of plasma amino acid levels, including a marked decrease in many of these amino acids (e.g. [64,65]). Whilst the mechanisms by which HIV might alter amino acid levels warrant a dedicated review in themselves, it is possible that HIV also activates GCN2 through this elicited depletion in amino acids (see IIb, Figure 3a). A significant reduction in several plasma amino acids has also been found after infection with Sandfly Fever Virus (SFV),[66] Murine Hepatitis Virus 3 (MHV-3),[67] and murine Influenza A Virus (IAV),[68] suggesting that amino acid depletion could be a more widespread route of GCN2 activation by viruses. The reduction in serum amino acids may lead to depletion of these amino acids intracellularly where it can be sensed by GCN2. Further experiments are required to determine whether infected patients exhibiting low plasma levels of amino acids indeed show intracellular amino acid depletion and GCN2 activation.

The difference in codon usage between virus and host genes may lead to increased levels of uncharged tRNAs (Figure 3b). Given that viruses rely on the translation machinery of the host, one would anticipate the codon usage of viral genes to have evolved to match that of its host. This resemblance would promote the efficient use of the host's tRNA pool, and the rapid aminoacylation of uncharged tRNAs by the matched pool of tRNA synthetases, leading to abundant translation of viral proteins. However, several viruses have been reported to exhibit codon usage patterns that diverge from those of their hosts.[69] In these cases, viral protein production is limited by the available pool of rare aminoacylated tRNAs. As the cellular pool of aminoacyl tRNA synthetases may not be able to charge cognate tRNAs at the rate at which they are utilised in translation, this could also lead to a build-up of uncharged tRNAs and subsequent GCN2 activation. In this scenario, the moderated synthesis of viral proteins would not overburden the host's translation machinery, enabling survival of its host, albeit at the cost of a reduced rate of viral replication.[69] Whilst there are differing views on whether these differences in codon usage emerged as a consequence of its impact on the host's translation machinery, or simply due to the virus' inherent nucleotide preference in its genetic material,[69,70] this debate is beyond the scope of our review.

In one example, while the total cellular tRNA composition remains unchanged, infection by VV or IAV leads to dramatic changes in the population of polysome-associated tRNAs. This strongly correlates with viral codon usage, suggesting the existence of localised tRNA pools tailored for efficient viral translation.[71] As the host cell is not well-equipped to aminoacylate the rare tRNAs, their aminoacylation may not occur as quickly, and this may serve as the activating starvation signal for GCN2 (see IIb, Figure 3b).

In the case of HIV-1, the codon usage of its early genes is similar to that of highly expressed host genes, whilst the codon usage of its late genes differs largely from that of its host.[72,73] Interestingly, tRNA microarray studies revealed that HIV-1 packages a variety of tRNAs, suggesting that the packaged tRNAs aid in changing the tRNA pool composition in the infected host to accommodate the virus's codon usage and improve the translation efficiency of the late viral RNAs.[73,74] This selective alteration of the tRNA pool would favour the translation of HIV-1 genes over that of the host genes.[73] However, the biased composition of the aminoacyl tRNA synthetases in the host cell may not be well-suited to efficiently charge the altered tRNA pool, leading to a build-up of uncharged tRNAs and thus GCN2 activation (Figure 3b). It remains to be ascertained whether a delay in tRNA aminoacylation can cause GCN2 activation, and this warrants further investigation.

IIIb) GCN2 detecting viral RNA

Another mechanism of GCN2 activation is by viral RNA binding to the HisRS-like domain of GCN2 (Figure 3c). Analogous to uncharged tRNAs binding to the HisRS-like domain, viral RNA can bind to this domain and lead to the stimulation of the GCN2 protein kinase (PK) domain. For example, in *in vitro* kinase assays, SV RNA is

Table 1 Overview of viruses for which GCN2 activation has been reported, and the mechanism of GCN2 stimulation. The hosts known to be infected as well as the diseases / symptoms caused are indicated. The content of the table is primarily sorted by the mechanism of GCN2 activation, followed by the genetic material used by the virus to generate mRNA (Baltimore classification[167]), and then grouped into their specific viral family. For more detail see text.

Proposed mechanism of GCN2 activation	See section in review	Baltimore class	Viral family	Virus (abbreviation)*	Host	Disease / Symptoms / Relevance	Source**
Viral infection leads to amino acid depletion (Figure 3a)	IIIa	+ ssRNA	*Flaviviridae*	Yellow fever virus (YFV)	Humans	Abrupt onset of fever, chills, malaise, headache, back pain nausea and dizziness. Can lead to haemorrhagic manifestations and kidney failure in severe cases.	b
	IIIa	− ssRNA	*Bunyaviridae*	Sandfly fever virus (SFV)	Humans	Fever, headache, fatigue, joint, muscle and abdominal pain.	[168]
	IIIa	+ ssRNA	*Coronaviridae*	Murine hepatitis virus 3 (MHV-3)	Mice	Causes hepatitis	[169]
	IIIa	− ssRNA	*Orthomyxoviridae*	Influenza A virus (IAV)	Humans, pigs, horses	Causes influenza, symptoms include sudden onset of fever, headache, body aches, fatigue and a dry cough.	[170]
	IIIa	+ ssRNA-RT	*Retroviridae*	Human immunodeficiency virus 1 (HIV-1)	Humans	Can cause severe diseases such as AIDS, secondary infections, and lymphomas. Acute phase symptoms include fever, headache, rash, sore throat weight loss, diarrhoea	b
Viral infection leads to accumulation of uncharged tRNAs (Figure 3b)	IIIa	+ ssRNA-RT	*Retroviridae*	Human immunodeficiency virus 1 (HIV-1)	Humans	Can cause severe diseases such as AIDS, secondary infections, and lymphomas. Acute phase symptoms include fever, headache, rash, sore throat weight loss, diarrhoea	b
Viral RNA binds to GCN2 HisRS-like domain (Figure 3c)	IIIb	+ ssRNA	*Picornaviridae*	Poliovirus	Humans	Causes poliomyelitis (polio). Symptoms include fever, fatigue, stiffness in the neck and in rare cases paralysis or death.	b
	IIIb	+ ssRNA	*Togaviridae*	Sindbis virus (SV)	Humans, birds	Humans: Exanthema over trunk and limbs, joint symptoms. Sometimes nausea, general malaise, headache, and muscle pain. Can be asymptomatic.	g
	IIIb	+ ssRNA-RT	*Retroviridae*	Human immunodeficiency virus 1 (HIV-1)	Humans	Can cause severe diseases such as AIDS, secondary infections, and lymphomas. Acute phase symptoms include fever, headache, rash, sore throat weight loss, diarrhoea	b
Viral infection enhances ribosome-stalling (Figure 3d)	IIIc, IVg	dsDNA	*Herpesviridae*	Epstein-Barr virus (EBV)	Humans	Can be asymptomatic. Possible symptoms include tiredness, fever, sore throat, headaches and body aches, swollen lymph nodes, swelling in the liver and/or spleen, rash, causing mononucleosis.	b, f, i, h
	IVg	dsDNA	*Herpesviridae*	Human *Cytomegalovirus* (HCMV)	Humans	Can be asymptomatic. Possible symptoms include fever, sore throat, fatigue, swollen glands. Occasionally mononucleosis or hepatitis.	f, h
	IIIc	dsDNA	*Poxviridae*	Vaccinia virus (VV)	Humans, cattle	Symptoms are usually very mild to non-existent in humans but may produce localised skin infection.	j
	IIId	− ssRNA	*Rhabdoviridae*	Vesicular stomatitis virus (VSV)	Humans, livestock, wild deer, rodents	Human: Flu-like symptoms that can lead to encephalitis Livestock: lesions in the mouth and other parts of the body (e.g. feet, ears, udder, ventral abdomen), secondary infections.	f,e

(*continued on next page*)

Table 1 (*continued*)

Proposed mechanism of GCN2 activation	See section in review	Baltimore class	Viral family	Virus (abbreviation)*	Host	Disease / Symptoms / Relevance	Source**
GCN2 exhibits an antiviral effect, GCN2 activation mechanism unknown	I	+ ssRNA	*Caliciviridae*	Murine *Norovirus* (MNV)	Mice	Affects immunocompromised mice, clinical signs include wasting, diarrhoea and death. Also, may develop hepatitis, peritonitis and interstitial pneumonia.	a
	IVa	+ ssRNA	*Flaviviridae*	Dengue virus 2 (DENV-2)	Humans	Symptoms can include fever, headache, retroocular pain, myalgia, arthralgia, exanthema, and prostration with or without haemorrhage. Can be asymptomatic.	h
	IIId	+ ssRNA-RT	*Retroviridae*	Murine leukaemia virus (MLV)	Mice, rats	Cancer in mice, may induce neurodegenerative disorders and paralytic diseases.	[171]

* Note that there may be more viruses than those mentioned that have the same effect on GCN2 activation, for more see text.
** For source of information, see references (publications) or bottom of Table 2 (internet links).

sufficient to enhance GCN2 auto-phosphorylation as well as eIF2α phosphorylation.[11] Two short non-contiguous sequences called GCN2-Activating RNA (GAR) in the SV RNA were found to be sufficient for stimulating GCN2. Given that denatured GAR has no effect, this suggests that its secondary structure is essential for stimulating GCN2. *In vitro* northwestern assays revealed that GAR can bind to GCN2, but not to GCN2 with a mutated m2 motif.[11] Since the detection of uncharged-tRNAs also requires the m2 motif in the HisRS-like domain[17] this suggests that the same parameters mediate SV RNA binding in the HisRS-like domain as those found for tRNA binding. It is possible that this bipartite GAR adopts a structure resembling that of a tRNA that allows its detection by the HisRS-like domain.[11]

HIV-1 RNA has also been found to activate GCN2 *in vitro*. As found above, the GCN2 m2 mutation prevents HIV-1 RNA from eliciting phosphorylation of GCN2 and eIF2α,[75] supporting the idea that HIV-1 RNA also activates GCN2 by binding to the HisRS-like domain. It will be interesting to validate whether this mechanism of GCN2 activation also occurs *in vivo*. Furthermore, the HIV-1 RNA sequence activating GCN2 remains to be determined.

It is possible that GCN2 could also detect viral RNA following poliovirus infection. Initially, it was reported that poliovirus does not elicit an increase in eIF2α phosphorylation,[76] but three independent studies found that poliovirus leads to GCN2 activation, or to an increase in eIF2α phosphorylation.[11,16,77] It was suggested that the initial finding could have been due to differences in experimental procedure.[77] With the advancement of technology and improvements in experimental methods since the initial report, this may be the reason why the effect of poliovirus is now detectable. Infection of HeLa cells with poliovirus leads to an increase in eIF2α phosphorylation, which is not as strong in HeLa cells infected with a mutant poliovirus.[77] This correlates with an accumulation of dsRNA in cells, which occurs faster in cells infected with wild-type poliovirus than in cells infected with the mutant, suggesting that dsRNA is the activating ligand.[77] The eIF2α-kinases activated in this scenario likely include GCN2, given that the poliovirus RNA genome activates GCN2 *in vitro*,[11] although these findings have not been published yet. It would be interesting to confirm whether GCN2 is indeed activated *in vivo*, and whether this is mediated by poliovirus RNA.

