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Viruses, when entering their host cells, are met by a fierce intracellular immune defense. One prominent antiviral pathway is the integrated stress response (ISR). Upon activation of the ISR — typically though not exclusively upon detection of dsRNA — translation-initiation factor eukaryotic initiation factor 2 (eIF2) becomes phosphorylated to act as an inhibitor of guanine nucleotide-exchange factor eIF2B. Thus, with the production of ternary complex blocked, a global translational arrest ensues. Successful virus replication hinges on effective countermeasures. Here, we review ISR antagonists and antagonistic mechanisms employed by picorna- and coronaviruses. Special attention will be given to a recently discovered class of viral antagonists that inhibit the ISR by targeting eIF2B, thereby allowing unabated translation initiation even at exceedingly high levels of phosphorylated eIF2.

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**Integrated stress response**

The ISR is an ancient salvaging pathway allowing cells to cope with physiological changes brought about by extrinsic and intrinsic stress factors such as hypoxia, amino acid deprivation, glucose deprivation, or accumulation of unfolded proteins in the ER (reviewed in [1,3]). These stressors are sensed by a family of protein kinases, that is, heme-regulated eIF2α kinase (HRI), general control nonderepressible 2 (GCN2), and PKR-like ER kinase (PERK). Vertebrates acquired another eIF2α kinase, the dsRNA-dependent protein kinase-R (PKR), which plays a key role in antiviral defense [3]. Upon detection of their specific stimuli, these protein kinases homodimerize, activate through autophosphorylation to then converge on phosphorylation of the alpha-subunit of eukaryotic initiation factor 2 (eIF2α). This has significant implications for translation initiation, which critically relies on the availability of ternary complexes (TCs) comprised of eukaryotic initiation factor 2 (eIF2), guanosine-5'-triphosphate (GTP), and initiator methionyl-tRNA (Met–tRNAi). Upon delivery by TC of Met–tRNAi to initiating ribosomes, GTP is hydrolyzed, necessitating continuous replenishing of eIF2-GDP into its active form via nucleotide exchange. This process is catalyzed by guanine nucleotide-exchange factor (GEF) eIF2B, a twofold
symmetric heterodecameric complex comprised of two copies each of subunits designated α through ε (see [4], and references therein). Phosphorylated eIF2, eIF2(p), acts as a competitive inhibitor and, because cellular concentrations of eIF2B are limiting, increasing levels of eIF2(p) decrease TC levels and thereby canonical translation initiation [5]. Recent structural studies revealed eIF2α and eIF2α(p) bind eIF2B at distinct, nonoverlapping sites and that eIF2β–eIF2 complexes occur in two widely different conformations, an enzymatically active A-state and a GEF-inactive I-state (Figure 3) [6–9]. Phosphorylation of eIF2α proposedly shifts the equilibrium to the I-state to the effect that binding of eIF2 is reduced and nucleotide-exchange activity diminished.

The ISR-induced drop in TC levels results in translational reprogramming. While global translation is strongly reduced, leaky-scanning-dependent translation of select mRNAs is favored, including that of activating transcription factor 4 (ATF4), a key regulator of stress-responsive genes. ATF4 drives expression of growth arrest and DNA damage-inducible protein 34 (GADD34), which together with the catalytic subunit of protein phosphatase 1 (PP1c), assembles into the eIF2α phosphatase complex. This complex initiates a feedback loop to terminate the ISR, thus allowing cells to resume normal protein synthesis and to return to homeostasis once stress is alleviated. Upon persistent stress and/or failure to restore homeostasis, ATF4 signaling promotes apoptosis [1].

Polysome dissociation, ensuing activation of the stress response, results in an excess of stalled 48S preinitiation complexes, which are stored in cytoplasmic membrane-less organelles called stress granules (SGs) [10]. The SGs serve as deposits from which mRNAs can be rapidly retrieved, poised for translation through their remaining association with critical components of the translation machinery. SGs, however, are also considered a coordinating platform for antiviral signaling since many antiviral sensors such as OAS and the RLRs retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) are recruited to ISR-induced SGs [11].

