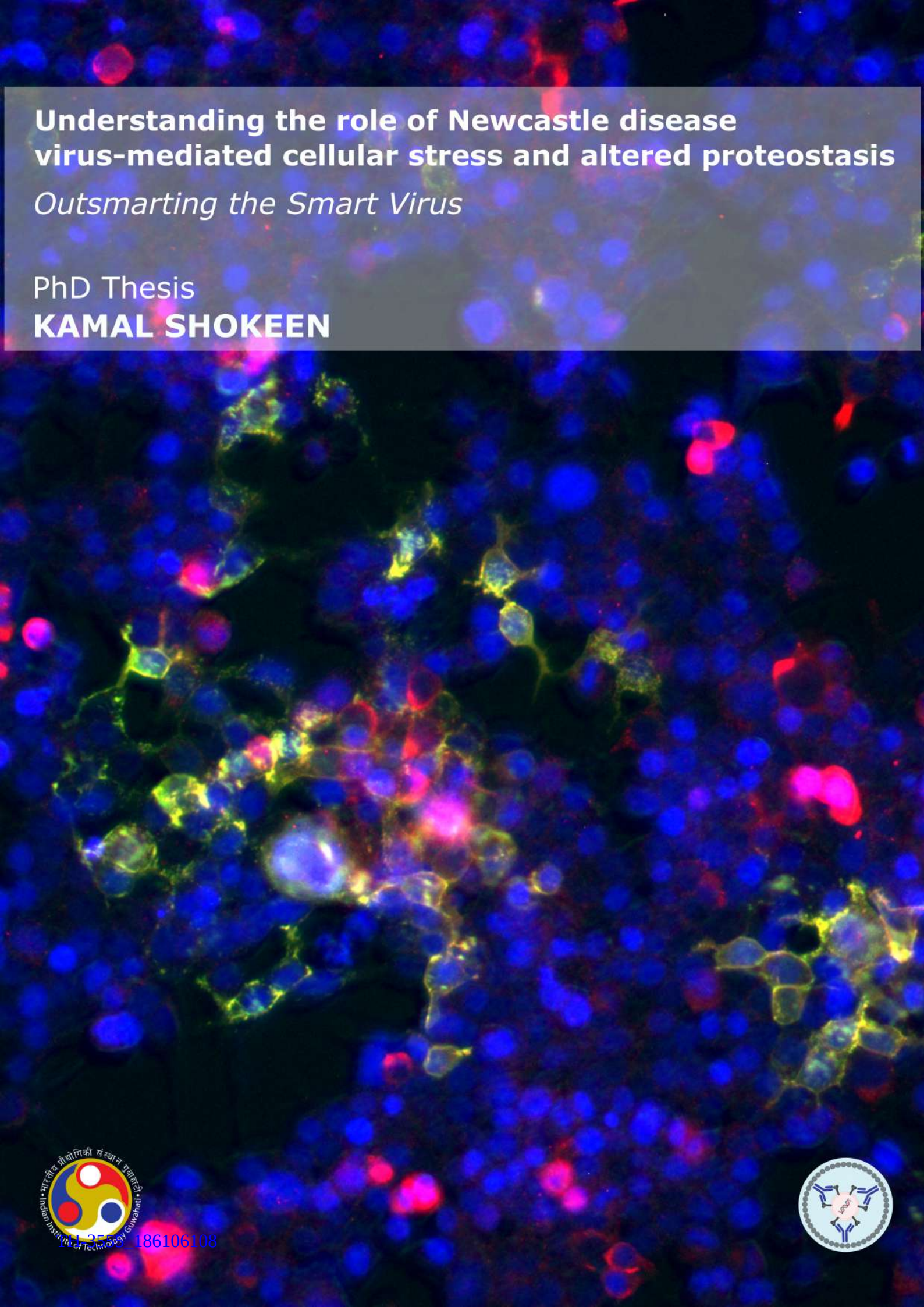
Understanding the role of Newcastle disease virus-mediated cellular stress and altered proteostasis

Outsmarting the Smart Virus

PhD Thesis

KAMAL SHOKEEN



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Understanding the role of Newcastle disease virus-mediated cellular stress and altered proteostasis

"Outsmarting the Smart Virus"

Thesis submitted in partial fulfilment of the requirements
for the award of the degree of

Doctor of Philosophy

by

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Under the Guidance of

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Dedicated to all those who have enriched me through their teachings and guidance. I couldn't have done it without you.

DECLARATION

I do hereby declare that this written submission represents my original ideas in my own words. Where others' ideas and words have been included, I have adequately cited and referenced the original source. I declare that I have adhered to all academic honesty and integrity principles and have not misrepresented, fabricated, or falsified any data in my submission.

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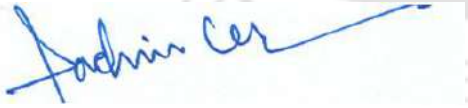
CERTIFICATE

This is to certify that the thesis entitled **"Understanding the role of Newcastle disease virus-mediated cellular stress and altered proteostasis: Outsmarting the Smart Virus"**, submitted by **Kamal Shokeen** to the Indian Institute of Technology Guwahati, for the award of the degree of **Doctor of Philosophy** in the Department of Biosciences and Bioengineering, is a record of the original research work carried out by him under my supervision and guidance. The thesis has reached the standards fulfilling the requirements of the regulations related to the award of the degree.

The results contained in this thesis have not been submitted in part or in full to any other University or Institute for the award of any degree or diploma to the best of my knowledge.



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"*Acknowledging the good that you already have in your life is the foundation for all abundance.*"— Eckhart Tolle, A New Earth: Awakening to Your Life's Purpose.

This thesis is a collective achievement, reflecting the invaluable efforts of all those who have helped and supported me along the way. I am blessed to have these many people in my life to whom I want to express my sincere thanks. The depth of gratitude I feel for all the love, energy, and intelligence they have poured into me is difficult to put into words. But I'll try.

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"*It's in those quiet little towns, at the edge of the world, that you will find the salt of the earth people who make you feel right at home.*" — Aaron Lauritsen (100 Days Drive: The Great North American Road Trip)

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Thank you to every one of you. Your blessings move the fingers that hold my pipette.

Signing off,

Kamal

"We go about our daily lives with limited understanding of ourselves, giving little thought to the functioning of our bodies. There is a lot of stuff happening inside that is unknown, waiting to be discovered."

Abstract

Viruses are known to instigate a variety of stresses in their host cells, including endoplasmic reticulum (ER) and oxidative stress, to affect normal cell functioning and their replication cycle. The virus-induced stress subsequently triggers cellular stress responses by activating numerous stress-responsive proteins (SRPs). Afterward, all the viral proteins and SRPs substantially exacerbate ER protein flux, eliciting the unfolded protein response (UPR). This can be characterized by the modulation of Glucose-Regulated Protein 78 (GRP78) as an ER stress sensor. Moreover, viruses have also been shown to induce oxidative stress by disrupting the balance of reactive oxygen species (ROS) inside the cells. However, the purpose of virus-induced stresses and the precise role of SRPs in infection remain elusive. The present work utilizes the Newcastle disease virus (NDV) to investigate the role of cellular stress and the function of specific SRPs in pathogenesis.
Firstly, the thesis deals with the instigation of ER and oxidative stress following NDV infection. Here, we show that NDV induces ER stress, confirmed by the GRP78 upregulation, and oxidative stress, established by the accumulation of intracellular ROS levels. Additionally, the results demonstrate that NDV infection modulates the expression of key oxidative stress-responsive genes, including Nrf2, HO-1, and SOD-1. The members of the sirtuin (SIRT) family were also found to be differentially modulated due to NDV infection.
Secondly, the thesis specifically focuses on establishing the function of SIRT7 protein in NDV pathogenesis. SIRT7 primarily functions as a NAD^+-dependent histone deacetylase enzyme, thus regulating multiple biological processes, including genome stability, metabolic pathways, and stress responses. However, its enzymatic activity, physiological function, and target proteins in virus-infected cells remain unexplored. Here, the detailed mechanistic studies reveal that elevated expression of NDV-mediated SIRT7 protein metabolizes the NAD^+ to deacetylate the host proteins, thus contributing to high virus replication. Considering the results, we establish the constructive role of SIRT7 in NDV replication.

The heightened production of viral proteins and SRPs can eminently disrupt protein homeostasis, prompting the cells to activate specific proteolytic pathways. These pathways employ post-translational modifications (PTMs) to direct ubiquitin-mediated proteasomal degradation of aberrantly accumulated proteins. Among various PTMs, the addition of Arg to the N-terminus of target proteins (Arginylation) by arginyltransferase 1 (ATE1) enzyme remains unexplored mainly in virus pathogenesis. Lastly, the thesis delves into the mechanism of arginylation on host proteins and the engagement of proteasomal degradation machinery. The results show that NDV alters proteostasis, prompting the cells to activate the ATE1 enzyme for arginylation. Here, we also explored the HN protein of NDV as a presumable target for arginylation-mediated degradation.

The thesis findings collectively indicate that NDV infection induces stress within cells, resulting in the upregulation of numerous SRPs. Among these SRPs, the SIRT7 protein was observed to facilitate NDV infection by deacetylating the host proteins, thereby impeding the cellular antiviral response. Subsequently, excessive protein production leads to proteostasis imbalance, which is further mitigated by the activation of proteolytic pathways. We also confirmed the active participation of the arginylation process in infected cells. Finally, this process can be employed to degrade the HN-like viral proteins, thereby establishing a unique host defense mechanism.

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CHAPTER 1

INTRODUCTION

1.1 Preamble

Cells typically carry out their regular physiological activities within a controlled environment, and any deviation from the normal conditions causes multiple types of stress. Stress is usually defined as any physical, chemical or biological factor that disrupts cellular homeostasis, thus triggering stress responses via the activation of several signaling pathways [54]. The cell's ability to activate such pathways in response to stress is crucial for maintaining cellular homeostasis [225]. Various defense mechanisms are activated by cells depending on the mode and extent of stress they experience [141]. Stress mainly prompts the cells to induce cell repair mechanisms, initiate temporary adaptation responses, induce autophagy or trigger cell death [138]. These responses are made possible through the modulation of various proteins known as stress-responsive proteins (SRPs) [225, 281]. These highly conserved SRPs are diverse, with specific functions to establish dysfunctional states and prevent disease development [226]. The coordinated activation of SRPs represents all the defense strategies cells employ to ameliorate the damage caused by the stressors. Prolonged exposure to any stressor, such as toxins, lack of nutrients, heavy metals, or pathogens [305, 92], leads explicitly to endoplasmic reticulum (ER) stress and oxidative stress.

ER is a complex organelle that functions as a protein synthesis, folding, modification, and transport site [73]. The protein flux through ER is closely monitored for

abnormalities such as unfolded or misfolded protein accumulation. It has sophisticated networks that allow it to respond to the aberrant buildup of misfolded proteins. These networks of signal transduction pathways, collectively termed unfolded protein response (UPR), restore normal cellular homeostasis and may also play critical roles in pathogenesis [360, 253]. UPR often leads to ER chaperones' upregulation, protein translation reduction and subsequent degradation of misfolded proteins [12]. It is mediated by three transmembrane ER-proximal regulators: PKR-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring element 1 (IRE1) [197, 361]. Under normal conditions, the master ER chaperone glucose-regulated protein 78 (GRP78) binds these proteins in an inactive state [163, 242]. However, during UPR, it dissociates from these sensors to facilitate protection against the overwhelming load of misfolded proteins and produce the molecules necessary for restoring the ER equilibrium [287, 265, 473]. It also subsequently binds to the accumulated misfolded proteins and initiates ER-associated degradation (ERAD) responsible for UPR regulation (Fig. 1.1) [69].

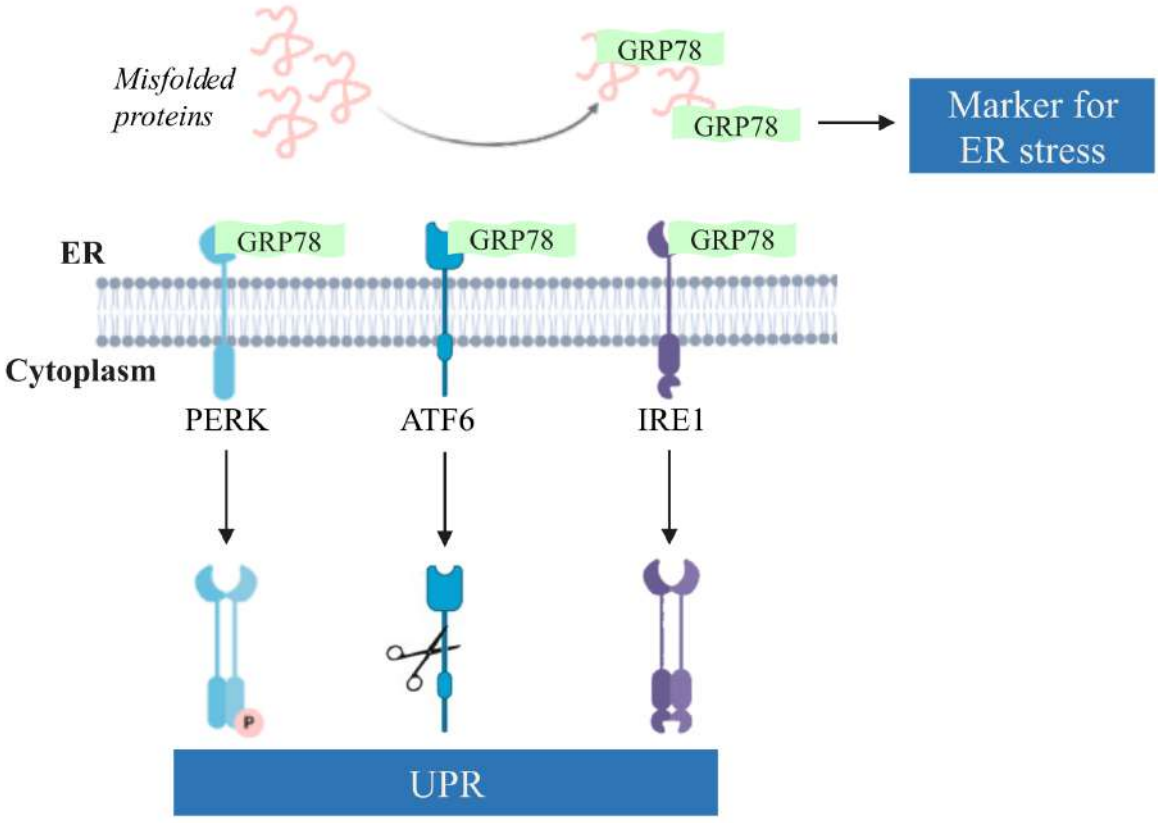

Figure 1.1: Schematic representation of unfolded protein response and GRP78 functioning.

Additionally, stressors instigate oxidative stress inside the cells by disturbing the balance between producing reactive oxygen species (ROS) and antioxidant defenses [140, 359]. Mitochondria are a primary source of intracellular ROS production during the electron-transport chain [272, 293]. Specifically, the electron leakage from respiratory complexes I and III of the respiratory chain generates ROS. The most reactive free radicals are superoxide anion ($O_2^{\cdot}$-) and the hydroxyl radical ($OH^{\cdot}$) [412, 413]. Increased levels of free radicals can induce mitochondrial DNA mutations, alter the membrane permeability, damage the mitochondrial respiratory chain, and influence Ca_2^+ homeostasis [31, 46]. Mitochondria generally utilizes a multi-layer network of systems that produce various antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), for protection from oxidative damage (Fig. 1.2) [304]. SODs catalyze the removal of $O_2^{\cdot}$- by dismutating it into H_2O_2 and O_2, which inhibits the formation of $OH^{\cdot}$ by metal-catalyzed Haber-Weiss reaction [202, 55]. High ROS also influences several signaling pathways, including NF-κB, MAPKs, Keap1-Nrf2-ARE, and PI3K-Akt, ion channels and transporters (Ca_2^+ and mPTP) [71, 383, 220].