IIIc) Viral infection eliciting ribosome stalling

To enable viral replication, viruses exploit the host's translation machinery which can be heavily burdened by the viral load, leading to an increase in ribosome stalling and collisions (Figure 3d). Consistent with this idea, it appears that VV infection aggravates ribosome collisions due to

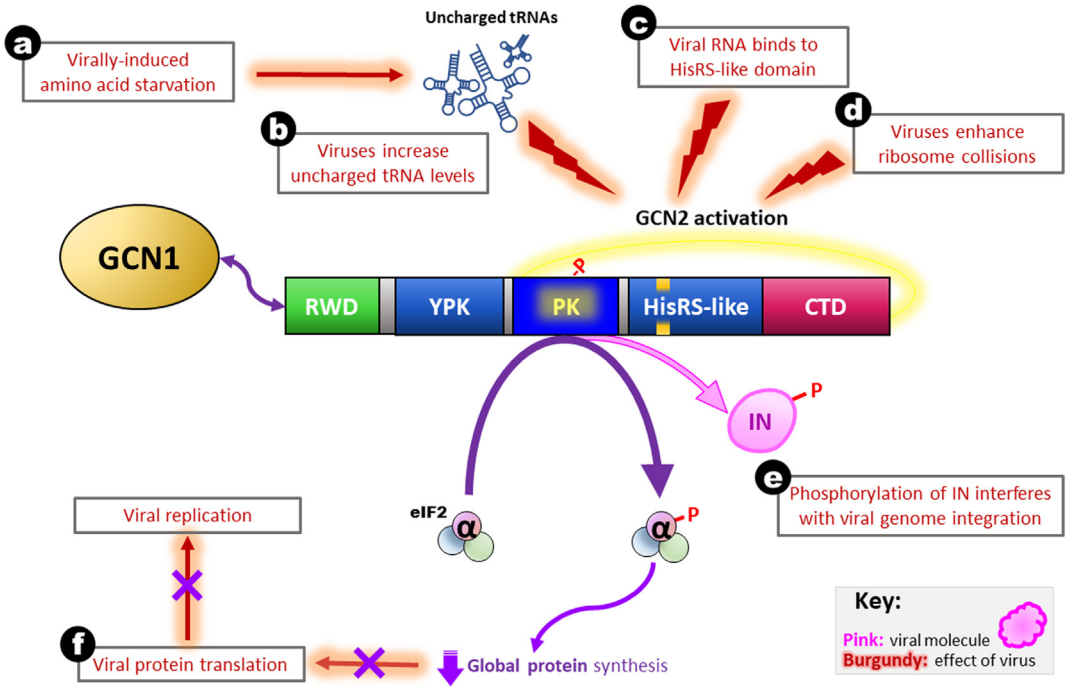

Figure 3. Mechanisms of GCN2 activation by viruses and the downstream anti-viral effects. a-d. Viruses can activate GCN2 through several proposed mechanisms. a. Depletion of the host's intracellular amino acid reserve by viral replication elicits the cell's GCN2-mediated amino acid starvation response. **b.** Increase in the abundance of intracellular uncharged tRNAs are detected by the GCN2 HisRS-like domain. **c.** Viral RNA binds to the GCN2 HisRS-like domain, leading to GCN2 activation. **d.** Virally caused promotion of ribosome collisions leads to GCN2 activation. **e-f. Activated GCN2 elicits several anti-viral activities. e.** GCN2 can directly phosphorylate the Integrases (IN) of viruses belonging to the *Retroviridae* family. This interferes with viral integration into the host genome, ultimately resulting in reduced viral load and infectivity. **f.** The reduction in global protein synthesis may limit the synthesis of viral proteins and thus hamper viral replication. For more see text.

both the increased burden on the translation machinery and the virally-induced reduction of eIF2α-phosphorylation. Signifying that the translation machinery is burdened, VV propagation enhances ubiquitination of a ribosomal protein. This is indicative of an increased burden on the RQC pathway,[78] which is required for resolving stalled/collided ribosomes (see IIb). Furthermore, ribosome collisions during infection are more severe when eIF2α is rendered unable to be phosphorylated.[78] This strongly implicates one of the eIF2α-kinases in responding to and managing the increased translational burden caused by VV infection. GCN2 is the most likely candidate, given that GCN2 is activated by conditions which promote ribosome collisions (see IIb).[54] This highlights the possibility that GCN2 senses VV infection indirectly through the concomitant increase in ribosome collisions.

In further support of the idea that viruses can trigger GCN2 activation by promoting ribosome collisions, Epstein-Barr Virus (EBV) activates GCN2 in conjunction with reducing the RQC response.[79] EBV produces a large tegument protein called BPLF1 which harbours a Ubiquitin Deconjugase (vDUB) at its N-terminus. BPLF1 reduces the ubiquitination of the 40S ribosome usually associated with the RQC response,[56] and this is dependent on its vDUB catalytic activity.[79] This suggests that BPLF1 counteracts the host's RQC response and thus impairs the host's ability to resolve stalled ribosomes. Intriguingly, the BPLF1-mediated impairment of the host's RQC is associated with an increase in eIF2α phosphorylation levels, and this is dependent on GCN2 activity.[79] Together, this suggests that the increased level of stalled ribosomes – resulting from RQC inhibition – are sensed by GCN2, leading to subsequent GCN2 activation (Figure 3d).

More studies are required to further investigate the potential interplay between viral infections, GCN2 activation, and the RQC. Whilst the mechanisms of GCN2 activation in VV and EBV infection remain to be uncovered, the findings so far, along with the indication that GCN2 can be activated by ribosome collisions (see IIb), suggest that a virally-induced increase in ribosome collisions could be another avenue for GCN2 activation by viruses (Figure 3d).

IIId) Anti-viral mechanisms involving GCN2 but not eIF2α phosphorylation

GCN2 activation, and the subsequent eIF2α phosphorylation, result in impaired global protein synthesis, thereby hampering the translation of both host and viral proteins (Figure 3f). Since viruses are entirely dependent on the host's translation machinery, its inaccessibility will inevitably suppress viral replication. Interestingly, in response to VSV and HIV infections, GCN2 has been observed to also elicit anti-viral effects independently of eIF2α phosphorylation.

GCN2 counteracts VSV infection without the need for eIF2α phosphorylation. Importantly, VSV infected GCN2$^{-/-}$ MEFs produce more viral proteins than wildtype MEFs, indicating that GCN2 is relevant for the defence against VSV.[80,16] Interestingly though, wildtype MEFs and MEFs containing eIF2α-S51A (to render eIF2α non-phosphorylatable) show similar amounts of viral protein production. This would indicate that the phosphorylation of eIF2α by GCN2 may not be essential for the defence against VSV, or that GCN2 phosphorylates a substrate other than eIF2α that is relevant to VSV defence. Supporting this idea, GCN2 has been reported to phosphorylate proteins other than eIF2α.[81] Further research is necessary to uncover the precise mechanism by which GCN2 supports the anti-viral defence against VSV. It also needs to be verified whether the GCN2 catalytic activity *per se* is required to convey these anti-viral effects.

Another example centres around the fact that GCN2 can bind to and phosphorylate the Integrase enzyme (IN) of HIV-1 (Figure 3e).[13,82] IN mediates the integration of retroviral DNA into the host genome, an essential step for retrovirus replication.[83] Studies revealed that GCN2 phosphorylates IN at two highly conserved positions – Ser-24 and Ser-255 – with Ser-255 being the main phosphorylation site.[82] IN S255A substitution (to render Ser-255 non-phosphorylatable) leads to an increase in infectivity as well as viral DNA integration. Strikingly, the same effect was observed with wildtype IN but in host cells lacking GCN2. Together, this supports the idea that GCN2-mediated phosphorylation of IN hampers integration efficiency.[82] GCN2 can also phosphorylate IN produced by the retroviruses HIV-2, Murine Leukaemia Virus (MLV) and Avian Sarcoma Virus (ASV).[82] Notably, ASV poses a significant threat to the poultry industry and economy, especially considering that chickens are a major food source worldwide. Interestingly, GCN2 is not able to efficiently phosphorylate the IN of the retrovirus Prototype Foamy Virus (PFV) which contains a Gly at the position equivalent to Ser-255.[74] Thus, it is tempting to speculate that PFV has acquired this mutation to counteract this particular anti-viral mechanism of GCN2. More studies are warranted to further validate the IN as a GCN2 substrate and to uncover the biological relevance of IN phosphorylation. Unravelling the exact mechanisms by which viral infections lead to GCN2 activation could provide valuable insights into viral pathogenesis, and potentially lead to the discovery of novel drugs that help enhance GCN2-mediated viral defence in patients.

IV) Viral Mechanisms Counteracting GCN2

Thus far, the majority of the viral virulence factors targeting the ISR have been researched in relation to PKR, but some of these also have the potential to inhibit GCN2. In addition, viral strategies have been uncovered that specifically counteract GCN2 function in viral defence (Figure 4). In the interest of providing a comprehensive review, here we discuss any viral mechanism able to inhibit GCN2, whether they are specific to GCN2 or also target other eIF2α-kinases. Table 2 provides an overview of these mechanisms.

IVa) Mimicking the eIF2α-kinase substrate eIF2α

A pseudosubstrate imitates the real substrate of an enzyme but does not undergo the typical chemical reaction or process that the intended substrate would. Because the pseudosubstrate competes with the actual substrate for binding to the enzyme, it hinders the enzyme's catalytic activity. Interestingly, viral proteins have been found that mimic eIF2α, and several of these act as *bona fide* eIF2α pseudosubstrates (Figure 4d).

A critical characteristic of a pseudosubstrate is its ability to imitate the part of the substrate that typically binds to the enzyme. Studies suggest that eIF2α-kinases recognise a large surface region of eIF2α.[29] This includes residues flanking the Ser-51 phosphorylation site and the KGYID motif located ~ 30 residues C-terminal of the Ser-51 phosphorylation site (~20 Å distance to Ser-51).[29,84] These residues are part of the Oligonucleotide Binding (OB) fold domain (residues 1–89/1–87 in yeast/human),[85,86] and studies suggest that its 3-dimensional structural integrity is relevant for substrate-enzyme interaction.[25] Considering that eIF2α-kinases share a common substrate, it is anticipated that eIF2α pseudosubstrates should hinder the activity of all eIF2α-kinases, including GCN2.

VV expresses the eIF2α pseudosubstrate called K3L which inhibits all four eIF2α-kinases, including GCN2.[87–90] The K3L protein mimics eIF2α in that it contains an OB-fold and a KGYID motif which is critical for kinase binding.[86,91,92] As one would expect from a pseudosubstrate, studies have shown that K3L-mediated inhibition of PKR depends on residues that are conserved between K3L and eIF2α.[84,92] For example, the *S. cerevisiae* model system was employed to gain more insight

