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REVIEW

Epigenetic mechanisms influencing COVID-19[1]

Rwik Sen, Michael Garbati, Kevin Bryant, and Yanan Lu

Abstract: The COVID-19 pandemic is one of the most significant public health threats in recent history and has impacted the lives of almost everyone worldwide. Epigenetic mechanisms contribute to many aspects of the SARS-CoV-2 replication cycle, including expression levels of viral receptor ACE2, expression of cytokine genes as part of the host immune response, and the implication of various histone modifications in several aspects of COVID-19. SARS-CoV-2 proteins physically associate with many different host proteins over the course of infection, and notably there are several interactions between viral proteins and epigenetic enzymes such as HDACs and bromodomain-containing proteins as shown by correlation-based studies. The many contributions of epigenetic mechanisms to the viral life cycle and the host immune response to infection have resulted in epigenetic factors being identified as emerging biomarkers for COVID-19, and project epigenetic modifiers as promising therapeutic targets to combat COVID-19. This review article highlights the major epigenetic pathways at play during COVID-19 disease and discusses ongoing clinical trials that will hopefully contribute to slowing the spread of SARS-CoV-2.

Key words: epigenetics, COVID-19, ACE2, DNA methylation, histone modifications, clinical trials.

Résumé : La pandémie de la COVID-19 constitue l'une des plus importantes menaces à la santé publique de l'histoire récente et a touché les vies de presque tous à l'échelle planétaire. Des mécanismes épigénétiques contribuent à plusieurs aspects du cycle de réplication du SRAS-CoV-2, incluant le niveau d'expression du récepteur viral ACE2, l'expression des gènes codant pour les cytokines qui participent à la réponse immunitaire de l'hôte, et l'implication de plusieurs modifications des histones touchant de nombreux aspects de la COVID-19. Les protéines du SRAS-CoV-2 se lient physiquement avec plusieurs protéines de l'hôte au cours de l'infection et, notamment, des études de corrélation ont révélé de nombreuses interactions entre des protéines virales et des enzymes épigénétiques telles que les HDAC et des protéines à bromodomaine. Les nombreuses contributions des mécanismes épigénétiques dans le cycle vital du virus et dans la réponse immunitaire de l'hôte ont fait en sorte que les facteurs épigénétiques sont vus comme étant des biomarqueurs émergents pour la COVID-19 et laissent entrevoir que des modificateurs épigénétiques pourraient constituer des cibles thérapeutiques intéressantes pour combattre la COVID-19. Cet article de synthèse met en lumière les principaux sentiers épigénétiques qui jouent un rôle dans la maladie de la COVID-19 et les auteurs discutent des essais cliniques en cours qui contribueront possiblement à ralentir la propagation du SRAS-CoV-2. [Traduit par la Rédaction]

Mots-clés : épigénétique, COVID-19, ACE2, méthylation de l'ADN, modifications des histones, essais cliniques.

Introduction

Epigenetic modifications, such as DNA methylation and post-translational modifications of histone tails, contribute to the regulation of almost all biological processes, and are also involved in many different human diseases (Cantone and Fisher 2013; Eccleston et al. 2013; Zoghbi and Beaudet 2016). Epigenetic changes have previously been observed in patients infected with SARS-CoV-2, the virus responsible for the COVID-19 pandemic, suggesting that therapeutics targeting epigenetic mechanisms might help treat SARS-CoV-2 infections. This article reviews the current state of the literature on the relationships between epigenetic modifications and COVID-19 and speculates on some areas that will provide the greatest chance to help slow or end this pandemic.

Epigenetic mechanisms allow cells to rapidly respond to changes in the environment and alter gene expression profiles to adapt to environmental stimuli. In particular, changes in levels or patterns of histone, DNA, or RNA modifications are often observed in human diseases (Flavahan et al. 2017; Zhang and Cao 2019; Zoghbi and Beaudet 2016). In some cases, these changes are due to the affected cell trying to respond to the disease state

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and restore normal function. In other cases, mutations in epigenetic enzymes or proteins that recognize the epigenetic modifications lead to the observed changes and are directly responsible for driving the disease phenotype.

Viral infection is an especially important stimulus that cells detect and respond to. Almost immediately after infection, many host cells undergo significant changes in an attempt to block or slow virus replication. This includes induction of both the innate and adaptive immune responses, and potentially also apoptosis in extreme cases. In many cases, viruses have co-evolved with their host species and have developed their own mechanisms to combat or evade the cellular antiviral responses. These mechanisms can include reprogramming the host cell to produce an environment suitable for virus replication and essentially turning infected cells into virus-producing factories. The host and virus have an "arms race" of sorts where the cell tries to inhibit the virus, but the virus tries to counter this attack and manipulate the host cell for its own needs.

Infection of cells by many viruses initiates epigenetic changes as part of the arms race. Epigenetic mechanisms both regulate the expression of antiviral genes and the expression of host factors that the virus uses for efficient replication and spread. Therefore, therapeutic strategies that target epigenetic mechanisms are promising approaches to treat COVID-19.

We will highlight some of the main epigenetic mechanisms at play during SARS-CoV-2 infection, cover the roles of these epigenetic pathways in the viral lifecycle, and discuss how epigenetic enzymes and proteins might be targeted to slow the spread of COVID-19 and contribute to ending the pandemic. In particular, we review the role of epigenetics in the expression of the viral receptor ACE2, expression of cytokine genes, and links between histone citrullination and COVID-19.

Overview of SARS-CoV-2 life cycle

The novel coronavirus SARS-CoV-2 genome is a positive-sense single-stranded RNA that is 29.9 kilobases (kb) in length (F. Wu et al. 2020), and the virion contains a nucleocapsid with helical symmetry. The SARS-CoV-2 RNA genome contains a 5′ methylated cap and a 3′ polyadenylated tail, resembling mRNAs of the host cells (Astuti and Ysrafil 2020; Fehr and Perlman 2015).

Once inside the host cell, the viral coat is shed to release viral genome into the cytoplasm. The viral RNA genome undergoes replication and transcription by its own enzymes, and progeny viral RNA are translated by host ribosomes into structural and accessory proteins of the virus (Tables 1 and 2). Initial translation results in two large overlapping polyproteins, pp1a and pp1ab, which are cleaved by virally encoded proteases into proteins and enzymes that are essential for viral replication (Astuti and Ysrafil 2020).