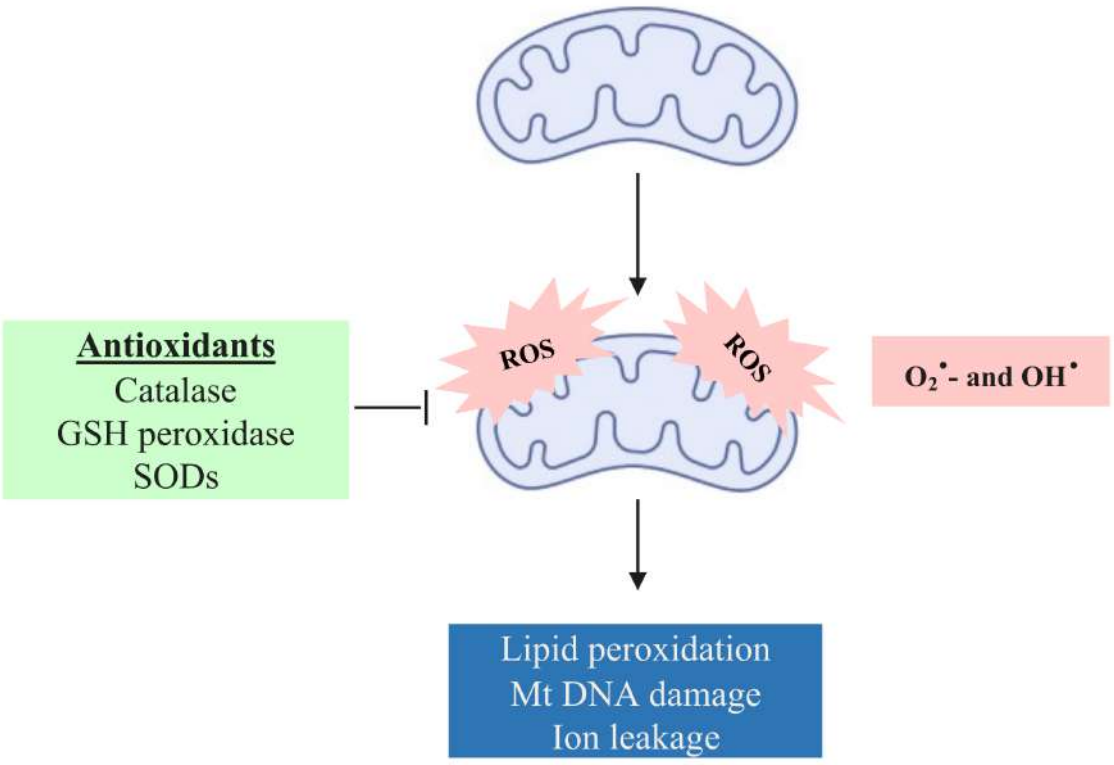

Figure 1.2: Schematic representation of ROS and its harmful effects.

The most common and potent stressors are pathogens, specifically viruses, that are always trying to enter the host cells [150]. These invading pathogens trigger a variety of stress that subsequently activate the cellular stress responses at multiple levels, including ER, mitochondria (oxidative stress), and cytoplasmic (stress granules formation) [465, 362, 440]. Virus-mediated production of SRPs generally has two functions: facilitating virus replication and recognition by the immune system when expressed on virus-infected cells for consequent elimination of the infected cells [190, 90]. However, viruses have evolved numerous strategies to manipulate these host responses and exploit these stress-related pathways, thereby ensuring their replicative success. Viral replication generally occurs in close association with ER, which is sensitive to perturbations in cellular homeostasis [89]. More specifically, the overproduction of viral proteins and numerous SRPs during infection increases the load in the ER lumen, thereby leading to the aberrant accumulation of proteins, consequently triggering ER stress [253, 245, 134]. Simultaneously, the progress of virus infection in cells also promotes oxidative stress to manipulate antioxidant systems, leading to chronic diseases. The oxidative damage subsequently induces RNA damage, allowing viruses to escape from immune systems and antiviral drugs [362, 32]. Hence, precisely how cellular stress and the triggered pathways function in viral pathogenesis is of considerable interest.

Moreover, the rapid progress of virus infection places pressure on proteostasis machinery, thereby disrupting the well-balanced proteome of the cells [19]. Proteostasis, or protein homeostasis, is how cells maintain a healthy protein life cycle, from synthesis to degradation. Disruptive proteostasis during virus infection often leads to severe pathologic conditions; hence, its integrity is crucial for maintaining a healthy cell environment [206, 267, 288]. Cells' defense strategies include the induction of molecular chaperones like heat shock proteins (HSPs) to reestablish the proper folding of misfolded proteins and the activation of proteolytic pathways that remove the surplus proteins via the ubiquitin-proteasomal system (UPS) [186, 130, 165]. For UPS-mediated degradation, the targeted proteins must be linked with ubiquitin, which is then recognized by the proteasome [211, 298]. This whole process requires the active involvement of three distinct enzymes: Ubiquitin-activating enzyme (UBE1), Ubiquitin-conjugating enzyme (E2), and Ubiquitin ligase (E3) [323, 185]. This post-translational ubiquitin linkage to targeted proteins drives them for proteasomal degradation [183, 165]. Post-translational modifications (PTMs) are enzyme-mediated covalent linkage of functional groups to proteins during or after synthesis. Numerous PTMs to proteins act as destabilizing

signals that drive their proteolytic degradation, ensuring proteostatic balance [232, 424].

Protein arginylation is one such type of PTM, in which specific target proteins are added with Arg from tRNA via arginyltransferase 1 (ATE1) enzyme [381, 25, 173] (Fig. 1.3). This transfer can occur either at the N-terminal or side chains of a protein having aspartic acid (Asp) and glutamic acid (Glu) at the 2^{nd} position. Arginylation has been established as a global regulator of protein degradation in the cellular stress response [354, 441]. It is also essential in multiple physiological processes, including aging, angiogenesis, heart, and embryo development [229, 472, 356, 228].

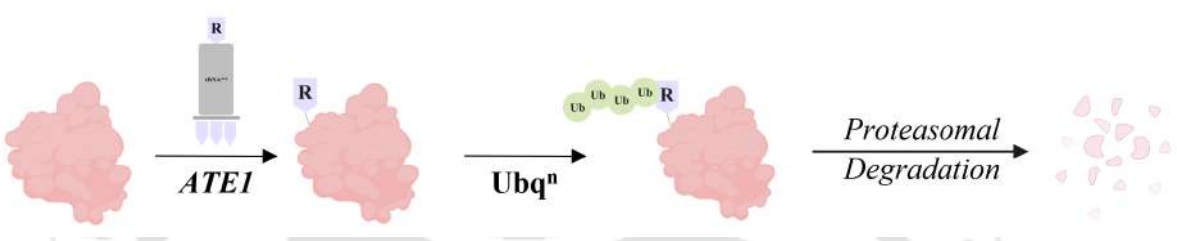

Figure 1.3: Schematic representation of arginylation-mediated degradation of proteins.

This thesis utilizes Newcastle disease virus (NDV) as a model to investigate the role of cellular stress and altered proteostasis in pathogenesis. It is a causative agent of Newcastle disease, a highly infectious disease that can infect 240 species of birds and spreads primarily through direct contact between infected and healthy birds. The virus belongs to the genus *Orthoavulavirus* of the subfamily *Avulavirinae* under the family *Paramyxoviridae* [345]. NDV strains are classified into three pathotypes based on their pathogenicity: velogenic, mesogenic, and lentogenic [7, 159]. Virulent strains are endemic in poultry in many parts of the world [3, 283], causing massive damage in the poultry industry with a 100% mortality rate. It is infectious for almost all avian species, domestic and wild; however, the virulence varies vastly with the host type [6]. Chickens are highly vulnerable to infection among all poultry species [1]. Primary clinical features include respiratory distress, watery eyes, inappetence, neurological symptoms, and greenish or white diarrhea, which in advanced stages, evident by paralysis and torticollis, which ultimately leads to death [6, 273, 282].

In this thesis, we primarily investigate the role of induced cellular stress in the progress of viral replication. Additionally, we aim to identify the SRPs positively involved in the replication and elucidate their precise part in modulating virus infection. Furthermore, we aim to study the activation of protein arginylation due to virus-mediated

proteostasis imbalance while exploring the viral hemagglutinin protein of NDV as a presumable target for arginylation-driven proteasomal degradation.

1.2 Literature Review

1.2.1 Stress and Cellular Stress response

Cells-activated stress response pathways are universally controlled by diverse SRPs that mitigate the damage and reestablish homeostasis [225]. These signal transduction pathways lead to the activation of several cytoprotective proteins, acting as early responders. Major responsive pathways having their specific prototypic downstream protein include heat shock, ER stress, and oxidative stress response.

1.2.2 ER Stress

ER ensures the standard functionality and proper folding of newly synthesized proteins, maintaining ER homeostasis. Several conditions, like high demand for protein secretion and missense mutations, lead to protein misfolding and ultimately cause ER stress [240]. Adaptation of ER to stress is crucial for the cell's normal functioning and survival [430, 144]. The UPR activated by ER helps maintain the functional integrity of this organelle [197, 361]. UPR signaling is initiated by three ER-membrane-associated proteins: ATF6, IRE1, and PERK [253, 287]. An essential molecular chaperone, GRP78, is generally found to be linked with these three proteins via their luminal domain under normal conditions [301, 242]. After the stress induction, the dissociation of these sensors from GRP78, followed by further activation, induces several downstream cascades to control the aberrant protein accumulation [395, 466]. The release of GRP78 from the PERK lumen domain initially prompts its homodimerization and autophosphorylation. The activated PERK then phosphorylates eukaryotic translation-initiation factor 2α (eIF2α) to cease the protein synthesis transiently [126, 274]. Next, the release of GRP78 triggers the activation of IRE1α by autophosphorylation, which then initiates the removal of the 26-base intron from mRNA encoding X-box binding protein 1 (XBP1) [380, 4]. The activated XBP1, along with ATF6, function mainly as transcription factors to trigger the upregulation of molecular chaperones and other folding enzymes (GRP78, GRP94, GRP178, protein disulfide isomerase, peptidyl-prolyl isomerases) [44, 230, 437, 297].

In extreme conditions, the ER-associated degradation (ERAD) machinery targets all the misfolded proteins to overcome the ER load [279]. Finally, if the stress cannot be alleviated, IRE1 recruits TNF receptor-associated factor 2 (TRAF2), further activating c-Jun N-terminal kinase (JNK) pathways through apoptosis signal-regulating kinase 1 (ASK1), leading to apoptosis [416].

1.2.3 ER stress and Viruses

The induction of ER stress due to viral protein production was first shown by overexpressing influenza hemagglutinin protein, leading to the modulation of ER chaperones [222]. Later, numerous viruses, including Zika [284], herpes [191], Japanese encephalitis (JEV) [296, 367], and dengue (DENV) [114, 241] were reported to induce ER stress during their infection (Table 1.1). Various viruses trigger the activation of distinct pathways by modulating specific proteins. Specifically, JEV and DENV activate UPR pathways, enhancing protein folding abilities [389]. Studies have also revealed that the influenza virus's HN protein is transiently associated with GRP78 protein, inhibiting its transportation to the plasma membrane [176]. Additionally, the ATF6-induced expression of chaperones helps viral proteins fold and prevent protein aggregation. Furthermore, activated ATF4 enhances HIV replication [67], ATF6 promotes ASFV and LCMV replication [139, 316], and GRP78 facilitates DENV, HCMV and JEV replication [250, 52, 443]. Similar linkage of GRP78 with glycoproteins of paramyxovirus, VSV, and HCV have been reported previously [299, 439, 83].

1.2.4 Oxidative Stress

Oxidative stress is triggered by accumulating free radicals or ROS, causing lipid peroxidation and DNA damage. They are highly unstable and reactive, generated during the metabolic processes continually occurring inside the cells [129]. In response to increased ROS levels, several oxidative stress sensors and protein kinases are activated to reduce stress through diverse mechanisms [55]. The oxidative stress sensors, including activator protein-1 (AP1), p53, and NF-κB, are majorly activated due to oxidative damage [271]. Under stress-free conditions, NF-κB is generally linked with the inhibitory subunit (IκB). After the stress, the dissociation of IκB activates the NF-κB, further enhancing pro-inflammatory cytokine expression [87]. The NF-κB pathway can be linked with the increased levels of interleukin (IL)-1β, IL-2, pro-inflammatory enzyme cytochrome c

Table 1.1: Table listing various viruses alongside their distinct ER sensors and associated pathways.

Virus	Modulated ER Sensor	Specific Pathways	References
DENV	JNK, PERK, eIF2α, and XBP1	Calcium Signaling Pathways, Autophagy, and UPR	[415, 241, 114]
Ebola virus	PERK, IRE1, and ATF6	UPR	[351, 38]
HBV	PERK, IRE1, ATF6, IFN, SDF-1, COX-2, and eIF2α	Autophagy, Inflammatory response, and Apoptosis	[30, 81, 82]
HCV	PERK, CHOP, JNK, XBP1, ATF6, MCH1, and eIF2α	ERAD and UPR	[396, 428, 346, 450, 364]
Influenza A virus	ERp57 and IRE1	UPR and ERAD	[348, 161, 134]
JEV	ATF6, XBP1, PERK, and IRE1	Autophagy, ERAD, Apoptosis, and UPR	[367, 436, 389]
SARS-CoV-2	GRP78, JNK, PERK, IRE1, and ATF6	UPR and Inflammatory response	[445, 365, 256, 373, 28]
Zika	CHOP, IRE1, XBP1, eIF2α, JNK, and MAPK	Apoptosis and UPR	[284, 294, 411]

oxidase subunit II (COX2), and (TNF-α) [87, 88]. Besides, stress response also activates MAPK species JNK and p38, which induces apoptosis [9, 116, 155], and serine/threonine kinase protein kinase C (PKC-α), which is involved in the cell cycle regulation [216]. Additionally, nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a nuclear transcription factor that controls the expression of several antioxidant genes having an antioxidant-responsive element (ARE) in the promoter region [214, 203]. Under normal conditions, it binds to Kelch ECH associating protein 1 (Keap1) and remains inactivated. However, this complex undergoes disruption under oxidative stress, and Nrf2 gets activated and translocates to the nucleus, where it dimerizes with small Maf proteins. The Nrf2-Maf heterodimers interact directly with ARE to induce the expression of target genes such as Heme oxygenase-1 (HO-1), Glutathione S-transferase A2 (GST), and NAD(P)H quinone oxidoreductase (NQO1). Subsequently, these genes act as ROS scavengers and limit the

oxidative damage [203, 258, 199].

Studies have also linked ER stress to the generation and accumulation of ROS [410]. The cellular stress response to these stresses has also been found to have a strong interactive relationship with interlinked response elements [98, 463]. As one of the ER stress sensors, IRE1 is responsible for activating the NF-κB pathway through the phosphorylation of inhibitor IκB and JNK pathway [352]. Additionally, the NF-κB can also be influenced by the inhibition of eIF2α [464]. This crosstalk between ER stress and oxidative stress shows the complexity of cellular stress signaling networks.

1.2.5 Oxidative stress and Viruses

The instigation of oxidative stress by the virus was first reported for Sendai virus infection [320]. Since then, many viruses including HIV [125], DENV [325], Influenza [219], respiratory syncytial virus (RSV) [169], rhinoviruses [39], and Hepatitis [137] have shown to modulate oxidative stress and specific oxidant-sensitive pathways (Table 1.2). Although these pathways trigger an antiviral inflammatory response, several studies have shown that increased ROS levels can alter the cells' redox state, which could benefit the virus's replication [278, 177, 11]. Studies revealed that oxidative stress can positively contribute to the pathogenesis of respiratory viral infections, influenza, flaviviruses and alphaviruses, while antioxidant treatment can substantially inhibit their replication [58, 254, 471, 350]. DENVs are known to trigger NOX-dependent oxidative stress, leading to the activation of IRF3, IRF7, and STAT1 and NF-κB-mediated antiviral responses [308]. Although different viruses induce ROS differentially, they modulate a common pathway of Nrf2/ARE to defend against oxidative stress. They can impede the activation of Nrf2 protein to inhibit the synthesis of antioxidant enzymes such as SOD, CAT, and GPx, thereby maintaining the oxidative environment [337, 24, 238]. Additionally, direct interactions of viral proteins with mitochondrial membranes have also been shown to depolarize the mitochondrial transmembrane potential (MMP), resulting in ROS generation [122, 91]. HBV X protein, HCV Core, E1, E2, NS3 proteins, and enterovirus A71 proteins are a few viral proteins that induce mtROS generation through interactions with mitochondrial membranes [91, 166, 79].

The regulation of oxidative stress during virus infection has been established; however, the underlying mechanisms remain unclear. Accumulating evidence has extensively linked oxidative stress and viral pathogenesis, which suggests that the endogenous

Table 1.2: Table listing various viruses associated with induction of oxidative stress and its role in replication.

Virus	Induction of Oxidative Stress	Associated Pathways with Proteins	Role of ROS in Replication	References
DENV	Expression of pro-inflammatory cytokines	Inhibition of NOX, Activation of NF-κB, Increased activity of GST, Activation of Nrf2	Non-Supportive	[205, 308, 458]
HCV	Increases ROS, Inflammation	Reduction of SOD activity, Upregulation of NOX, Lipid peroxidation, Mitochondrial dysfunction (Cytochrome c)	Supportive	[180, 307, 217, 322]
HSV	Increase ROS	Activation of Nrf2, Activation of NF-κB, Lipid Peroxidation	Supportive	[181, 201]
Influenza virus	Increases ROS production	Downregulation of SOD-1, Upregulation of NOX, Deactivation of Nrf2, Reduction of G6PD activity	Supportive	[372, 252, 11, 100]
HIV	Increase ROS,	Upregulation of NOX, Decrease in SOD activity, Lipid peroxidation, Deactivation of Nrf2	Supportive	[156, 20, 147, 181]
Zika	Increase ROS, expression of pro-inflammatory cytokines	Activation of Nrf2, Lipid peroxidation, protein carbonylation, Decrease in SOD activity	Uncertain	[10, 237, 68]

antioxidant host pathways and exogenous therapeutic antioxidants could help attenuate the redox-mediated pathways that drive pathogenesis.