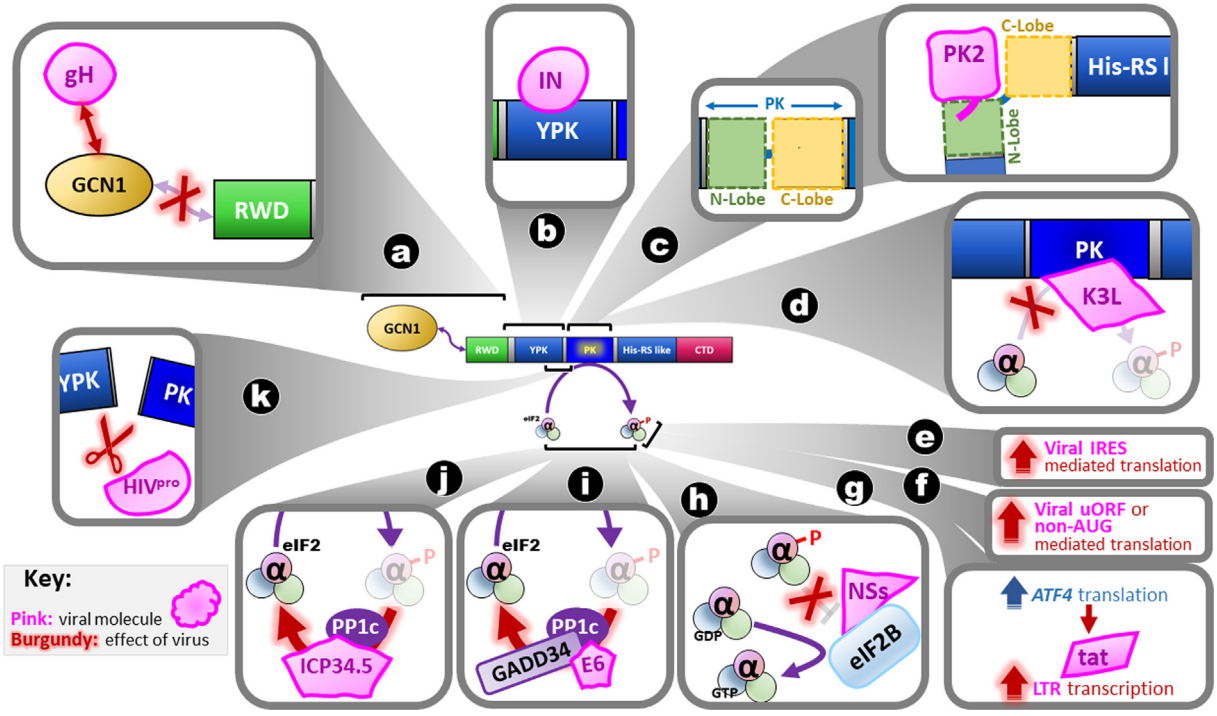

Figure 4. Overview of viral mechanisms counteracting or exploiting GCN2 and the Integrated Stress Response (ISR). Viruses have developed various mechanisms to counteract or even exploit GCN2 activation. For example: **a.** The Herpes Simplex Virus 1 (HSV-1) glycoprotein H (gH) sequesters GCN1, thereby preventing endogenous GCN1-GCN2 interactions and GCN2 activation. **b.** Integrase (IN) from the *Retroviridae* family of viruses binds to the pseudokinase domain (YPK). The significance of this interaction with the YPK is not yet fully understood. Potentially, IN-YPK binding could impede the interaction between the YPK and Protein Kinase (PK)-domain, which is essential for facilitating the stimulation of the GCN2 catalytic activity. However, this remains to be experimentally tested. **c.** Nuclear polyhedrosis virus Protein Kinase-like 2 (PK2) interacts with the GCN2 PK-domain in a way that hinders activation of the PK-domain. The N- and C-lobe of the PK-domain are depicted (green and yellow) to illustrate the binding of the PK2 N-terminal extension (pink line) to the N-lobe**.** The PK2 C-lobe-mimic domain (pink square) takes the place of the C-lobe, resulting in a hybrid PK-domain consisting of the eIF2α-kinase N-lobe and the catalytically-inactive PK2 C-lobe-mimic domain. **d.** Vaccinia Virus (VV) protein K3L acts as a pseudosubstrate that competes with eIF2α for GCN2 binding, thereby hampering eIF2α phosphorylation. **e.** Viruses such as Hepatitis C Virus (HCV) capitalise on the eIF2α-P-mediated reduction in global translation to enhance Internal Ribosome Entry Site (IRES)-mediated translation of viral RNA. **f.** Viruses such as Ebola Virus (EBOV) exploit elevated eIF2α-P levels to promote upstream Open Reading Frame (uORF)-dependent translation of a viral gene, while in Sindbis Virus (SV) non-AUG mediated translation is enhanced. **g.** Viruses such as Human Immunodeficiency Virus 1 (HIV-1) can take advantage of GCN2-mediated ISR activation by exploiting the concomitant increase in ATF4 levels for enhancing transcription of its own genetic material, the Long Terminal Repeats (LTRs). **h.** Non-structural protein S-segment (NSs) from the *Phleboviruses* Rift Valley Fever Virus (RVFV) selectively hinders the binding of eIF2B to phosphorylated eIF2α, but not to unphosphorylated eIF2α, thereby allowing unimpeded GDP-GTP exchange on eIF2α**. i.** Early protein 6 (E6) from Human Papilloma Virus (HPV) type 18 binds to the host proteins PP1c and GADD34 to enhance eIF2α-P dephosphorylation. **j.** Protein ICP34.5 from HIV-1 recruits the host's phosphatase PP1c to promote dephosphorylation of eIF2α-P, thereby counteracting GCN2-mediated eIF2α phosphorylation. **k.** The HIV protease (HIV^{Pro}) removes a GCN2 N-terminal portion that contains the binding site for GCN1 which is essential for mediating GCN2 stimulation. Note that viruses other than those mentioned in this figure also utilise one or more of these mechanisms. For more see text.

into the mechanism of K3L mediated inhibition of PKR.[92] This system uses a yeast strain lacking its only eIF2α-kinase, Gcn2, and instead human PKR is expressed from a galactose-inducible promotor. In yeast, PKR is hyperactive, leading to eIF2α hyper-phosphorylation and a concomitant severe reduction in translation.[93] As a result, the insufficient production of proteins leads to a severe reduction in growth rate. Thus, the growth rate in this system inversely correlates with the level of PKR activity. Using this system, PKR expression was shown to dampen yeast growth, whilst additional expression of K3L reverts this growth defect.[92] This correlates with high eIF2α-P levels in strains

Table 2 Viral proteins with the potential to inhibit or exploit GCN2 or the GCN2 pathway. Overview of viral mechanisms known to counteract / exploit GCN2 or the GCN2 pathway. The content of the table is primarily sorted by the mechanism of GCN2 activation, followed by the genetic material used by the virus to generate mRNA (Baltimore classification)[167], and the viral family. For more information on the extent to which GCN2 has been shown to be involved thus far, please refer to the text.

Proposed mechanism: for counteracting GCN2		See section in review	Baltimore class	Viral molecule	Viral family	Virus* (abbreviation)	Host	Disease / symptoms	Source **
	Sequesters GCN1 (Figure 4a)	IVc	dsDNA	gH	*Herpesviridae*	Herpes simplex virus 1 (HSV-1)	Humans	Causes cold sores in and around the mouth, fever, and swollen lymph nodes.	b
Reduces levels of functional GCN2	Cleaves GCN2 (Figure 4k)	IVd	+ ssRNA-RT	HIV^{Pro}	*Retroviridae*	Human immunodeficiency virus 1 (HIV-1)	Humans	Can cause severe diseases such as AIDS, secondary infections, and lymphomas. Acute phase symptoms include fever, headache, rash, sore throat, weight loss, and diarrhoea	b
						Human immunodeficiency virus 2 (HIV-2)	Humans (more prevalent in West Africa)	Similar to HIV-1 but less virulent, i.e. slower disease progression.	[172]
	Enhances GCN2 degradation, mechanism unknown	IVd	+ ssRNA	Unknown	*Coronaviridae*	Severe acute respiratory syndrome coronavirus (SARS-CoV)	Humans. Occasionally also dogs, cats, mink, gorilla, bats, pangolin	Causes severe acute respiratory syndrome (SARS), presents in humans as respiratory and flu-like symptoms	b, c
Hampers GCN2 catalytic activity	Forms a non-functional kinase domain (Figure 4c)	IVb	dsDNA	PK2	*Baculoviridae*	*Autographa californica* multiple Nucleopolyhedrovirus (AcMNPV)	Winged insects	Interferes with insect development	d
	Acts as pseudosubstrate (Figure 4d)	IVa	dsDNA	vIF2α	*Iridoviridae*	Ranavirus	Amphibians, fish	Affects kidneys, spleen, lungs, and other tissues depending on the species. Great concern for wildlife conservation and aquaculture industry due to mass mortality events	e
			dsDNA	K3L and its orthologues***	*Poxviridae*	Vaccinia virus (VV)	Humans, cattle	Symptoms are usually very mild to non-existent in humans, but may produce localised skin lesions.	i
						Camelpox virus (CMLV)	Camels	Fever and local or generalized pox lesions on the skin, mouth and respiratory tracts	e
						Myxoma virus (MYXV)	Rabbits, hares	Causes Myxomatosis which is lethal in European rabbits. Symptoms include skin lesions, respiratory issues, and death.	e, l
						Swinepox virus (SPV)	Pigs	Causes swinepox, symptoms include mild fever, inappetence, and dullness.	f

Table 2 (*continued*)

Proposed mechanism: for counteracting GCN2	See section in review	Baltimore class	Viral molecule	Viral family	Virus* (abbreviation)	Host	Disease / symptoms	Source **
					Variola virus (VARV)	Humans	Causes smallpox. Symptoms include lesions in the mucous membranes of the nose and mouth as well as face and extremities, fever, fatigue, severe back pain, abdominal pain, and vomiting.	h
		+ ssRNA-RT	Integrase	*Retroviridae*	Avian sarcoma virus (ASV)	Chickens and other birds	Inappetence, weakness, diarrhoea, dehydration and emaciation. Can lead to tumour formation and decreased fertility.	f
					Human immunodeficiency virus 1 (HIV-1)	Humans	Can cause severe diseases such as AIDS, secondary infections, and lymphomas. Acute phase symptoms include fever, headache, rash, sore throat, weight loss, and diarrhoea	b
					Human immunodeficiency virus 2 (HIV-2)	Humans (more prevalent in West Africa)	Similar to HIV-1 but less virulent, i.e. slower disease progression.	[172]
					Murine leukaemia virus (MLV)	Mice, rats	Cancer in mice, may induce neurodegenerative disorders and paralytic diseases.	[171]
		+ ssRNA	E2	*Flaviviridae*	Hepatitis C virus (HCV)	Humans	Causes hepatitis, hepatocellular carcinoma, and cirrhosis.	h
Counteracts eIF2α-P	Attenuates eIF2α-P-mediated eIF2B inhibition. (Figure 4h)	IVf	+ ssRNA	AcP10	*Coronaviridae*	Beluga whale Coronavirus (Bw-CoV)	Beluga whale	May cause pulmonary disease and liver disease.	[173]
			+ ssRNA	AiVL	*Picornaviridae*	Aichi virus (AiV)	Humans	Causes gastroenteritis	h
			- ssRNA	NSs	*Phenuiviridae*	Rift valley fever virus (RVFV)	Humans, ruminants	Humans: ranges from mild flu-like illness to severe haemorrhagic fever Livestock: fever, listlessness, anorexia, abortion, high mortality rates in neonates.	b, e
						Sandfly fever Sicilian virus (SFSV)	Humans, possibly other animals	Fever, headache, photophobia, malaise, myalgia, and retro-orbital pain.	[174]
	Promotes PP1c-mediated eIF2α-P dephosphorylation, various mechanisms (Figure 4i-j)	IVe	dsDNA	IE180	*Herpesviridae*	Pseudorabies virus (PRV)	Pigs	Affects the central nervous, respiratory, and reproductive systems.	f
			dsDNA	E6	*Papillomaviridae*	Human papilloma virus (HPV)	Humans	Can cause cervical, vulval, vaginal, and penial cancers and warts	h
			+ ssRNA	Unknown	*Coronaviridae*	Infectious bronchitis virus (IBV)	Chickens, peafowl, non-galliform birds	Decreased vitality and appetite, nasal discharge, sneezing, coughing, gasping, declined egg production.	f
			dsDNA	ICP34.5, its orthologues, and similar proteins***	*Ascoviridae*	*Trichoplusia ni* Ascovirus 2c (TNAV2c)	Cabbage looper *Trichoplusia ni* (Hübner)	Opaque yellow-white discoloration on the larval body, incomplete shedding of the molted cuticle, decreased feeding activity, and slower growth rate alongside prolonged larval lifespan. Can be lethal.	[179]

(*continued on next page*)

Table 2 (*continued*)

Proposed mechanism: for counteracting GCN2	See section in review	Baltimore class	Viral molecule	Viral family	Virus* (abbreviation)	Host	Disease / symptoms	Source **
							Loopers pose a threat to crops such as cabbage, broccoli, and cauliflower.	
				Asfarviridae	African swine fever virus (ASFV)	Domestic pigs, wild boar	High fever, haemorrhages in the reticuloendothelial system, high mortality rate.	e
				Herpesviridae	Herpes simplex virus 1 (HSV-1)	Humans	Causes cold sores in and around the mouth, fever, and swollen lymph nodes.	b
					Herpes simplex virus 2 (HSV-2)	Humans	Causes genital herpes, symptoms include bumps, blisters, and ulcers around the genitals or anus. May be accompanied by fever, headache and swollen lymph nodes.	b
					Macropodid herpesvirus (MaHV)	Kangaroos, wallabies	Rhinitis, conjunctivitis, pneumonia, cloacal ulceration, splenic, pulmonic, and hepatic necrosis.	j
				Hytrosaviridae	*Glossina pallidipes* salivary gland hypertrophy virus (GpSGHV)	Tsetse fly *Glossina pallidipe*	Hampers tsetse fly fertility and production of offspring Tsetse flies are the vector of the pathogenic African trypanosomes that cause human and animal sleeping sickness in sub-Saharan Africa	[180]
				Iridoviridae	*Anopheles minimus* Iridovirus (AMIV)	Mosquito *Anopheles minimus*	Causes cytopathic damage, leading to the reduction of body size, fecundity and longevity Mosquito is a major Southeast Asian malaria vector. Can pose a threat to humans as infected mosquitoes are a vector for malaria.	[177,178]
				Poxviridae	Canarypox virus (CNPV)	Birds	High mortality rate, has three forms: Cutaneous form: wart-like lesions typical on the face, beak and legs Diphtheritic form: lesions on the mucosa of the oral cavity and respiratory tract Septicaemic form: internal lesions affecting the respiratory and GI tracts	f
					Amsacta moorei Entomopoxvirus "L" (AmEPV)	Some Lepidoptera such as the salt marsh moth *Estigmene acrea*	Colour changes of body, decreased feeding, very characteristic is the extremely extended longevity of infected insects. Salt marsh moth caterpillars pose a threat to agriculture in North America, since it feeds on e.g. cabbage, cotton, walnuts, apple, tobacco, pea, potato, clovers, and maize	[175,176]
Reduces eIF2α-P levels, mechanism unknown	IVe	dsDNA	IE63	*Herpesviridae*	Varicella zoster virus (VZV)	Humans	Causes varicella (chickenpox) and shingles, which presents as lesions concentrated on the chest and back.	h
	IVa	+ ssRNA	Unknown, potentially NS2A / NS4A	*Flaviviridae*	Dengue virus 2 (DENV-2)	Humans	Causes Dengue fever and Dengue haemorrhagic fever, symptoms can include fever, headache, retroocular pain, myalgia, arthralgia, exanthema, and prostration with or without haemorrhage. Can also be asymptomatic.	h

Table 2 (*continued*)