Table 1. SARS-CoV-2 structural proteins.

Structural protein	Characteristics and functions
S	Has S1 and S2 subunits. S1 forms head of spike, contains receptor binding domain (RBD). S2 forms stem to anchor into viral envelope, triggers fusion upon protease activation. S is cleaved at two sites, which exposes a peptide to begin fusion with host receptor ACE2. (Fehr and Perlman 2015; Guo et al. 2020; Walls et al. 2020)
E	Integral membrane protein that forms viral envelope. Translocates along secretory pathway to Golgi intermediate compartment. (Astuti and Ysrafil 2020; Bianchi et al. 2020; Fehr and Perlman 2015)
M	Contributes to viral envelope, responsible for majority of protein–protein interactions that assemble the viruses following their binding to nucleocapsid. Translocates to Golgi intermediate compartment. (Astuti and Ysrafil 2020; Bianchi et al. 2020; Fehr and Perlman 2015)
N	Multiple copies of N protein bind to RNA genome in a continuous beads-on-a-string pattern to form the nucleocapsid inside viral envelope. (Astuti and Ysrafil 2020; Fehr and Perlman 2015)

Table 2. SARS-CoV-2 accessory proteins.

Accessory protein	Functions
3a	Ion channel, endoplasmic reticulum stress, apoptosis
3b	Inhibition of antiviral response, cell cycle arrest at G0/G1
6	Immune evasion, DNA synthesis
7a	Apoptosis, cytokine production, overexpression causes G0/G1 cell cycle arrest
7b	May act as attenuating factor in vivo
8a, 8b	Upregulation of ER chaperones, activation of ATF6 branch of unfolded-protein response
9b	Apoptosis, interaction with exportin 1

Sources: Liu et al. (2014) and Narayanan et al. (2008).

The virus also contains a third category of proteins called nonstructural proteins (NSPs, Table 3), which are derived from the self-cleavage of viral replicase polyprotein (pp1ab) to form the replicase-transcriptase enzyme complex. On the other hand, structural proteins help to assemble new viruses that are released through secretory vesicles by exocytosis to infect other cells (Astuti and Ysrafil 2020; Bianchi et al. 2020; Fehr and Perlman 2015). The SARS-CoV-2 life cycle and replication mechanisms has been reviewed in detail elsewhere (Astuti and Ysrafil 2020; Bianchi et al. 2020; Fehr and Perlman 2015).

Table 3. SARS-CoV-2 nonstructural proteins.

Non-structural protein	Functions
NSP1	Blocks host immune response
NSP2	Binds prohibitin proteins
NSP3	Blocks host immune response
NSP4	Transmembrane scaffolding
NSP5	Cleaves viral polyprotein
NSP6	Transmembrane scaffolding
NSP7	RNA Pol II processivity
NSP8	Primase activity, some roles like NSP7
NSP9	Binds RNA
NSP10	Exoribonuclease, 2′-*O*-methyltransferase
NSP11	Predicted interaction with human TBCA
NSP12	RNA-dependent RNA polymerase (RdRp)
NSP13	RNA helicase, 5′ triphosphatase
NSP14	Exoribonuclease, N7 methyltransferase
NSP15	Endoribonuclease
NSP16	2′-*O*-methyltransferase for 5′ mRNA cap

Sources: Astuti and Ysrafil (2020) and Fehr and Perlman (2015).

Interaction between the viral spike (S) protein on the surface of the virus and the host receptor angiotensin-converting enzyme 2 (ACE2) on the surface of the target cell begins the process of viral entry inside host. ACE2 is a type I membrane receptor located across several cell types including endothelial cells of blood vessels, type II alveolar cells, small intestine enterocytes, and arterial smooth muscle cells of cerebral cortex, brainstem, and many organs (Kim et al. 2020). The S1 subunit of S contains a receptor binding domain (RBD) that binds to ACE2 for entry, and the binding is significantly enhanced by a single N501T mutation of ACE2, which indicates that screening for this mutation in populations can possibly determine proclivity to COVID-19 (Y. Wan et al. 2020). The widespread distribution of ACE2 receptors across so many cell types poses additional challenges to therapeutic developments against COVID-19.

The S protein that is attached to the host receptor is cleaved by a host protease or pro-protein convertase called furin and activated by transmembrane serine protease 2 (TMPRSS2) (Fig. 1), leading to viral entry by endocytosis or direct membrane fusion (Bestle et al. 2020; Hoffmann et al. 2020*a*).

Targeting the cellular entry process of SARS-CoV-2 is a promising therapeutic strategy. Specifically, many researchers are developing neutralizing antibodies that bind to the S protein to prevent its interaction with ACE2 (Amanat and Krammer 2020; Premkumar et al. 2020; Ravichandran et al. 2020; J. Wan et al. 2020). In this direction, ACE2 inhibitors are currently under clinical trials (NCT04367883, NCT04467931, NCT04318418) (Table 4). Since furin and TMPRSS2 play a crucial role in viral entry into the host cell, an inhibitor against TMPRSS2 is in clinical trials (NCT04470544, NCT04321096). Further, studies have proposed that combinatorial inhibitors targeting both furin and TMPRSS will engender a more potent antiviral activity (Barile et al. 2020; Hoffmann et al. 2020*b*). However, furin is also essential for development, hence its inhibition needs to be temporally optimized (Hoffmann et al. 2020*a*).

Immune response to SARS-CoV-2 infection: inflammation and cytokine storm

COVID-19 patients are characterized by dysregulated immune response and acute inflammation in response to SARS-CoV2 infection (Konig et al. 2020; Qin et al. 2020; Ye et al. 2020). The inflammatory response causes various immune cells to release chemical messengers called cytokines. Cytokines are signaling proteins that are secreted by cells and bind to receptors on target cells leading to the recruitment of immune cells at the site of infection (Ragab et al. 2020).