1.2.6 Sirtuins

Sirtuins (SIRTs) are nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylases that catalyze the deacetylation of histone as well as non-histone proteins, including several transcription factors and metabolic enzymes [170]. They are known to regulate aging, cell cycle, differentiation, inflammation, metabolism, senescence, and stress resistance [302]. SIRTs derive their name from the Silent Information Regulator 2 (SIR-2), first identified in *Saccharomyces cerevisiae* [212]. They primarily catalyze targeted protein deacetylation at the side chain amino group of lysine residues through the hydrolysis of NAD^+ to generate nicotinamide (NAM) and O-acetyl-ADP-ribose alongside the resulting deacetylated proteins [358]. Seven distinct sirtuins have been identified so far, named numerically as SIRT1-7. SIRT1, SIRT6, and SIRT7 are primarily located in the nucleus; SIRT2 is a cytoplasmic protein, whereas SIRT3, SIRT4, and SIRT5 are distributed in mitochondria [153, 170, 280, 314]. They share a chemically and structurally conserved catalytic domain entailing a NAD^+ binding pocket [456]. Initially, SIRTs were primarily identified as deacetylase enzymes [178]. However, in mammals, their enzymatic activity has substantially diversified (Table 1.3), such as MARylation by SIRT1, SIRT4 and SIRT6, as well as long chain deacylation by SIRT4, SIRT5, SIRT6 and SIRT7. More specifically, SIRT1-3 exhibits strong deacetylase activities [462], while SIRT4 has ADP-ribose transferase activity and reduces insulin secretion response [157]. Additionally, SIRT5 regulates the PTMs, including lysine succinylation, malonylation, and glutarylation [338, 117]. SIRT6 can act as NAD^+-dependent monoADP-ribosyl transferase and long-chain fatty acyl deacetylases [189]. Conversely, the SIRT7 protein is the least studied protein that functions as a deacetylase enzyme.

Recent studies have presented the function of SIRTs in glycolysis as well. SIRT1 hinders glycolysis by activating proliferator-activated receptor co-activator 1-α (PGC1α), thereby inhibiting the expression of several glycolytic genes [192]. Moreover, SIRT1, SIRT3, and SIRT6 downregulate the transcription factor HIF1α, resulting in decreased glycolysis and enhanced oxidative metabolism [209], whereas SIRT3 activates SOD2 to impede ROS-mediated stabilization of HIF1α [131]. Additionally, the SIRT family members are shown to mediate antioxidant and redox signaling pathways by

Table 1.3: Table listing sirtuin proteins with their enzymatic activities and their specific roles.

SIRTs	Location	Enzymatic activity	Substrate	Specific Pathways	References
SIRT1	Nucleus	Deacetylase	p53, PGC1α, PPARγ, NF-κB, AKT, FOXO, HIF1α, PARP-1	Aging, Cell cycle, DNA repair, Glycolysis, Notch signaling, Oxidative stress, RNA splicing	[76, 459, 384, 5]
SIRT2	Cytoplasm	Deacetylase, Demyristoylase	α-tubulin, FOXO3a, H4K16, JNK, NF-κB, p65, PEPCK, SOD	Aging, Autophagy, DNA repair, Infection, Inflammation, Metabolism, Oxidative stress, Tumor suppression	[461, 442]
SIRT3	Mitochondria	Deacetylase	AT1, FOXO3, GDH, HIF1α, JNK, NRLP3, PARP1, SOD-2, RIPK1, RIPK3	Apoptosis, Autophagy, Inflammation, Metabolism, Oxidative stress	[462, 77, 363, 15]
SIRT4	Mitochondria	ADP-ribosyltransferase, Deacetylase, Lipoamidase	GDH, Lsd1, PDH	Insulin secretion response, Metabolism, Oxidative stress	[363, 15, 270, 405]
SIRT5	Mitochondria	Deacetylase, Deglutarylase, Demalonylase, Desuccinylase	Caspase 3, CPS1, KLF6	Cancer, Metabolism, Oxidative stress	[363, 15, 255]
SIRT6	Nucleus	ADP-ribosyltransferase, Deacetylase, Demyristoylase, Depalmitoylase	Bax, H3K9, HIF1α, Keap, LDH, NF-κB, TNFα	DNA repair, Inflammation, Oxidative stress, Telomere maintenance	[363, 15, 120, 213]
SIRT7	Nucleus	Deacetylase, Desuccinylase	ATM, CDK9, FOXO3, GABPβ1, GATA4, H3K122, NPM1, p53, PAF53, H3K18	DNA Repair, Oxidative stress, RNA polymerase activity, Tumor suppression	[41, 447, 133, 227, 399, 243]

directly deacetylating several transcription factors that regulate antioxidant genes [379]. Specifically, SIRT1, SIRT3, and SIRT5 have been reported to protect the cells from excessive ROS, whereas SIRT2, SIRT6, and SIRT7 modulate several essential oxidative stress genes [277]. SIRT1 regulates the FOXO family of Forkhead transcription factors to promote the expression of SOD2 [50]. It also promotes mitochondrial biogenesis by activating peroxisome PGC-1α [192], which increases the mitochondrial mass and upregulates the stress-responsive genes, including SOD, CAT, and GPx [385]. SIRT2 also deacetylates FOXO3a in response to oxidative stress and caloric restriction, thus improving the cellular resistance to H_2O_2 [432]. Additionally, SIRT3 has been reported to boost the SOD enzymatic activity through direct deacetylation [333]. SIRT6 mono-ADP ribosylates the PARP1 to stimulate DNA double-strand break repair in response to oxidative stress [264].

1.2.7 Sirtuin and Viruses

Numerous viruses are reported to control the host epigenetic and transcription machinery by modulating SIRT-regulated pathways [121]. The table mentioned the list of viruses modulating the specific SIRT proteins during their replication cycle (Table 1.4). However, the majority of research predominantly focused on SIRT1 protein. During HIV infection, SIRT1 regulates the transcription via Tat deacetylation, which consequently inhibits its deacetylase activity, thus activating NF-κB-responsive gene transcription and promoting $CD4^+$ T-cell hyperactivation [312, 231]. SIRT1 has also been extensively reported to impact other viruses, including HBV, HCV, HSV, influenza, and SARS-CoV-2 [105, 460, 268, 33, 468]. Additionally, HSV-1 modulates SIRT1 interaction and colocalization with p53 to inhibit apoptosis and restore the energy status in neural cells [268]. Similarly, HBV also activates SIRT2, deacetylating critical genes and promoting HBV transcription [80]. Another study shows that SIRT1 regulates HBV transcription and replication by targeting transcription factor AP-1 [343]. Interestingly, a study demonstrated the activation of HBV transcription by all SIRT1-7 proteins and the abundant detection of SIRT1 protein in HBV-infected tissues [105]. From the host perspective, several studies have presented SIRTs as essential defense factors against viral infection [53]. For example, SIRT1 stimulates PML sumoylation in VSV-infected cells, mediating antiviral activity [60]. Furthermore, SIRT3 deacetylase activity is also necessary for suppressing HCMV production, as it helps maintain mitochondrial pH and membrane

potential during infection [368]. SIRT6 also negatively regulates inflammatory responses during DENV infection, as it inhibits RIG-I-like receptor (RLR) and Toll-like receptor 3 (TLR3) mediated NF-κB activation [244]. Recently, NDV has been shown to induce SIRT3 degradation in damaged mitochondria via PINK1-PRKN-dependent mitophagy to reprogram energy metabolism, thereby promoting viral replication [151]. Majorly, viruses are observed to utilize the SIRT1's modulated cellular mechanisms, but the precise role of other SIRTs has not yet been fully explored.

Table 1.4: Table listing sirtuin proteins modulated during virus infection.

SIRTs	Viruses	References
SIRT1	HBV, HCV, HIV, HSV, Influenza, LCMV, RSV, SARS-CoV-2	[231, 208, 475, 207, 311, 43, 435, 105]
SIRT2	HBV, HCMV, HIV	[349, 37, 119]
SIRT3	HBV, HCMV, Influenza, NDV	[151, 215, 457, 368]
SIRT4	HBV, WSSV	[215, 397]
SIRT5	BmNPV, HBV, SARS-CoV-2	[429, 467, 328]
SIRT6	HCV, DENV	[84, 244]
SIRT7	HBV, SVCV	[453, 248]

1.2.8 Sirtuin 7

SIRT7 is the least explored protein among all members as there is a lack of understanding about its substrates and specificity, which makes its precise role unclear. However, recent studies have shown that it can deacetylase the histone as well as non-histone proteins, including ATM, GATA4, p53, PAF53, and SMAD4 [399, 418, 447, 74, 246]. The deacetylation of non-histone substrate suggests that it regulates numerous cellular processes. It can deacetylase PAF53 and U3-55k proteins, thus controlling the pre-rRNA processing [75]. It can also improve mitochondrial function by deacetylating the GA-binding protein β1 (GABPβ1) [353]. Moreover, it can also activate CDK9's kinase activity to facilitate transcription elongation [41]. Contrarily, it has also been observed to have histone (H3K122) desuccinylase to regulate chromatin remodeling during DNA repair [243]. Till now, its function in DNA repair, maintenance of genomic stability, regulating rRNA transcription, and stress responses have been established by various studies [425, 426]. SIRT7 protein levels were found to be elevated in ER stress conditions

induced by thapsigargin treatment. Precisely, XBP1 activation upregulates the SIRT7 protein, reducing the expression of ER stress-responsive proteins. It can also inhibit mRNA translation under ER stress by inhibiting the synthesis of ribosomal proteins (RPS20 and RPS14) [371]. Like the other family proteins, it has also been linked with a cellular oxidant–antioxidant imbalance as it plays a substantial role in homeostasis [218]. Research showed it directly interacts with hypoxia-inducible factors (HIF-1α and HIF-2α) to inhibit their activity [175]. It has also been demonstrated to increase cardiomyocytes' stress resistance and prevent apoptosis and inflammatory cardiomyopathy [418]. Studies suggest that it does play a role in the resistance to different oxidative stress conditions; however, there is a lack of knowledge about the target substrates and their mechanisms.

1.2.9 SIRT7 and Viruses

A recent observation has linked SIRT7 to viral infection, as its activity increased during SVCV infection. This study marks the first report of a correlation between SIRT7 activity and virus infection, suggesting its potential role in suppressing the cellular antiviral responses [248]. Afterward, another study was published demonstrating the involvement of SIRT7 in HBV infection [453], showing that it can bind to cccDNA through interaction with HBV core protein, where it can catalyze H3K122 desuccinylation. Considering these studies, it could restrict as well as enhance virus replication via the deacetylation of a different set of proteins. Hence, SIRT7-mediated deacetylation and its precise role in virus replication are yet to be understood.

1.2.10 NDV

NDV is an enveloped virus with a single-stranded negative-sense RNA genome of approximately 15 kb in size [103, 223]. The genome encodes six structural proteins, namely, the nucleocapsid (N), the phosphoprotein (P), the matrix (M), the fusion (F), the hemagglutinin-neuraminidase (HN) and the large polymerase (L), as well as two non-structural proteins, V and W [233, 357, 387]. NDV infection is initiated by binding of HN glycoprotein to the sialic acid receptors outside the host cell membrane, which further triggers the fusion of the virus envelope with the host cell membrane via F protein [72]. Following the entry into the plasma membrane, the nucleocapsid dissociates from the M protein, and the large polymerase complex transcribes the negative-sense RNA genome into positive-sense mRNA, which is then translated into viral proteins [115]. The HN and

F are the major immunogenic proteins and are essential in determining the virulence of NDV. They have been extensively explored as antigens for various vaccine formulations [174, 210, 407].

The principal strategy to prevent the spread of NDV in poultry is vaccination. The avirulent strains of NDV like LaSota and B1 have been routinely used as live attenuated vaccines in various countries of North America, Europe, Africa, and Asia [93, 97, 112, 476]. Other strains like R2B and F are more commonly used in India and around to control clinical disease [70, 107]. Despite these, regular outbreaks have been reported even in vaccinated populations worldwide [48, 111, 266]. The emergence of genotype variants is the primary cause of its outbreaks in poultry worldwide [34, 401, 403]. The other reason for the disease outbreak is virus shedding from the vaccinated birds, as it has already been reported that the vaccination strategies only protect the vaccinated birds and do not prevent the virus shedding [196, 420]. In addition, inadequate vaccination practices, faulty farm management conditions, failure to maintain the cold chain, stress, and immunosuppression are the other probable reasons for NDV outbreaks and the emergence of its virulent strains

1.2.11 NDV and associated stress

Studies have revealed the initiation of ER stress and the subsequent UPR after infection with recombinant NDV expressing the rabies virus glycoprotein (rL-RVG) [51]. Initially, NDV NP and P proteins were reported to trigger autophagy via the ER stress-related UPR by regulating the transcription of ER chaperones [78]. However, recent studies have shown that both glycoproteins (HN and F) could also cause ER stress [344]. Additionally, the oxidative imbalance with reduced antioxidant enzyme activity has been described in NDV-infected chickens [341, 342]. It has also been observed that NDV replication instigates the impairment of the cellular antioxidant defense system, and vitamin E and C supplementation can help ameliorate oxidative damage, improving the immune response and reducing the mortality rate [391]. Additionally, NDV infection in chicken bursa could induce oxidative stress by high nitric oxide, leading to organ damage [224].

1.2.12 Protein Homeostasis

Cells employ a proteostasis network comprising numerous maintenance pathways to ensure a healthy protein life cycle from synthesis to proper folding to degradation [330, 184]. The mass production of viral proteins during infection could disrupt the proteome integrity of the host cells. In response to surplus proteins, the cell produces molecular chaperones to fold them correctly [334]. Molecular chaperones are the critical regulators in balancing protein homeostasis [235, 303]. They interact with aggregated proteins and prevent the further interaction of partially folded protein intermediates. The family of HSPs includes HSP60s [164], HSP70s [127] and HSP90s [319], which are majorly involved in protein folding [234, 96]. HSP70 and HSP90 are well reported to be engaged during viral infection as they can effectively interact with the components of both adaptive and innate immune systems. They are often found to be overexpressed under stress conditions [160, 200]. However, their capacity to fold proteins is limited; therefore, cells cannot control the excessive proteins, which severely damages proteostasis. In addition to the molecular chaperones, the activation of proteolytic pathways also ensures the degradation of surplus proteins in the cytoplasm [110]. These pathways primarily require the cooperative action of three ubiquitin enzymes that mediate UPS to degrade proteins. UBE1 first activates the ubiquitin, which is further conjugated with E2, followed by the ligation by E3 to the lysine residue of the target protein. This PTM to the targeted protein leads to their proteolytic degradation [85].

UPS is one of the central protein degradation machineries in eukaryotic cells that manages protein homeostasis [298]. It comprises more than 700 enzymes involved in ubiquitin addition, which works in coordination with the major chaperones for the selective degradation of abnormal proteins. Specific PTMs on targeted protein function as a degradation signal by recruiting the proteosomes, thus initiating the N-end rule pathway [21, 423]. Adding destabilizing signals marks them for the N-recognin ubiquitin receptors (UBRs). Later, the UBRs bind to the signal residue and further direct their ubiquitination, followed by proteolytic degradation [422]. The UBR binding substrates include positively charged amino acids, including arginine, lysine, and histidine [27]. Several studies have revealed Arg's role as an N-degron to initiate the N-end rule pathway [113, 300, 400, 423]. Additionally, these PTMs can produce various changes to protein function, affecting their stability, conformation, dynamics, and activity [106]. However, despite their significance, all the well-explored PTMs constitute merely a

portion of protein modifications observed in cells [263].

1.2.13 Protein Arginylation

Protein arginylation is one such type of PTM in which the targeted proteins are added with Arg residue from tRNA either at the N-terminal or side chains having Asp and Glu [25, 173]. The Arg transfer is mediated by the ATE1 enzyme [86]. ATE1 was initially discovered in *E. coli* as an enzyme that does not require ribosomal machinery to incorporate amino acids into protein [285]. Later, the gene encoding for this enzyme was identified in *Saccharomyces cerevisiae*, cloned and further characterized [25]. The arginylation of the targeted proteins first requires their preprocessing by Met-aminopeptidases that mediate the endoproteolytic cleavage of nascent Nt-Met residues [45, 135]. If the exposed residue is Asn, Gln, or Cys (tertiary destabilizing residues), it further requires a second processing step. Asn and Gln are enzymatically deamidated by Asn-amidohydrolase (NTAN) and Gln-amidohydrolase (NTAQ) to yield Asp and Glu, respectively, (secondary destabilizing residues) [62, 315]. However, Cys reside is oxidized by nitric oxide to produce either Cys-sulfinic acid ($CysO_2H$) or Cys-sulfonic acid ($CysO_3H$) (secondary destabilizing residues) [172]. Next, these secondary residues are recognized by the ATE1 enzyme that adds Arg, making them the primary destabilizing residue. Subsequently, N-recognin family proteins involving E3 ligases of UPS bind to the primary destabilizing residues and drive their ubiquitination and proteasomal degradation [424].

