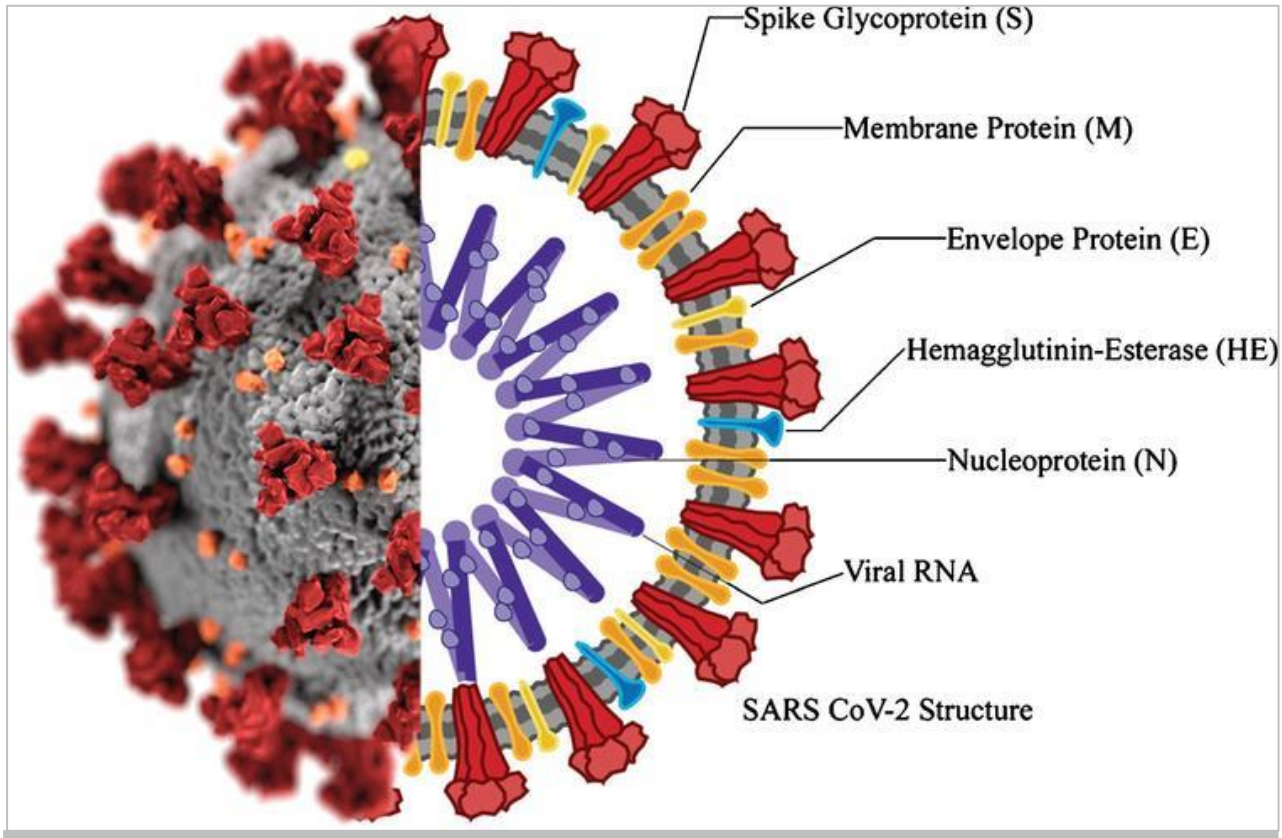
A molecular approach towards treating SARS-COV-2.

Understanding and disrupting the Liquid-Liquid phase separation of the SARS-CoV-2 nucleocapsid protein as a possible target for novel drugs.

BSc thesis written by: Bindert Algra, S3188485

Supervisor: dr. Maarten Linskens



Structural overview of the SARS-CoV-2 virion. (Yamamoto et al., 2020)

Table of contents

Preface

This report is intended as a final thesis for the author's bachelor. While it may be taken as an informational provision, do read with caution. Since the insights and knowledge about SARS-CoV-2 are currently being investigated and might be revised or need to be interpretated differently in light of new research. Due to the high rate of research into this area new insights may be obtained that make certain statements obsolete. Therefore, always put this thesis in contrast with the current knowledge about SARS-CoV-2.

Abstract

Due to antigenically different variants the current vaccines against SARS-CoV-2 might become partially or completely obsolete. Drugs that have been approved for emergency use against SARS-CoV-2 are not specifically developed for targeting the virus and no specifical drugs are approved for clinical use yet. The host cell has a wide variety of antiviral responses as for example the interferon response and the stress granule response, but SARS-CoV-2 seems to antagonise both. The nucleocapsid protein of SARS-CoV-2 undergoes RNA induced liquid-liquid phase separation in a manner that it colocalises with stress granule associated proteins G3BP1/2. Subsequently the nucleocapsid protein disassembles stress granules, which benefits the reproduction of SARS-CoV-2.
Current drugs such as Remdesivir block the replication of RNA dependent RNA polymerase complexes. However RdRp targeting drugs do not remove the dampening effect of the virus on the innate immune response of the host cell. Compounds such as gallocatechin gallate inhibit Liquid-liquid phase separation of the nucleocapsid protein. Targeting the nucleocapsid protein of SARS-CoV-2 seems to be a promising lead towards the development of specific drugs against the virus.

Introduction

The Covid-19 pandemic, caused by SARS-CoV-2, has struck a large part of the world. In May 2021 there were 143.000.000 confirmed cases of COVID-19, including 3.000.000 deaths (Harvey et al., 2021). Due to its high infection rate the virus has spread worldwide and is the cause of a global health crisis (Zhao et al., 2021). SARS-CoV-2 belongs to the genus *Coronavirus* and the order *Nidovirales* , similar as the middle eastern respiratory syndrome (MERS), and severe acute respiratory syndrome (SARS) (Ju et al., 2021).
However, SARS-CoV-2 is more infective than SARS-CoV (Chen et al., 2020). The SARS-CoV-2 virus is an enveloped, positive-sense RNA virus (Fig. 1). It contains a non-segmented single-stranded RNA genome of roughly 30,000 nucleotides (Zhu et al., 2020).

Vaccines

Multiple new vaccines are being or have been developed for the pandemic. In November 2020, 198 vaccines were being developed of which 44 were already undergoing clinical evaluation (Kennedy et al., 2020). Currently of the multiple vaccines in development only four have been approved by the European Medical Agency (EMA) for use in the European union.
Vaccines are developed to boost the humoral immunity by exposing the body to either an attenuated virus, as dead virus, parts of the virus or a harmless virus such as the adenovirus carrying similar spike proteins (Del Giudice et al., 1998). By exposing the immune system, to these viral agents or proteins, the body develops antibodies and specific T-cells, preparing it for an infection with the real SARS-CoV-2 virus.
SARS-CoV-2 enters the host cell through interactions of the spike protein with the

angiotensin-converting enzyme 2 (ACE2) receptor. This is the same pathway the SARS-CoV virus uses. Moreover, both viruses share roughly 80% sequence similarity (Hoffmann et al., 2020; Wang et al., 2020).

However, since the onset of SARS-CoV-2 there have already been more than 10,000 single mutations recorded in comparison with the reference genome collected on January 5, 2020 (Chen et al., 2020). When comparing the Spike protein of SARS-CoV-2 with SARS-CoV the protein has 725 mutations over its 1255 residues, of which 89 mutations are on the Receptor Binding Domain (RBM) consisting of a total of 194 residues (Chen et al., 2020). The RBM is immunodominant but there is evidence that the N-terminal domain (NTD) is also an area of interest for antigenicity (Harvey et al., 2021).