The different classes of viral integrated stress response antagonist proteins and strategies

Attesting to the importance of the PKR branch of the ISR as an antiviral defense mechanism, DNA and RNA viruses encode proteins that specifically counteract this pathway to avert translational arrest. These can be divided into distinct categories based on their mode of action (Figure 1) [12••]. Class-I antagonists act at the level of the stressor, sequestering or degrading dsRNA to prevent PKR activation. Class-II antagonists rather affect the sensor, in some cases by inhibiting PKR phosphorylation, in other cases by inactivating PKR, either through cleavage by viral proteases or by triggering its degradation. Class-III antagonists leave PKR unaffected but instead induce eIF2(p) dephosphorylation to prevent inhibition of eIF2B GEF activity. Finally, the newly discovered class-IV antagonists act at the level of eIF2B, allowing continued TC formation and canonical translation initiation even at exceedingly high concentrations of eIF2(p). Of note, the class-III and -IV antagonists counter the ISR, irrespective of the initiating kinase. Some viral proteins act even further downstream, countering ISR-induced SG formation directly, possibly to block SG-promoted activation of other antiviral pathways. For a recent review on PKR antagonists see [13].

In addition to these protein-based strategies, viruses may employ mechanisms in which specific RNA structures promote noncanonical, TC-independent translation initiation. For example, alphaviruses rely on an RNA hairpin loop structure located downstream the start codon. This structure stalls the ribosomes on the correct site for initiation of translation, which bypasses the requirement for a functional eIF2, and therefore renders alphavirus translation unconstrained by the ISR [14,15].

Picornavirus integrated stress response antagonists

Picornaviruses are small, naked +RNA viruses (> 150 species grouped in 68 genera) that can infect a wide variety of hosts and that have important clinical and socioeconomic impact. Their genome contains an internal ribosome entry site (IRES) in the 5′UTR and encodes a single polyprotein that is processed by viral proteases to yield capsid proteins and several non-structural proteins (NSPs) (Figure 2a). Among the NSPs, the Leader (L), which is present in many but not all picornaviruses, and the 2A proteins play important roles in antagonizing host innate antiviral responses [16]. Although these ‘security proteins’ share common names based on their position in the polyprotein, they are often structurally and biochemically unrelated, likely acquired via independent evolutionary acquisition [16]. Below, we will discuss how members of three different genera of picornaviruses (Enterovirus, Aphthovirus, and Cardiovirus) antagonize or circumvent the ISR and/or SG formation.

Enteroviruses (e.g. poliovirus, coxsackievirus, echovirus, numbered enteroviruses such as EV-A71 and EV-D68, and rhinovirus) are important pathogens for humans. The 2A protein, which is a protease in enteroviruses, plays an important role in controlling translation and stress responses in infected cells. 2A(pro) rapidly shuts off host mRNA translation, proposedly to prevent
expression of antiviral proteins, by cleaving eIF4G, an initiation factor that is important for cap-dependent host mRNA translation but not viral IRES-mediated translation [17–19]. Additionally, enteroviruses, such as other picornaviruses, can activate PKR and PERK, by producing significant amounts of dsRNA and by modifying ER and Golgi functions to build viral replication organelles, respectively, resulting in eIF2α phosphorylation and further repression of host translation [20–22]. Importantly, eIF2α phosphorylation does not affect viral translation because 2Apro, through an unknown mechanism but dependent on its proteolytic activity, confers eIF2 independence to IRES-driven translation [23,24].

The ISR in picorna- and coronavirus-infected cells

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Figure 1

Schematic representation of integrated stress response and different classes of viral antagonists. Canonical translation initiation critically requires eIF2–GTP–Met–tRNAi TCs. The formation of TCs requires the activity of the guanine nucleotide-exchange factor eIF2B, which catalyzes the nucleotide exchange of eIF2–GDP into its active form eIF2–GTP. The integrated stress response is constituted by a set of four kinases, which, in response to a range of stimuli, phosphorylates eIF2α. Phosphorylated eIF2α is a competitive inhibitor of eIF2B, thereby downregulating TC formation resulting in translational inhibition. The buildup of inactive mRNA-ribosome complexes finally culminates in the formation of SGs. Viruses have evolved a wide range of antagonists and antagonistic mechanisms to counteract the ISR at different levels. These can be assigned to specific classes based on their mechanism of action.
Aphthoviruses (e.g. foot-and-mouth disease virus, FMDV) encode for an L protein with proteolytic activity. Through its proteolytic activity, $L^{\text{pro}}$, such as enterovirus 2A$^{\text{pro}}$, shuts off host translation by cleaving eIF4G [34], confers eIF2 independence to IRES-driven translation [35], and interferes with the formation of SGs, the latter exemplified by the formation of SGs during infection with $L^{\text{pro}}$-defective FMDV but not wt FMDV [36]. $L^{\text{pro}}$ can cleave SG scaffold proteins G3BP1 and G3BP2 [36], but evidence that this cleavage is essential for the inhibition of SG formation is lacking. Hence, it cannot be excluded that the inhibition of SG formation by $L^{\text{pro}}$ is due to cleavage of other cellular factors and/or disruption of the interaction between eIF4G and G3BP, as described above. The viral protease 3C$^{\text{pro}}$ may also play a role in suppressing the ISR by triggering the lysosomal degradation of PKR, although the importance hereof is unknown as it occurs relatively late in infection [37]. Moreover, like enterovirus 3C$^{\text{pro}}$, FMDV 3C$^{\text{pro}}$ also cleaves G3BP1, but it is unknown whether this activity contributes to inhibit SG formation or to counter the direct antiviral activity of G3BP1 to suppress genome replication by binding to the IRES [38,39].