Various studies have presented the ATE1-mediated post-translational arginylation as a critical regulator of cellular homeostasis [419]. Additionally, the crucial role of arginylation in protein degradation, autophagy, and cell migration has been explored profoundly [382, 187, 29]. The physiological significance of arginylation has also been shown in aging, cancer metastasis, cardiovascular maturation, embryonic development, and germ cell development [228, 335, 386]. Numerous proteins, such as α-synuclein, β-catenin, calreticulin, GRP78, RGS4, RGS5, and RGS16, have been reported to undergo arginylation [472, 149, 65, 318, 369, 239, 99]. Studies suggest that ATE1 can help protect against heat stress by regulating the stability of HSPs, specifically HSP40, HSP70.1, and HSP70.3 [104]. Additionally, the arginylation of calreticulin during ER stress results in stress granule formation in cells [64]. It can also promote actin polymerization and cell migration by modifying β-actin [318, 198]. Moreover, the arginylation of α-syn in the brain negatively impacts its pathological aggregation, suggesting a neuroprotec-

tive function [472]. Interestingly, several proteins in the retina, including Gαt1, Gβ1, RGS6, and RGS7, are elevated in Ate1 knockout, implying its role in G-protein-mediated responses in the retina [128]. The reduced levels of ATE1 expression in melanomas can affect cellular responses to mTOR inhibitors and serum deprivation [236]. Studies have also highlighted the arginylation-dependent endophagy/autophagy during endo-lysosomal proteostasis perturbations [431]. Recently, mid-chain arginylation on Glu and Asp residues of certain proteins has been reported, substantially broadening the potential targets and the biological processes that can be regulated by arginylation [260, 434, 124].

Despite the emerging studies identifying its new target proteins and expanding its biological significance, its regulation in infectious diseases, especially viruses, remains to be established. SARS-CoV-2-mediated arginylation has recently unveiled its plausible relationship with viral pathogenesis. Arginylation of calreticulin, HSPA5, and protein disulfide isomerase was detected in SARS-CoV-2-infected cells, aiming to restore ER equilibrium. Additionally, diminishing the activity of ATE1 could also lead to a reduction in viral load [124]. There have been no reports, aside from this study, highlighting the significance of protein arginylation in viral pathogenesis.

1.3 Rationale of the Study

Although significant advances have been made to understand how various viruses instigate ER and oxidative stress, there are still ambiguities and gaps in our understanding of the role of stress response pathways and SRPs in virus replication. The virus-mediated stress elicits several transcriptional and translational outputs that trigger the cellular survival pathways, leading to the production of SRPs. Here, the molecular mechanisms of crosstalk between cellular stress and virus replication cycle are not well explored. Previous studies describing how different viruses induce stressful conditions may only offer a partial understanding, as they may overlook the broader perspective of the precise role of SRPs in pathogenesis. Further works are needed to address the SRPs' specific biology in viral pathogenesis. Determining the role, either explicit or implicit, of SRPs in virus replication and protein homeostasis maintenance will not only help us better understand the virus's strategies to sustain in the host while perturbing the cell's normal functionality but also teach us how our cell fights the virus infection and how the viruses get away from their resistance. This knowledge comprehensively opens up new

possibilities for disease interventions. Discovering the detailed mechanisms of stress responses will help develop better therapeutic avenues for viral diseases significantly influenced by persistent stress.

Additionally, various proteolytic pathways have been reported to be activated to maintain protein homeostasis during virus infections. However, protein arginylation and its precise role remain unexplored in infectious diseases. Recently, studies exploring these pathways in disease intervention have gained rapid momentum as they offer a promising approach to developing unique therapeutic avenues. The pharmacological modulation of arginylation could be a simple solution to combat viral diseases. However, achieving this goal requires a comprehensive understanding of the protein arginylation and the underlying mechanism.

1.4 Study Objective

Based on current research needs, the following are the research objectives defined below:

1. Understanding the role of cellular stress-responsive proteins in viral replication.
2. Understanding the molecular mechanism of processes involved in maintaining virus-modulated proteostasis imbalance.
3. Targeting the viral proteins using host proteostasis maintaining processes.

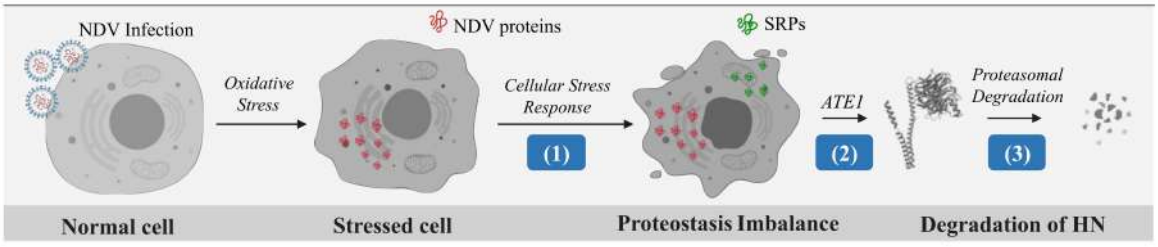

Figure 1.4: Schematic representation of the thesis objectives.

1.5 Thesis Organization

This thesis consists of 6 chapters, including the present chapter of introduction to the research topic. This chapter also provides a review of literature, the rationale of

the study, objectives, and a brief thesis outline. Chapter 02 briefly describes the detailed methodology adopted to fulfill the current objectives. Chapter 03 describes about the types of stress instigated by NDV infection. Chapter 04 delves into the biology of SIRT7 protein and role of its deacetylation activity in NDV pathogenesis. Chapter 05 explores the activation of protein arginylation process and its utilization in proteasomal degradation of HN protein. Chapter 06 summarizes the conclusions and research contributions of the current work and presents practical implications.

CHAPTER 2

STUDY METHODOLOGY

2.1 Cell culture and reagents

Chicken embryo fibroblast (DF-1) cells were purchased from ATCC (CRL-12203™), whereas baby hamster kidney fibroblast (BHK-21), and human embryonic kidney (HEK-293T), cells were obtained from National Centre for Cell Science (NCCS), Pune, India. The cells were cultured in high glucose Dulbecco's modified eagle's media (DMEM) supplemented with 1% antibiotic-antimycotic solution (HiMedia) and 10% fetal bovine serum (FBS, Gibco) in a humidified incubator at 37°C in the presence of 5% CO_2.

The following reagents used in the experiments were procured from Sigma-Aldrich: Bortezomib (BTZ) (#5.04314), Bromine (#1.01945), Cycloheximide (CHX) (#239763), Dichloromethane (DCM) (#34856), Diethyl Ether (#309966), DIPEA (#03439), Dimethyl furamate (DMF) (#242926), N,N-Dimethylformamide (N-DMF) (#270547), Glycine (Gly) (#50046), L-arginine (Arg) (#A8094), Lithium chloride (LiCl) (#203637), 3-Maleimidobenzoic acid N-hydroxysuccinimide ester (MBS) (#M2786), MG132 (#M8699), N-Acetyl-L-cysteine (NAC) (#A7250), 1-Napthol (#N1000), Piperidine (#411027), Pyrrolidine dithiocarbamate (PDTC) (#P8765), Tannic acid (TA) (#403040), Trifluoroacetic acid (#302031), and Triethylsilane (#230197).

The following reagents were purchased from SRL: β-nicotinamide adenine dinucleotide (β-NAD) (#64257), β-nicotinamide mononucleotide (β-NMN) (#63509), and nicotinamide (NAM) (#93517). All the compounds were dissolved in ethanol, dimethyl

sulfoxide (DMSO), or phosphate buffer saline (PBS) to prepare stock solutions and further diluted in a culture medium for experiments. The cell-permeant ROS probe Dichlorodihydrofluorescein diacetate (DCFH-DA) (#D6883, Sigma, USA) was also diluted in DMSO. Rink amide MBHA resin, all the protected amino acids, and the coupling agents were procured from GL Biochem, China.

2.2 Virus Infection and Quantification

The velogenic strain of NDV Bareilly, previously characterized in our laboratory, and mesogenic strain R2B were used for conducting all the experiments, while the recombinant NDV expressing GFP available in the laboratory was utilized for the fluorescent-based study [289]. The virus stock was prepared by inoculating in nine-day-old specific-pathogen-free embryonated chicken eggs. NDV containing allantoic fluid was collected 48 hrs post-inoculation, and its presence was confirmed by hemagglutination assay (HA) using 1% chicken RBC. Furthermore, the infectious titer of NDV was determined by plaque assay and $TCID_{50}$ as per the standard procedure [22, 336, 340]. The seeded cells were infected with NDV at 0.1 and 0.01 multiplicity of infection (MOI) and incubated for 2 hrs at 37°C for virus adsorption. The unbound virus particles were removed by washing the cells' monolayer with PBS twice. Then, infection was allowed to proceed for different time points in the presence of DMEM with 2% FBS.

Moreover, for *in ovo* study, nine-day-old specific-pathogen-free embryonated eggs were used for NDV inoculation (4 HA units) and drug treatments. The allantoic fluid was collected 48 hrs post-NDV inoculation, and the presence of the virus in infected allantoic fluids was confirmed by HA, and further NDV was titrated by plaque assay. The embryo tissue samples were also collected from the respective groups and used for gene and protein expression studies.

2.3 Cell Viability Assay

The mean lethal dose of compounds was determined by MTT assay, as described earlier [290]. Briefly, DF-1 cells were seeded in a 96-well plate at a density of $1x10^4$ cells per well and incubated overnight for attachment. The cells were then treated with different doses of respective compounds. After incubation for 24 hrs, 3-(4,5-dimethylthiazol-2-yl)-

2,5-diphenyl tetrazolium bromide (SRL, India) was added each well and then incubated at 37°C for another 3 hrs. The MTT reagent was removed from the wells, and formazan crystals were dissolved with DMSO. The absorbance was measured at 570 nm using multiskan Go (Thermo Scientific, USA).

Further, the viability of the DF-1 cells in respective compound's presence was also analyzed using calcein dye (Thermo Scientific, USA). DF-1 cells were seeded in a 12-well plate and incubated overnight to form the monolayer. Varying concentrations of compounds were added to the monolayer and then incubated at 37°C for 24 hrs. Cells were stained with calcein dye, and the fluorescence was recorded under the microscope.

2.4 Drug Treatments

Initially, For LiCl treatment and virus-related studies, the DF-1 cells were treated with LiCl at 0 and 6 hrs post-infection with NDV. Additionally, two different conditions with DMF (12 hrs pre-treatment) and PDTC (12 hrs post-treatment) were used to examine the effects of oxidative stress on NDV infectivity. For pre-treatment experiments, cells were seeded with $5x10^5$ cells/well density in 6-well plates. After 6 hrs, the cells were pre-treated with DMF (1 μM) and incubated for another 12 hrs in a humidified 37°C incubator under 5% CO_2. After incubation, the monolayer was infected with 0.01 MOI of NDV; subsequently, cells and supernatant were collected at 24, 48, and 72 hrs post-infection. For post-treatment experiments, the cell monolayer was infected with 0.01 MOI of NDV, and the cells were treated with PDTC (5 μM) 12 hrs post-infection. Finally, cells and supernatant were collected at 24, 48, and 72 hrs post-infection. The viral quantification was done using $TCID_{50}$ and plaque assay as per the standard protocol. Similarly, for the SIRT7 activity assessment study, cells were subjected to two conditions with β-NMN (1 mM) (12 hrs pre-treatment) for activation and NAM (1 mM) (12 hrs post-treatment) for inhibition.

For ATE1 activity assessment experiments, DF-1 cells were pre- and post-treated with a 2 μM concentration of TA, a known inhibitor of ATE1. For pre-treatment experiments, cells were seeded in 6-well plates. After 6 hrs, the cells were pre-treated with TA and incubated for another 12 hrs at 37°C. After incubation, the monolayer was infected with NDV for 2 hrs at a MOI of 0.1; afterward, cells and supernatant were collected 48 hrs post-infection. Furthermore, the cell monolayer was initially infected with 0.1 MOI of NDV for post-treatment experiments, and cells were treated with TA

12 hrs post-infection. Finally, cells and supernatant were collected 48 hrs post-infection. Collected samples were subjected to RT-qPCR, immunoblotting, and plaque assay. NDV-infected cells were also treated with different doses of Arg and Gly (1 and 2 mM). Briefly, DF-1 cells were initially infected with NDV for 2 hrs, which was then substituted with fresh 2% DMEM. After 36 hrs of infection, the media was replaced with fresh 2% DMEM containing Arg or Gly and again incubated for 12 hrs. Lastly, cells were washed with PBS and harvested for further analysis.

To conduct protein stability experiments, firstly, cells were infected with NDV and incubated for 48 hrs. After incubation, the medium was replaced, and cells were cultured in fresh 2% DMEM with 2 mM of Arg and translation inhibitor CHX of 50 μg/ml for 6 hrs before harvesting. Additonally, ATE1-overexpressing cells were infected with NDV and then incubated for 48 hrs. Subsequently, the cells were treated with CHX and harvested for further analyses after 6 hrs. Similarly, for proteasomal inhibition experiments, the media of NDV-infected cells was discarded after 36 hrs, and then cells were grown in the presence of 2 mM of Arg and 1 nM of BTZ for 12 hrs before harvesting.

2.5 Plasmid and siRNA Transfection

To achieve the heterologous expression, *Gallus gallus* origin SIRT7 gene (NM_001291971.1) (C-terminally 3xFLAG tagged) under the control of a CMV promoter in pcDNA3.1 was commercially synthesized (GenScript, USA). The expression plasmid encoding ATE1 (RF-88-Ate1) was a gift from Richard Fahlman (Addgene plasmid #79187). For transfection experiments, cells were seeded in 6-well plates and then transfected with 2 μg of expression vector using Lipofectamine 3000 (Invitrogen, USA) following the manufacturer's instructions. Cells were then incubated for 24 and 48 hrs before harvesting.

For knockdown experiments, two small interfering RNAs (siRNA) were designed to target SIRT7 and ATE1 mRNA. Cells were transfected with a mixture of siRNAs (25 pmol each) using RNAiMAX reagent (Invitrogen, USA). The two siRNAs used for SIRT7 knockdown (siSIRT7) in this study were s1 (GCAAAGCAGAUGUGAUUCU) and s2 (GCAUCAGCACUACAGAUCU). The two siRNAs used for ATE1 knockdown (siATE1) were s3 (UAUGGGUUUCUACAUUC) and s4 (CUGUGUAUCAUCUGUGUAU). The non-target siRNA (siNT) (#SIC001, Sigma, USA) was used as a negative control. The knockdown was performed for 24 hrs before analyses.

2.6 Measurement of ROS levels

The generation of intracellular ROS was determined using DCFH-DA dye. Cells were seeded in 96-well plates and incubated overnight in a humidified 37°C incubator under 5% CO_2. DMF pre-treatment and PDTC post-treatment assays, along with virus infection, were done, as discussed earlier. At 24, 48, and 72 hrs post-NDV infection, the growth media was aspirated from each well, and cells were incubated with DCFH-DA dye at a final concentration of 10 μM for 30 min at 37°C in the dark. Post-incubation, cells were washed three times with PBS, and subsequently, the fluorescence intensity was measured using the Tecan Infinite 200Pro device with excitation at 493 nm and emission at 523 nm. Mean fluorescence intensity was calculated to plot the intensity graph. Changes in the emitted fluorescence intensity post-virus infection and compound treatment allowed interpretations for the production of ROS in mitochondria.

2.7 NAD^+ and NAM detection

Nine-day-old chicken embryonated embryos were inoculated with 100 μl of NDV with 10^6 PFU/ml and subsequently incubated in a 37°C incubator for two days. Equal weights (500 mg) of embryonic tissue samples were collected from control and infected groups. After the trituration in an equal volume of plain DMEM, the samples were vortexed thoroughly to mix the cells evenly. Tissue samples were added with pre-chilled 10% perchloric acid ($HClO_4$) at a 1:10–50 ratio (tissue weight: $HClO_4$ volume) and kept on ice for 15 min. After centrifuging the samples at 22,000g at 4°C for 10 min, the supernatant was transferred to a new tube and subsequently added with 3 M potassium carbonate (K_2CO_3) and vortex rigorously. The mixture was then incubated on ice for 15 minutes and centrifuged at 22,000xg at 4°C for 10 min. Finally, the supernatant was transferred to HPLC glass vials. Tissue NAD^+ and NAM levels were determined using a High-Performance Liquid Chromatography (HPLC) system (Shimadzu, Kyoto, Japan) with a C18 column (5 μm, 4.6x250 mm) by absorbance at 261 nm. Mobile phase A consisted of 0.05 M phosphate buffer (pH 7.0), and mobile phase B was 100% methanol. The following linear gradient was run for 30 min at a flow rate of 1 μl/min with the running program already described previously [451]. Multiple known concentrations of NAD^+ and NAM were prepared in PBS and used to plot the standard curve. Lastly, the injection volume was 100 μl per sample, and all the samples were kept at 4°C in the

autosampler tray prior to injection.