The circulatory system of COVID-19 patients show an abnormal acute elevation of many proinflammatory cytokines like IL-1β, IL-6, IL-2 R, IL-4, IL-7, IL-8, IL-10, TNF-α, IFNγ, and G-CSF (G. Chen et al. 2020; Feldmann et al. 2020; Huang et al. 2020; Konig et al. 2020; McGonagle et al. 2020; Mehta et al. 2020; Pedersen and Ho 2020; Ye et al. 2020; Zhou et al. 2020). Another proinflammatory cytokine upregulated upon SARS-CoV-2 infection is GM-CSF, which is currently a therapeutic target in clinical trials against COVID-19 (Lang et al. 2020) (NCT04400929, NCT04341116). Novel therapies against elevated cytokines and overall therapeutic implications for COVID-19 are discussed in detail elsewhere (Atlante et al. 2020; Bradshaw et al. 2020; Crimi et al. 2020). In summary, the type of immune response described above is referred to as a "cytokine storm" which causes excessive influx of immune cells at the site of infections. The acute influx disrupts normal cell to cell interactions and cellular structures leading to tissue damage, multi-organ failure, and death, which are often seen in COVID-19 patients (Ragab et al. 2020).

Several proinflammatory pathways are activated as a result of this cytokine storm but, in particular, the JAK-STAT pathways are common nodes through which some of these cytokines signal (Feldmann et al. 2020; Huang et al. 2020; Zhou et al. 2020). Secretion of one of these cytokines, IL-6, correlates with the severity of the disease and its downstream effectors include the JAK proteins and STAT3 (Garbers et al. 2018; Kang et al. 2019; Tanaka et al. 2016). As such, the IL-6 receptor neutralizing antibody Tocilizumab has been used in the clinic with patients with severe COVID-19 pneumonia (Luo et al. 2020; Michot et al. 2020; Xu et al. 2020) (NCT04445272, NCT04359667, NCT04356937, NCT04479358).

Levels of expression of the cytokines responsible for the cytokine storm are increased by epigenetic mechanisms. The epigenetic modifications that contribute to the abnormal expression of these cytokines and ACE2 are discussed in detail below.

Fig. 1. SARS-CoV-2's host receptor ACE2 is epigenetically modified and interacts with chromatin modifying enzymes. S protein of SARS-CoV-2 binds to ACE2 receptor on host cells for viral entry into the cell. The S protein attached to ACE2 is cleaved by host protease furin, and activated by transmembrane serine protease 2 (TMPRSS2) for viral entry. Epigenetic modifications associated with ACE2 are DNA methylation, histone H3K27me3 by EZH2, and the interaction of ACE2 with HAT1 (histone acetyltransferase), HDAC (histone deacetylase), and KDM5 (lysine demethylase). To prevent viral infections, several therapies using antibodies and inhibitors are aimed at interrupting the binding of S protein to ACE2. →, promotion; ↔, interaction; –|, interruption.

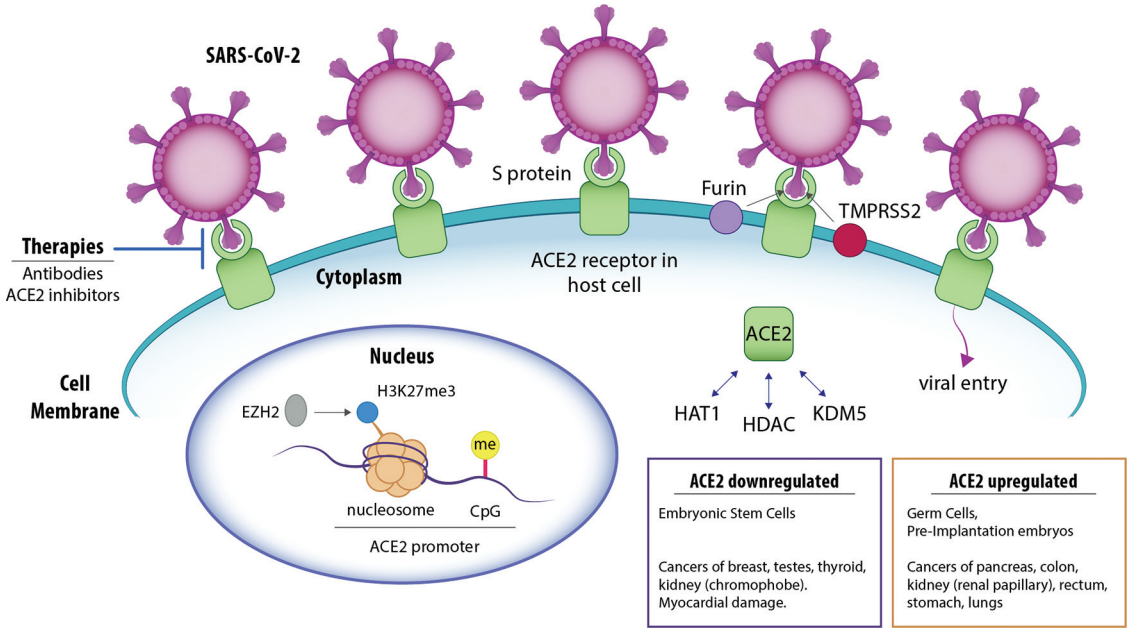


Table 4. Clinical Trials (in order as they appear in text).