It has been reported that sera from convalescent patients who have recovered form SARS-CoV-2 or from vaccinated individuals still shows neutralization activity towards SARS-CoV-2 variants of concern (VOC), al be it in a decreased manner (Harvey et al., 2021; Noh et al., 2021; Planas et al., 2021). Depending on the VOC and the vaccine given the neutralization activity of the antibodies may range from 96,6% (Novavax against the wild-type) to 57% (single-dose Janssen against the B.1.351 variant) (Harvey et al., 2021). Clearly, the mutations of the spike protein provides a challenge still.

Where VOCs may partially nullify the effects of antibodies there still remains the SARS-CoV-2 specific $CD4^+$ and $CD8^+$ T-cell response, which is much harder to evade. However, there have been mutations reported that negate the binding to major histocompatibility complexes (Noh et al., 2021).

Besides vaccines, currently, there are no specially developed drugs for SARS-CoV-2 infection. Only existing drugs, such as Chloroquine and derivatives, Arbidol, Teicoplanin, Favipiravir, Ribavirin and Remdesivir that have been approved for emergency use. Most of these drugs prevent either RNA replication or host cell entry (Li et al., 2020).

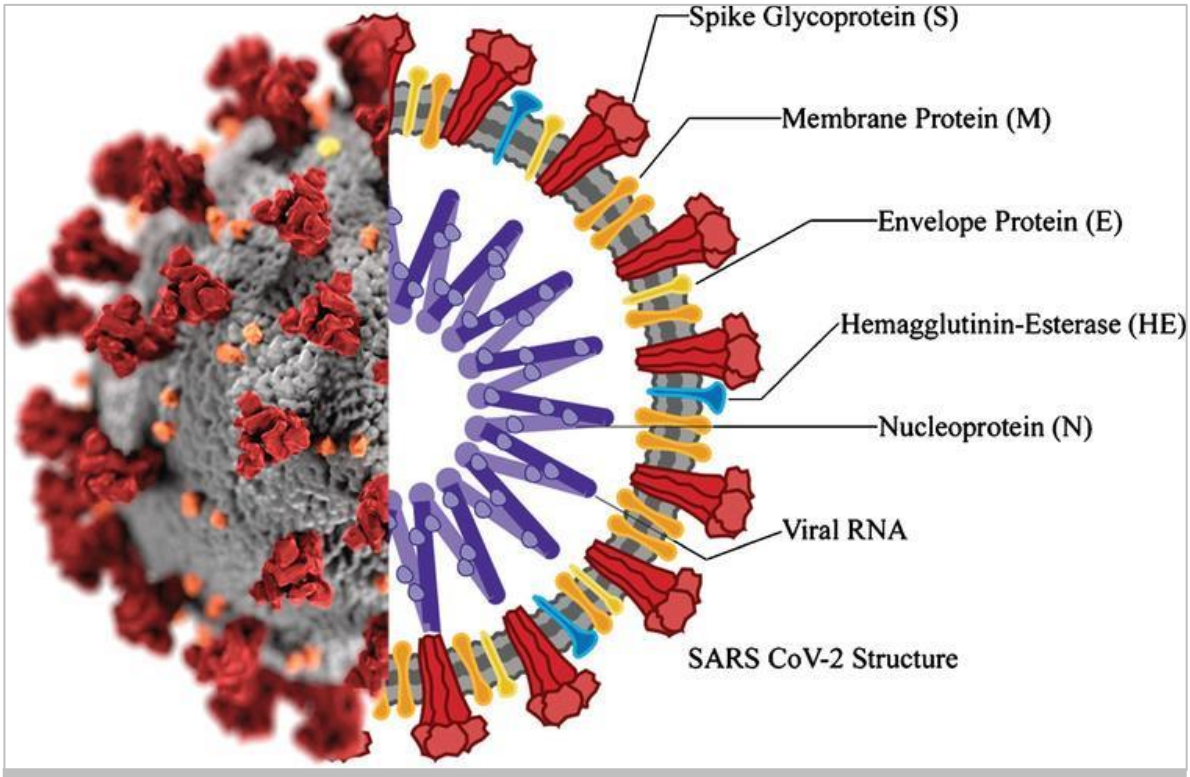


Figure 1. structural overview of the SARS-CoV-2 virion. (Yamamoto et al., 2020)

Remdesivir

Remdesivir is one of the earliest drugs approved for emergency clinical treatment. It is one of the most used medicines against SARS-CoV-2 infection (Jiang et al., 2021). Remdesivir works through the inhibition of RNA-dependent RNA polymerase (RdRp). RdRp is essential for the replication of RNA viruses, which makes it functional against multiple viruses including SARS-CoV-2 (Jiang et al., 2021). This functionality makes it a broad-spectrum antiviral medicine. Remdesivir is a 1'-cyano-substituted adenosine nucleotide analog prodrug, and it has to be metabolized by the body into its active triphosphate metabolite (RDV-TP). The RDV-TP can be incorporated by RdRp and subsequently forms interactions with the primer strand and two hydrogen bonds with the template strand. This enables RDV-TP to block the RdRp complex and attenuate or stop viral transcription. (Jiang et al., 2021).
Targeting the RdRp complex has the expected benefit that probably off target effects are circumvented. This is because the viral RdRp complex and the host cell RdRp complex are substantially different from each other. Additionally, since the active catalytic motif of the RdRp is highly conserved among RNA viruses, it is an attractive broad-spectrum drug (Jiang et al., 2021).

However, SARS-CoV-2 antagonises the innate immune system in other ways than these drugs can prevent. The Nucleocapsid protein of SARS-CoV-2 is able to colocalise with certain regulatory proteins through Liquid-liquid phase separation. The process by which the Nucleocapsid protein does this and the relevance of it shall be discussed in this thesis. Additionally, in this thesis the information at hand regarding the biomolecular antiviral immune response of the cell will be laid down. This will be put in contrast with articles that elucidate how the nucleocapsid protein of SARS-CoV-2 hijacks the host cell.
Subsequently, the molecular mechanism of interaction with host proteins such as G3BP1/2 and interferon beta, and their role within the immune response is explained. A compound and its possible application towards treatment of the SARS-CoV-2 virus is discussed.
Finally, it will be assessed whether the nucleocapsid protein of SARS-CoV-2 is an appropriate druggable target.

Cellular Antiviral Response

The innate antiviral response of the cell is dependent on the capacity to sense viral nucleic acids and instigate an appropriate response to these non-self-entities (Eiermann et al., 2020). Recognition is mediated through specific features called pathogen-associated molecular patterns (PAMPs) (Goubau et al., 2013). Once activated these immune sensors trigger an antiviral response in the cell, such as the production of pro-inflammatory cytokines and interferons (IFN) (Ma et al., 2018). Additionally, the increase of viral double-stranded (ds) RNA in the cytosol or the accumulation of viral proteins in the endoplasmic reticulum (ER) triggers more stress sensors such as PKR-like endoplasmic reticulum kinase (PERK) and protein kinase R (PKR) (Donnelly et al., 2013). The activation of these kinases initiates the integrated stress response (ISR), which results in a translational initiation stop due to polysomes disassembly (Pakos-Zebrucka et al., 2016).
Viral infection is not the only trigger for a translational halt, cells can respond to a variety of unfavourable conditions. When exposed to conditions such as ER stress, oxidative stress, hypoxia, amino acid starvation or UV irradiation, the cell responds with rapid attenuation of translation initiation rates. With this self-imposed limitation the cell can discern between translation of non-critical mRNAs, e.g. housekeeping, and essential mRNAs encoding for survival factors or stress-regulatory proteins (Spriggs et al., 2010).