2.8 Quantification of Arg Utilization

DF-1 cells were infected with NDV and incubated in 2% DMEM for 48 hrs at 37°C. Media was aspirated from mock-infected and NDV-infected cells, and cells were incubated with a 4 mM concentration of Arg in PBS for 1 hr and collected for Arg quantification. Arg detection was performed using the Sakaguchi test [49, 406]. Briefly, 10 μl of the sample was mixed with 30 μl of 1-naphthol (in 95% aqueous ethanol) and 30 μl of 10% aqueous NaOH solution. The sample was vortexed thoroughly and incubated in an ice bath for 5 min. 1.5 μl of sodium hypobromite solution (freshly prepared by dissolving 1 ml of bromine in ice-cold 10% NaOH solution (20 ml)) was taken in the microtiter plate well and diluted with 40 μl of water. Following the incubation period, an aliquot of 60 μl of the incubated sample was mixed with the sodium hypobromite solution in the wells. 30 μl of 40% aqueous urea solution was added to quench excess NaOBr. The reaction mixture was incubated at 37°C for 2 min and analyzed by measuring the absorbance at 500 nm.

2.9 Gene expression Analysis

Total RNA was isolated from cells using RNAiso Plus reagent (TaKaRa) to study the gene expression. 2 μg of isolated RNA was reversely transcribed to synthesize cDNA using the high-capacity cDNA reverse transcription kit (Thermo Scientific). Real-time PCR reactions were carried out in triplicates using PowerUp SYBR Green Master Mix (Applied Biosystems) according to the manufacturer's recommended cycling conditions in Quant Studio 5 (Applied Biosystems). Transcript levels were quantified using host and NDV gene-specific primers and normalized to expression levels of GAPDH. With final normalization to untreated and uninfected controls, the comparative cycle threshold ($2^{-\Delta\Delta CT}$) method was used to calculate relative fold change values [257].

2.10 Immunoblot Analysis

For whole-cell protein extraction, cells were lysed in 2x SDS Sample buffer supplemented with β-mercaptoethanol and spun down at 13,000 rpm for 10 min. The supernatants were boiled at 95°C for 10 min, and then equal amounts of each sample were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis to resolve the proteins. Proteins were transferred to a 0.4 µm nitrocellulose membrane (HiMedia) at 30 V for 30 min via Bio-Rad Trans-blot Turbo transfer for immunoblotting. Subsequently, the blots were blocked with 5% non-fat dry milk in 1x TBST buffer for 2 hrs at room temperature. Blots were then incubated overnight at 4°C with specific primary antibodies, followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling Technology) for 2 hrs at room temperature. Protein blots were developed with an ECL Detection kit (Bio-Rad) and images using ChemiDocTM XRS System (Bio-rad, USA). The blots were quantified using the ImageJ tool. The intensity of different lanes was then normalized to the control lane, which was set to one. The following antibodies were used in 1:5000 dilution: Acetylated lysine, ATE1, β-actin, FLAG, GAPDH, GRP78, Hsp90β, N-terminal arginylation, SIRT1, SIRT7, SQSTM, Ubiquitin, and NDV polyclonal (available in our laboratory).

2.11 Flow cytometric Analysis

DF-1 cells were cultured in a 6-well plate at $5x10^5$ cells/well for flow cytometric assays and subjected to virus infection and compound treatments. At the end of the infection and treatment, cells were harvested, and the cell pellet was washed with ice-cold PBS twice to remove the media. Finally, cells were resuspended in 2% FBS-PBS and analyzed by a CytoFLEX flow cytometer (Beckman Coulter, USA). The final results from three independent experiments were averaged and shown in the histogram.

For ATE1 detection, NDV-infected cells were harvested, and the cell pellet was washed with ice-cold PBS twice to remove the media. The cells were then fixed using 4% paraformaldehyde, permeabilized utilizing 0.2% Triton X-100, and blocked in 4% goat serum for 1 hr. Following the blocking step, samples were incubated overnight at 4°C in a moist chamber, immersed in primary ATE1 antibody mixed in blocking solution at 1:400 dilution. Subsequently, the cells were subjected to Alexa Fluor 488 anti-mouse IgG (1 μg/ml) (Invitrogen) for 1 hr at room temperature in the dark. Finally, the cells were

washed thrice and resuspended in 2% FBS-PBS for analysis.

2.12 Molecular Docking

The crystal structure for chicken SIRT7 (AlphaFold DB with UniProt ID: F1NC39) and NDV Matrix (PDB ID: 4G1G) were retrieved. The interaction between the SIRT7 and matrix protein was performed by molecular docking. Docking was performed using the ZDOCK protein-protein docking web server [324]. The three-dimensional structure of chicken ATE1 was predicted and generated using I-TASSER (Iterative Threading ASSEmbly Refinement) server, whereas full-length NDV HN structure was generated using AlphaFold [193]. The quality of the final models was further validated using the PROCHECK-Ramachandran plot after their energy minimization using Yasara software. Additionally, the crystal structure for β-actin (PDB id: 3J0S), ubiquitin (PDB id: 6K2U), and GAPDH (PDB id: 6IQ6) were retrieved from the protein data bank. The interaction between ATE1 and β-actin, ubiquitin, GAPDH, HN, and N protein was performed by molecular docking using Cluspro 2.0 protein-protein docking software [417, 221]. The PDB sum generator server was used to find the interacting amino acid residues spanning the ATE1 and target proteins [101]. Finally, the docked models were analyzed for Z-score using the DockScore tool [261].

2.13 Molecular Dynamics Simulations

The docked complexes were subjected to molecular dynamics (MD) simulations to study the conformational stability using the GROMACS v5.1.4 software program [2]. Each system was enclosed within a solvated dodecahedron box, ensuring a minimum distance of 1.2 nm from the boundary. The systems were filled with single-point charge water and neutralized by counter 14 cations (Na^+). Later, the solvated systems were energy minimized using the steepest descent method [321]. This was followed by a 100 ps equilibrium simulation using NPT and NVT ensembles to optimize the system's density and orientation. The final equilibrated systems were then used as starting conformations to run the MD simulations for 100 ns. Finally, the output trajectories were obtained, and the estimation of Root Mean Square Deviation (RMSD), Root Mean Square Fluctuation (RMSF), Solvent Accessible Surface Area (SASA), and Radius of Gyration (Rg) were done

using GROMACS packages. The final graphs were analyzed using XMGRACE software and plotted with GraphPad Prism software.

2.14 Co-Immunoprecipitation Assay

293T cells were transfected with expression plasmids and incubated for 48 hrs. Following the manufacturer's instructions, the cells were lysed with Pierce IP Lysis/Wash Buffer (Pierce, USA). Antibodies against specific proteins were added to lysates and incubated overnight at 4°C with continuous mixing. The A/G magnetic beads were washed twice with IP Lysis/Wash Buffer and subsequently added to the antigen-antibody mixture for incubation at room temperature for 1 hr. Magnetic beads were separated from the samples and washed with Lysis/Wash Buffer three times. Incubation of beads in low-pH elution buffer for 10 min was done to collect the supernatant containing the antigen-antibody complex. The samples were further analyzed by Immunoblot analysis.

2.15 Peptide Synthesis and Purification

The arginylated-HN peptide, RDRAVNRVVLENEEC, was prepared by Solid Phase Peptide Synthesis (SPPS) technique using standard Fmoc (9-fluorenylmethoxycarbonyl) chemistry on Rink Amide MBHA resin. For standard coupling, 3 equiv. of Fmoc protected amino acid (with respect to the loading of the resin), 3 equiv. of Hexafluorophosphate Benzotriazole Tetramethyl Uronium (HBTU), 6 equiv of N, N-Diisopropylethylamine (DIPEA) were taken in 5 ml of N-DMF and stirred for 5 min before the addition of the mixture to the resin having free amine group on its surface. The resin was shaken for 1 hr, followed by washing with N-DMF several times to ensure complete removal of the coupling cocktail. The Fmoc deprotection was achieved by 20% piperidine in an N-DMF solution. The coupling steps and Fmoc-deprotection were repeated to get the desired sequence via C to N terminal propagation. After building the entire sequence on the resin, it was treated with a freshly prepared cleavage solution of Trifluoroacetic acid/Triethylsilane/phenol/water (85:5:5:5). Finally, the resin was washed several times with DCM, and all the washings were combined and concentrated to a minimum volume under reduced pressure. The cleaved peptide was then precipitated from a large excess volume of cold dry diethyl ether and centrifuged to collect the peptide. Purification of the

peptide was achieved on a semipreparative HPLC (Thermo Scientific, Dionex Ultimate 3000) using a Luna 5 μm C18(2) 100 Å column (Phenomenex) using the acetonitrile-water system as eluent. A program, starting from 5% ACN/H_2O, reaching 100% ACN in 20 min, was used for purification. The retention time of the peptide was 7.59 min. Furthermore, MALDI analyses were performed with a Daltonics—autoflex™ speed MALDI-TOF instrument (Bruker, Billerica, MA, USA). The pure lyophilized peptides were obtained in 90% yield. MALDI-TOF, calculated for $C_{72}H_{125}N_{27}O_{25}S$: 1802.02 $[M+H]^+$, was obtained 1803.58.

2.16 Preparation of arginylated-HN-KLH Conjugate

The antigen peptide was coupled with Mariculture Keyhole Limpet hemocyanin (Imject™ mcKLH) (Thermo Scientific). Briefly, 5 mg of KLH was dissolved in 0.5 ml of 0.01 M phosphate buffer (pH 7), and 3 mg of MBS was dissolved in 200 μl N-DMF. Subsequently, 70 μl of the MBS solution was added to 0.5 ml of KLH solution, and the mixture was incubated at room temperature for 30 min on a rotary shaker. Additionally, 5 mg of arginylated-HN peptide was dissolved in 100 μl of N-DMF, then rapidly added with 1 ml of KLH/MBS. The solution was vigorously shaken, and 11 μl of 2 N NaOH was immediately added. The pH was checked using pH paper and adjusted to be 7-7.2. The reaction mixture was further incubated for 3 hrs at room temperature with gentle shaking, then stored at 4°C. Finally, 3 ml of ammonium bicarbonate (0.1 M) was added before lyophilizing the reaction solution.

2.17 Rabbit Immunization

The arginylated-HN peptide was used for the production of anti-R-HN antibody as the antigen, as per the standard protocol. Briefly, 5 mg of arginylated-HN-KLH conjugate was dissolved in 500 μl PBS and mixed thoroughly. 500 μg of antigen was mixed with complete Freund's adjuvant in a 1:1 ratio and rabbits were immunized subcutaneously. The rabbits received two booster doses after 21 and 35 days from first injection. Lastly, after 14 days from 2nd booster dose, serum was collected for further analysis.

2.18 Statistical Analysis

All the values are presented as mean±standard deviation (SD). The values $p<0.05$ (*), $p<0.01$ (**), and $p<0.001$ (***) exhibited significance in one-way analysis of variance (ANOVA) and Student's t-test. Statistical analyses were carried out using GraphPad Prism v5. All experiments were reproduced at least three independent times.

CHAPTER 3

INSTIGATION OF ER AND OXIDATIVE STRESS BY NDV INFECTION

The articles based on this chapter are published as follows:

Lithium chloride functions as Newcastle disease virus-induced ER-stress modulator and confers anti-viral effect.
Shokeen K, Srivatsan A, Kumar S. **Virus Res.** (2021)

Newcastle disease virus regulates its replication by instigating oxidative stress-driven Sirtuin 7 production.
Shokeen K, Kumar S. **J Gen Virol.** (2024)

Abstract

ER stress-modulated, GRP78/Bip, a molecular chaperone, is actively involved in numerous viruses' replication cycles. Moreover, the accumulation of ROS inside the cells instigates oxidative stress, activating stress-responsive genes. The viral strategies for promoting stressful conditions and utilizing the induced host proteins to enhance their replication remain elusive. This chapter investigates the instigation of ER and oxidative stress during NDV infection. Additionally, we explore the impact of oxidative stress-induced ROS on virus replication. The results demonstrate that NDV-induced ER stress leads to substantial upregulation of GRP78 protein *in vitro* and *ovo*. Subsequently, the treatment of NDV-infected cells with LiCl can significantly reduce the levels of GRP78.

Moreover, the results also show the involvement of accumulated intracellular ROS in maintaining a stressed environment and activating the oxidative stress response, which helps the NDV replicate better.

3.1 Introduction

Cells usually perform all their normal physiological functions in a controlled environment, and any deviation from it causes cellular stress. The high impact of stress on cells' normal functioning makes stress management a critical factor in managing internal homeostasis. Cells respond to stress by inducing several stress-mediated signaling pathways to mitigate its effect [141]. Further, the stress-activated signal transduction pathways modulate SRPs, maintaining protein homeostasis [138]. Prolonged exposure to pathogens, mainly viruses, causes ER and oxidative stress [82, 92, 182, 394, 458, 470], leading to increased stress protein production in the host. Interestingly, the virus-initiated stress has been demonstrated to exacerbate its pathogenesis, resulting in stress-induced cell death [179, 313].

ER plays a vital role in protein folding and processing in eukaryotic cells. Accumulation of unfolded or misfolded proteins in the ER lumen causes ER stress, which further triggers the UPR to decrease the load of unfolded proteins [360]. The UPR is identified by the activation of three distinct ER stress sensors: ATF6, IRE1, and PERK, which were initially linked to GRP78. The dissociation of GRP78 from these sensors leads to the activation of processes that restore protein homeostasis [12]. Zika [284], Herpes [191], and Japanese encephalitis [296] viruses have already been shown to be involved in inducing ER stress. Several studies reported the activation of UPR under the influence of recombinant NDV expressing the rabies virus glycoprotein (rL-RVG) [51]. Specifically, NDV N and P proteins were shown to be potent ER-stress inducers to regulate the transcription of the ER chaperones against ER malfunction [78].

Moreover, Cells produce free radicals or ROS as a by-product of energy production [108, 272, 412]. Subsequently, antioxidants remove or neutralize the ROS to maintain the proper balance [275]. ROS are reactive chemical molecules that carry electron-deficient oxygen in the forms of superoxides and peroxides [17]. Effective cellular sectionalization for oxygen, extensive expressions of SODs, and GST keep the superoxide level low in the cells [55, 471]. Oxidative stress-driven production of several stress-responsive proteins helps in maintaining redox homeostasis. One central regu-

lator of oxidant exposure is Nrf2, which functions as a transcription factor driving the expression of antioxidant enzymes such as HO-1 and NQO1 [332]. In addition, heat shock proteins (majorly HSP90), which are ATP-dependent chaperones, are also reported to be actively involved in oxidative stress [94, 276].

Viruses are the most common and potent stressors, which trigger various pathways to create a stressed environment [150]. Studies demonstrate that viruses, including HIV [125, 269], DENV [325, 377], influenza [219], and hepatitis [137, 182] virus, modulate oxidative stress and specific oxidant-sensitive pathways. NDV has also been reported to cause oxidative damage and induce stress response [224, 306, 392]. Studies showed oxidative imbalance and decreased antioxidant activity, including SODs, GST, and catalase, in chickens infected with NDV [341]. Furthermore, it has also been observed that the antioxidant defense system is impaired following NDV infection and vitamin E supplementation helps restore it [342, 391, 392]. Accumulating ROS and oxidative damage play a crucial role in viral diseases; thus, establishing their precise part is a prerequisite to revealing new insights into cell defense strategies while identifying new therapeutic targets.

In this chapter, we primarily focus on the influence of NDV infection on ER stress and oxidative stress. We also aim to elucidate the role of intracellular ROS in NDV infection by studying its replication kinetics in cells with active oxidative stress response.

3.2 Results

3.2.1 Drug cytotoxicity

LiCl is a widely prescribed drug for the treatment of bipolar disorder, acute brain injuries, and chronic neurodegenerative diseases. It has also been repurposed as an effective anti-viral drug for some viral infections. DMF has been shown to activate antioxidant systems by inducing the upregulation of antioxidant gene expression in the cells, whereas PDTC is a well-established ROS scavenger as it helps to neutralize harmful levels of free radicals produced within the cell. In our study, we utilized LiCl as a potential anti-NDV agent, DMF as a regulator of oxidative stress response, and PDTC as a ROS scavenger. Firstly, the cytotoxicity assessment of LiCl, DMF and PDTC in DF-1 cells was performed using MTT calorimetry assay to determine the working concentration.