Type	Identifier	Link to clinical trial
ACE2 inhibitor	NCT04367883	https://clinicaltrials.gov/ct2/show/NCT04367883
ACE2 inhibitor	NCT04467931	https://clinicaltrials.gov/ct2/show/NCT04467931
ACE2 inhibitor	NCT04318418	https://clinicaltrials.gov/ct2/show/NCT04318418
TMPRSS2 inhibitor	NCT04470544	https://clinicaltrials.gov/ct2/show/NCT04470544
TMPRSS2 inhibitor	NCT04321096	https://clinicaltrials.gov/ct2/show/NCT04321096
GM-CSF	NCT04400929	https://clinicaltrials.gov/ct2/show/NCT04400929
GM-CSF	NCT04341116	https://www.clinicaltrials.gov/ct2/show/NCT04341116
Tocilizumab	NCT04445272	https://clinicaltrials.gov/ct2/show/NCT04445272
Tocilizumab	NCT04359667	https://clinicaltrials.gov/ct2/show/NCT04359667
Tocilizumab	NCT04356937	https://clinicaltrials.gov/ct2/show/NCT04356937
Tocilizumab	NCT04479358	https://clinicaltrials.gov/ct2/show/NCT04479358
Stem cells	NCT04486001	https://clinicaltrials.gov/ct2/show/NCT04486001
Stem cells	NCT04348435	https://clinicaltrials.gov/ct2/show/NCT04348435
Stem cells	NCT04349631	https://clinicaltrials.gov/ct2/show/NCT04349631
Stem cells	NCT04355728	https://clinicaltrials.gov/ct2/show/NCT04355728
Stem cells	NCT04365101	https://clinicaltrials.gov/ct2/show/NCT04365101
Stem cells	NCT04362189	https://clinicaltrials.gov/ct2/show/NCT04362189
Stem cells	NCT04371393	https://clinicaltrials.gov/ct2/show/NCT04371393
Stem cells	NCT04367077	https://clinicaltrials.gov/ct2/show/NCT04367077
Stem cells	NCT04445402	https://clinicaltrials.gov/ct2/show/NCT04445402
Epigenetics	NCT04403386	https://clinicaltrials.gov/ct2/show/NCT04403386
Epigenetics	NCT04411563	https://clinicaltrials.gov/ct2/show/NCT04411563

DNA methylation and its effects on ACE2 expression levels

Since the onset of the COVID-19 pandemic, patients with certain underlying conditions, age, and gender are found to be at a greater risk with higher morbidity and mortality. One such condition seen in COVID-19 patients is hypertension (Richardson et al. 2020). In this direction, the epigenetic regulation of *ACE2* by DNA methylation and histone modifications can shed significant insights on how they affect the involvement of ACE in

diseases (Chlamydas et al. 2020). A comparison of DNA methylation profiles across five genomic loci of the *ACE2* promoter in 96 patients with essential hypertension (EH) show loci-based and sex-based differences in methylation patterns, underscoring the significance of subtle nuances of epigenetic regulations at play (Fan et al. 2017). DNA methylation in a promoter CpG island is a known mark of transcription repression, meaning that higher levels of CpG DNA methylation generally lead to lower levels of gene expression (Deaton and Bird 2011). Conversely, lower promoter CpG island methylation is associated with higher expression and methylation of the *ACE2* promoter is lower in the lung epithelium than the gut, the liver, the pancreas, the brain, and blood according to a study in preprint by M.J. Corley and L.C. Ndhlovu. Additionally, higher expression of ACE2 in the lungs is correlates with higher disease severity (Leung et al. 2020; Pinto et al. 2020). Hypomethylation of ACE2 and its contribution to higher susceptibility to COVID-19 has been further reviewed in a recently published article (Pruimboom 2020). More vulnerability to COVID-19 is also observed in cancer patients, where DNA methylation at the *ACE2* locus may play a role (Curigliano 2020; Indini et al. 2020; Kuderer et al. 2020). Analysis of cancer databases revealed that *ACE2* promoter is hypomethylated, and *ACE2* mRNA is highly expressed in cancers of colon, kidney, pancreas, rectum, lung, and stomach (Chai et al. 2020).

X-chromosome inactivation and COVID-19 severity

Apart from CpG island methylation of the *ACE2* promoter, X-chromosome inactivation, which depends in part on DNA methylation, also plays a role in ACE2 expression. A sex-related analysis from Italian Istituto Superiore di Sanità (ISS) on 239 709 patients reported the infection rate of COVID-19 to be higher in females than in males, because 54.2% of the cases are found in females. The *ACE2* locus is on a portion of the X-chromosome that can escape X-inactivation, which may explain the higher ACE2 expression observed in females and the above-mentioned higher rate of infection in females (Benetti et al. 2020; Berletch et al. 2011; Posynick and Brown 2019; Tukiainen et al. 2017). *TLR7*, which encodes Toll-like receptor 7, is another X-chromosome gene and plays a major role in the innate immune response, activating type I and II interferon responses (Du et al. 2000). Like *ACE2*, *TLR7* evades X-inactivation and there are similar gene dosage effects in females versus males (Souyris et al. 2018). Of relevance to COVID-19, males with loss-of-function variants of *TLR7* had extremely severe disease states after being infected by SARS-CoV-2 as compared with age and sex matched COVID-19 patients without *TLR7* mutations (van der Made et al. 2020). Consistent with these findings, COVID-19 patients with impaired interferon responses tend to have more severe disease and, as such, epigenetic mechanisms that downregulate these responses can influence COVID-19 severity (Arunachalam et al. 2020; Blanco-Melo et al. 2020; Hadjadj et al. 2020).

Connections between ACE2 and histone modifications

In addition to DNA methylation, histone modifications also epigenetically regulate *ACE2*. Post-translational modifications occur at amino acid residues on N-terminal tails of histones (Ramakrishnan 1997). Based on the nature of histone modifications, chromatin structure either opens up for accessibility to transcription factors to facilitate transcription, or, chromatin condenses and becomes inaccessible to transcription factors, leading to transcription repression. One such repressive mark is H3K27me3, which is catalyzed by the histone methyltransferase EZH2. In the absence of functional EZH2, H3K27me3 levels are reduced, causing an increase in ACE2 expression in a mouse germ cell line (Li et al. 2020).

Since ACE2 is enriched in human preimplantation embryos, germ cells, and repressed by EZH2 in human embryonic stem cells, further knowledge in this direction will contribute to ongoing anti-COVID-19 clinical trials focusing on ACE2, stem cells, and epigenetics (Li et al. 2020).