Cardioviruses (e.g. Encephalomyocarditis virus (EMCV) and Theiler’s encephalomyelitis virus) are equipped with L and 2A proteins but these lack protease activity, and infection with these viruses does not lead to eIF4G cleavage and host-translation shutoff. These viruses actively suppress the ISR via their L protein [36,40,41•], which, as a class-II antagonist, interferes with PKR activation, eIF2$\alpha$ phosphorylation, and SG formation. L inhibits PKR activation via a novel and indirect mechanism. Unlike other viral class-II ISR antagonists, L does not interact with PKR. Instead, it interferes with dsRNA association to PKR by binding and hijacking host kinases, RSKs [41•,42••]. How this suppresses PKR activation is unknown, but this observation adds to the notion that regulation of PKR activation is more complex than generally assumed, as also recently proposed by others [43,44]. Additionally, it has been suggested that L interferes with the interaction between eIF4G and G3BP to suppress SG formation [27•]. Like in cells infected with enteroviruses and aphthoviruses, cardiovirus translation becomes eIF2-independent.
during infection [45]. Both the identity of the viral protein responsible for this effect and the underlying mechanism are unknown.

Coronavirus integrated stress response antagonists

Coronaviruses (subfamily Orthocoronavirinae with genera alpha-, beta-, delta-, and gammacoronavirus) are enveloped positive-strand RNA viruses of mammals and birds. Compared with picornaviruses, coronaviruses not only have a much larger genome (~30Kb, roughly four times larger than that of poliovirus), but also a far more elaborate replication. Upon entry, the genome is translated into two large polyproteins, pp1a and pp1ab, which are autocatalytically cleaved to yield 15–16 NSPs and an unknown number of functional intermediates (Figure 2b). NSP-induced remodeling of host cell membranes results in the formation of replication neo-organelles (ROs) comprised of double-membrane vesicles (DMVs). These ROs harbor the macromolecular NSP complexes required for genome multiplication and, later during infection, the synthesis of a 3’ coterminal nested set of subgenomic mRNAs (sgmRNAs) [46,47]. The latter encode subfamily-wide conserved structural proteins and various accessory proteins (AcPs) that are (sub)genus and occasionally even virus-species and which function mainly to counteract cellular defenses [47]. Genome replication and sgRNA synthesis from dedicated minus-strand templates occurs within the confines of the DMVs, such that dsRNA would be largely excluded from cytoplasmic sensors, with pores allowing ssRNA export into the cytosol [48–50].

To prevent activation of the PKR branch of the ISR, the OAS–RNase-L system, and type-1 IFN responses, coronaviruses encode a range of specific antagonists. One of these, NSP15, a universally conserved endoU-ribonuclease, counteracts activation of all three pathways. It cleaves both ssRNA and dsRNA species 3’ of uridine bases to antagonize antiviral signaling [51••–56]. Localizing to ROs, apparently in transient association with replication-transcription complexes (RTCs), NSP15 may act as a gatekeeper to prevent escape of dsRNA from the protective surroundings of the ROs [51••,52,57,58]. Upon catalytic inactivation of NSP15, an increase in dsRNA levels was observed during infection [51••]. Moreover, the dsRNA appeared to be more dispersed throughout the cytosol [52]. NSP15-defective viruses are severely attenuated due to activation of the ISR, the OAS–RNase-L system, and IFN signaling [51••,53,59,60•]. Consequently, NSP15-defective murine hepatitis virus (MHV) (genus Betacoronavirus subgenus Embeccovirus) was rapidly cleared and avirulent in a murine model, in stark contrast to the wild type (wt) virus [52].