Firstly, the results indicated that higher doses of LiCl affect DF-1 cell viability. The mean lethal dose of LiCl in DF-1 cells was calculated to be 83.20 mM (Fig. 3.2.1A). The cell viability analysis using calcein stain showed minimal cell morphological differences between LiCl-treated and mock-treated cells when lower concentrations of LiCl (5-30 mM) were used (Fig. A.1A). These analyses suggested that 5-20 mM concentrations of LiCl are almost non-toxic, except for the highest concentration of 30 mM, which is moderately toxic to DF-1 cells after 24 hrs of treatment. All further experiments in the study were performed at 5, 10, 20, and 30 mM concentrations of LiCl. Moreover, there were no impacts on the growth of the DF-1 cell line till 5 μM of DMF and PDTC, whereas the maximum toxicity recorded at 20 μM concentration was about 20% inhibition value for DMF and 50% for PDTC (Fig. 3.2.1B and C).

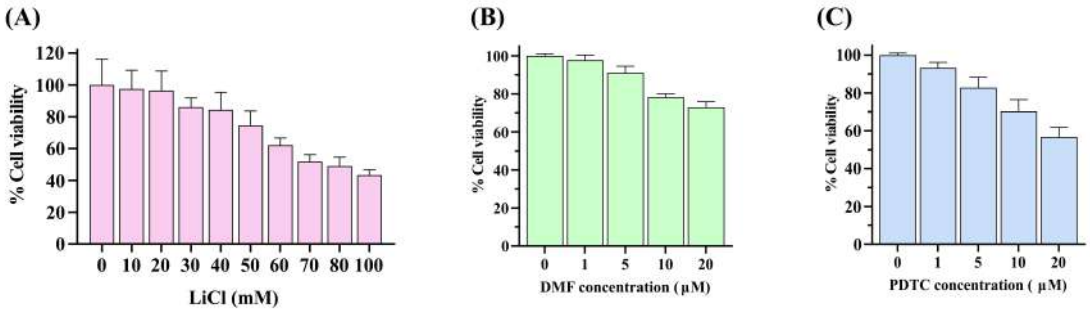

Figure 3.1: Cytotoxicity of drugs using MTT assay.
Graph representing the percentage cell viability after LiCl, DMF, and PDTC treatment **(A)(B)(C)**.

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3.2.2 NDV modulates ER-stress

The expression levels of GRP78 were analyzed to assess the NDV proliferation effects on ER stress. The mRNA copies of GRP78 were clearly seen to be modulated following infection. The mRNA copy number of GRP78 in DF-1 cells was comparatively down-regulated following 36 hrs post-infection with NDV compared to control. However, the copy number was increased to 3-fold 60 hrs post-infection with NDV (Fig. 3.2A). Similarly, the evident upregulation in the protein expression of GRP78 in a time-dependent manner as a consequence of NDV infection was observed (Fig. 3.2A). As the NDV infection proceeds with time, there is a gradual increase in the protein level of GRP78.

The transcript levels of GRP78 were measured to determine the influence of LiCl on NDV-triggered ER stress. The elevated levels of GRP78 mRNA and protein were significantly reduced when NDV-infected cells were treated with different concentrations of LiCl (5-30 mM). The results showed up to 16-fold higher mRNA copies of GRP78 in the NDV-infected than control cells. Subsequently, these higher mRNA levels were reduced up to 8-fold by the LiCl treatment (Fig. 3.2B). In line with the transcriptional expression of GRP78, we investigated the protein levels in NDV-infected cells when treated with LiCl. A substantial increase in the GRP78 protein level was observed upon NDV infection, which was reduced by the treatment (Fig. 3.2B).

Furthermore, to substantiate the GRP78 modulation induced by NDV infection, mRNA expression was also evaluated in the chicken embryos. The results suggested a high copy number of GRP78 mRNA in the NDV-infected embryo tissues and a reduction of up to 14-fold when treated with LiCl (Fig. 3.2C). Similar patterns of GRP78 down-regulation were reflected in the protein level as well (Fig. 3.2C).

3.2.3 Intracellular ROS production post-NDV infection

The ROS induction and scavenging were tested at 1 and 5 μM of DMF and PDTC (Fig. A.1A and B). Further, the results showed that 1 μM of DMF and 5 μM of PDTC are optimal for the study. Subsequently, intracellular ROS induced upon NDV infection were detected using fluorescent DCFH-DA dye. A persistent increase in fluorescence intensity was observed, up to 140%, till 72 hrs post-NDV infection (Fig. 3.3A). This effect was almost equivalent to DMF-treated cells. Furthermore, LiCl, an antiviral drug for NDV, was used to observe the effects on ROS levels due to NDV replication inhibition, the results presented the reversion of ROS levels inside the cells as the increased fluorescence intensity was diminished to 65% after LiCl on NDV-infected cells (Fig. 3.3C). In contrast, known ROS scavengers PDTC and NAC were also used to confirm the modulation in NDV-infected cells. The post-treatment of NDV-infected cells with these drugs exhibited an impairing effect on the NDV-induced ROS, reaching more than 50% in PDTC and almost 100% by NAC, respectively (Fig. 3.3B and D).

3.2.4 Replication kinetics of NDV in oxidative condition

The cells were pre-incubated with DMF for 12 hrs to induce the oxidative stress response. Subsequently, they were infected with NDV to investigate the potential impacts

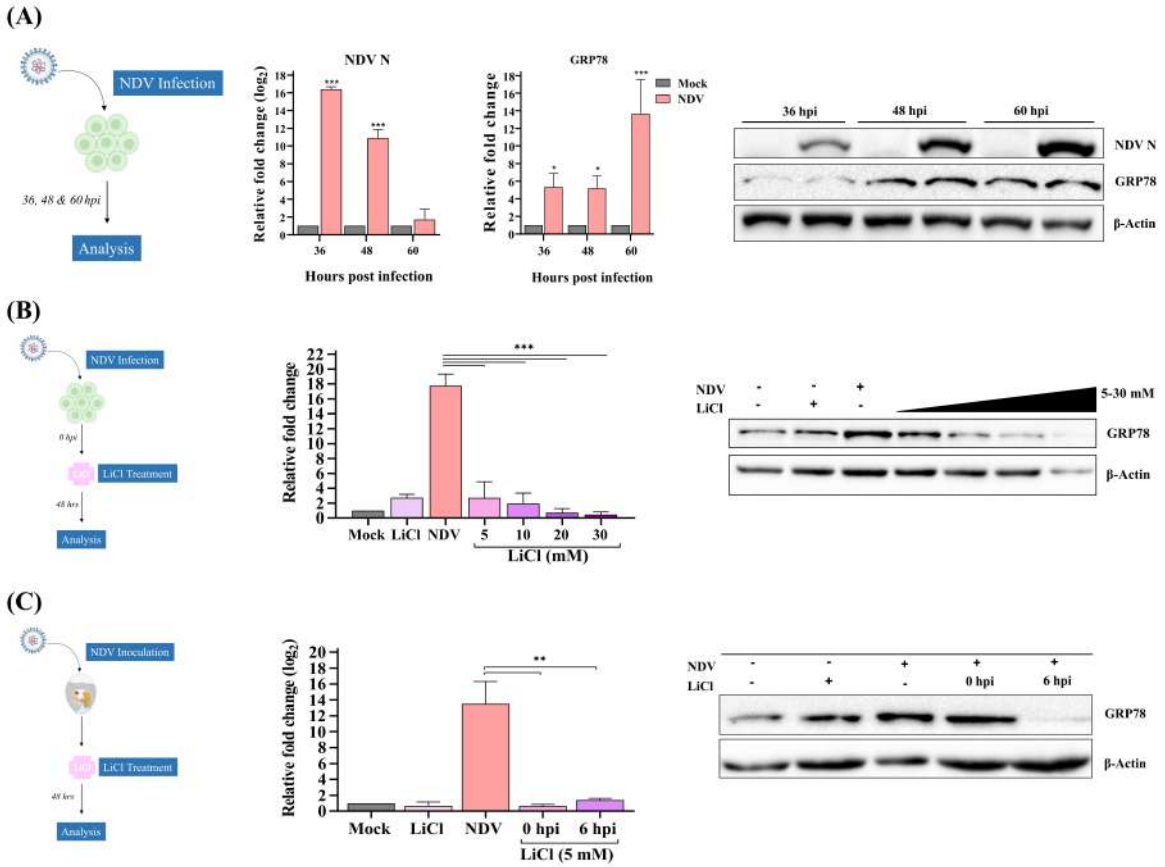


Figure 3.2: NDV modulates ER-stress. The workflow of the experiment designed for GRP78 expression evaluation. Graph shows the relative change in NDV N transcript levels and second graph shows the relative change in GRP78 transcript levels after 36, 48, and 60 hrs post-NDV infection. Immunoblot analysis showing the modulation of GRP78 with NDV infection **(A)**. The workflow of the experiment designed *in vitro* for GRP78 expression evaluation after NDV infection and LiCl treatment. Relative fold change in GRP78 transcript level in NDV infected and LiCl treated cells. Immunoblot analysis of GRP78 regulation in NDV infected and LiCl treated cells **(B)**. The workflow of the experiment designed *in ovo* for GRP78 expression evaluation after NDV infection and LiCl treatment. Graph shows the relative fold change in the GRP78 mRNA level after LiCl treatment in the NDV-infected chicken embryos. Immunoblot analysis of NDV infected embryo tissue samples using GRP78 specific antibody **(C)**.

of an active stress response on NDV infection. The gene and protein expression experiments showed that DMF-treated cells had higher virus transcript and protein levels than untreated groups (Fig. 3.4A and B), and the viral kinetic profile was consistent at all time intervals. The overall NDV titer was also compared with untreated groups using $TCID_{50}$ and plaque assay with similar conditions. The difference in titer was noted at 48 hrs post-NDV infection, where the mean viral load was 7.42 $\log_{10}$ $TCID_{50}$/ml in DMF-treated

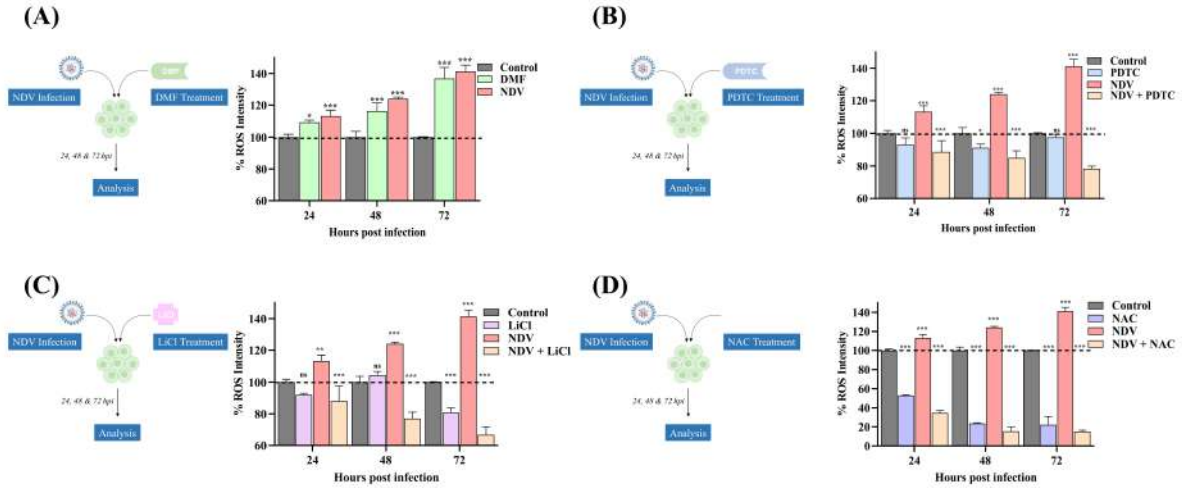


Figure 3.3: Intracellular ROS production post-NDV infection. The workflow of the experiment designed for estimation of intracellular ROS due to NDV with DMF **(A)**, PDTC **(B)**, LiCl **(C)**, and NAC **(D)**. Generation of intracellular ROS due to NDV with DMF **(A)**, PDTC **(B)**, LiCl **(C)**, and NAC **(D)** through fluoroscopic measurements determined using DCFH-DA dye. The statistical significance in all the figures shows the significance levels between the control and test of the same time point.

cells, compared to 6.66 $\log_{10}$ $TCID_{50}$/ml in control cells (Fig. 3.4C). Similarly, 7.9x10^4 PFU/ml were observed at 48 hrs post-NDV infection, compared to 3.4x10^4 PFU/ml in control cells (Fig. 3.4D).

Contrarily, the effects of reduction of ROS generation on NDV pathogenesis were investigated to establish their roles, where cells were post-treated with ROS scavenger drug. 12 hrs post-NDV infection PDTC treatment significantly inhibited virus replication (up to 8-fold) and protein production (Fig. 3.4E and F). In congruence with the above results, the $TCID_{50}$ and plaque assay subsequently revealed the reduced titer of NDV in PDTC-treated cells. The mean viral load was reduced to 5.6 $\log_{10}$ from 6.8 $\log_{10}$ $TCID_{50}$/ml in control groups at 48 hrs post-infection (Fig. 3.4G). Similarly, a notable decline of NDV plaques from 4.15x10^4 to 1.0x10^4 PFU/ml was observed at 48 hrs (Fig. 3.4H).

3.2.5 Modulation of oxidative stress-response genes

The expression profiles of key oxidative stress-responsive genes in NDV-infected cells were analyzed and compared with mock-infected DF-1 cells, to investigate the induction of oxidative stress and the cellular response. At first, the relative fold change

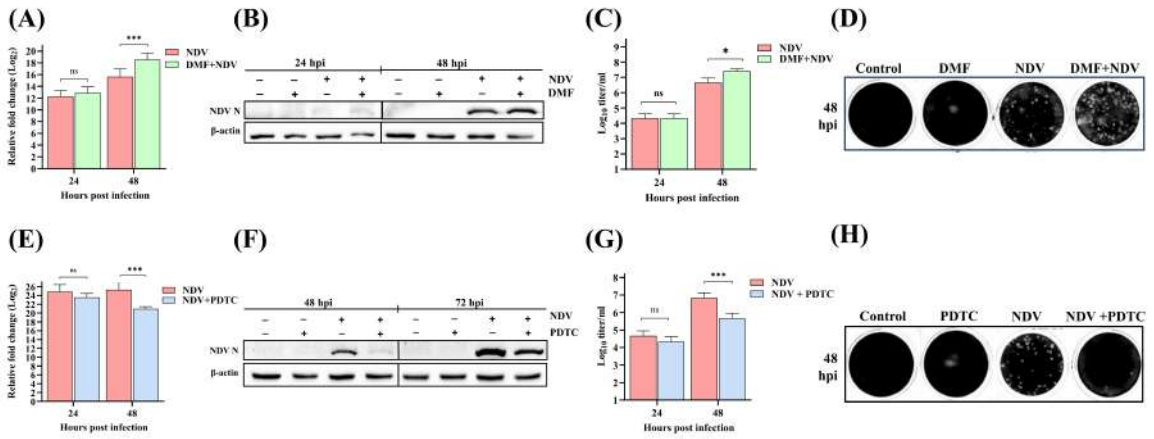


Figure 3.4: Replication kinetics of NDV influenced by ROS generation. Graph shows the relative fold change in NDV N gene mRNA in cells with and without DMF **(A)**. Immunoblot analysis of NDV proteins in DMF pre-treated cells **(B)**. Assessment of NDV titer in DMF pre-treated cells using $TCID_{50}$ assay **(C)**. Assessment of NDV titer in DMF pre-treated cells using plaque assay **(D)**. Graph shows the relative fold change in NDV N gene mRNA in cells with and without PDTC **(E)**. Immunoblot analysis of NDV proteins in PDTC post-treated cells **(F)**. Assessment of NDV titer in PDTC post-treated cells using $TCID_{50}$ assay **(G)**. Assessment of NDV titer in PDTC post-treated cells using plaque assay **(H)**.

of the NDV N gene was calculated at 24, 48, and 72 hrs to confirm the progress of virus replication (Fig. 3.5A). Subsequently, Nrf2, HO-1, SOD-1, and Hsp90b expression profiles were studied with or without NDV at different time points. The Nrf2 mRNA expression was decreased from 1.0 to 0.3-fold (Fig. 3.5B), followed by HO-1 inhibition from 1.0 to 0.25-fold after 72 hrs of NDV infection (Fig. 3.5C). At the same time, SOD-1 was upregulated up to 3-fold at 24 hrs post-infection but showed a marked drop at 48 hrs when NDV replication reached its peak (Fig. 3.5D). Concurrently, Hsp90b over-expression was progressive and time-dependent, getting a 4-fold increment (Fig. 3.5E). Furthermore, mRNA variation levels of sirtuin family genes (SIRT1-7) instigated by NDV were also calculated. NDV-infected cells demonstrated significant up-regulation of SIRT1 (from 1.0 to 4.5-fold at 24 hrs), SIRT4 (from 1.0 to 1.5-fold at 48 hrs), SIRT6 (from 1.0 to 2.2-fold at 48 hrs) and SIRT7 (from 1.0 to 11.0-fold at 48 hrs) expression levels as well as substantial down-regulation of SIRT2 (from 1.0 to 0.56-fold at 48 hrs), SIRT3 (from 1.0 to 0.22-fold at 48 hrs) and SIRT5 (from 1.0 to 0.15-fold at 72 hrs) expression levels compared to the control groups as measured by qPCR (Fig. 3.5F-L)