Epigenetic modifiers like HAT1, HDAC2, and KDM5B are predicted as potential regulators of *ACE2* from correlation and network analyses of lung transcriptome from 700 patients with high expression of *ACE2* and comorbidities for severe COVID-19 (Pinto et al. 2020). Further, proteomics analysis has also predicted high-confidence interaction between NSP5 of SARS-CoV2 and human HDAC2 (Gordon et al. 2020*b*). These findings, however, are correlative and further investigation is required to elucidate whether these or other epigenetic writers can directly affect ACE2 expression. Hence, deeper understanding of epigenetic regulations of *ACE2* through DNA methylation, histone modifications, and chromatin modifying enzymes (Fig. 1) will help in the development of epigenetics-based therapies and deciphering the pathophysiology of severe COVID-19 in patients with comorbidities. Indeed, omics-based studies for drug repurposing in COVID-19 are being pursued (Mousavi et al. 2020). However, an important open question in this context arises as to where viral proteins and their predicted host interaction partners are located inside the host cell. In this direction, immunofluorescence localization analysis of all 2×Strep-tagged SARS-CoV-2 proteins in HeLaM cells have been reported (Gordon et al. 2020*a*). The localization of NSP5 is in the cytoplasm and plasma membrane, while the other very important S protein localizes to the endoplasmic reticulum and plasma membrane (Gordon et al. 2020*a*). On the other hand, NSP5's predicted interaction partner, HDAC2, is located in the nucleus. It will be interesting to compare the localization of viral proteins with that of their predicted host interaction partners, and also investigate how significant differences in location between viral and host protein partners are overcome during their interactions.

Regulation of inflammatory pathway genes by histone modifications

Histone modifications regulate genes associated with mounting an immune response to coronavirus infection. During infection, host pathogen recognition receptors (PRRs), e.g., Toll-like receptors (TLR), recognize viral pathogen-associated molecular patterns (PAMPs), leading to a cascade of innate immune response reactions (Schafer and Baric 2017). Dendritic cells and macrophages are mainly responsible for sensing pathogenic signals that activate these cells to initiate a rapid, persistent, and specific immune response.

One of the mechanisms of the response's transduction is the activation of interferons (IFNs) and tumor necrosis factor (TNF). IFNs and TNF elicit primary responses as key genes of the innate immune system. They are rapidly activated, causing IFNs to induce expression of interferon-stimulated genes (ISG). These proteins are some of the key players in the cytokine storm that is a hallmark of severe COVID-19 (Wilk et al. 2020).

Histone modifications have been implicated in the regulation of IFNs, TNFs, and ISGs, and hence innate immune response. In COVID-19 patients, IFN-γ, TNF-α, and ISGs are highly upregulated (Costela-Ruiz et al. 2020; Wilk et al. 2020). An accumulation of histone acetylation and RNA polymerase II (RNAPII), both of which are hallmarks of transcription activation, are observed at specific promoters in activated macrophages and dendritic cells (De Santa et al. 2010; Kim et al. 2010). Often, promoters of TLRs are marked with H3K4me3, which is a transcription-activating histone modification. The promoters of IFNs and TNF are usually in a poised state, often containing CpG islands that are not favorable for transcription activation (Stender and Glass 2013). The poised promoters generally show the presence of transcription-activating H3K4me3 and transcription-repressing H3K27me3 marks.

Another histone modification seen on promoters of type I interferon is H3K9me2, a repressive chromatin mark. It promotes heterochromatin formation, and prevents histone acetylation that favors euchromatin, through the recruitment of heterochromatin protein 1 (HP1) family, which is a transcription repressor (Fang et al. 2012). Since H3K9me2 is a transcription repressive mark, its occupancy of IFN promoters in dendritic cells shows inverse correlation with ISG expression (Aevermann et al. 2014; Fang et al. 2012).

The epigenetic landscape of ISGs are different from IFNs and TNFs because they show reduced presence of transcription activating histone modifications like H3K4me3, H4Ac, and decreased RNAPII occupancy (Agalioti et al. 2000). Usually, ISGs require additional stimulation from transcription factors and chromatin remodelers like ATP-dependent chromatin remodeling complex SWItch/sucrose non-fermentable (SWI/SNF) to initiate transcription (Busslinger and Tarakhovsky 2014; Smale et al. 2014). Hence, a combination of histone marks and chromatin modifiers are involved in innate immune response to SARS-CoV-2 infection (Mantovani and Netea 2020) (Fig. 2).

The link between histone citrullination and COVID-19

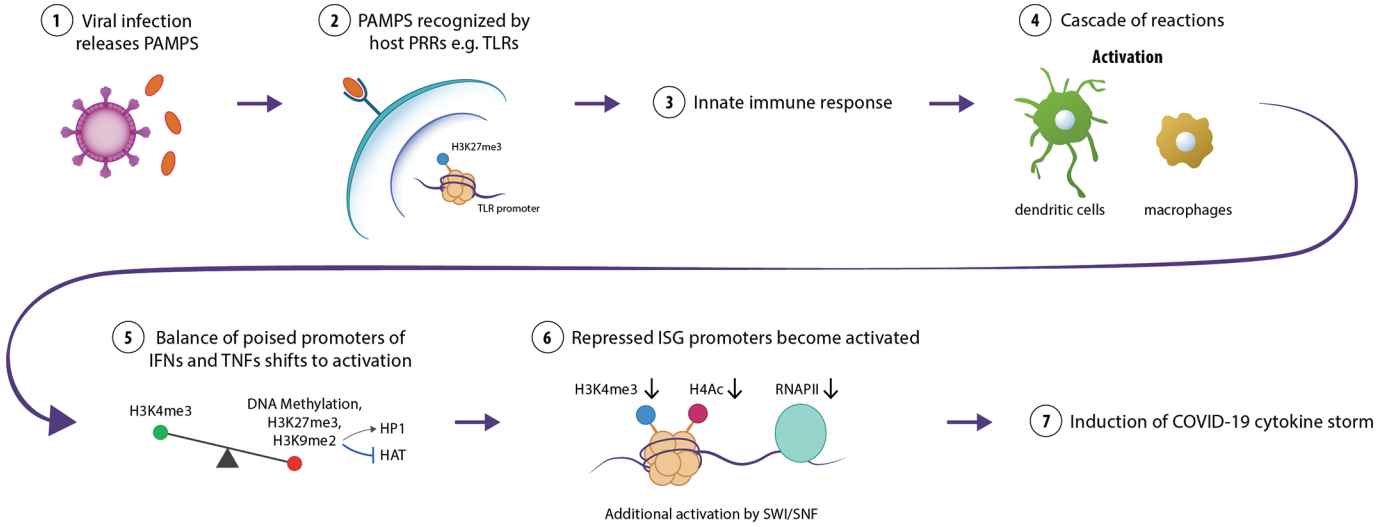
Citrullination of histone H3 (Cit-H3) is another epigenetic modification detected in COVID-19 patients. Citrullination, or deamination, of arginine residues on histones decondenses chromatin structure to render it more accessible for transcription (Christophorou et al. 2014; Leshner et al. 2012; Li et al. 2012; Wang et al. 2009). Cit-H3 is a specific marker of neutrophil extracellular traps (NETs), which is an immune response to infection (Wang et al. 2009). Composed of granule proteins and decondensed chromatin, NETs are a concentrated source of localized microbicides and antioxidants (Brinkmann et al. 2004; Porto and Stein 2016; Thiam et al. 2020; Twaddell et al. 2019).