Proposed mechanism: for exploiting GCN2	See section in review	Baltimore class	Viral molecule	Viral family	Virus* (abbreviation)	Host	Disease / symptoms	Source **
Exploitation of the GCN2 / ATF4 axis	Exploits the GCN2 / ATF4 axis to promote translation of viral EBNA1 protein, mechanism to be elucidated	IVg	dsDNA	vDUB-containing proteins***	*Herpesviridae*	Epstein-Barr virus (EBV)	Humans	Causes mononucleosis, also known as glandular fever. Symptoms include tiredness, fever, sore throat, headaches and body aches, swollen lymph nodes, swelling in the liver and/or spleen and rash. Can also be asymptomatic.	b, f, i, h
						Human *Cytomegalovirus* (HCMV)	Humans	Can occasionally develop into mononucleosis or hepatitis. Possible symptoms include fever, sore throat, fatigue, swollen glands. Can also be asymptomatic.	f, h
						Kaposi's sarcoma-associated Herpesvirus (KSHV)	Humans	In patients with weakened immune systems, can lead to Kaposi sarcoma, primary effusion lymphoma, and plasma cell variant of multicentric Castleman disease. Can also be asymptomatic.	h, k
	Exploits GCN2 / ATF4 axis to activate the transcription of long terminal repeats (LTR) (Figure 4g)	IVg	+ ssRNA-RT	Transactivator proteins***	*Retroviridae*	Human immunodeficiency virus 1 (HIV-1)	Humans	Can cause severe diseases such as AIDS, secondary infections, and lymphomas. Acute phase symptoms include fever, headache, rash, sore throat weight loss, diarrhoea.	b
						Human T cell leukaemia virus 1 (HTLV-1)	Humans	Can be asymptomatic or induce adult T-cell leukaemia and associated myelopathy / tropical spastic paraparesis, neurological diseases.	b
			+ ssRNA-RT	Unknown	*Retroviridae*	Simian immunodeficiency virus (SIV)	Non-human primates	Mostly non-pathogenic but may develop AIDS-like symptoms if chronically infected.	e
	Exploits eIF2α-P to promote uORF-dependent translation of viral genes (Figure 4f)	IVg	-ssRNA	Unknown	*Filoviridae*	Ebola virus (EBOV)	Humans, great apes, monkeys, antelopes, bats	Causes Ebola, symptoms in humans include fever, weakness, muscle pain, impaired liver and kidney function, internal and external bleeding, death.	c
	Exploits eIF2α-P to mediate enhanced non-AUG mediated translation (Figure 4f)	IVg	+ ssRNA	Unknown	*Togaviridae*	Sindbis virus (SV)	Humans, birds	Exanthema over trunk and limbs, joint symptoms. Sometimes nausea, general malaise, headache, and muscle pain. Can be asymptomatic.	g
	Exploits eIF2α-P to promote IRES-dependent translation of viral genes (Figure 4e)	IVg	+ ssRNA	Unknown	*Flaviviridae*	Hepatitis C virus (HCV)	Humans	Causes hepatitis, hepatocellular carcinoma, and cirrhosis.	h

* Note that there may be additional viruses beyond those listed above that share the same mechanism, for more see text.
** For source of information, see references (publications) or below (internet links).

*** These viral molecules have been classified by group. For the individual names of the molecules in each species listed, please refer to text.
Internet sources:
a. Charles River website: https://www.criver.com/products-services/research-models-services/research-animal-diagnostics/infectious-agent-technical-info/murine-norovirus-mnv?region = 3701.
b. World Health Organization (WHO) mainly addresses human viral disease symptoms: https://www.who.int/.
c. ViralZone has information on genome, host range and short notes on diseases: https://viralzone.expasy.org/.
d. Autographa californica: https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/autographa-californica.
e. World Organization for Animal Health (WOAH): https://www.woah.org/en/what-we-do/animal-health-and-welfare/disease-data-collection/.
f. MSD manual for diseases in animals: https://www.msdvetmanual.com/generalized-conditions/vesicular-stomatitis-in-large-animals/vesicular-stomatitis-in-large-animals/?autoredirectid = 22922.
g. European Centre for Disease Prevention and Control (ECDC), details on disease symptoms: https://www.ecdc.europa.eu/en.
h. Centers for Disease Control and Prevention (CDC), details on disease symptoms: https://www.cdc.gov/.
i. Medscape, site commonly used by medical practitioners to learn about infections and diseases: https://emedicine.medscape.com/.
j. Prevalence and Clinical Significance of Herpesvirus Infection in Populations of Australian Marsupials: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4519311/.
k. Mayo Clinic: https://www.mayoclinic.org/ .
l. WOAH Terrestrial Manual 2021: https://www.woah.org/fileadmin/Home/fr/Health_standards/tahm/3.07.01_MYXO.pdf.

expressing PKR, and reduced eIF2α-P levels in strains expressing both PKR and K3L. This demonstrates that K3L can effectively inhibit PKR activity *in vivo*. Using this heterologous yeast assay system, a single amino acid substitution, H47R, was found to render K3L even more potent in reverting the growth defect, which correlates with a further reduction in eIF2α-P levels.[92] This suggests that this K3L variant is more potent in PKR inhibition, likely because the eIF2α mimicry has been improved. Supporting this idea, the H47R substitution increases the homology of K3L to eIF2α close to the Ser-51 phosphorylation site. Conversely, decreasing the eIF2α mimicry of K3L should reduce or abolish K3L's potency in inhibiting PKR. In fact, single amino acid substitutions in the KGYID motif conserved between K3L and eIF2α proteins, such as Y76A, fail to revert the growth defect associated with PKR expression, and this correlates with the inability to reduce eIF2α-P levels. Together, these studies support the idea that K3L mimics the substrate eIF2α. Consistent with the idea that K3L is a bona fide pseudosubstrate, pulldown assays revealed that K3L binds to PKR. The H47R substitution enhances this interaction, while the Y76A substitution has the opposite effect. Similarly to PKR, constitutively active yeast Gcn2 also leads to a growth defect in yeast, and K3L reverts this growth defect.[92] The fact that K3L inhibits Gcn2 in *in vitro* kinase assays, binds to the Gcn2 kinase domain, and reduces eIF2α-P levels in yeast cells,[88] supports the idea that K3L inhibits GCN2 via the same mechanism as found for PKR (Figure 4d).

K3L orthologs have also been found in viruses belonging to the poxvirus family, such as C8L from swinepox virus,[94] C3L from Variola Virus (VARV, causes smallpox) and CMLV032 from camel pox virus.[95] These orthologues contain the KGYID motif as found for K3L, and studies suggest that these proteins exert similar inhibitory effects on eIF2α-kinases as K3L.[94,95]

The PKR PK-domain shows substantial sequence variability across species.[96] This reduces the ability of viral K3L-type inhibitors to bind to PKR from species outside their natural hosts.[96–98] Because of this, host organisms are likely to have a degree of innate resistance to poxvirus strains from other species, reducing the risk of severe zoonotic disease. Curiously, this sequence variability in the PK-domain is not seen to that extent in GCN2,[96] which would suggest that GCN2 may be more susceptible to these pseudosubstrates irrespective of the natural host.

Curiously, not all poxviruses contain K3L orthologues with a KGYID motif. One example is the Myxoma Virus (MYXV) which causes myxomatosis (a lethal disease in rabbits). This virus was released in Australia in the 1950s in an attempt to control the rabbit population.[99] Its K3L orthologue, called M156R, has been shown to be a competitive inhibitor of PKR.[100] Curiously, M156R is also an efficient substrate of PKR *in vitro* even though it does not contain a Ser at a position equivalent to eIF2α Ser-51.[100] It is not known yet whether M156R phosphorylation has any biological relevance in terms of M156 function. M156R contains the motif YVD instead of KGYID, while still being a structural mimic of eIF2α,[100] suggesting that not every residue within the KGYID motif is critical for PK-domain docking. In fact, structural analysis of PKR suggests that the Tyr and Asp in YVD are critical determinants for binding to the PK-domain.[100] This is consistent with a prior discovery, where an Ala-substitution of Lys, Tyr, or Asp within the KGYID motif hampers K3L's ability to inhibit PKR, and the Ala-substitution of Tyr also impairs K3L-PKR interaction.[84] Additionally, the naturally occurring M156R-L98P mutation, which is proximal to the YVD motif, leads to a loss of function M156R protein unable to inhibit rabbit PKR.[101] This substitution for Pro likely disrupts the structural features of the YVD motif necessary for kinase docking, highlighting the importance of the amino acids surrounding the KGYID motif to adopt the correct conformation for PKR binding.

The pox-like Ranaviruses are pathogens of lower vertebrates such as fish, amphibia and reptiles. Their human-induced spread and emergence has become a great concern for wildlife conservation and aquaculture industry, given that they can cause mass mortality events.[102] Ranaviruses produce a pseudosubstrate, the viral mimic of eIF2α (vIF2α), which acts in a similar fashion to K3L.[103] As found for K3L, vIF2α has high sequence homology to the N-terminus of eIF2α, but in the KGYID motif the amino acid Ile is not absolutely conserved. This would suggest that the Ile in this motif is not critical for vIF2α function in inhibiting PKR,[103,104] in agreement with the findings in M156R and K3L where not Ile, but the two neighbouring amino acids are critical for function.[92,100]

As has been mentioned previously, HIV-1 IN is a substrate of GCN2 (see IIId). In *in vitro* assays, GCN2 can phosphorylate IN produced by various *Orthoretrovirinae* including HIV-1, HIV-2, MLV, and ASV,[82] supporting the idea that IN is a GCN2 substrate. Interestingly, IN contains an SGYIE motif, reminiscent of the KGYID motif in eIF2α, as well as a Ser residue downstream at a position that approximately corresponds to that of eIF2α Ser-51.[13,82] This may suggest that IN functions as a pseudosubstrate. Supporting this idea, *in vitro* kinase assays show that phosphorylation of IN decreases with increasing amounts of eIF2α added.[82] Curiously however, IN phosphorylation occurs at Ser-24 and Ser-255 *in vitro* and *in cellulo* but not at Ser-57, which would be the equivalent to eIF2α Ser-51.[82] It would be interesting to repeat the *in vitro* kinase assay with constant eIF2α levels but increasing amounts of IN in order to test whether IN can hamper eIF2α phosphorylation, which would

further support the idea that IN acts as a true pseudosubstrate.

The Hepatitis C Virus (HCV) Envelope protein 2 (E2) enables the virus to evade PKR-mediated anti-viral activities.[105] Unlike the previously mentioned K3L orthologs, E2 lacks the KGYID motif, at least according to the HCV genome polyprotein sequence (Accession number Q9WMX2, Gibbs & Sattlegger unpublished). Instead, it was found that in most HCV isolates, E2 shows sequence homology to portions of both eIF2α and PKR. The homology to eIF2α includes Ser-51 (Ser-280 in E2) and residues located immediately upstream to eIF2α Ser-51 (amino acids 277–279 in E2 and 48–50 in eIF2α), while the homology to PKR includes again the Ser-280 residue in E2 plus downstream amino acid sequences (amino acids 280–287 in E2 and 83–90 in PKR) (Figure 5).[105] The respective PKR segment is located in the RNA-binding domain of PKR, and within a region found to harbour an auto-phosphorylation cluster (PKR amino acids 81–107, contains six auto-phosphorylation sites).[105,106] It was suggested that this PKR-eIF2α Phosphorylation Homology Domain (PePHD) is important for PKR binding and inhibition, given that deletion of a portion of E2 that also removes the PePHD significantly reduces E2-PKR interaction in *in vitro* co-precipitation assays.[105] Furthermore, *in vitro* kinase assays have demonstrated that E2 inhibits PKR auto-phosphorylation. Similarly, PKR auto-phosphorylation is hampered by E2 *in vivo*, but not by an E2 variant lacking a specific region that includes PePHD.[105] Repeating these assays with E2 only lacking the PePHD would provide definitive evidence that this motif is critical for PKR binding and inhibition.

Co-precipitation assays suggest that E2 also binds PERK, and it has been shown that this interaction is mediated by the PK-domain.[107] Similarly to PKR, E2 inhibits PERK *in vitro*, and *in vivo* it prevents the translational repression associated with PERK overexpression.[107] As with PKR, E2 is less effective in counteracting PERK-mediated translational repression when a portion of E2 is missing, which also includes the removal of PePHD. Similar observations were made with an E2 variant that contains amino acid substitutions in the PePHD, reducing the homology to eIF2α (SELS substituted by GQQH).[107] This supports the idea that it is in fact the PePHD that is responsible for counteracting PERK. It will be interesting to investigate whether this substitution also hampers E2′s ability to bind PERK and hamper PERK activation at a molecular level. Nevertheless, given that E2 can inhibit PERK as well as PKR, and that E2 binds to the PK-domain, it is likely that E2 can inhibit all eIF2α-kinases including GCN2. Additional experiments are needed to verify whether E2 acts as a true pseudosubstrate, or alternatively inhibits eIF2α-kinase activity by a different mechanism.