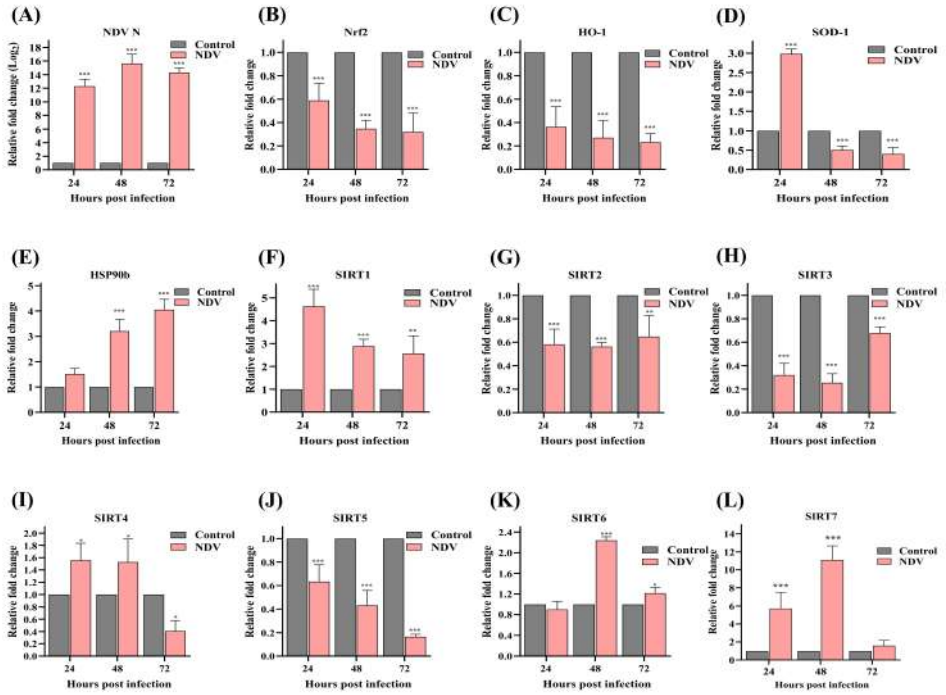


Figure 3.5: Modulation of oxidative stress-response genes post-NDV infection. Graphs show the relative fold change in the NDV N **(A)**, Nrf2 **(B)**, HO-1 **(C)**, SOD1 **(D)**, HSP90b **(E)**, SIRT1 **(F)**, SIRT2 **(G)**, SIRT3 **(H)**, SIRT4 **(I)**, SIRT5 **(J)**, SIRT6 **(K)**, and SIRT7 **(L)** transcript levels after 24, 48, and 72 hrs post-infection with NDV.

3.3 Discussion

ER ensures the standard functionality and proper folding of newly synthesized proteins, maintaining ER homeostasis. Several conditions, like high demand for protein secretion and missense mutations, lead to protein misfolding and ultimately cause ER stress [240]. Numerous viruses, including Zika [284], herpes [191], JEV [296], and DENV [114] are involved in inducing ER stress and the UPR during their infection. GRP78 is a molecular chaperone involved in maintaining ER integrity, and its overexpression can be directly linked with the induction of ER stress [378]. All the viral proteins synthesized in the cytoplasm must be folded appropriately to perform their usual functions. Here, we investigated the induction of ER stress following NDV infection by studying the modulation of GRP78. The GRP78 protein levels showed upregulation during the infection to overcome the ER stress induced by the high level of unfolded viral proteins in the ER. We also demonstrated that LiCl treatment protects the cells from NDV-induced ER stress as GRP78 was downregulated after the LiCl treatment. As per the previous studies, both

upregulation [295, 366] and downregulation [8, 145, 162] of ER-stress-related proteins were shown in the presence of lithium. Our results collectively indicate the downregulation of GRP78 at both transcript and protein levels in both cell culture and embryonated chicken eggs.

Mitochondria are ubiquitous multifunctional organelles that assist the cells' survival through metabolism and biosynthesis. As a principal ROS source in cells, mitochondria play a pivotal role in modulating oxidative stress and innate immune responses [56, 129, 293]. Any abnormality in a typical cellular environment can cause an imbalance in the ROS production and removal system, resulting in higher accumulation [16]. ROS are often considered etiologic in inducing cellular injury by altering the cell's redox state. Various viruses, including HIV, DENV, influenza, and SARS-CoV-2, are reported to exploit or manipulate mitochondrial functions to induce oxidative stress, thus promoting their replication [59, 292, 362, 394, 458]. It brings attention to the crosstalk mechanism of oxidative stress and the pathogenesis of viral infections, which remains hither and thither. Oxidative stress typically triggers the host's antiviral innate response; however, recent studies have described that elevated ROS levels may favor viral replication [132, 278, 362, 404]. Although the instigation of oxidative stress during NDV infection has been reported several times, the cellular stress-responsive protein's direct function in virus replication is yet to be well understood. This study highlights the plausible stress-responsive proteins involved in viral pathogenesis while exploring the detailed molecular mechanisms of crosstalk between the activated cellular protein and the progress of the NDV replication cycle. Moreover, previous studies describing how different viruses modulate cellular stress may not fully reflect the complete picture of viral strategies.

First, we tested the intrinsic impact of NDV infection on oxidative stress and intracellular ROS production. The results reflect the high generation of ROS temporally as the NDV infection cycle progressed. Simultaneously, comparing these elevated levels in infected cells with DMF-treated and ROS scavenger (PDTC and NAC)-treated cells confirms the presumption of ROS induction due to NDV infection. It was further validated via post-LiCl treatment, lowering ROS levels by inhibiting NDV replication. LiCl affects the activity of the GSK3, subsequently triggering the degradation of the Nrf2 protein [444], potentially leading to ROS levels' depletion. Here, we aimed to show that the ROS generation was induced by NDV replication. It is clear from the results that ROS intensity reduction after LiCl treatment alone declines significantly only at 72 hrs.

However, a substantial decrease in ROS intensity was observed as early as 24 hrs in LiCl-treated NDV-infected cells. From this, we concluded that at an early time point, LiCl had a more prominent role as an anti-NDV rather than modulating the GSK3 activity.

Further, to determine the plausible significance of the NDV-induced increased ROS levels in the pathogenesis, we studied the propensity of NDV to replicate in cells with active oxidative stress response. Several studies showed that DMF activates the Nrf2 pathway, thus leading to an increase in HO-1 protein levels [158, 262]. Most importantly, it has been shown to upregulate the antioxidant gene expression in the cells [63, 249], aligning precisely with our intended focus. The initial results showed that DMF pre-incubated cells had higher NDV mRNA and protein expression, reflecting comparatively inflated virulence than control DF-1 cells. The viral quantification assays also produced similar higher trends in virus titer. Additionally, PDTC attenuated the ROS generated in NDV-infected cells through direct scavenging, thus making a corresponding sharp decrease in NDV titer. In conjunction, these results affirm the involvement of intracellular ROS generation in maintaining a stressed environment and activating the oxidative stress response, which helps the NDV replicate better.

Cellular response to the virus-imposed stress is mainly controlled by the universally conserved stress-responsive proteins, which are upregulated depending upon the type of stress the cell is experiencing [225]. Viruses often manipulate the cellular response to maintain the oxidative stress environment throughout the infection cycle by inhibiting the synthesis and activity of antioxidant enzymes [10, 136, 171, 390]. Therefore, we determined the expression patterns of crucial oxidative stress-responsive genes to examine the persistence of oxidative stress during infection. The suppression of Nrf2 and its downstream effector HO-1 suggested that NDV interferes with the Nrf2/Keap1-signalling pathway, concording with previous similar reports [66, 309, 317]. Interestingly, SOD-1 activation was seen to be appreciable in the early stages of infection, insinuating that the infected cells might benefit from their activation to confer increased resistance against the virus-induced oxidative stress. However, the SOD-1 activity might be unfavorable for replication, and NDV reduces its expression drastically, as shown previously [252]. The cellular gene expression profile data confirmed the modulation of oxidative stress-related genes post-NDV infection, thus implying their implicit or explicit role in viral pathogenesis.

Additionally, expression profiles of SIRT family genes were also studied, and expectedly, they are found to be differentially altered due to NDV infection. The mag-

nitude of the variations in expression levels was strong enough to suggest their active participation in viral pathogenesis. In recent years, SIRTs have been mainly studied for their regulatory roles in cell metabolism, aging, and neurodegenerative diseases [61, 146, 188, 347]. Moreover, numerous studies support their implication in mediating oxidative stress response to protect the cells from excessive ROS by directly deacetylating several key transcription factors, thus regulating antioxidant gene expression [50, 277, 379, 449]. Hence, the prominent activation of SIRT1 and SIRT7 could be directly linked to the oxidative stress induced by NDV. Similar results are also reported, showing their overexpression and constructive role in the progress of virus infection [105, 248, 326, 446]. However, SIRT3 and SIRT5 were inhibited by NDV, which corroborates with the recent research work showing the reprogramming of energy metabolism in infected cells after their degradation. The SIRT4 and SIRT6 modulation has not been previously reported in virus infection [151]. Moreover, the involvement of SIRT proteins in stress-related pathways has unvaryingly strengthened the knowledge of viral pathogenesis, especially when the holes in our understanding of pathogenesis and host-response hindering strategies are perceived to be significant.

3.4 Conclusion

Taken together, the data obtained in the study suggest that NDV induces ER and oxidative stress inside the cells. High intracellular ROS generation and the resulting oxidative stressed environment are also necessary for effective NDV replication. Moreover, the NDV-modulated stress elicits several transcriptional and translational outputs that activate stress-responsive proteins, which the virus utilizes for better replication.

CHAPTER 4

FUNCTION OF SIRT7 PROTEIN IN NDV PATHOGENESIS

An article based on this chapter is published as follows:

Newcastle disease virus regulates its replication by instigating oxidative stress-driven Sirtuin 7 production.
Shokeen K, Kumar S. **J Gen Virol.** (2024)

Abstract

SIRT7 protein functions as a NAD^+-dependent histone deacetylase enzyme, thus, regulating multiple biological processes including genome stability, metabolic pathways, and stress responses. This chapter investigates the expression patterns of SIRT7 protein, and then further explores its activity. The results demonstrate that its activity is associated with the positive regulation of cellular protein deacetylation. A detailed mechanistic study in vitro and ovo was also conducted utilizing SIRT7 activity modulators to decipher the underlying role in infection, either constructive or destructive. Lastly, we concluded that the elevated expression of NDV-mediated SIRT7 protein with enhanced activity metabolizes the NAD^+ to deacetylase the host proteins, thus contributing to high virus replication.

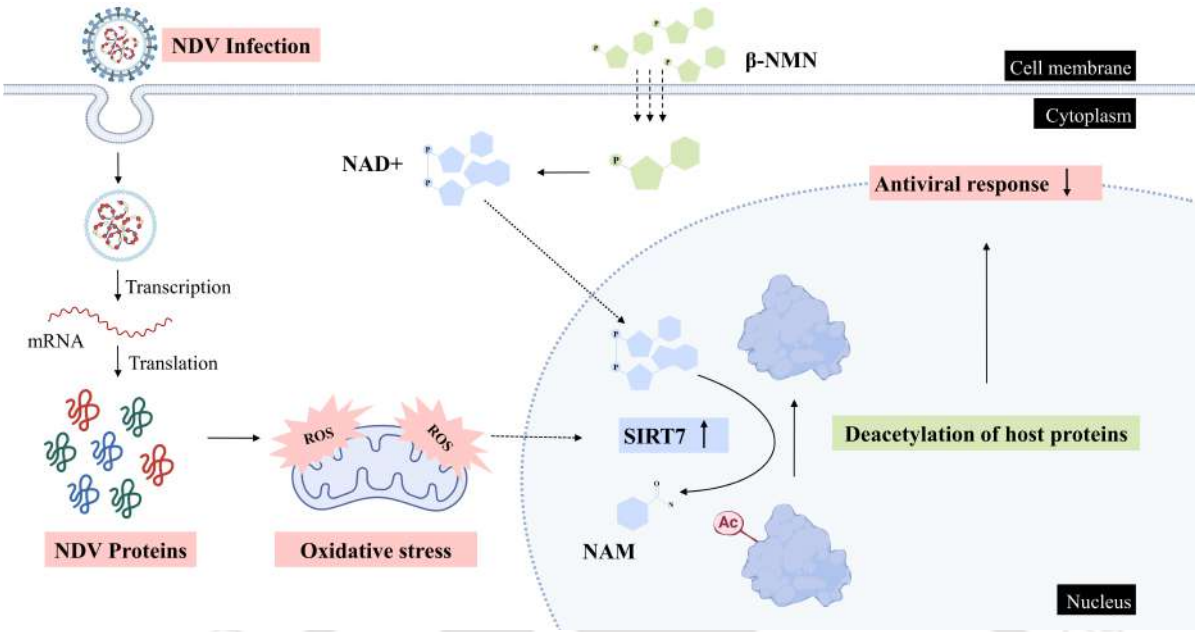


Figure 4.1: Graphical abstract of the chapter highlighting the SIRT7 activity.

4.1 Introduction

SIRTs, comprising seven different proteins, are a family of NAD^+-dependent histone deacetylases identified as essential for cell survival [170]. They are ubiquitously expressed, evolutionarily conserved, and actively involved in DNA repair, autophagy, aging, cardiovascular diseases, and neurodegenerative diseases [347]. SIRT1, SIRT6, and SIRT7 are primarily located in the nucleus; SIRT2 is a cytoplasmic protein, whereas SIRT3, SIRT4, and SIRT5 are distributed in mitochondria [153, 170, 280, 314, 421]. Studies have presented SIRTs as an integral part of cellular defense mechanisms against oxidative stress and ROS formation [379]. More specifically, SIRT1, SIRT3, and SIRT5 have been reported to be involved in the protection of cells from excessive ROS, whereas SIRT2, SIRT6, and SIRT7 modulate several essential oxidative stress genes [277]. Additionally, they are linked with oxidative stress-driven cellular responses triggered to curtail the stress via deacetylation of various substrates [109, 454, 469]. Deacetylation of FOXO3a by SIRT2 and SIRT3 leads to increased expression of its downstream genes, which are responsible for maintaining mitochondrial homeostasis, anti-apoptosis, and anti-oxidative stress [409, 432]. SIRTs are reportedly as an essential defense against several viral infections [339, 326, 453], while others suggested their constructive participation in their pathogenesis [312, 327, 343, 448].

SIRT7 is the least explored protein among all members as its enzymatic activity, physiological function, and target proteins remain elusive. The genetic sequence of the chicken SIRT7 gene exhibits an 72.4% similarity to that of the corresponding human gene. It belongs to class III histone deacetylase (HDAC) and is a critical regulator of host gene expression [370]. However, recent studies have revealed its deacetylase function on several non-histone proteins, including ATM [399], p53 [418], GATA4 [447], and PAF53 [74]. Presumably, it may restrict as well as enhance virus replication via the deacetylation of a different set of proteins [248, 453]. However, recent studies focus on deciphering its role as an essential cellular regulator and utilizing it as a therapeutic target to explore future therapies for multiple diseases [247]. SIRT7-mediated deacetylation and its involvement in virus replication are yet to be understood. Most importantly, the molecular mechanisms of crosstalk between SIRT7 protein and the virus replication cycle are not well explored. Therefore, many previous studies describing how different viruses modulate the SIRT7 expression may not fully reflect the bigger picture of their participation in viral pathogenesis.

This chapter primarily focuses on elucidating the role of SIRT7 protein in NDV infection in cells with active oxidative stress response. We also aim to establish the exact part of SIRT7 protein deacetylation activity in viral pathogenesis.

4.2 Results

4.2.1 Expression of SIRT7 due to oxidative stress

As the over-expression of SIRT1 and SIRT7 genes was notably higher than other SIRTs (as described in previous chapter), their protein modulation upon NDV infection were studied temporally. The results showed dramatic overexpression of SIRT1 upon NDV infection at 72 hrs (Fig. A.2A) and SIRT7 at 48 hrs (Fig. 4.2A). Additionally, NDV-infected cells were treated with varying concentrations of DMF (1 and 5 μM) to confirm the overexpression of SIRT7 in response to oxidative stress. The immunoblot analyses showed that high levels of SIRT7 were induced by DMF-induced antioxidant response (Fig. 4.2B). In contrast, the effects of PDTC on SIRT7 mRNA were also examined further to validate the influence of ROS on SIRT7 expression. The results revealed that SIRT7 mRNA was around 80% lower (from 1.0 to 0.22-fold at 24 hrs) in treated cells at 24 hrs (Fig. 4.2D). To verify the PDTC-specific effects on NDV-induced SIRT7, its expression was

studied in the presence of NDV and in combination with PDTC. While the up-regulation of SIRT7 at the transcriptional and translational level was observed in NDV alone, the PDTC post-treatment data showed that SIRT7 expression induced by NDV was substantially suppressed (Fig. 4.2E).