In COVID-19 patients, levels of citrullinated histone H3 (Cit-H3) are elevated (Zuo et al. 2020), which positively correlates with increased cytokine IL-8, leukocyte, and granulocyte counts in COVID-19 (Leppkes et al. 2020). COVID-19 patients show an elevation of NETs in plasma, tracheal aspirate, and lung epithelium and tissue including alveolar space (Middleton et al. 2020; Skendros et al. 2020; Veras et al. 2020; Zuo et al. 2020). Interestingly, COVID-19 patients show damage to lungs, alveoli, and airway epithelia, cytopathy, squamous metaplasia, endothelial swelling, small fibrinous thrombi, and neutrophilic pneumonia (Veras et al. 2020). Microthrombus formation in COVID-19 patients are proposed to elicit autophagy and a coagulation cascade that likely lead to excessive NETosis (Cicco et al. 2020; Hidalgo 2020).

Severe COVID-19 patients show apoptosis of lung epithelial and endothelial cells (Veras et al. 2020). Since neutrophils have short life spans and cell death processes are linked to the formation of NETs or NETosis, activation of cell death processes are predicted to be one of the many contributing factors toward increased Cit-H3 in COVID-19, but detailed investigation of such connection is warranted (Zuo et al. 2020).

COVID-19 patients show increased release of NETs from their neutrophils, and sera from those patients stimulate NETosis when added to control neutrophils (Zuo et al. 2020). The observation is supported by a previous study showing a dramatic elevation in histone citrullination, chromatin decondensation, and NETosis upon treatment of neutrophils with proinflammatory cytokines (Wang et al. 2009); and SARS-CoV-2 infection causing a cytokine storm. Collectively, the above studies indicate that SARS-CoV-2 infection and viral proteins likely induce neutrophils directly to release NETs (Cicco et al. 2020; Veras et al. 2020). These NETs cause several detrimental effects including lung epithelial apoptosis (Veras et al. 2020). Although a specific reason for

Fig. 2. Epigenetic modifications are implicated during the innate immune response to SARS-CoV-2 infection. Viral infection releases pathogen-associated molecular patterns (PAMPs), which are recognized by pattern recognition receptors (PRRs), e.g., Toll-like receptors (TLRs). A cascade of reactions is set off as innate immune response. Dendritic cells and macrophages are activated. Promoters of interferon (INFs) and tumor necrosis factor (TNFs) exist in poised state, showing both transcription-activating histone marks like H3K4me3 and repressive marks like CpG methylation, H3K27me3, and H3K9me2. H3K9me2 further recruits heterochromatin protein 1 (HP1) and represses histone acetyltransferase (HAT) activity. Activated IFNs lead to the activation of interferon-stimulated genes (ISGs). Epigenetic landscape of promoters of ISGs show reduced activating marks like H4K4me3 and H4 Ac (acetylation) and RNA polymerase II (RNAPII). ATP-dependent chromatin remodeler SWI/SNF contributes to the activation of ISG promoters. ↓, downregulation; →, recruitment; –|, repression.



enhanced NETosis in COVID-19 is not yet established, several causes are proposed to trigger it.

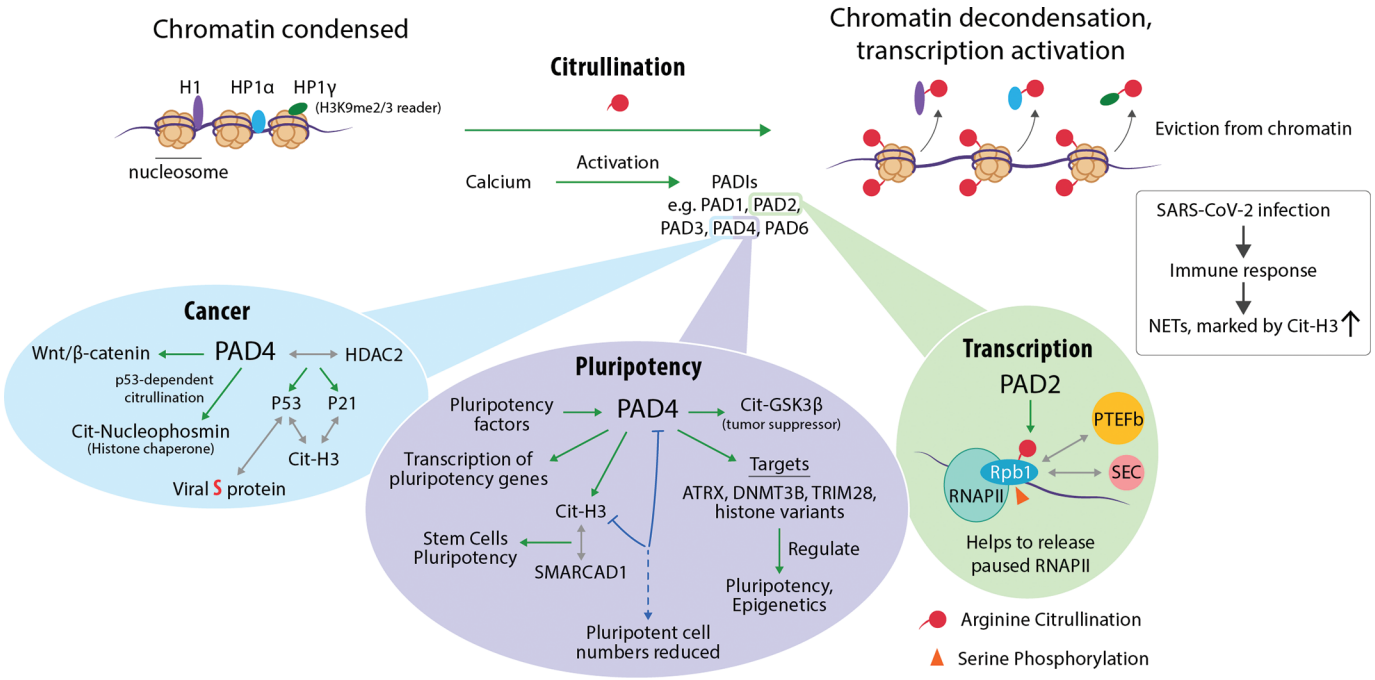
Zuo et al. report a positive correlation between the levels of Cit-H3 and platelet counts, indicating the role of abnormal platelet counts as one of the possible reasons of NETs in COVID-19 patients (Zucoloto and Jenne 2019; Zuo et al. 2020). In this direction, transcriptomics analysis by RNA sequencing of lung samples of COVID-19 patients show about 5.5-fold upregulation of platelet factor 4, which is implicated in platelet-induced NETosis (M. Wu et al. 2020). Cytokine storm in COVID-19 is likely another major contributor for elevation of NETs because cytokines enhance the recruitment and activity of neutrophils for NETosis (Cicco et al. 2020).