Stress Granules

An integral part of the host stress response is the assembly of stress granules which are now considered to be signalling platforms that aid with the coordination of cellular processes during stress. Stress granules are believed to play an important role with metabolic control, cell growth, apoptosis and the antiviral defence (McCormick & Khaperskyy, 2017).

Stress granules are an example of biomolecular condensates, structures within the cell without a membrane surrounding it. These condensates often come with specific functions due to their nature that within the condensate certain biomolecular reactions can be more favourable than elsewhere in the cell (Laflamme & Mekhail, 2020). Biomolecular condensates are aggregates of RNA binding proteins (RBP) and mRNAs mediated through interactions between the different constituents. This condensing into a liquid-like dense phase is defined as Liquid-Liquid phase separation (LLPS). Effectively demixing the proteins and mRNA based on favourable intra- and intermolecular interactions, such as hydrophobic, electrostatic and pi-pi contacts (Laflamme & Mekhail, 2020).

Certain RBPs have an essential role in the formation of stress granules, for example Ras GTPase-activating protein-binding protein 1 (G3BP1) (Ivanov et al., 2019). Overexpression of the protein G3BP1 can result in stress granule formation, indicating that shifting the equilibrium between this protein and solubilizing proteins is sufficient to induce LLPS (Eiermann et al., 2020).

Stress granules are dynamic structures able to assemble and disassembly rapidly when stress is present or is remediated. Their size can also vary depending on the type of stress present and the proteins that constitute them. Within their function to aid with attenuating translation, stress granules are also able to exchange proteins and mRNAs with processing bodies (PB), which are in turn able to degrade RNA and function in mRNA silencing (Hubstenberger et al., 2017).

G3BP1, G3BP2, and Interferon-beta

G3BP1 and G3BP2 (G3BP1/2) are not only involved with the formation of stress granules but also functions within the innate interferon response (Eiermann et al., 2020). Interferon-beta (IFN-b) expression is regulated by proteins that sense pathogenic cytosolic DNA, such as RIG-I (retinoic acid-inducible gene I), DDX41, c-GAS, or STING.

It was found that when G3BP1/2 was induced in unison with RIG-I, an increased expression of INF-b was present. Cells stimulated with transfected poly(I.C), to mimic a viral RNA infection, containing both RIG-I and G3BP1/2 showed higher levels of expression of IFN-b in comparison to cells with only RIG-I or G3BP1/2 (Kim et al., 2019). Consequently, implying that G3BP1/2 is able to physically interact with RIG-I and upregulate the IFN-b response.

Through immunoprecipitation, it was shown that G3BP1/2 and RIG-I strongly

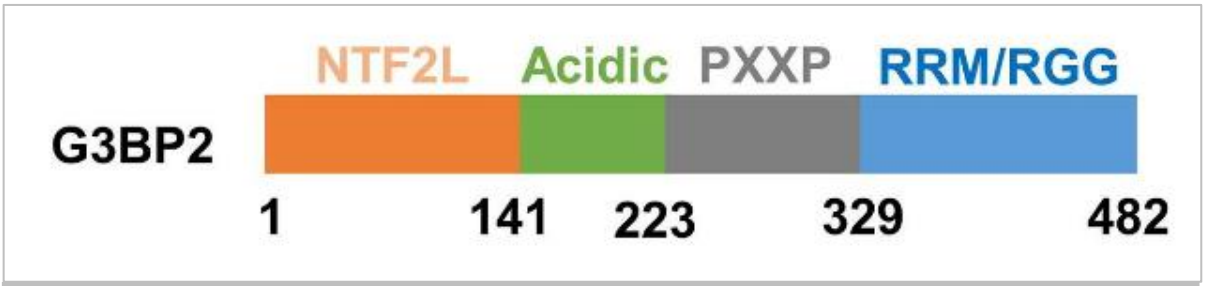


Figure 2. Schematic overview of the G3BP2 protein. nuclear transport factor 2-like, an acidic rich region, a Proline rich region (PXXP), an RNA recognition motif and an arginine and glycine repeat (Luo et al., 2021)

coimmunoprecipitate. Interestingly, the two proteins do not strongly colocalise in cytosolic structures unless transfected poly(I.C) was added. The addition of transfected poly(I.C) leads to a diffuse distribution of both proteins throughout the cell (Kim et al., 2019).

G3BP1 and G3BP2 consist of a nuclear transport factor 2-like, an acidic rich region, a Proline rich region (PXXP), an RNA recognition motif and an arginine and glycine repeat (Fig. 2). Utilising truncated versions it was shown that the arginine and glycine repeat (RGG) was necessary for IFN-b upregulation. This lead to the postulation that the RGG domain is required for biochemical interactions with RIG-I and subsequently IFN-b synthesis (Kim et al., 2019). Additionally, it was shown that the RGG domain of G3BP1 was able to bind ds-RNA as well (Kim et al., 2019).

Concludingly, the G3BP1/2 protein is able to bind ds-RNA and the regulator protein RIG-I. Via these interactions, the G3BP1/2 protein is able to upregulate the IFN-b response of the cell.

Nucleocapsid protein

The nucleocapsid protein of SARS-CoV-2 is necessary for the encapsulation of the viral RNA, as such it is critical for virion assembly (Savastano et al., 2020).

It is a 46 kDa protein, similar as the nucleocapsid protein of SARS-CoV (Zhao et al., 2021). The protein consists of a N-terminal domain (NTD), a RNA binding domain (RBD), a LINKER, a Homodimerization domain (HDD), and a C-terminal domain (CTD) (Fig. 3) (Cubuk et al., 2021). The NTD, LINK and CTD domains are all intrinsically disordered in comparison to the RBD and the HDD (Luo et al., 2021).

The nucleocapsid protein encoded by the SARS-CoV-2 genome is the only protein expected to undergo LLPS (Zhao et al., 2021). This is expected since the protein is necessary for RNA protection within the cellular environment. Additionally, the nucleocapsid protein is of importance for the formation of RNA & RdRp complexes (Fig. 4), which replicate the viral genome (Savastano et al., 2020).

The nucleocapsid protein of SARS-CoV has been shown to antagonise the interferon response and by doing so, hamper the innate immune response of the host cell (Lu et al., 2011). This antagonising effect has been established for the nucleocapsid protein of SARS-CoV-2 as well (Zhao et al., 2021).