The *Flaviviridae* Dengue Virus 2 (DENV-2) has also been found to inhibit eIF2α phosphorylation.[108] While DENV-2 infection of 2fTGH (a human fibrosarcoma cell line) prevents PERK-mediated eIF2α phosphorylation in response to ER stress, it can also hamper GCN2 auto-phosphorylation and eIF2α phosphorylation in response to Leu starvation.[108] This suggests that DENV-2 contains a molecule capable of inhibiting GCN2, which is intriguing considering GCN2′s involvement in immune signalling during DENV-2 infection.[109] It was suggested that DENV-2 may produce an inhibitor akin to that in HCV, given their phylogenetic link.[108] If that is true, then the DENV genome should encode a protein equivalent to E2 which is identifiable by its PePHD domain. A protein Basic Local Alignment Search Tool (BLAST) search query against all DENV protein sequences was con-

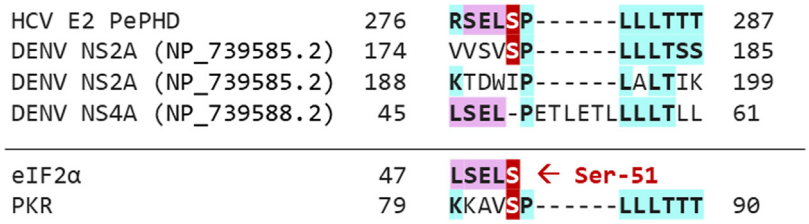

Figure 5. Viral proteins share sequence identity with eIF2α and PKR. The Hepatitis C Virus (HCV) Envelope protein 2 (E2) harbours the PKR-eIF2α Phosphorylation Homology Domain (PePHD) that shares sequence homology with both, eIF2α and PKR. The homology with eIF2α covers the eIF2α phosphorylation site Ser-51 (burgundy, Ser-280 in E2), and the immediate N-terminal three residues (pink, residues 48–50 in eIF2α, residues 277–279 in E2). The homology with PKR includes again the Ser-280 residue in E2 (burgundy, Ser-83 in PKR). Additionally, it encompasses the immediate downstream residues located in the RNA-binding domain of PKR (cyan, residues 281–287 in E2, residues 84–90 in PKR). This region is known to harbour an auto-phosphorylation cluster which contains six auto-phosphorylation sites (PKR amino acids 81–107). An NCBI protein BLAST search was conducted querying the PePHD sequence in E2 against all Dengue Virus (DENV) proteins (sequences derived from the DENV polyprotein sequence, accession NC_001474.2). Regions in the DENV Non-structural proteins 2A and 4A (NS2A and NS4A) were found that show some amino acids conservation to those encompassing PePHD, as indicated. The accession numbers of the respective DENV proteins are indicated in the figure.

ducted, and the Non-structural proteins 2A and 4A (NS2A and NS4A) were found to contain sequence similarity to the PePHD of E2 (Figure 5; Gibbs & Sattlegger, unpublished). The NS2A protein harbours two nearby sequences resembling the PKR-like part of the PePHD but lacks the part with resemblance to eIF2α. The NS4A protein contains a region with similarity to both the eIF2α-like and PKR-like regions of PePHD, but with two notable differences. Firstly, NS4A lacks the Ser-51 equivalent. Secondly, there is a small insertion in the PKR-like section of this sequence. Hence, judging from this sequence analysis, it is not apparent that DENV antagonises GCN2 in a similar fashion as E2 from HCV. This underscores the need for further experiments to unveil how DENV inhibits eIF2α phosphorylation.

One function of the Transactivator of transcription (Tat) protein of HIV-1 is likely that of a pseudosubstrate.[110,111] PKR has been shown to bind Tat *in vitro* and *in vivo*, and activated PKR was found to phosphorylate Tat.[110,111] *In vitro*, increasing amounts of Tat clearly leads to reduced levels of PKR-mediated eIF2α phosphorylation in a dose-responsive manner.[111] When doing the reverse experiment using increasing amounts of eIF2α, a dose-dependent decrease in PKR-mediated Tat phosphorylation was not as pronounced.[111] Nevertheless, these findings support the idea that Tat competes with eIF2α for PKR binding, as one would expect from a pseudosubstrate. Further supporting this idea, the region in Tat required for PKR binding (amino acids 40–58) shows homology to the area in eIF2α surrounding Ser-51.[112] Given that PKR-Tat interaction requires a portion in Tat that encompasses the RNA-binding region,[111] and that PKR binds dsRNA, one cannot exclude the possibility that the PKR-Tat interaction is mediated by RNA. The authors reported (as data not shown) that treatment of either PKR or Tat with RNase prior to interaction assays did not affect the interaction.[111] These findings would agree with the idea that PKR-Tat interaction is not bridged by RNA. However, one cannot exclude the possibility that after an RNase digest, some RNA has remained that can potentially bridge a protein–protein interaction. Conclusive evidence could be provided by using Tat with point mutations that specifically abolish RNA binding. Since Tat acts as a pseudosubstrate, it is reasonable to assume that Tat may be able to inhibit eIF2α-kinases other than PKR, such as GCN2. However, this needs to be tested experimentally.

IVb) Mimicry of the protein kinase C-lobe

A viral protein has been reported that mimics a portion of the PK-domain of eIF2α-kinases and can likely inhibit any eIF2α-kinase including GCN2. Best studied is the imitation Protein Kinase 2 (PK2) from baculovirus *Autographa californica* Multiple Nucleopolyhedrovirus (AcMNPV), which is highly pathogenic to a variety of larval lepidopteran insects (insects with scaly wings such as butterflies and moths). PK2 contains an N-terminal extension that is unique to PK2, but otherwise bears closest homology to the C-lobe of the highly conserved eIF2α-kinase PK-domain itself.[113,114] The so-called C-lobe-mimic domain shares homology to C-lobe subdomains VI – XI, just that several of these subdomains vary from the consensus sequence typical for Ser/Thr kinases.[23,113–115] Together with the fact that the phosphorylation loop and potential auto-phosphorylation sites are lacking,[116] this strongly suggests that the C-lobe-mimic domain is catalytically inactive.

Heterologous yeast assays have revealed that PK2 functions as an eIF2α-kinase inhibitor. In this assay, the growth defect of yeast caused by the hyperactivity of overexpressed PKR is alleviated when PK2 is co-expressed.[116] This is consistent with PK2 also reversing the hyperphosphorylation of eIF2α associated with hyperactive PKR. Similar findings were obtained for yeast cells expressing hyperactive yeast Gcn2, suggesting that PK2 can also inhibit Gcn2. In agreement with the idea that PK2 inhibits eIF2α phosphorylation, infected insect SF9 cells (a clonal isolate of *Spodoptera frugiperda* Sf21 cells) show reduced eIF2α-P levels as compared to uninfected cells, while this is not found in SF9 cells infected with a PK2-deficient virus.[116]

The C-lobe-mimic domain contains an αG helix that corresponds to the one in the C-lobe of the eIF2α-kinases' PK-domain known to mediate eIF2α binding.[113,114,25] Surprisingly though, PK2 does not bind to eIF2α. Instead, it binds to PKR, as determined for example by *in vitro* pulldown assays using purified proteins, or by *in vivo* co-immunoprecipitation assays using yeast expressing PK2 as well as PKR.[115,116]

The PKR PK-domain N-lobe, but not the C-lobe, directly interacts with PK2, as determined via *in vitro* pulldown and yeast 2-hybrid assays.[115,116] Curiously, the 22 amino acid long N-terminal extension of PK2 is necessary, but not sufficient, for binding to the PK-domain of PKR *in vitro*, as determined by pulldown assays. This suggests that the PK2 C-lobe-mimic domain has a supporting role in PKR binding. Within the N-terminal extension, a single F18A substitution abolishes the interaction between PK2 and the PKR kinase domain. This correlates with the inability to inhibit PKR, underscoring the importance of the PK2 N-terminal extension for PKR inhibition.[115] Together with results from additional studies, this led to the so-called lobe-swapping model[115] (Figure 4c), in which the PK2 N-terminal extension mediates high-affinity binding to the eIF2α-kinase N-lobe. The PK2 C-lobe-mimic domain may displace and take on the place of the eIF2α-kinase domain C-lobe. This would result in a hybrid protein kinase domain consisting of the eIF2α-kinase N-lobe and the catalytically inactive PK2 C-lobe-mimic domain, rendering the

kinase unable to bind ATP and phosphorylate eIF2α. It has been shown that PK2 also hampers HRI, suggesting that PK2 can inhibit any eIF2α-kinase.[115]

PK2-devoid baculovirus is less able to replicate in *Bombyx mori* insect cells, and this correlates with higher eIF2α-P levels as compared to cells infected with wildtype baculovirus. Furthermore, PK2-devoid baculovirus takes 12 hrs longer to kill host larval insects. Together, these findings agree with the idea that PK2 is critical for the virus to counteract the host's viral defence.[115] PK2-devoid baculovirus regains the ability for budded virus production in cells where HRI is knocked down, but not in cells where GCN2 or PERK is knocked down, suggesting that HRI is the major eIF2α-kinase that counteracts AcMNPV infection.[115] In agreement with this idea, PK2 shares the highest sequence homology with HRI, in particular insect HRI.[115] It was suggested that PK2 evolved from an insect HRI-like kinase. It is possible that within the HRI PK-domain there are residues specific to HRI but not to other eIF2α-kinases. These residues, while not critical for the kinase's enzymatic activity *per se,* may aid and enhance the interaction with PK2, thereby rendering PK2 more potent in inhibiting the eIF2α-kinase HRI. While these findings suggest that GCN2 may not be the primary target of PK2, one could imagine that the inhibitory effect of PK2 on other eIF2α-kinases is part of a welcome side effect in hampering host defence.

IVc) Sequestering GCN1, a protein required for GCN2 activation

The Herpes Simplex Virus 1 (HSV-1) expresses a protein called glycoprotein H (gH) which has been shown to hamper the host's ISR response.[117] Specifically, in Vero kidney cell lines, overexpression of gH is sufficient to reduce eIF2α-P levels, demonstrating that gH can hamper eIF2α phosphorylation in the absence of any other viral proteins or molecules.[117] MEFs infected with HSV-1 show increased eIF2α-P levels, while this is hampered in MEFs knocked-down for GCN1. This suggests that GCN1 is critical for the defence against HSV-1. Accordingly, given that GCN1 is the effector protein for GCN2 and no other eIF2α-kinase, one would conclude that GCN2 phosphorylates eIF2α in response to HSV-1 infection. Interestingly, GCN1 co-immunoprecipates with transiently expressed gH in tandem affinity purification of 293 T cell extracts.[117] Similarly, gH expressed in 293 T or Human Epithelioma 2 (HEp-2) cells co-immuniprecipitates GCN1, suggesting that GCN1 and gH reside in the same protein complex.

According to immunofluorescence microscopy, GCN1 is mainly found in the cytoplasm of mock-infected HEp-2 carcinoma cell lines, while in HSV-1 infected cells GCN1 predominantly colocalises with gH in the nuclear rim.[117] This aligns with the notion that GCN1 and gH interact, and that this interaction relocates GCN1 to a different area of the cell.[117] Collectively, these findings suggest that gH binds and sequesters GCN1 away from GCN2, thereby preventing GCN2 activation (Figure 4a). It remains to be investigated whether the gH-GCN1 interaction is direct or mediated by a third molecule, and whether gH directly blocks the GCN1-GCN2 interaction.

IVd) Reducing GCN2 protein levels

Proteolytic cleavage of an enzyme is a way of permanently quashing its activity. In MT-2 human T-lymphocyte cell lines infected with HIV-1, it was found that GCN2 protein levels are dramatically reduced.[75] The decrease in GCN2 levels is mitigated in the presence of saquinavir, a specific inhibitor of the HIV-1 protease (HIV-1^{Pro}), suggesting that GCN2 is subject to proteolytic cleavage. In fact, HIV proteases HIV-1^{Pro} and HIV-2^{Pro} cleave both human and mouse GCN2 *in vitro* at one specific site.[75] The amino acid sequence of the cleavage site is ^{560}YVETVIP^{566} for human GCN2 and ^{559}YIETVIP^{565} for mouse GCN2 respectively, with the cleavage site being between the first and second position.[75] Such amino acid sequences are not shared in the other eIF2α kinases. Proteolysis removes the N-terminal end of GCN2 which encompasses the binding site for its effector protein GCN1.[39] Since the GCN1-GCN2 interaction is essential for GCN2 activation, this suggests that the removal of the GCN1-binding site by HIV^{Pro} is a way of hampering GCN2 activation (Figure 4k). Demonstrating that HIV-1-mediated protease cleavage indeed renders GCN2 inactive, incubation of GCN2 with HIV-1^{Pro} prior to an *in vitro* kinase assay abolishes eIF2α phosphorylation.[75]

Interestingly, when conducting multiple sequence alignments of GCN2 proteins from a selection of organisms, we found that the Y[I/V]ETVIP cleavage sequence is not conserved throughout all eukaryotes (Figure 6). Within the organisms tested, the sequence of the protease site is almost completely conserved in placental mammals, while the first position of the site is only conserved in placental and pouched mammals. It appears that the conservation of the protease site decreases as a species becomes more distantly related to placental mammals. It remains to be determined whether any of the altered cleavage sequences can still be recognised by an HIV protease. In *Drosophila melanogaster* (representative of insects), *Arabidopsis thaliana* (representative of plants), and *S. cerevisiae* (representative of fungi), no sequence could be found that resembles the protease site (data not shown).