Still, NSP15 alone does not seem to be entirely sufficient to prevent innate immune signaling. The nucleocapsid protein (N), shared by all CoVs and expressed in large amounts from sgmRNAs later during infection, has also been implicated in antagonizing dsRNA-dependent signaling. Like NSP15, N is a class-I ISR antagonist and aside from its main function in genome packaging, prevents PKR activation, ISR-induced translational arrest, SG formation, as well as induction of beta-interferon by sequestering dsRNA. Recent findings in our laboratory showed that this activity can be assigned to the dsRNA-binding domain N2b (Aloise et al., BioRxiv DOI = 10.1101/2022.09.02.506332). Interestingly, the continued evolution of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2, genus Betacoronavirus, subgenus Sarbecovirus) has given rise to variants that express a truncated C-terminal portion of N, designated N-iORF3, from a novel sgRNA species. N-iORF3, encompassing the C-terminal N2b domain and surrounding linker regions, inhibits the dsRNA-dependent IFN response (Mears et al., BioRxiv DOI = 10.1101/2022.04.20.488895), and overexpression experiments in our laboratory showed that it also blocks the ISR (Aloise et al., BioRxiv DOI = 10.1101/2022.09.02.506332). In interactome studies, SARS-CoV-2 N was found to bind to SG assembly factors G3BP1 and G3BP2 via its N-terminal domain N1a, suggesting that it may also directly suppress SG formation [61–67•], although this may depend on the cell type [82].

Aside from the conserved structural and NSPs, coronaviruses also encode a wide range of highly divergent AcPs, some of which have been found to inhibit the ISR. For example, the AcP 4a of Middle East respiratory syndrome coronavirus (MERS-CoV; genus Betacoronavirus, subgenus Merbecovirus) sequesters dsRNA through its RNA-binding domain to antagonize dsRNA-dependent signaling [68–70]. The observation that both NSP15 and 4a act as class-I antagonists raises questions about their individual contribution in countering antiviral responses. Infection with MERS-CoV lacking a functional 4a protein resulted in the formation of SGs in HeLa cells, but not in Vero cells [68,69]. Thus, redundancy might have evolved to effectively counteract antiviral responses in the context of certain hosts and cell types. Moreover, different antagonists may function at distinct times and subcellular locations over the course of the viral life cycle. A recent study showed that MERS-CoV NSP15, 4a, and AcP 4b — an antagonist of the OAS–RNase L pathway [71] — jointly act to suppress dsRNA-mediated innate immunity during infection of primary lung and nasal epithelial cultures [60•], demonstrating the necessity of redundancy to oppose the host defense in these tissues.

A class-III ISR inhibitor was identified in members of the alphacoronavirus subgenus Tegacovirus. The AcP 7, a
functional homolog of GADD34, acts as a scaffold to recruit PP1c and eIF2α(p) to promote eIF2α(p) dephosphorylation [72]. Finally, our laboratory recently identified an AcP, AcP10, of a cetacean gammacoronavirus as the first example of a class-IV ISR inhibitor [12••]. The mode of action of AcP10 and other class-IV inhibitors will be discussed in more detail below. Coronavirus class-III and -IV inhibitors may also contribute to counter translational arrest due to activation of other ISR kinases, such as PERK, which may be activated in response to ER stress as caused by virus-induced membrane reorganization or high levels of envelope glycoproteins in the secretory pathway of infected cells.

**Class IV: a novel class of integrated stress response antagonists**

Class I through III ISR inhibitors, preventing or reversing eIF2α phosphorylation, have been described for many different RNA and DNA viruses (recently reviewed in [13]). Studies in our laboratory provided first evidence for the existence of yet another class of ISR antagonists acting at a more downstream level [12••]. While studying the AcP10 of Beluga Whale coronavirus-SW1 (BW-CoV SW1; genus Gammacoronavirus, subgenus Cegacovirus), we observed that its transient expression averted ISR-associated translational arrest. Remarkably, AcP10 inhibited the activation of the ISR by both PKR and HRI. Moreover, excluding a class-III mechanism, AcP10 prevented translational arrest even at very high cellular levels of eIF2(p). In mass spectrometry-based immunoprecipitation proteomics experiments with AcP10 as bait, all five eIF2B subunits were identified. Reciprocal pulldowns confirmed AcP10–eIF2B association. Interestingly, AcP10–eIF2B complexes also contained eIF2 but not eIF2(p). The data pointed to a novel mechanism in which AcP10 competes with eIF2(p) but not with eIF2, for binding to the eIF2B complex. Thus, continued eIF2B GEF activity and TC formation is ensured even under stress conditions in which the majority of the cellular eIF2 pool is phosphorylated. The selective inhibition of eIF2(p)–eIF2B association also led us to conclude eIF2 and
eIF2(\(p\)) must differ in their interaction with eIF2B, as since confirmed by structural analysis [4,6–9].