The mode by which the nucleocapsid protein seems to block the interferon response is assumed to be the sequestering of viral RNA's

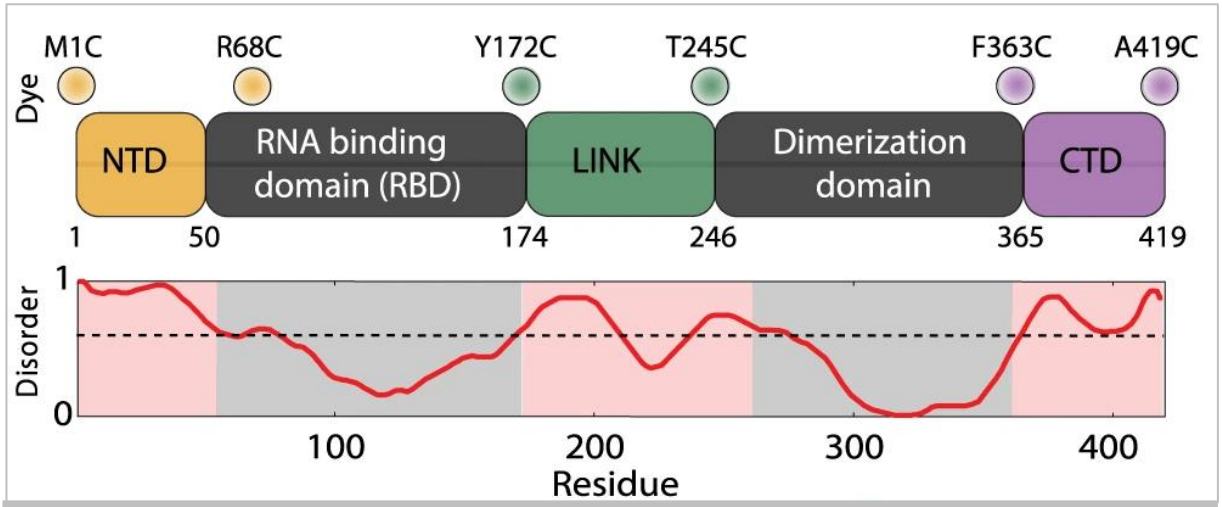


Figure 3. Schematic overview of the SARS-CoV-2 nucleocapsid protein. Left to right; N-terminal domain (NTD), RNA binding domain (RBM), LINK, Homodimerization domain (HDD), and the C-terminal domain (CTD). Showing the order-disorder relative between each domain of the protein (Cubuk et al., 2021).

into stress granules where they are more difficult to be detected (Luo et al., 2021; Zhao et al., 2021).

SARS-COV-2 Nucleocapsid protein RNA induced Phase Separation

The nucleocapsid protein of SARS-CoV-2 shows to phase separate independently of other viral proteins (Luo et al., 2021). However, at increasing protein concentrations, the nucleocapsid protein retained low absorbance (Savastano et al., 2020). Indicating that additional requirements are necessary to stimulate LLPS. Upon adding PolyU (a mimic for viral RNA) to the mixture it was shown that the turbidity strongly increased (Luo et al., 2021). Turbidity is used to quickly assess whether droplet formation was started by LLPS.

The droplets formed were resistant to the addition of aliphatic alcohol but disassembled fast under the addition of NaCl. This indicates that the electrostatic interactions between the PolyU and nucleocapsid protein are of importance for the phase separation (Luo et al., 2021; Savastano et al., 2020; Zhao et al., 2021).

The serine and arginine region (SR) of the nucleocapsid protein were shown, with molecular dynamics (MD), to be important for the RNA binding functionality (Savastano et al., 2020).

Next to the SR region, the RBD also binds RNA through electrostatic interactions, which is crucial for viral infectivity (Luo et al., 2021). The HDD folds into a beta-sheet and is expected to aid in binding RNA. The LINK is essential for multimerization. Various fragment versions of the nucleocapsid protein were tested to determine which region was pivotal for the LLPS characteristics. NTD was found to be essential for LLPS, and the RBD was found to contribute to this (Luo et al., 2021).

Other fragments lacking LINK, CTD or the HDD were found to still undergo LLPS but in an attenuated fashion (Luo et al., 2021). Interestingly deletion of the c-terminal HDD and CTD facilitated diffusion of the nucleocapsid protein into the droplet, indicating that those regions contribute to the condensate stabilisation of the viral particle (Luo et al., 2021).

Also note that when any domain of the nucleocapsid protein was deleted, the protein lost its ability to bind RNA almost altogether (Liu et al., 2019).

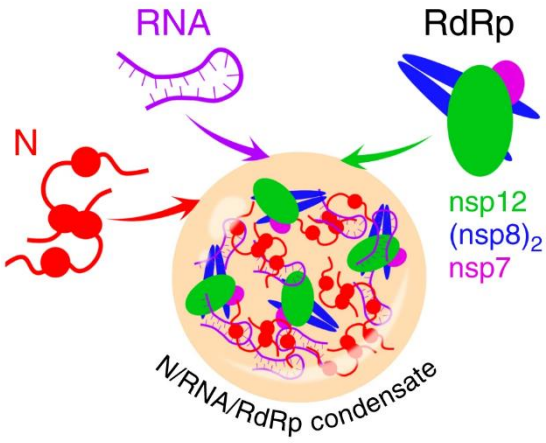


Figure 4. Nucleocapsid protein & RNA & RdRp condensate. The condensate formation is an essential step in the replication cycle of the virus (Savastano et al., 2020).

SARS-CoV-2 Nucleocapsid protein colocalises with G3BP1 and G3BP2

G3BP1/2 are of particular interest to viruses, which aim to disrupt the stress granules formation. Examples of viruses that interfere with G3BP1/2 are HCV (Garaigorta et al., 2012), Chikungunya virus (Scholte et al., 2015), Semliki forest virus (Panas et al., 2012), SINV (Frolova et al., 2006), and SARS-CoV-2 (Luo et al., 2021).

The affinity of the nucleocapsid protein with G3BP1/2 was shown early on in the pandemic by means of a protein analysis (Fig. 5) (Gordon et al., 2020; Luo et al., 2021).

The nucleocapsid protein of SARV-CoV-2 and other SARS-CoV viruses were shown to localise in stress granules.

Upon stress granule induction, the interaction between the nucleocapsid protein and G3BP1/2 was enhanced (Gordon et al., 2020; Luo et al., 2021; Savastano et al., 2020).
The localisation of G3BP1/2 and the nucleocapsid protein to stress granules was further enhanced when RNA was added. Interestingly, when both the nucleocapsid protein and G3BP1/2 were present upon RNA addition the phase separation happened in an increased manner than when only the nucleocapsid protein or G3BP1/2 was present (Luo et al., 2021). This led to the postulation that both proteins are able to perform enhanced co-LLPS under the influence of RNA.
Furthermore, the NTF2L domain of G3BP1/2 showed to be essential for interaction with the SARS-CoV-2 nucleocapsid protein (Luo et al., 2021). The domain of the nucleocapsid protein that interacted with the NTF2L domain was concluded to be the NTD (Luo et al., 2021).