Together, these findings suggest the possibility that the Y[I/V]ETVIP protease site has arisen progressively during evolution. This prompts the question of why this site has emerged – and still exists – in placental mammals, given the resulting

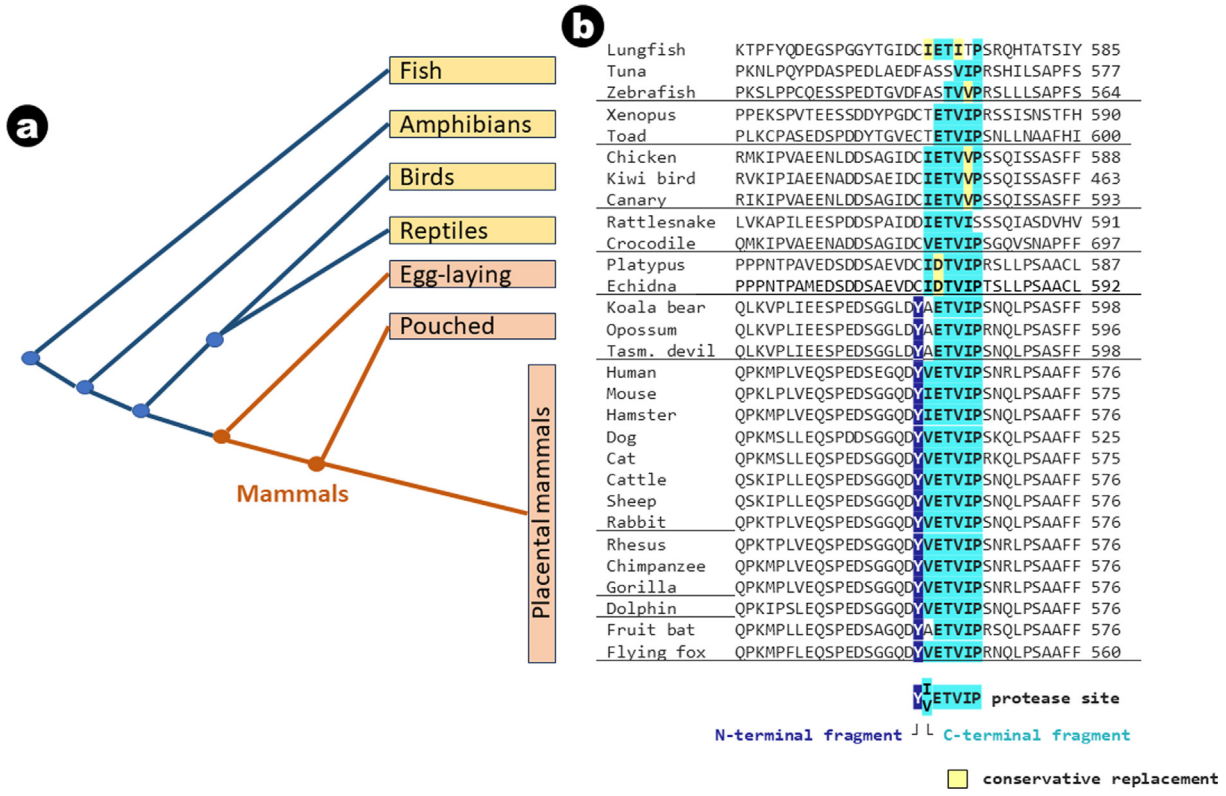

Figure 6. The HIV protease recognition site is conserved in GCN2 of placental mammals. a. Cladogram depicting the relationships among the indicated species. **b.** Multiple sequence alignment of GCN2 from different species as indicated. Only the portion of the alignment is shown that encompasses the HIV protease site in mouse and human, and immediate upstream and downstream amino acid sequences. For each sequence the positional number of the last shown residue is indicated. The protease recognition site is indicated below the multiple sequence alignment. The GCN2 sequences and accession numbers, from top to bottom are: West African Lungfish (*Protopterus annectens*, XP_043929767.1), yellowfin tuna (*Thunnus albacares*, XP_044230996.1), zebrafish (*Danio rerio*, XP_017209968.2), African clawed frog (*Xenopus laevis*, XP_041429235.1), common toad (*Bufo bufo*, XP_040267430.1), Chicken (red junglefowl; *Gallus gallus*, XP_040527909.1), kiwi bird (Okarito brown kiwi; *Apteryx rowi*, XP_025946508.1), wild canary (S*erinus canaria*, XP_018763042.3), tiger rattlesnake (*Crotalus tigris*, XP_039198591.1), saltwater crocodile (C*rocodylus porosus*, XP_019406887.1), duck-billed platypus (*Ornithorhynchus anatinus*, XP_028933853.1), short-billed echidna (*Tachyglossus aculeatus*, XP_038597019.1), koala bear (*Phascolarctos cinereus*, XP_020834905.1), opossum (grey short-tailed opossum; *Monodelphis domestica*, XP_007480090.1), Tasmanian devil (*Sarcophilus harrisii*, XP_031807935.1), human (*Homo sapiens*, NP_001013725.2), mouse (mouse isoform 1; *Mus musculus*, NP_038747.2), golden hamster (*Mesocricetus auratus*, XP_040601966.1), domestic dog (*Canis lupus familiaris*, XP_005638286.1), domestic cat (*Felis catus*, XP_023111271.2), cattle (*Bos taurus*, XP_010807512.1), domestic sheep (*Ovis aries*, XP_004010488.2), European rabbit (*Oryctolagus cuniculus*, XP_002718031.1), rhesus monkey (*Macaca mulatta*, XP_014997405.2), chimpanzee (*Pan troglodytes*, XP_001140245.1), gorilla (*Gorilla gorilla*, XP_018866377.1), common bottlenose dolphin (*Tursiops truncatus*, XP_019790833.1), Jamaican fruit bat (*Artibeus jamaicensis*, XP_037017581.2), and large flying fox (*Pteropus vampyrus*, XP_011353450.1).

susceptibility of GCN2 to HIV proteases. It will be interesting to investigate possible correlations between HIV susceptibility and the presence of the HIV protease site in GCN2.

Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) also elicits a reduction in GCN2 levels. Specifically, it has been found that during late stages of SARS-CoV infection, the GCN2 protein level is strongly reduced. RT-PCR studies suggest that this reduction is not due to reduced GCN2 mRNA levels, in line with the idea that this virus also elicits enhanced GCN2 protein degradation, though the exact mechanism remains to be elucidated.[118]

IVe) Counteracting eIF2α phosphorylation

Viruses have developed strategies that enhance the rate of eIF2α-P dephosphorylation (Figure 4i, j). Since eIF2α phosphorylation is a consequence

of the activation of eIF2α-kinases, the reversal of eIF2α phosphorylation counteracts the anti-viral activities of all eIF2α-kinases, including GCN2.

After the resolution of cell stress, eIF2α needs to return to its basal phosphorylation level. This is achieved by a negative feedback loop which is explained here briefly. Increased eIF2α phosphorylation leads to enhanced synthesis not only of the ATF4 protein, but also of the Growth Arrest and DNA Damage-inducible protein 34 (GADD34).[119] GADD34 is a regulatory subunit within the Protein Phosphatase 1 (PP1) complex. This complex contains the PP1 catalytic subunit (PP1c) that can dephosphorylate eIF2α-P.[120] Increased levels of GADD34 promote its binding to PP1c, thereby amplifying the rate of eIF2α-P dephosphorylation.[121] Following the return of eIF2α-kinases to their latent state, this enhanced dephosphorylation effectively restores eIF2α-P levels back to the basal level observed in non-stressed cells.

Infection by the *Coronaviridae* Infectious Bronchitis Virus (IBV) was shown to lead to increased levels of GADD34 protein, but not infection by UV-inactivated IBV.[122] It was found that IBV replication in Vero cells is reduced upon treatment with the phosphatase inhibitor okadaic acid (at concentrations necessary to inhibit PP1c), and this correlates with an increase in eIF2α-P levels.[122] This suggests that IBV counteracts host cell-mediated eIF2α-phosphorylation by inducing GADD34 expression, thereby enhancing the rate of eIF2α-P dephosphorylation. The mechanism by which IBV triggers an increase in GADD34 levels remains to be elucidated.

HSV-1 produces Infected Cell Protein 34.5 (ICP34.5), a protein that shares homology with a section in GADD34 encompassing both the PP1-binding motif and the region required for eIF2α binding.[123,124] In fact, it has been shown that ICP34.5 can bind to both eIF2α and PP1, bringing these two proteins in close enough proximity to promote eIF2α-P dephosphorylation by PP1c.[124–126] Hence, ICP34.5 mimics the function of GADD34 already present in the host cell (Figure 4j). The importance of the catalytic activity of PP1c in dampening the host's anti-viral response during HSV-1 infection was shown in experiments with infected SK-N-SH cells (a neuroblastoma cell line). In the presence of okadaic acid, protein synthesis is fully blocked, while the inhibitor has no detectable effect in mock-infected cells.[126] Further showing the importance of ICP34.5 in lowering eIF2α-P levels, HeLa cells infected with an HSV-1 variant that lacks ICP34.5 show increased eIF2α-P levels, while infection with the wildtype virus only elicits a weak increase in eIF2α-P levels. The latter correlates with enhanced synthesis of viral proteins as well as a higher viral titre. Moreover, in HeLa cells expressing a mutated version of ICP34.5 that is unable to bind eIF2α or PP1, additional infection with the ICP34.5-negative virus elicits an unhampered increase in eIF2-P levels.[125] Together, this demonstrates that ICP34.5 is relevant for counteracting the host viral defence, and that binding of ICP34.5 to PP1c as well as eIF2α is critical for this function.

As found for ICP34.5, the African Swine Fever Virus (ASFV) protein DP71L shares homology with the C-terminal part of GADD34 required for PP1c and eIF2α binding.[124,127] *In vivo*, DP71L was shown to bind to PP1c as well as eIF2α, and to reduce eIF2α-P levels in a PP1c-dependent manner,[127] as found for ICP34.5. Proteins with sequence similarity to ICP34.5 have also been found in several other viruses, including Canarypox Virus (CNPV), *Macropodid Herpesvirus* (MaHV, infecting marsupials), human HSV-2, and many invertebrate viruses. Invertebrate viruses include *Amsacta moorei* Entomopoxvirus L" (AmEPV, infects certain lepidopteran insects such as the salt marsh moth which poses a threat to crops such as cabbage, cotton, walnuts, apple, tobacco, maize), *Anopheles minimus* Irodovirus (AMIV, infects the *Anopheles minimus* mosquitos which are a major Southeast Asian malaria vector), *Trichoplusia ni* Ascovirus 2c (TNAV2c, infects cabbage loopers which poses a threat to crops such as cabbage, broccoli, and cauliflower), and *Glossina pallidipes* Salivary Gland Hypertrophy Virus (GpSGHV, infects tsetse flies which pose a threat to humans and animals as a vector for African trypanosomiasis). Given that they also harbour both the PP1c- and eIF2α-binding motifs, this suggests that they revert eIF2α phosphorylation in the same manner as ICP34.5 does.[124]

The Human PapillomaVirus (HPV) type 18 expresses the Early protein 6 (E6) that also mediates enhanced eIF2α-P dephosphorylation, but interestingly this is not by way of mimicking the role of GADD34.[128] Instead, E6 binds to GADD34 and PP1c. This was determined by *in vivo* co-immunoprecipitation assays using HeLa cells transiently transfected with FLAG-tagged GADD34 or E6.[128] These E6-immunoprecipitates are capable of dephosphorylating eIF2α-P, as determined by dephosphorylation assays using ^{32}P-labelled eIF2α-P. Together, this suggests that E6 associates with the GADD34-PP1c complex to enhance eIF2α-P dephosphorylation (Figure 4i). The exact mechanism by which E6 promotes eIF2α-P dephosphorylation remains to be elucidated. HPV persists in the body post-infection, with certain serotypes causing cancers later-on, such as cervical cancer.[129] Current vaccines target the most prevalent cancer-causing strains of HPV.[130] However, due to ongoing evolution, these vaccines require regular adjustments to effectively prevent HPV-related cancers. Exploring alternative drug targets that HPV cannot readily evade could be an intriguing avenue for research.