In parallel, we discovered a class-IV ISR inhibitor in picornaviruses. We showed that the L protein of Aichivirus (AiV, genus Kobuvirus), such as AcP10, prevents translation arrest during conditions of strong eIF2\(\alpha\) phosphorylation by binding to eIF2B [12••]. Replication of L-defective AiV was severely hampered and resulted in formation of SGs, which were absent in wild-type AiV-infected cells. Upon knockout of PKR, no SGs were formed and replication was rescued, underscoring the importance to the virus of blocking the PKR-activated ISR pathway. Recently, another class-IV ISR inhibitor, was identified in a negative-stranded RNA virus, the NSP NSs of Sandfly Fever Sicilian virus (SFSV), a member of the arthropod-transmitted genus Phlebovirus (family Phenuiviridae, order Bunyavirales) [73]. Notably, this function is not conserved in NSs from Rift Valley fever virus, another phlebovirus [74]. The SFSV NSs, BW-CoV AcP10 and AiV L proteins, do not share any similarity in sequence or predicted structure [12••,75••,76••], indicating that they arose independently via convergent evolution. Although they all prevent eIF2B inactivation by eIF2(\(p\)), it is unknown whether they do so via the same molecular mechanism. Structural analysis of a NS–eIF2 complex showed that NSs block the eIF2(\(p\))-binding site (Figure 3). However, whereas binding of eIF2(\(p\)) forces the eIF2B heterodecamer into an GEF-inactive conformation, NS binding preserves GEF activity by maintaining eIF2B’s conformation in its active state, thus allowing binding of eIF2 and continued GDP/GTP exchange (Figure 3a) [75••,76••]. Currently, no structures are available of AiV L or AcP10-bound eIF2B. Cross-linking mass spectrometry experiments suggest that AcP10 binds as a multimer that would cover a large area encompassing the eIF2(\(p\))-binding site (i.e. cross-links were found with both the eIF2B\(\alpha\) and eIF2B\(\epsilon\) subunits, including the catalytic C-terminal domain of eIF2B) [12••]. Future studies should determine whether AcP10 and AiV L sterically inhibit eIF2(\(p\)) binding via an NS-like mechanism or rather induce allosteric alterations to keep eIF2B in the active conformation, as shown for the small compound integrated stress response inhibitor (ISRIB) [77–80]. Conceivably, additional class-IV ISR antagonists will be identified. One potential candidate is the above-described L protein of cardiovirus, which, apart from directly inhibiting PKR, also suppresses SG formation induced by thapsigargin and arsenite (i.e. activators of PERK and HRI, respectively) without preventing eIF2\(\alpha\) phosphorylation [40,41•].

**Concluding remarks**

Compared with coronavirus, picornaviruses have a limited repertoire of classical ISR antagonists, which may be related to their relatively small genome size and the expression of their genes via a single polyprotein. They largely rely on L and/or 2A security proteins, but these differ widely in structure and function from one picornavirus to another. The large coronavirus genome size and complex genome organization, with structural and AcPs expressed from sgRNAs, provides genetic flexibility to readily accommodate new genes. This may account for the wide variety in CoV antagonists that target one or more branches of the innate antiviral response, but at the same time prompts the question of why individual coronaviruses encode multiple antagonists with similar activity as is the case for MERS-CoV, while others seem to flourish with a more limited set of inhibitors. Expression levels of antiviral effector and sensor molecules are highly variable between different host species, tissues, and cell types. Conceivably, the number and nature of antagonists encoded by a given coronavirus may reflect the conditions met during natural infection in its current host species as well as those met in reservoir hosts from which the virus emerged. Host and cell tropism may drive selection for additional, sometimes even redundant, capabilities to counteract these responses, as is evidenced by the presence of multiple class-I ISR antagonists in MERS-CoV. Interestingly, the MERS-CoV 4a protein, conserved in isolates from dromedary camels, is not required for zoonotic infection and subsequent transmission among humans, as demonstrated by a documented hospital outbreak with a 4a-defective MERS-CoV variant [81]. The redundancy in CoV ISR antagonists, with NSP15 expressed from the genome and others from sgRNAs, may also reflect different requirements during different stages of the viral replication cycle and a necessity to block the ISR once large amounts of sgRNAs are produced. Future studies should consider the dynamics of the interplay between viruses and their hosts. Moreover, it would be interesting to elucidate functions and antagonistic mechanisms employed by other picornavirus security proteins and coronavirus AcPs, many of which have not yet been characterized. Given that viruses also induce ER-, oxidative, and metabolic stress, such studies may even identify yet poorly explored viral antagonists of PERK-, HRI-, and/or GCN2-mediated ISR activation. Hence, studying viral antagonistic mechanisms may reveal many novel aspects of ISR regulation, which is essential for understanding its role in health and disease.

**Data availability**

No data were used for the research described in the article.

**Conflict of interest statement**

The authors declare no conflicts of interest.
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