Stress Granule disassembly by the Nucleocapsid protein

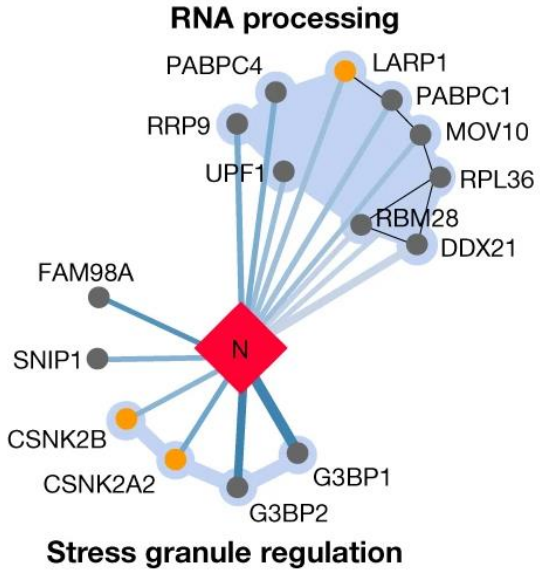


Figure 5. Protein interaction map between the Nucleocapsid protein (N) of SARS-CoV-2 and host cell proteins. (Gordon et al., 2020)

It has been shown that the nucleocapsid protein strongly inhibited stress granule formation in cells, whether induced via heat shock, poly(I.C) (mimics Viral infection) or Dithiothreitol (DTT) (mimics ER stressor) (Luo et al., 2021).
With immunoprecipitation and western blotting it was observed that higher amounts of the nucleocapsid protein compromise interactions between G3BP1/2 and other stress granule associated proteins (Luo et al., 2021).
Through this competition the nucleocapsid protein prohibits complete stress granule formation, and disassembles them completely when enough protein copies are present (Luo et al., 2021).
Although, the LLPS which the nucleocapsid protein under goes might be due to its function to form viral envelopes. It has also been proposed that the virus is able to use LLPS to localise in the cell where all the translational machinery is stored, and thus can increase its replication rate (Fig. 6) (Cubuk et al., 2021; Luo et al., 2021; Savastano et al., 2020).
Similarly, it has been proposed that the strong LLPS might contribute to viral mRNA evading the innate cellular immune response. Mutant versions of the nucleocapsid protein with higher propensity to undergo LLPS, also have a higher inhibitory effect on the IFN response (Zhao et al., 2021).

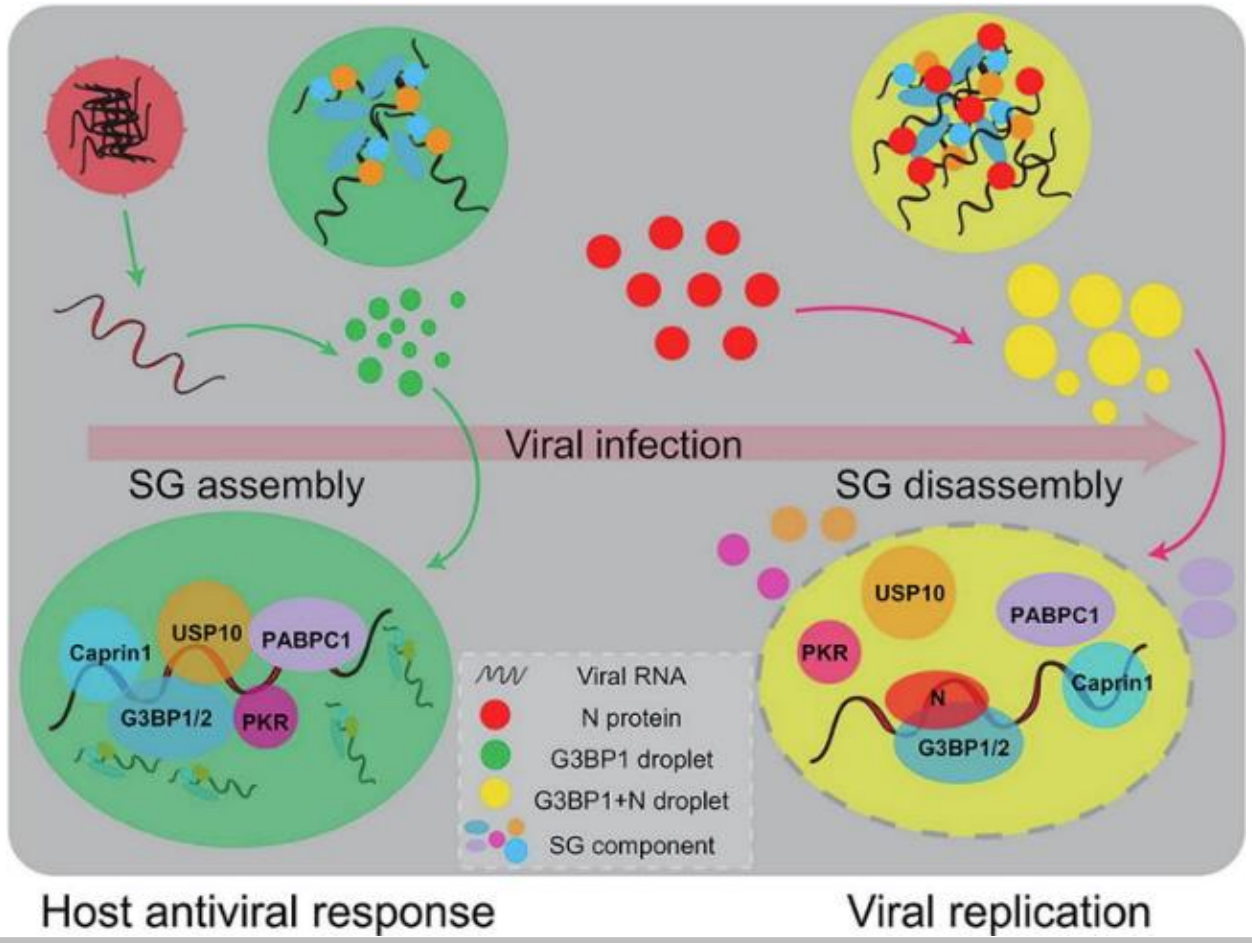


Figure 6. schematic overview of the normal formation of SG (right) versus how the N protein disrupts SG assembly (left). The nucleocapsid protein competes with the other SG associated protein for interaction with G3BP1/2 thus preventing regular SG assembly. (Luo et al., 2021)

The Nucleocapsid protein as a target for medication

The nucleocapsid protein is a promising target for new drug development. Of all the proteins encoded by the SARS-CoV-2 genome only the nucleocapsid protein is expected with a high degree of certainty to undergo LLPS (Zhao et al., 2021). Making it therefore hard to be replaced by another protein, encoded in the RNA template of the virus.

Besides that the nucleocapsid protein is expected to be difficult to replace, it is the only protein of the genome that markedly inhibited stress granule assembly (Luo et al., 2021).

Additionally, The nucleocapsid protein of SARS-CoV-2 has been shown to antagonise the interferon response and by doing so, hamper the innate immune response of the host cell (Zhao et al., 2021). The mode by which the nucleocapsid protein seems to block the interferon response is assumed to be the sequestering of viral RNA's into stress granules where they are more difficult to be detected (Luo et al., 2021; Zhao et al., 2021).Thus targeting the nucleocapsid protein might alleviate the antagonising effect on the cellular immune system caused by SARS-CoV-2.

Furthermore, *Zhao et al*, showed that the nucleocapsid protein was highly conserved throughout all the samples, except within for the SR region of the protein, which showed the highest mutation variability of the protein (Fig. 7). When compared to the SARS-CoV virus, the nucleocapsid protein of SARS-CoV-2 showed 90% similarity (Zhao et al., 2021). This might indicate that the nucleocapsid protein is a stable target for newly developed drugs with a limited chance of mutations.

With a transcomplementational model, a newly developed cell culture system to model the SARS-CoV-2 life cycle, mutants were derived of the nucleocapsid protein (Ju et al., 2021). With these mutants it was shown that functionality of the wild type nucleocapsid protein is essential for viral infectivity (Luo et al., 2021). This was confirmed by disrupting the LLPS of the nucleocapsid protein, which also led to a loss of infectivity and lower concentrations of viral RNA copies (Zhao et al., 2021). Loss of any domain of the protein resulted in loss of LLPS (Zhao et al., 2021).