The porcine *Alphaherpesvirinae* Pseudorabies Virus (PRV) is the causative agent of Aujeszky's

disease, which impacts the central nervous, respiratory, and reproductive systems. This virus is a large concern for the economy of livestock farming, given that infection leads to a nearly 100% mortality rate among newborn and suckling pigs.[131] Furthermore, PRV naturally infects ruminants, carnivores, rodents, and lagomorphs, causing disease with fatal outcomes.[132] PRV infection produces the protein Immediate Early 180 (IE180), which also interferes with eIF2α phosphorylation levels in a manner dependent on phosphatase activity.[133] For example, Swine Testicular (ST) cells were transfected with a plasmid expressing only IE180 and then treated with thapsigargin to elicit PERK-mediated eIF2α phosphorylation. In these conditions, eIF2α-P levels are reduced as compared to cells transfected with a control plasmid expressing enhanced Green Fluorescent Protein (eGFP), suggesting that IE180 counteracts the effects of PERK activity. The same was observed with differentiated 50B11 cells (embryonic rat dorsal root ganglion neuron-derived cells). Supporting the idea that IE180 exerts this effect through its phosphatase activity, the eIF2α-P levels are again elevated when cells are treated with the PP1c specific inhibitor 'Inhibitor 2' prior to and during infection. This suggests that PRV reduces eIF2α-P levels via enhancing eIF2α-P dephosphorylation. As yet, the precise mechanism of how IE180 reduces eIF2α-P levels remains to be uncovered.

Varicella Zoster Virus (VZV; causes chicken pox and shingles) produces a protein called Immediate Early protein encoded by ORF63 (IE63), and this has been shown to reduce eIF2α-P levels. For example, ectopic expression of IE63 in CV-1 in Origin with SV40 genes (COS) cells leads to a dramatic decrease in eIF2α-P levels.[134] This suggests that IE63 is sufficient to either hamper eIF2α phosphorylation or enhance eIF2α-P dephosphorylation. The mechanism remains to be elucidated.

IVf) Counteracting the inhibitory effect of eIF2α-P on eIF2B

As for all eIF2α kinases, following GCN2 activation, phosphorylated eIF2α impedes the function of eIF2B in recycling inactive eIF2α-GDP to eIF2α-GTP (see IIc). Some viruses have developed a strategy to neutralise the host's ISR by rendering eIF2B resistant to the inhibitory effect of eIF2α-P, thereby allowing for continuous ternary complex formation and mRNA translation.

For example, infection experiments were conducted with HeLa cells using the *Phleboviruses* Rift Valley Fever Virus (RVFV) and Sandfly Fever Sicilian Virus (SFSV).[135] It was found that viruses lacking Non-structural protein S segment (NSs) exhibit reduced replication. However, this effect is reversed in HeLa cells knocked-down for PKR, suggesting that NSs antagonises PKR. Infection of A549 cells (a human lung cancer cell line) with recombinant RVFV containing NSs from SFSV revealed that this NSs does not affect PKR auto-phosphorylation or eIF2α phosphorylation. Instead, NSs interacts with eIF2B as determined by co-immunoprecipitation assays, suggesting that NSs neutralises the inhibitory effect of eIF2α-P on eIF2B. Cryoelectron microscopy data along with additional experiments suggest that NSs competes with eIF2α-P for binding to eIF2B because they utilise overlapping binding sites on eIF2B, while NSs and unphosphorylated eIF2α can bind eIF2B simultaneously (Figure 4h).[136,137] Whilst this competitive binding was not observed by previous studies,[122] this may be due to the experimental conditions employed. A model was proposed in which NSs-binding (and the requisite blocking of eIF2α-P binding) locks eIF2B into the 'productive' conformational state,[123] preserving the activity of eIF2B in mediating GDP-GTP exchange on eIF2α even in the presence of eIF2α-P. This would allow these *Phleboviruses* to continue to utilise the translation machinery of the cell for its replication. Although these studies identified PKR as the relevant eIF2α kinase, as NSs acts downstream of eIF2α phosphorylation they would be able to antagonise other eIF2α kinases, including GCN2.

Additional proteins were found to also bind to eIF2B.[138] The ORF10-encoded Accessory Protein (AcP10) of Beluga whale Coronavirus (Bw-CoV) strain SW1 was found to directly interact with eIF2B. When associated with AcP10, eIF2B preferentially binds the unphosphorylated form of eIF2α, as judged by co-precipitation studies. This supports the idea that AcP10 selectively prevents the interaction of eIF2B with the inhibitory eIF2α-P, promoting the continuation of translation. This mechanism would be effective against any activated eIF2α kinase, including GCN2, due to it acting downstream of eIF2α phosphorylation. Aichivirus (AiV, a human gastroenteric *Picornaviridae*) expresses the Leader protein (AiVL) that exhibits a similar role as AcP10 in preventing the association of eIF2α-P with eIF2B.[138] Interestingly, AiVL and AcP10 have no sequence homology, suggesting their emergence through convergent evolution.

IVg) Other mechanisms

This chapter highlights additional mechanisms by which viruses may hamper the anti-viral activity of GCN2, or leverage the GCN2/ATF4 axis to support viral replication.

Could the HIV IN protein impede the interaction between the YPK and PK-domains in GCN2? As previously mentioned, GCN2 phosphorylates IN to hamper its function of integrating the HIV DNA into the host genome, thus impairing HIV replication (see IIId). IN and GCN2 interact with each other, and this interaction is mediated by amino acids 350–500 in yeast Gcn2, as determined by yeast 2-hybrid assays.[13] This GCN2 portion encompasses part of the GCN2 YPK but not the PK-domain[1] (Figure 4b). However,

to allow GCN2-mediated IN phosphorylation, one would expect that IN binds to the GCN2 PK, and not to the YPK which is catalytically inactive. It is possible that YPK-bound IN is sufficiently close to the PK-domain for it to be phosphorylated. Alternatively, given that the YPK domain does share homology to the PK-domain, it is possible that IN interacts with a region conserved between these two domains. Indeed, IN contains an area similar to the region of eIF2α involved in PK docking (see IVa), supporting the hypothesis that it is able to bind to both of these domains. No extensive efforts have been made yet to test whether IN does contact the PK-domain, highlighting this as an intriguing area of inquiry.

Studies in yeast have revealed that in Gcn2, the YPK domain must interact directly with the PK-domain to facilitate the stimulation of its catalytic activity.[34] This raises the possibility that IN interferes with the process of GCN2 activation. In this scenario, the binding of IN to the YPK domain would prevent the intramolecular interaction between the YPK and PK-domains, thereby impeding GCN2 stimulation. More studies are necessary to fully understand the molecular relevance of IN-YPK interaction. Since the other eIF2α-kinases do not possess a YPK, this IN-YPK interaction is specific to the eIF2α-kinase GCN2.

Some viruses exploit the GCN2/ATF4 axis to their benefit. Activation of an eIF2α-kinase ultimately leads to the increased translation of transcription factor ATF4. This in turn brings about a shift in the cell's gene expression profile, enabling the cell to adapt to the stress condition.[1] Interestingly, HIV leverages the early anti-viral defence mediated by this GCN2/ATF4 axis to establish viral replication. Moreover, HIV utilises ATF4 expression to induce HIV reactivation.[15,139] For example, it has been found that in $CD4^+$ Jurkat T cells, HIV-1 infection results in a marked increase in *ATF4* mRNA and protein levels. This in turn leads to elevated levels of HIV proviral DNA and a higher titre of retroviral particles.[139] Additionally, in 293 T cells, siRNA mediated knockdown of *ATF4* leads to a decrease in HIV particles.[140] These findings support the idea that ATF4 supports HIV replication.

Given the inhibitory effect of the ISR on viral replication, how could increased ATF4 protein levels in fact be beneficial to HIV? Increased HIV-replication depends on the transcriptional activation of its Long Terminal Repeat (LTR), a DNA element that contains the HIV enhancer and promotor.[141] ATF4 was found to enhance the activity of the HIV protein Trans-activator of transcription (Tat) in LTR activation,[139,140] suggesting that HIV takes advantage of virally-induced GCN2-activation to leverage ATF4 for enhanced viral replication (Figure 4g). Interestingly, ATF4 abundance is further enhanced by the HIV Viral protein U (Vpu) which appears to protect ATF4 from degradation, thereby increasing the ATF4 half-life.[142]

Similar mechanisms are indicated to exist for Simian Immunodeficiency Virus (SIV), and the Human T-cell Leukaemia Virus (HTLV).[143,144,15] The latter can cause the development of adult T-cell leukaemia, or a neurodegenerative disease termed tropical spastic paraparesis, also called HTLV-I-associated myelopathy.[145] As an example, in the case of HTLV, it was found that its protein Transactivator encoded by the X gene region (Tax) binds to ATF4 *in vitro and* enhances Tax-mediated transactivation of LTR in reporter gene assays.[143,144] This suggests that the GCN2/ATF4 axis is exploited by various *Lentiviruses*.

The GCN2/ATF4 axis also appears to be exploited by EBV.[79] As described previously, the activity of the EBV protein BPLF1 indirectly leads to GCN2 activation (see IIIc). Strikingly, it has been found that BPLF1 promotes the translation of EBV Nuclear Antigen 1 (EBNA1) in a manner that is dependent on GCN2. Two possible scenarios have been proposed by which this could be accomplished. One is based on the fact that the EBNA1 ORF could form G-quadruplex (G4) structures, which could function as 'roadblocks' for elongating ribosomes. It was proposed that BPLF1-mediated impairment of the RQC could somehow allow the ribosomes to overcome these G4 structures, though the exact mechanism of how this would be accomplished has not been expounded. The other proposed scenario is the increased cap-independent translation through EBNA1 IRESs and/or uORFs as a result of GCN2 function, resulting in enhanced synthesis of the EBNA1 protein.[146] In support of this, bicistronic reporter assays have shown that GCN2 can increase the level of HCV IRES-dependent translation, leading to an increased ratio of IRES-mediated versus cap-dependent translation (Figure 4e).[147] Furthermore, inhibitory uORFs have been described in Ebola Virus (EBOV) which allow it to produce more infectious particles when the cell mounts a response to stress.[148] Similar to the uORF-mediated upregulation of *ATF4* translation in response to stress (see IIc), reporter gene assays suggest that – in the presence of elevated eIF2α phosphorylation – the uORFs in EBOV RNA facilitate increased translation of a gene encoding a viral RNA polymerase (Figure 4f).[148] More work is necessary to reveal the exact role GCN2 plays in mediating enhanced EBNA1 translation. Nevertheless, it is apparent that GCN2 signalling is not only implicated in fighting viral infections, but in this case is advantageous for the production of certain viral proteins.

Whilst EBV utilises the vDUB activity of BPLF1 to exploit the GCN2 pathway for its benefit, this mechanism is not shared by all *Herpesveridae* vDUB-containing proteins.[79] However, Human *Cytomegalovirus* (HCMV) Unique Long region protein 48 (UL48) and Kaposi's Sarcoma-associated Herpesvirus (KSHV) ORF64 appear to utilise a similar mechanism to that of EBV.[79]

Non-AUG translation start sites which are unaffected by eIF2α phosphorylation, have also been reported to facilitate enhanced viral protein synthesis. One example is the efficient translation of the Sindbis Virus (SV) subgenomic mRNA (sgRNA) (Figure 4f).[149]

There are many more examples of viruses utilising nonstandard pathways to translation initiation that have not yet been explored in relation to GCN2 or the ISR,[146] but these are beyond the scope of this review.

Together, it is evident that GCN2 signalling is not only implicated in fighting viral infections, but in some cases actively supports viral replication. The latter could be a novel target for medical interventions. In this scenario, a drug would inhibit GCN2 to hamper the rate of viral replication. This intervention would be most appropriate in treating viruses that are resistant to the anti-viral effects of GCN2.

V) Conclusion and Perspectives

There are a vast number of viruses on our planet, and most of them have not been characterised yet,[150] suggesting that there are likely more mechanisms of GCN2 inhibition or exploitation that are yet to be uncovered. Furthermore, phylogenetic analyses suggest one of these inhibitory mechanisms has recently emerged,[115] underscoring the fact that viruses are constantly evolving and new virulence mechanisms against the ISR will likely continue to surface.