Proteomics analysis of lung samples of COVID-19 patients by LC-MS (liquid chromatography mass spectrometry) show enhanced expression of proinflammatory mediators like S100A8/A9 and S100A12, which induce cytokine secretion and are themselves released from neutrophils and macrophages (M. Wu et al. 2020). Hence, the observation supports enhanced neutrophils and NETosis in COVID-19 patients' lungs.

Possible other contributors of increased NETosis in COVID-19 patients could be cardiovascular complications, overall tissue damage, and sepsis. NETs affect vasculature including arteries, vein, and smaller vessels during cardiovascular disease, hence the cardiovascular and arterial pathologies seen in COVID-19 and associated end organ damage are likely linked to elevation of NETs (Cicco et al. 2020; Zhou et al. 2020; Zuo et al. 2020). Further, symptoms of sepsis like cytokine storm, organ damage, and increase of NETs in organs and circulation are reflected in COVID-19 where increased circulating neutrophil levels predict a worse outcome (T. Chen et al. 2020; Guan et al. 2020; Veras et al. 2020). Moreover, Cit-histone H3 levels are increased in the plasma samples of COVID-19 patients with sepsis (Lee et al. 2021).

At the molecular level, citrullination is caused by peptidylarginine deiminases (PADIs), namely PAD1, PAD2, PAD3, PAD4, and PAD6 (Beato and Sharma 2020), which are activated by calcium (Vossenaar et al. 2003). Increase in intracellular calcium by the treatment of HL-60 granulocyte cells with calcium ionophores causes citrullination of arginine residues of histone H3 and H4, leading to extensive chromatin decondensation (Wang et al. 2009, 2004). Citrullination of chromatin condensing proteins like histone H1 (Buttinelli et al. 1999; Juan et al. 1997; Laybourn and Kadonaga 1991), heterochromatin protein 1α (HP1α) (Sharma et al. 2012), and heterochromatin protein 1γ (HP1γ) (Wiese et al. 2019) cause their eviction from nucleosomes, leading to chromatin decondensation and transcription activation (Christophorou et al. 2014). PAD4-mediated citrullination of HP1γ, the principal reader of the repressive histone marks H3K9me2/3, reduces its chromatin-binding affinity to favor transcription (Wiese et al. 2019). Further, transcription is also promoted by citrullination of Rpb1, the largest subunit of RNAPII, which helps to release promoter-proximally paused

Fig. 3. COVID-19 patients show an upregulation of histone citrullination, which is also implicated in cancers, stem cell pluripotency, and transcription. COVID-19 patients show upregulation of citrullinated histone H3, which is a marker of NETs. Histone citrullination is also implicated in cancers, pluripotency, and transcription. All three of these processes have connections with COVID-19 as follows. The tumor suppressor p53, which has multiple associations with histone citrullination, is bioinformatically found to interact with viral S protein. Citrullination is implicated in pluripotency of stem cells, which are implemented in clinical trials against COVID-19. Histone citrullination decondenses chromatin which impacts transcription. COVID-19 patients show disrupted transcription of several genes, and since citrullination is upregulated in these patients, it is important to investigate if and how that affects the above three processes in COVID-19 patients. Cit, citrullinated; NETs, neutrophil extracellular traps; PADI, peptidyl arginine deiminase; H1, histone 1; HP1, heterochromatin protein 1; RNAPII, RNA polymerase II; PTEFb, positive transcription elongation factor; SEC, super elongation complex. →, regulation or promotion; ↔, interaction; –|, inhibition; – – –>, resulting effect.



RNAPII and facilitate its interactions with positive transcription elongation factor (PTEFb) and super elongation complex (SEC) (Sharma et al. 2019). It will be interesting to investigate if the elevation in Cit-H3 for NETosis in COVID-19 patients interfere with the roles of citrullination in chromatin and transcriptional regulation as described above.

As excessive NETs are inimical (Boeltz et al. 2019), NETs are proposed as therapeutic targets for COVID-19 (Barnes et al. 2020; Paraćkova et al. 2020; Tomar et al. 2020; Yaqinuddin et al. 2020). In this direction, Dipyridamole, an FDA-approved drug that inhibits NET formation, is on clinical trials against COVID-19 (Liu et al. 2020; Zuo et al. 2020) (NCT04391179). Also, new methodology to detect the pathophysiological role of NETs are being developed (Lv et al. 2020). Further, NETs in other pathologies like infection-related lung injury and periodontal disease pose increased risks to COVID-19 patients (Gupta and Sahni 2020). Detection of NETs in COVID-19 also indicates toward resurgence of a non-infectious inflammatory illness among convalescents, which is termed as "Post-COVID-19 Syndrome" (Sawadogo et al. 2020).