Possible treatment SARS-CoV-2: GCG

Gallocatechin gallate (GCG) is a polyphenol, derived from green tea. It was found to disrupts the LLPS of the nucleocapsid protein and subsequently inhibits SARS-CoV-2 replication.

A concentration of 12.5 µM was demonstrated to be sufficient at blocking the LLPS of the nucleocapsid protein. It was indicated that LLPS blocking started to occur at concentrations of 6–8 µM (Zhao et al., 2021).

Zhao et al. showed with a pull down assay and western blotting that GCG directly binds the nucleocapsid protein. And showed that upon addition of GCG to infected cells the viral titers were inhibited. It was concluded that increased amounts of GCG greatly impaired LLPS of the nucleocapsid protein. Viral replication was greatly inhibited and based on the data it was due to disruption of LLPS of the nucleocapsid protein (Zhao et al., 2021).

GCG is then also proposed as a lead compound towards the development of drugs against SARS-CoV-2.

Speculation on the mechanism of GCG inhibition

The precise mechanism by which GCG is able to disrupt the LLPS of the nucleocapsid protein is not yet elucidated. It is tempting to speculate that the compound GCG interacts with the protein G3BP1 in a similar fashion as the structural isomer EGCG.

EGCG disrupts the interaction between G3BP1 and cGAS, a regulatory protein of the IFN response, through the RGG and PXXP domain (Kim et al., 2019). These domains are necessary for G3BP1 to interact with RNA and interact with cGAS and RIG-I (Kim et al., 2019; Liu et al., 2019).

Possibly the interaction is also necessary for co-LLPS with the nucleocapsid protein, since co-

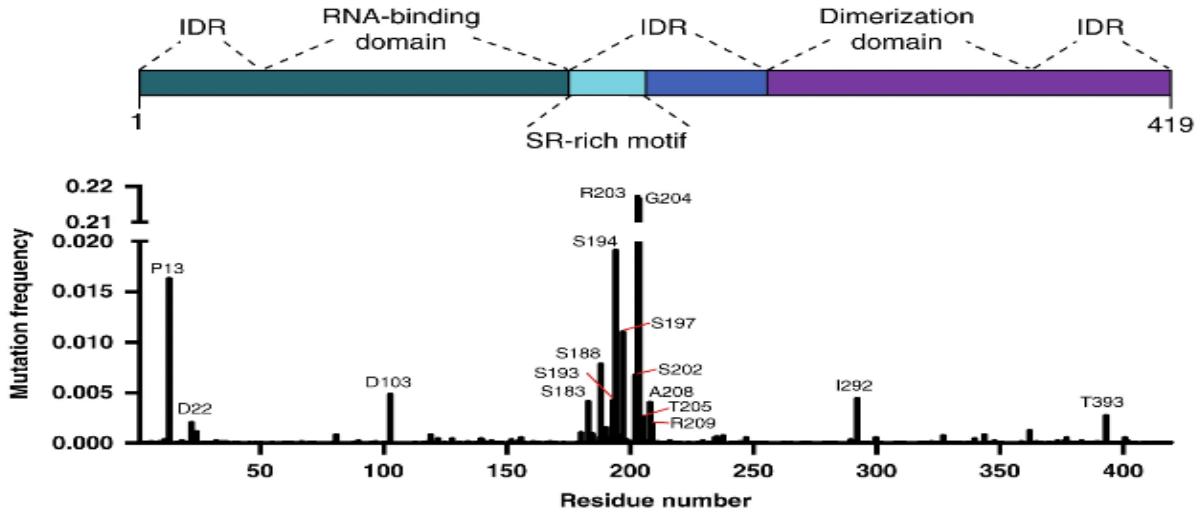


Figure 7. Frequency of mutations in the nucleocapsid protein in 42176 SARS-CoV-2 sequences from the China National Center for Bioinformation. (Savastano et al., 2020)

LLPS of the G3BP1 protein with the nucleocapsid protein is enhanced through addition of RNA. However more research is needed to determine this mechanism with certainty.
If GCG interacts with G3BP1 to disrupt binding of RNA, it could have a lowered innate immune response as a side effect. However, since it is assumed that the nucleocapsid protein evades the interferon response by sequestering viral RNA, this might not be a relevant problem.

Discussion

The innate immune response of the cell is elaborate, containing among others, the stress granules response and the interferon response. However, the nucleocapsid protein of the SARS-CoV-2 virus interacts with the G3BP1/2 proteins to circumvent both.
It has been shown that through enhanced colocalization the nucleocapsid protein uses G3BP1/2 as a vehicle to integrate with stress granules and prevent complete assembly. Thereby using the transcription factors present to replicate viral proteins and viral RNA.
By gathering its genomic RNA into stress granules the nucleocapsid protein can provide a way for the virus to elude sensory proteins that would otherwise instigate the interferon-beta response.
It was demonstrated that the proper structure of the nucleocapsid protein is essential for viral infectivity and reproduction (Luo et al., 2021; Zhao et al., 2021).
Notably, two papers showed different results. *Luo et all* showed that the IDR1 is absolutely necessary for LLPS (Luo et al., 2021). While *Zhao et all* showed that all domains are absolutely required for LLPS (Zhao et al., 2021). The exact importance of the different domains therefore still needs to be decisively determined.
Our current drugs against SARS-CoV-2 such as Remdesivir are useful, since their mode of operation is based on highly conserved structures within viral machinery. Yet targeting the replication machinery of RNA viruses does not abolish the antagonising effect that the virus has on the innate immune system.
Therefore, development of drugs based on compounds such as GCG, that inhibit the LLPS of viral proteins, can provide medication able to remove the dampening effect of the nucleocapsid protein altogether.
In clinical trials it has already been shown that upon treating patients with IFN-b, and thereby upregulating the innate immune response, the recovery time was shortened and patients needed less often additional care (Rahmani et al., 2020).
Beneficially, the domain of G3BP1/2 (NFT2L) that interacts with the nucleocapsid protein is different from the domains that interact with the regulatory proteins of the IFN response. Suggesting that disrupting the interactions between G3BP1/2 and the nucleocapsid protein probably will not have off target effects such as upregulating the IFN response throughout the whole body.

In my opinion the development of new medical strategies is essential to remain ahead of viruses that can cause an epidemic or a pandemic. The drugs that that are now approved for emergency use against SARS-CoV-2 usually either block the RdRp complexes or prevent the virion from entering the host cell. Currently we have almost no drugs that help the innate immune response with fighting the virus. While the drugs that we have already function great in their designed capacity, medical healthcare around the world is not always up to standard or available.
The vaccines that have been and are being developed work effectively towards the initial SARS-CoV-2 virus. But in the face of VOCs that undergo antigen change, they might lose functionality and this can lead to loss of faith in the vaccine strategy. Besides, we cannot create vaccines every year and expect them to work in the same capacity as the first ones that are developed. This is because pre-existing antibodies can interfere with vaccine efficacy (Dagan et al., 2010).

Additionally, since many vaccines are developed against the same spike protein of SARS-CoV-2, a mutation that leaves the virus resistant against one vaccine can result in a virus becoming resistant against all vaccines. This is referred to as 'collateral' or 'cross' resistance, which has been observed before with antimicrobial drugs (Kennedy et al., 2020). Therefore, examining other methods to treat the SARS-CoV-2 virus is something of substantial value. If in the near future new epidemics arise due to a novel *Coronavirus*, the medical community can only benefit from a multitude of options to treat the infection. The fact that the nucleocapsid protein appears to be vital to the replication cycle of SARS-CoV-2 and SARS-CoV, makes it an ideal target to use for the development of new medication.