Various viruses have developed a wide range of strategies to inhibit eIF2α-kinases, highlighting the crucial role of these kinases in viral defence. In addition, the fact that some viruses invest in multiple avenues to counteract eIF2α-kinase function underscores the significance of this process to the viral replication cycle. For example, in HSV-1 at least four different mechanisms have been uncovered that counteract the ISR: Unique short region protein 11 (Us11) antagonises PKR,[151,152] glycoprotein B (gB) regulates viral protein accumulation by physically associating with PERK,[153] gH interacts with GCN1 to hamper GCN2 activation (see IVc), and ICP34.5 counteracts all eIF2α-kinases by associating with PP1c to promote the dephosphorylation of eIF2α-P (see IVe). In the case of HIV, the Adenosine Deaminase Acting on RNA protein 1 (ADAR1) binds to and inhibits PKR, and high levels of *Trans*-activating response RNA (TAR) element also inhibits PKR.[154] HIV proteases specifically cleave GCN2 (see IVd), and ATF4 is exploited for transactivation of the viral LTR (see IVg). These examples demonstrate that, while PKR is currently considered the primary eIF2α-kinase in viral defence, the other eIF2α-kinases – such as GCN2 – also play significant roles in anti-viral defence.

This review has highlighted the underlying role of GCN2 in the anti-viral response. Interestingly, several viruses have developed various mechanisms to counteract – or even exploit – GCN2. A notable example is the specific cleavage and inactivation of GCN2 by HIV^{Pro} (see IVd). An elegant back-and-forth rivalry unfolds between HIV and GCN2, as GCN2 is required to fight against HIV infection (see I, IIIb). Part of the anti-viral role of GCN2 is independent of eIF2α phosphorylation but instead relies on the GCN2-mediated phosphorylation of IN, which hampers the ability of HIV to integrate into the host genome (see IIId). Conversely, translation of *ATF4*, which occurs downstream of GCN2 activation, bolsters viral replication, highlighting that viruses also exploit GCN2 for their own benefit. In the case of HIV infection, it is still unclear whether GCN2 can be better described as a friend or a foe of the virus. One might envisage that a delicate balance exists between these contradictory roles of GCN2 in HIV infection, where on one hand the GCN2/ATF4 axis hampers viral integration but on the other hand supports HIV replication. As viruses are known for their ability to mutate rapidly, it is possible that the answer to this differs between viral strains, alongside the specific host organism or tissue infected and the stage of infection. Thus, it is important to scrupulously consider the experimental conditions in research settings, as this will ensure the nuanced relationship between viruses and the GCN2/ISR axis is captured in full detail.

It is evident that, when considering anti-viral strategies targeting GCN2, it is crucial to first ascertain whether the infecting virus is susceptible to GCN2 activity or if it exploits GCN2 for its own benefit. The same is likely true for the other eIF2α-kinases. However, just because a virus can both be hampered by and benefit from GCN2, this does not mean that GCN2 cannot be used as an anti-viral target. For example, compounds are currently being investigated that increase the GCN2-IN interaction and GCN2-mediated IN phosphorylation (see IIId), which will aid the host in more effectively hampering retroviral integration into the host genome.[155] Targeting GCN2 in this way decreases the chance of supporting viral replication, as such a compound is unlikely to lead to increased eIF2α phosphorylation. In another example, it has been suggested to stimulate the ISR to actively reverse HIV latency, thereby reducing the HIV reservoir which poses a significant obstacle to eradicate HIV infection in a patient.[156] The idea is to substantially decrease the size of the HIV reservoir, thereby delaying or even preventing HIV rebound following anti-retroviral treatment. ISR stimulation leads to LTR activation (see IVg), concomitant transcription of the viral genes, and ultimately to viral replication. This reactivated HIV is susceptible to anti-retroviral drugs, as it requires

the reverse transcriptase to replicate and integrate into the host genome. In addition, ISR stimulation can lead to enhanced apoptosis of HIV infected cells *in cellulo*,[156] and it will be interesting to investigate whether the same can be achieved in the context of an organism. These above examples demonstrate that even leveraging the ways viruses exploit GCN2 could present new therapeutic possibilities. Therefore, the subject of GCN2 aiding viral replication is a largely underexplored but promising topic for further research and developing novel antiviral treatment measures.

The emergence of the next pandemic is not a matter of "if" but "when". Given the recurring emergence of pandemics and their profound impact on lives, society, and the economy (e.g. Spanish flu, Avian flu, Swine flu, Middle East Respiratory Syndrome/MERS, Ebola, Zika, COVID19), it is crucial to prioritize the treatment and prevention of such communicable diseases.[157–160] Anti-viral treatments should be researched proactively, rather than waiting for a crisis to prompt their development. Only by doing so can we prevent the devastation that ensues. In this vein, understanding in detail the mechanisms by which viruses counteract host defence processes is an essential prerequisite for developing effective prevention and treatment strategies. This also includes viral strategies that thwart eIF2α-kinases like GCN2. Treatment measures could be used either alone or in combination with other treatments to help combat the development of treatment resistance.

Anti-viral treatments will not only benefit humans, but also other eukaryotic life forms such as economically indispensable livestock and crops. Therefore, it is imperative to not only research the molecular pathways involving human viruses but also those of other lifeforms. As many viral families infect both humans and other species, research into one viral species may indeed benefit research into those species infecting other hosts.

Aside from the obvious application of this knowledge to fighting viral infections, understanding the relationship between viruses and GCN2 could help develop treatments to unrelated diseases. Since eIF2α-kinases have been implicated in various non-viral diseases or disorders, a comprehensive understanding of viral inhibitory mechanisms against specific eIF2α-kinases holds the potential to guide the development of targeted treatments of human diseases. In the case of GCN2, these include cancer, Alzheimer's disease, autoimmune diseases such as systemic lupus erythematosus, pulmonary disorder, and metabolic disorders.[1,161–164] As the drug development pathway is lengthy and expensive, using the already established viral mechanisms of inhibiting GCN2 holds the promise of accelerating the development of much-needed treatments. This would bypass the early stages of drug design and development, reducing risks such as financial loss and medical futility. In this way, patients could have access to life-changing treatments years earlier than could otherwise be possible.

Viruses also infect plants, which can lead to large economic losses,[165] warranting research into measures to combat plant viruses. Plants harbour GCN2, and contradicting hypotheses have been reported as to whether plants contain a functional orthologue of PKR or not. Furthermore, on one hand studies suggest that GCN2 does not respond to viral infections, while other reports suggest a link between plant GCN2 and viral infection.[166] It is possible that – as found for mammalian viruses – only a selection of plant viruses elicit a GCN2 antiviral response. More work is necessary to ascertain a possible role of plant GCN2 in viral defence.

In summary, it has become evident that further research aimed at deciphering the detailed role of GCN2 in anti-viral defence, and how viruses counteract or exploit GCN2, holds many economic and medical benefits for society.

CRediT authorship contribution statement

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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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viral virulence factors

Abbreviations:
A-site, Aminoacyl tRNA binding site; **AcMNPV**, *Autographa californica* multiple Nucleopolyhedrovirus; **AcP10**, Open reading frame 10 (ORF10)-encoded accessory protein; **ADAR1**, Adenosine deaminase acting on RNA protein; **AiV**, Aichivirus; **AiVL**, Aichivirus leader protein; **AmEPV**, *Amsacta moorei* Entomopoxvirus L; **AMIV**, *Anopheles minimus* Irodovirus; **ASFV**, African swine fever virus; **ASV**, Avian sarcoma virus; **ATF4**, Activating transcription factor 4; **BLAST**, Basic local alignment search tool; **BMDCs**, Bone marrow derived dendritic cells; **Bw-CoV SW1**, Beluga whale Coronavirus strain SW1; **cat-1**, High affinity cationic amino acid transporter 1; **COVID-19**, Coronavirus disease 2019; **CMLV032**, Camelpox virus; **CNPV**, Canarypox virus; **CTD**, Carboxy-terminal domain; **COS**, CV-1 in origin with SV40 genes; **DENV**, Dengue virus; **dsRNA**, Double-stranded RNA; **E2**, Envelope protein 2; **E6**, Early protein 6; **EBNA1**, EBV nuclear antigen-1; **EBOV**, Ebola virus; **EBV**, Epstein-Barr virus; **eIF2α**, α subunit of eukaryotic translation initiation factor 2; **eIF2α-P**, Phosphorylated eIF2α; **EIF2AK4**, Eukaryotic translation initiation factor 2-alpha kinase 4; **eIF2B**, Eukaryotic translation initiation factor 2B; **eGFP**, Enhanced green fluorescent protein; **GADD34**, Growth arrest and DNA damage gene 34; **GAR**, GCN2 activating RNA; **gB**, Glycoprotein B; **GCN1 and Gcn1**, General control non-derepressable 1 from mammals and yeast respectively; **GCN2 and Gcn2**, General control non-derepressable 2 from mammals and yeast respectively; **Gcn4**, General control non-derepressable 4; **gH**, Glycoprotein H; **GpSGHV**, *Glossina pallidipes* salivary gland hypertrophy virus; **HCMV**, Human *Cytomegalovirus*; **HCV**, Hepatitis C virus; **HEK**, Human embryonic kidney cell line; **HeLa**, Henrietta Lacks cell line; **HEp-2**, Human epidermoid cancer cells 2; **HisRS**, Histidyl-tRNA synthetases; **HIV-1 and HIV-2**, Human immunodeficiency virus 1 and 2; **HIV-1^{Pro} and HIV-2^{Pro}**, HIV protease; **hmDCs**, Human monocyte-derived dendritic cells; **HPV**, Human papilloma virus; **HRI**, Haem-regulated inhibitor of translation; **HSV-1 and HSV-2**, Herpes simplex virus 1 and 2; **HTLV**, Human T cell leukaemia virus; **IAV**, Influenza A virus; **IBV**, Infectious bronchitis virus; **ICP34.5**, Infected cell protein 34.5; **IE63 and IE180**, Immediate early 63 and 180; **IN**, Integrase; **IRES**, Internal ribosome entry site; **ISR**, Integrated stress response; **KSHV**, Kaposi's sarcoma-associated Herpesvirus; **LC-MS**, Liquid chromatography-mass spectrometry; **LTR**, Long terminal repeats; **MaHV**, *Macropodid herpesvirus*; **MCMV**, Mouse *Cytomegalovirus*; **MEFs**, Mouse embryonic fibroblasts; **MHV-3**, Murine hepatitis virus 3; **MLV**, Murine leukaemia virus; **MNV**, Murine *Norovirus*; **MYXV**, Myxoma virus; **NCBI**, National Centre for Biotechnology Information; **NS2A and NS4A**, Non-structural proteins 2A and 4A **NSs**, Non-structural protein, S segment; **OB**, Oligonucleotide binding; **ORF**, Open reading frame; **PePHD**, PKR eIF2α phosphorylation site homology domain; **PEK**, Pancreatic endoplasmic reticulum kinase; **PERK**, Protein kinase R-like endoplasmic reticulum kinase; **PFV**, Prototype foamy virus; **PK**, Protein kinase; **PK2**, Protein resembling a protein kinase 2; **PKR**, Protein kinase R; **PP1**, Protein phosphatase 1; **PP1c**, PP1 catalytic subunit; **PRV**, Pseudorabies virus; **siRNA**, Small inhibitory RNA; **RQC**, Ribosome quality control; **RT-PCR**, Reverse transcription PCR; **RVFV**, Rift valley fever virus; **RWD**, Domain present in RING finger proteins, WD-repeat-containing proteins, and DEAD-like helicases; **SARS-CoV**, Severe acute respiratory syndrome coronavirus; **SF9**, *Spodoptera frugiperda* cell line; **SFSV**, Sandfly fever Sicilian virus; **SFV**, Sandfly fever virus; **SIV**, Simian immunodeficiency virus; **ST cells**, Swine testicular cells; **SV**, Sindbis virus; **TAR**, Trans-activating response; **Tat**, Transactivator of transcription; **Tax**, Trans-activator encoded by the X gene region; **TNAV2c**, *Trichoplusia ni* Ascovirus 2c; **tRNA**, Transfer RNA; **UL48**, Unique long region protein 48; **uORF**, Upstream ORF; **Us11**, Unique short region protein 11; **VARV**, Variola virus; **vDUB**, Viral ubiquitin deconjugase; **vIF2α**, Viral protein with homology to eIF2α; **Vpu**, Viral protein U; **VSV**, Vesicular stomatitis virus; **VV**, Vaccinia virus; **VZV**, Varicella zoster virus; **YF-17D**, Yellow fever strain 17D used as a live attenuated vaccine; **YFV**, Yellow fever virus; **YPK**, Pseudokinase

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