Histone citrullination has also been reported to play important roles in multiple other human diseases such as cancers, autoimmune diseases, and thrombosis. All these diseases are critical risk factors for more severe COVID-19 disease. Interestingly, bioinformatics analysis revealed a strong interaction between SARS-CoV-2 S protein and p53 (Singh and Bharara Singh 2020), which is a tumor suppressor that mediates citrullination of histone chaperone nucleophosmin (Tanikawa et al. 2009) and also interacts with citrullinated histone H3 (Beato and Sharma 2020; Li et al. 2010). Therefore, it is crucial to investigate how citrullination might impact epigenetic regulations and the pathophysiology of COVID-19 (Fig. 3) in COVID-19 patient populations who are vulnerable due to comorbidities like cancers, autoimmune diseases, and thrombosis.

Histone citrullination, stem cell pluripotency, and COVID-19

Histone citrullination is an important epigenetic modification that is required for pluripotency of stem cells (Buttinelli et al. 1999; Christophorou et al. 2014; Slade et al. 2014*a*, 2014*b*; Theunissen et al. 2011; Wiese et al. 2019; Xiao et al. 2017). Pluripotent cell count during early embryogenesis decreases upon inhibition of PAD4

or histone citrullination (Jones et al. 2012). In addition to pluripotency, PAD4 is also connected with cellular reprogramming through its interactions with Yamanaka transcription factors like Oct4, Klf4, and Sox2, which reprogram somatic cells into pluripotent cells (Yamanaka and Blau 2010). In murine stem cells, Oct4, Klf4, and Sox2 bind to the promoter of Pad4 (Christophorou et al. 2014).

The links between stem cell pluripotency, reprogramming, and histone citrullination are significant in the context of COVID-19. There are currently nine ongoing clinical trials in the United States focused on stem cell therapy against COVID-19 (NCT04486001, NCT04348435, NCT04349631, NCT04355728, NCT04365101, NCT04362189, NCT04371393, NCT04367077, NCT04445402). Understanding pluripotency and reprogramming are critical for COVID-19 patients regarding certain conditions, e.g., organ transplants, maternal transmission to neonates (Walker et al. 2020).

Interactions between human epigenetic factors and SARS-CoV-2 proteins

Human epigenetic factors have been revealed as interaction partners of SARS-Cov-2 proteins. In a recent study, 26 SARS-CoV-2 proteins were studied by affinity purification mass spectrometry (AP–MS) analysis to map the protein interactome between virus and host proteins (Gordon et al. 2020*b*). Three hundred and thirty-two human proteins emerged as binding partners for SARS-CoV-2 proteins, out of which eight are epigenetic modifiers. Interactions were revealed between viral NSP5 and human HDAC2, viral E protein and human BRD2-BRD4, and viral ORF10 and human CUL2 complex (Gordon et al. 2020*b*).

The study identified a cleavage site in HDAC2 between the HDAC domain and its nuclear localization sequence. Detection of the site indicates that NSP5 likely inhibits the nuclear localization of HDAC2, which probably impedes HDAC2-based mediation of inflammation and interferon responses. This possibility is derived from the fact that HDAC2 deacetylates H4K16 at ISG promoters for optimal expression of ISGs, but its interaction with NOS1 prevents HDAC2 from eliciting the inflammation response (Xu et al. 2019).

The viral E protein was found to interact with BRD2 and BRD4, which belong to the family of bromodomain and extra-terminal (BET) domain proteins that are readers for acetylated histones leading to transcription activation. It is likely that N-terminal region of histone H3, which is the site of interaction with BRD proteins, is mimicked by E protein's C-terminal region, which results in an interaction between E and BRD proteins.

The SARS-CoV-2 protein ORF10 interacts with CUL2, ELOB, ELOC, RBX1, and ZYG11B, which are members of human Cullin-RING E3 ubiquitin ligase complex. The complex ubiquitinates proteins for degradation by 26S proteasome (Berndsen and Wolberger 2014; Cai and Yang 2016). It is hypothesized that ORF10 binds CUL2 to hijack CUL2-mediated ubiquitination and degradation to benefit viral replication.

Epigenetic-based therapies and clinical trials

Given the key roles of epigenetic mechanisms in the regulation of multiple aspects of COVID-19, it is not surprising that epigenetic enzymes are being targeted as potential therapeutic strategies. There are already two clinical trials initiated that focus on epigenetic mechanisms. Many additional clinical trials are also underway with other strategies to treat COVID-19, but this review only focuses on those targeting epigenetics mechanisms. One of the clinical trials (NCT04403386) will explore biomarkers for exposure to smoking, immune cell profiles, and their transcriptional and DNA methylation patterns. The study will determine if there is a greater threat from SARS-CoV-2 infection-related morbidity and mortality faced by smokers compared to nonsmokers because smoking alters epigenetic signatures (McCartney et al. 2018).

The second study (NCT04411563) aims to understand the correlation between COVID-19 severity and associated circulating epigenetic markers like microRNAs and epigenetic signatures like DNA methylation in the presence or absence of pneumonia and severe acute respiratory syndrome (SARS). DNA methylation status is assessed for *ACE2*, *TMPRSS2*, and *PARP* genes, where PARP is a key player in activating interleukins for cytokine storm (Curtin et al. 2020). The study also investigates the benefits of prognostic epigenetic markers.

Conclusion and future directions

Multiple layers of epigenetic regulation, including DNA methylation and histone modifications, contribute to determining the outcome of SARS-CoV-2 infection. The host cell attempts to mount an effective immune response to viral infection that is mediated in part by epigenetic mechanisms, and the virus attempts to evade this response and reprogram the cell to create an environment that facilitates viral replication, assembly of new virus particles, and propagation to infect new cells.

COVID-19 has proven to be a difficult disease to fight because it is highly infectious and can spread quickly before symptoms emerge, which makes the slowing down of the virus by quarantining infected patients challenging. Therefore, new approaches and technologies are needed to beat this virus.

The therapeutic approaches that target epigenetic proteins can potentially shift the arms race in favor of the infected patient and block viral replication and spread. To be effective in ending the global COVID-19 pandemic, the therapies will need to be made accessible to populations worldwide in a quick and economical manner, which requires cooperation from government

administrations, manufacturing companies and institutions, and health insurance policies.

The studies highlighted in this review represent a great start in our understanding of SARS-CoV-2, but we still have a lot left to learn. More research on the basic biology of SARS-CoV-2 and related viruses are needed to stop this pandemic and to help ensure that another viral outbreak is not as disruptive in the future.

Conflict of interest statement

Authors declare no conflict of interest.

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