Concluding remarks

Vaccines can become obsolete due to mutations in the RBD and NTD of the spike protein. Resulting in SARS-CoV-2 eluding the humoral immunity (Harvey et al., 2021).
The nucleocapsid protein shows to be a promising lead since it is highly conserved and is shown to be essential for infectivity for SARS-CoV-2 (Luo et al., 2021). Additionally, it is the only protein of SARS-CoV-2 that is expected to undergo LLPS (Zhao et al., 2021).
The co-localisation of the nucleocapsid protein to stress granules can improve the speed at which the virus is replicated. Therefore finding drugs that hamper or block the LLPS of the nucleocapsid protein have the potential to attenuate viral replication and speed up recovery.
Compounds such as GCG provide disruption of SARS-CoV-2 LLPS. However the exact mechanism behind them are not yet know. More research is necessary into the inhibitory mechanism of these compounds and the life cycle of SARS-CoV-2, to obtain tailor-made solutions.
This can benefit global healthcare in the long run, since the SARS-CoV-2 virus is the third corona virus in twenty years that has caused either an epidemic or a pandemic (Chen et al., 2020; Paules et al., 2020).

Literature

Chen, J., Wang, R., Wang, M., & Wei, G. W. (2020). Mutations Strengthened SARS-CoV-2 Infectivity. *Journal of Molecular Biology*, *432*(19), 5212–5226. https://doi.org/10.1016/j.jmb.2020.07.009

Cubuk, J., Alston, J. J., Incicco, J. J., Singh, S., Stuchell-Brereton, M. D., Ward, M. D., Zimmerman, M. I., Vithani, N., Griffith, D., Wagoner, J. A., Bowman, G. R., Hall, K. B., Soranno, A., & Holehouse, A. S. (2021). The SARS-CoV-2 nucleocapsid protein is dynamic, disordered, and phase separates with RNA. *Nature Communications*, *12*(1), 1–17. https://doi.org/10.1038/s41467-021-21953-3

Dagan, R., Poolman, J., & Siegrist, C. A. (2010). Glycoconjugate vaccines and immune interference: A review. *Vaccine*, *28*(34), 5513–5523. https://doi.org/10.1016/j.vaccine.2010.06.026

Del Giudice, G., Pizza, M., & Rappuoli, R. (1998). Molecular basis of vaccination. *Molecular Aspects of Medicine*, *19*(1), 1–70. https://doi.org/10.1016/S0098-2997(98)00002-8

Donnelly, N., Gorman, A. M., Gupta, S., & Samali, A. (2013). The eIF2α kinases: Their structures and functions. *Cellular and Molecular Life Sciences*, *70*(19), 3493–3511. https://doi.org/10.1007/s00018-012-1252-6

Eiermann, N., Haneke, K., Sun, Z., Stoecklin, G., & Ruggieri, A. (2020). Dance with the Devil: Stress Granules and Signaling in Antiviral Responses. *Viruses*, *12*(9), 1–47. https://doi.org/10.3390/v12090984

Frolova, E., Gorchakov, R., Garmashova, N.,

Atasheva, S., Vergara, L. A., & Frolov, I. (2006). Formation of nsP3-Specific Protein Complexes during Sindbis Virus Replication. *Journal of Virology*, *80*(8), 4122–4134. https://doi.org/10.1128/jvi.80.8.4122-4134.2006

Garaigorta, U., Heim, M. H., Boyd, B., Wieland, S., & Chisari, F. V. (2012). Hepatitis C Virus (HCV) Induces Formation of Stress Granules Whose Proteins Regulate HCV RNA Replication and Virus Assembly and Egress. *Journal of Virology*, *86*(20), 11043–11056. https://doi.org/10.1128/jvi.07101-11

Gordon, D. E., Jang, G. M., Bouhaddou, M., Xu, J., Obernier, K., White, K. M., O'Meara, M. J., Rezelj, V. V., Guo, J. Z., Swaney, D. L., Tummino, T. A., Hüttenhain, R., Kaake, R. M., Richards, A. L., Tutuncuoglu, B., Foussard, H., Batra, J., Haas, K., Modak, M., … Krogan, N. J. (2020). A SARS-CoV-2 protein interaction map reveals targets for drug repurposing. *Nature*, *583*(7816), 459–468. https://doi.org/10.1038/s41586-020-2286-9

Goubau, D., Deddouche, S., & Reis e Sousa, C. (2013). Cytosolic Sensing of Viruses. *Immunity*, *38*(5), 855–869. https://doi.org/10.1016/j.immuni.2013.05.007

Harvey, W. T., Carabelli, A. M., Jackson, B., Gupta, R. K., Thomson, E. C., Harrison, E. M., Ludden, C., Reeve, R., Rambaut, A., Peacock, S. J., & Robertson, D. L. (2021). SARS-CoV-2 variants, spike mutations and immune escape. *Nature Reviews Microbiology*, *19*(7), 409–424. https://doi.org/10.1038/s41579-021-00573-0

Hoffmann, M., Kleine-Weber, H., Schroeder, S., Krüger, N., Herrler, T., Erichsen, S., Schiergens, T. S., Herrler, G., Wu, N. H., Nitsche, A., Müller, M. A., Drosten, C., & Pöhlmann, S. (2020). SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell*, *181*(2), 271-280.e8. https://doi.org/10.1016/j.cell.2020.02.052

Hubstenberger, A., Courel, M., Bénard, M., Souquere, S., Ernoult-Lange, M., Chouaib, R., Yi, Z., Morlot, J. B., Munier, A., Fradet, M., Daunesse, M., Bertrand, E., Pierron, G., Mozziconacci, J., Kress, M., & Weil, D. (2017). P-Body Purification Reveals the Condensation of Repressed mRNA Regulons. *Molecular Cell*, *68*(1), 144-157.e5. https://doi.org/10.1016/j.molcel.2017.09.003

Ivanov, P., Kedersha, N., & Anderson, P. (2019). Stress granules and processing bodies in translational control. *Cold Spring Harbor Perspectives in Biology*, *11*(5), 1–18. https://doi.org/10.1101/cshperspect.a032813

Jiang, Y., Yin, W., & Xu, H. E. (2021). RNA-dependent RNA polymerase: Structure, mechanism, and drug discovery for COVID-19. *Biochemical and Biophysical Research Communications*, *538*, 47–53. https://doi.org/10.1016/j.bbrc.2020.08.116

Ju, X., Zhu, Y., Wang, Y., Li, J., Zhang, J., Gong, M., Ren, W., Li, S., Zhong, J., Zhang, L., Zhang, Q. C., Zhang, R., & Ding, Q. (2021). A novel cell culture system modeling the SARS-CoV-2 life cycle. *PLoS Pathogens*, *17*(3), 1–23. https://doi.org/10.1371/journal.ppat.1009439

Kennedy, D. A., Read, A. F., & Kennedy, D. A. (2020). Monitor for COVID-19 vaccine resistance evolution during clinical trials. *PLoS Biology*, *18*(11), 1–5. https://doi.org/10.1371/journal.pbio.3001000

Kim, S. S. Y., Sze, L., & Lam, K. P. (2019). The stress granule protein G3BP1 binds viral dsRNA and RIG-I to enhance interferon-β response. *Journal of Biological Chemistry*, *294*(16), 6430–6438. https://doi.org/10.1074/jbc.RA118.0058

68

Laflamme, G., & Mekhail, K. (2020). Biomolecular condensates as arbiters of biochemical reactions inside the nucleus. *Communications Biology*, *3*(1), 1–8. https://doi.org/10.1038/s42003-020-01517-9

Li, C., Wang, L., & Ren, L. (2020). Antiviral mechanisms of candidate chemical medicines and traditional Chinese medicines for SARS-CoV-2 infection. In *Virus Research* (Vol. 286, p. 198073). Elsevier B.V. https://doi.org/10.1016/j.virusres.2020.198073

Liu, Z. S., Cai, H., Xue, W., Wang, M., Xia, T., Li, W. J., Xing, J. Q., Zhao, M., Huang, Y. J., Chen, S., Wu, S. M., Wang, X., Liu, X., Pang, X., Zhang, Z. Y., Li, T., Dai, J., Dong, F., Xia, Q., … Li, T. (2019). G3BP1 promotes DNA binding and activation of cGAS. *Nature Immunology*, *20*(1), 18–28. https://doi.org/10.1038/s41590-018-0262-4

Lu, X., Pan, J., Tao, J., & Guo, D. (2011). SARS-CoV nucleocapsid protein antagonizes IFN-β response by targeting initial step of IFN-β induction pathway, and its C-terminal region is critical for the antagonism. *Virus Genes*, *42*(1), 37–45. https://doi.org/10.1007/s11262-010-0544-x

Luo, L., Li, Z., Zhao, T., Ju, X., Ma, P., Jin, B., Zhou, Y., He, S., Huang, J., Xu, X., Zou, Y., Li, P., Liang, A., Liu, J., Chi, T., Huang, X., Ding, Q., Jin, Z., Huang, C., & Zhang, Y. (2021). SARS-CoV-2 nucleocapsid protein phase separates with G3BPs to disassemble stress granules and facilitate viral production. *Science Bulletin*, *xxxx*. https://doi.org/10.1016/j.scib.2021.01.013

Ma, Z., Ni, G., & Damania, B. (2018). Innate Sensing of DNA Virus Genomes. *Annual Review of Virology*, *5*, 341–362. https://doi.org/10.1146/annurev-virology-092917-043244

McCormick, C., & Khaperskyy, D. A. (2017). Translation inhibition and stress granules in the antiviral immune response. *Nature Reviews Immunology*, *17*(10), 647–660. https://doi.org/10.1038/nri.2017.63

Noh, J. Y., Jeong, H. W., & Shin, E. C. (2021). SARS-CoV-2 mutations, vaccines, and immunity: implication of variants of concern. *Signal Transduction and Targeted Therapy*, *6*(1), 3–4. https://doi.org/10.1038/s41392-021-00623-2

Pakos-Zebrucka, K., Koryga, I., Mnich, K., Ljujic, M., Samali, A., & Gorman, A. M. (2016). The integrated stress response. *EMBO Reports*, *17*(10), 1374–1395. https://doi.org/10.15252/embr.201642195

Panas, M. D., Varjak, M., Lulla, A., Eng, K. E., Merits, A., Hedestam, G. B. K., & McInerney, G. M. (2012). Sequestration of G3BP coupled with efficient translation inhibits stress granules in Semliki Forest virus infection. *Molecular Biology of the Cell*, *23*(24), 4701–4712. https://doi.org/10.1091/mbc.E12-08-0619

Paules, C. I., Marston, H. D., & Fauci, A. S. (2020). Coronavirus Infections-More Than Just the Common Cold. In *JAMA - Journal of the American Medical Association* (Vol. 323, Issue 8, pp. 707–708). https://doi.org/10.1001/jama.2020.0757

Planas, D., Veyer, D., Baidaliuk, A., Staropoli, I., Guivel-Benhassine, F., Rajah, M. M., Planchais, C., Porrot, F., Robillard, N., Puech, J., Prot, M., Gallais, F., Gantner, P., Velay, A., Le Guen, J., Kassis-Chikhani, N., Edriss, D., Belec, L., Seve, A., … Schwartz, O. (2021). Reduced sensitivity of SARS-CoV-2 variant Delta to antibody neutralization. *Nature*, *596*(August). https://doi.org/10.1038/s41586-021-03777-9

Rahmani, H., Davoudi-monfared, E., Nourian, A., & Khalili, H. (2020). *Since January 2020 Elsevier has created a COVID-19*

resource centre with free information in English and Mandarin on the novel coronavirus COVID- 19 . The COVID-19 resource centre is hosted on Elsevier Connect , the company ' s public news and information . January.

Savastano, A., Ibáñez de Opakua, A., Rankovic, M., & Zweckstetter, M. (2020). Nucleocapsid protein of SARS-CoV-2 phase separates into RNA-rich polymerase-containing condensates. *Nature Communications*, *11*(1). https://doi.org/10.1038/s41467-020-19843-1

Scholte, F. E. M., Tas, A., Albulescu, I. C., Žusinaite, E., Merits, A., Snijder, E. J., & van Hemert, M. J. (2015). Stress Granule Components G3BP1 and G3BP2 Play a Proviral Role Early in Chikungunya Virus Replication. *Journal of Virology*, *89*(8), 4457–4469. https://doi.org/10.1128/jvi.03612-14

Spriggs, K. A., Bushell, M., & Willis, A. E. (2010). Translational Regulation of Gene Expression during Conditions of Cell Stress. *Molecular Cell*, *40*(2), 228–237. https://doi.org/10.1016/j.molcel.2010.09.028

Wang, Q., Zhang, Y., Wu, L., Niu, S., Song, C., Zhang, Z., Lu, G., Qiao, C., Hu, Y., Yuen, K. Y., Wang, Q., Zhou, H., Yan, J., & Qi, J. (2020). Structural and Functional Basis of SARS-CoV-2 Entry by Using Human ACE2. *Cell*, *181*(4), 894-904.e9. https://doi.org/10.1016/j.cell.2020.03.045

Yamamoto, V., Bolanos, J. F., Fiallos, J., Strand, S. E., Morris, K., Shahrokhinia, S., Cushing, T. R., Hopp, L., Tiwari, A., Hariri, R., Sokolov, R., Wheeler, C., Kaushik, A., Elsayegh, A., Eliashiv, D., Hedrick, R., Jafari, B., Johnson, J. P., Khorsandi, M., … Kateb, B. (2020). COVID-19: Review of a 21st Century Pandemic from Etiology to Neuro-psychiatric Implications. *Journal of Alzheimer's Disease*, *77*(2), 459–504. https://doi.org/10.3233/JAD-200831

Zhao, M., Yu, Y., Sun, L.-M., Xing, J.-Q., Li, T., Zhu, Y., Wang, M., Yu, Y., Xue, W., Xia, T., Cai, H., Han, Q.-Y., Yin, X., Li, W.-H., Li, A.-L., Cui, J., Yuan, Z., Zhang, R., Zhou, T., … Li, T. (2021). GCG inhibits SARS-CoV-2 replication by disrupting the liquid phase condensation of its nucleocapsid protein. *Nature Communications*, *12*(1), 1–14. https://doi.org/10.1038/s41467-021-22297-8

Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., Zhao, X., Huang, B., Shi, W., Lu, R., Niu, P., Zhan, F., Ma, X., Wang, D., Xu, W., Wu, G., Gao, G. F., & Tan, W. (2020). A Novel Coronavirus from Patients with Pneumonia in China, 2019. *New England Journal of Medicine*, *382*(8), 727–733. https://doi.org/10.1056/nejmoa2001017