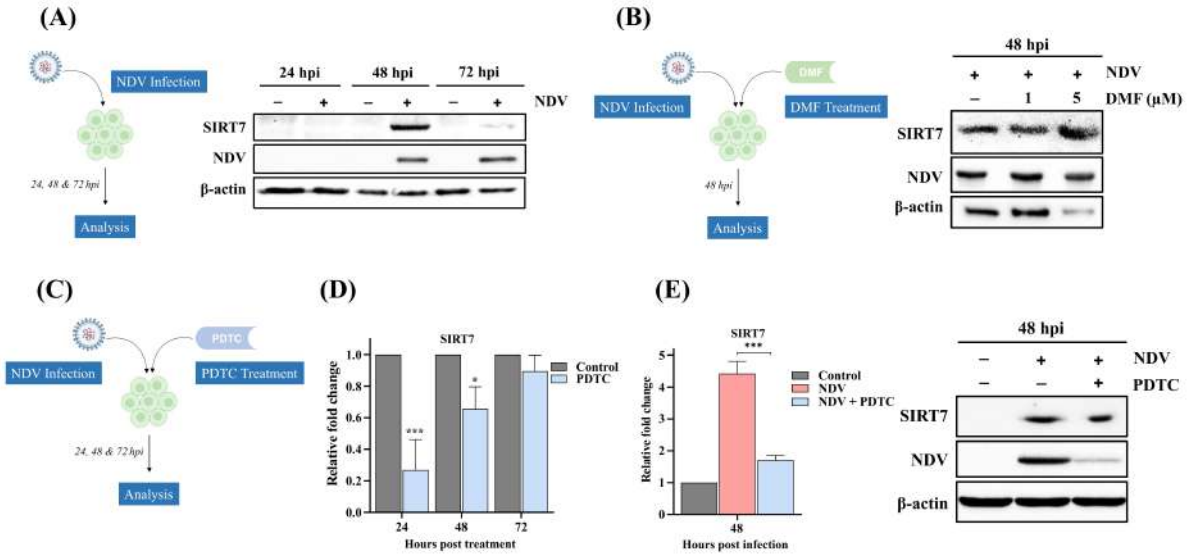


Figure 4.2: Expression of SIRT7 due to oxidative stress. The workflow of the experiment designed for SIRT7 expression evaluation. Immunoblot analysis of SIRT7, NDV, and β-actin in cells after 24, 48, and 72 hrs post-NDV infection **(A)**. The workflow of the experiment designed for SIRT7 expression evaluation after DMF treatment in NDV-infected cells. Immunoblot analysis of SIRT7, NDV, and β-actin after treatment with DMF in NDV-infected cells **(B)**. The workflow of the experiment designed for SIRT7 expression evaluation after PDTC treatment and NDV infection **(C)**. Graph shows the relative fold change in SIRT7 gene mRNA influenced by PDTC for different time intervals **(D)**. Graph shows the relative fold change in SIRT7 gene mRNA in cells with and without PDTC treatment **(E)**. Immunoblot analysis of SIRT7, NDV, and β-actin in cells with and without PDTC treatment **(E)**.

4.2.2 Function of SIRT7 in NDV replication

For the heterologous production, pcDNA3.1 expressing chicken SIRT7-FLAG was utilized. The expression was confirmed by immunoblot, which showed the presence of exogenous SIRT7 protein when detected with an anti-FLAG antibody (Fig. A.2B). To examine the effects of SIRT7 expression on NDV replication, DF-1 cells were transfected with the plasmid 12 hrs before NDV infection. The immunoblot results showed that the NDV protein production was considerably higher in SIRT7-overexpressing cells from

empty vector-transfected cells (Fig. 4.3B). Moreover, the NDV titer from plaque assay also revealed a significant increase in SIRT7-overexpressed cells (Fig. 4.3C). Additionally, NDV infection was also examined in cells transfected with GFP-expressing plasmid, and the results showed no difference in the NDV protein levels (Fig. A.2C). DF-1 cells were also transfected with NDV M- and N-expression plasmids to identify the individual viral protein responsible for SIRT7 induction. The immunoblot results evinced the induction of SIRT7 in pcDNA-M transfected cells (Fig. A.2D). Similar results were obtained for SIRT1 as well (Fig. A.2D).

In contrast with the overexpression experiments, DF-1 cells were depleted for SIRT7 using siRNA transfection. SIRT7 overexpressing cells were transfected with siSIRT7 to knockdown the gene. siSIRT7 transfection showed considerable silencing of overexpressed SIRT7 protein levels compared to siNT-treated cells (Fig. 4.3D). The mRNA levels also showed almost 70% reduction of endogenous SIRT7 gene relative to the siNT control (Fig. 4.3E). To confirm the SIRT7-specific modulation of NDV replication, cells were transfected with siSIRT7 6 hrs before NDV infection. The results indicated that NDV mRNA and protein levels were lower upon siRNA-mediated SIRT7 depletion in DF-1 cells (Fig. 4.3E and F).

Furthermore, to validate that enhanced NDV infection is specific to the cells overexpressing SIRT7 protein, we captured immunofluorescence images post-transfection and infection. The transfected cells stained with anti-Flag antibody exhibited red fluorescence, while infected cells stained with anti-HN antibody displayed a green fluorescence. The orange color in the merged panel corresponds to the overlapping region. The results showed that the expression of NDV proteins (green) was notably higher in cells specifically overexpressing SIRT7 compared to the control cells (Fig. 4.4).

4.2.3 SIRT7 activity involvement in NDV infection

To decipher the biological significance of SIRT7 for protein deacetylation (Fig. 4.5), global acetylation level was detected using an anti-acetylated lysine antibody in NDV-infected DF-1 cells. Interestingly, the virus infection significantly reduced acetylation levels time-dependently (Fig. 4.6A) by a maximum of 50% observed at 72 hrs. These changes in acetylation level were confirmed by infecting the cells with heat-inactivated NDV particles. Compared with the NDV-infected cells where the acetylated protein has completely vanished, it is evident that the deacetylation effect on cellular proteins was

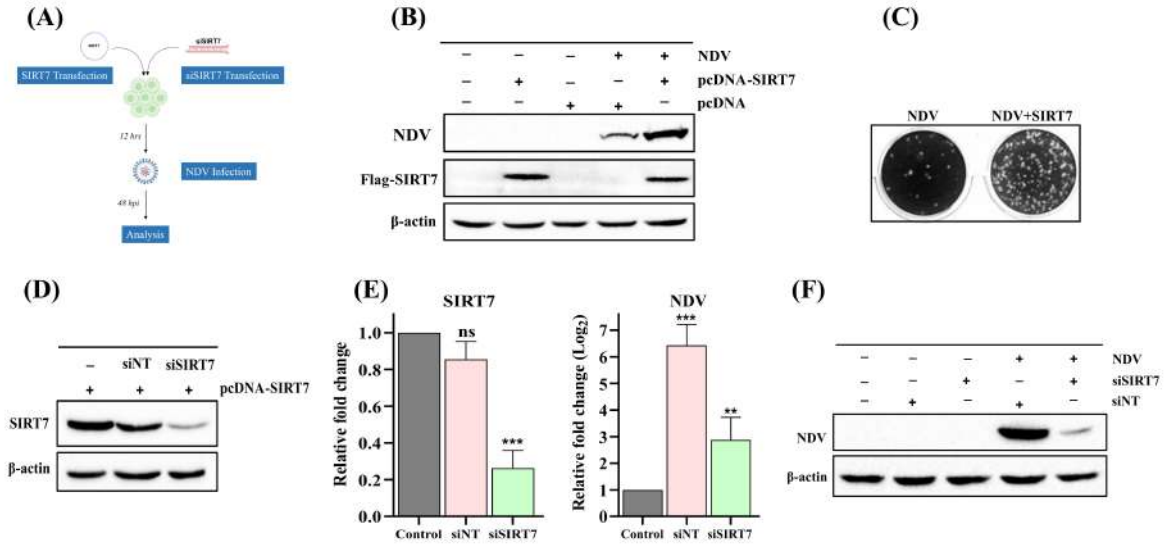


Figure 4.3: Function of SIRT7 in NDV replication. The workflow of the experiment designed for SIRT7 overexpression and silencing before NDV infection **(A)**. Immunoblot shows the production of NDV protein along with SIRT7 after over-expressing SIRT7 **(B)**. Assessment of NDV titer in SIRT7-overexpressed cells using plaque assay **(C)**. Immunoblot shows the expression of SIRT7 protein post siNT and siSIRT7 transfection in SIRT7 overexpressed cells **(D)**. Graph shows the relative fold change in SIRT7 mRNA post siNT and siSIRT7 transfection **(E)**. Graph shows the relative fold change in NDV N mRNA post siNT and siSIRT7 transfection **(E)**. Immunoblot shows the production of NDV protein along with β-actin in SIRT7-depleted cells **(F)**.

reverted to normal conditions in the case of inactivated NDV (Fig. 4.6B). Furthermore, the deacetylation activity was tested at 48 hrs in SIRT7-overexpressed cells. From the immunoblot analyses, excess SIRT7 in cells showed a similar trend in acetylation level compared to NDV-infected groups (Fig. 4.6C). Similarly, acetylated protein levels were also detected in SIRT7-overexpressing and NDV-infected cells. The results displayed that the cells subjected to both NDV infection and SIRT7 overexpression exhibited a more pronounced diminution in acetylated protein levels when compared to cells infected with NDV alone (Fig. 4.6D). Lastly, to further validate the current findings, β-NMN, a known SIRT7 activity enhancer, was utilized to monitor the acetylated protein levels. The addition of β-NMN in cells showed lower levels in corroboration with initial results (Fig. 4.6E).

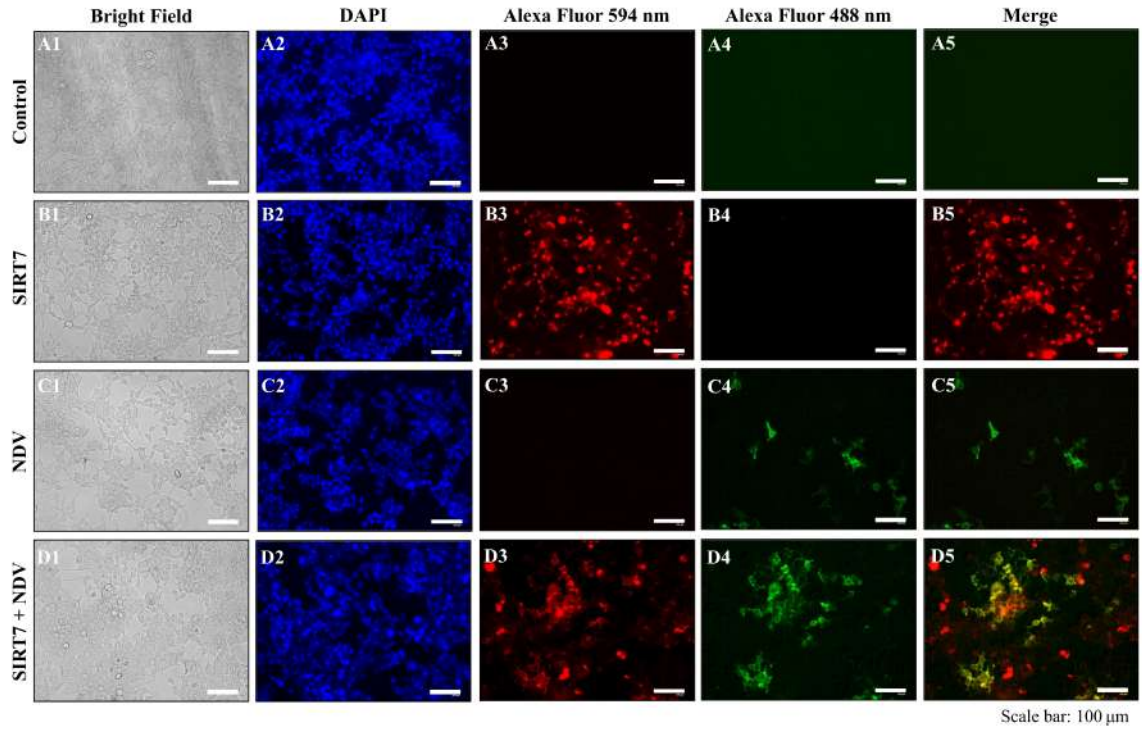


Figure 4.4: Immunofluorescence images of cells showing exogenous SIRT7 expression and NDV infection. Immunofluorescence staining of SIRT7, red fluorescence indicates positive exogenous expression; blue fluorescence indicates DAPI stained nuclei; and green fluorescence shows NDV HN expression. Representative images represent control cells **(A)**, SIRT7-transfected cells **(B)**, NDV infected cells **(C)**, and SIRT-transfected NDV-infected cells **(D)** stained with anti-Flag (SIRT7) (red) and anti-HN (green) antibody. Scale bar 100 μm.

4.2.4 *In vitro* and *ovo* SIRT7 activity assessment

To get a clear picture of the exact function SIRT7 plays in NDV replication, DF-1 cells were pre-incubated with β-NMN for 12 hrs before infection, which enhanced the SIRT7 activity. The immunoblot images showed that β-NMN treated cells exhibit higher viral protein production (Fig. 4.7A). On the contrary, the post-treatment of NAM, SIRT7 activity feedback inhibitor, was done 12 hrs after infection. The results demonstrated the virus-inhibitory effects of NAM in NDV-infected cells, with a notable decrease in viral proteins (Fig. 4.7A). The effects of the compounds were further validated by visualizing the number of cells expressing the rNDV-encoded GFP. As expected, cells with β-NMN post-treatment exhibit relatively more GFP fluorescence. Contrastingly, DF-1 cells with NAM pre-treatment showed reduced fluorescence intensity, representing the rNDV-GFP replication proportion in respective groups (Fig. 4.7B). To quantify the GFP expressed by

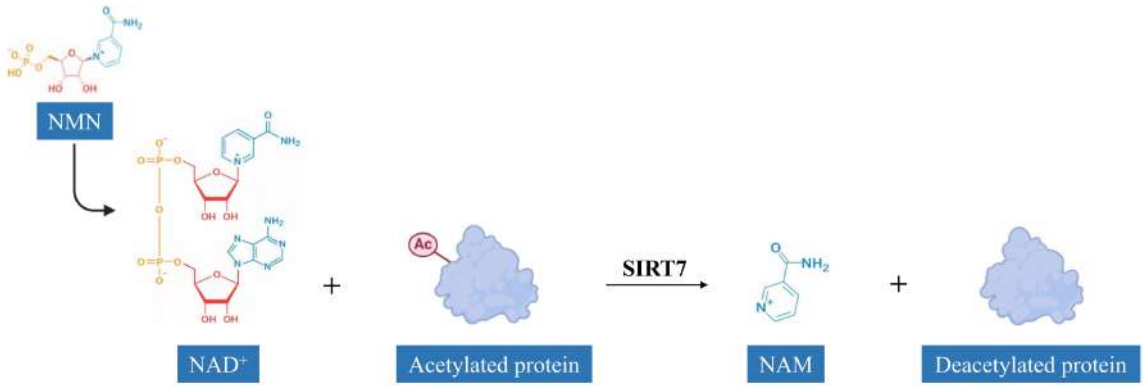


Figure 4.5: Deacetylation reaction of proteins mediated by SIRT7.

rNDV, infected cells were analyzed for GFP intensity by flow cytometry. Expectedly, the GFP-positive cells were more frequent in β-NMN post-treated infected cells (49.78%) but were rare in NAM pre-treated cells (23.99%) than in rNDV control (41.50%), confirming the participation of these compounds in viral pathogenesis quantitatively (Fig. 4.7C).

Considering a probable correlation between the NDV infection and SIRT7 activity, similar treatment studies were performed in *in ovo* system where 9-day-old embryonated eggs were pre- and post-treated with the respective compounds. NDV-inoculated chicken embryos without treatment were characterized by hemorrhages on the embryo body. The embryos with β-NMN pre-treatment and NDV inoculation died in 48 hrs and were found completely liquified, as shown in Fig. 4.7D; however, the embryos with β-NMN treatment alone did not exhibit any discernible hemorrhages on the body. NAM post-treated NDV-inoculated embryos showed minimal hemorrhages. The virus titer in the allantoic fluid collected from the embryos was also determined by standard plaque assay. The NDV titer in the β-NMN post-treated embryos was calculated to be 3.95×10^8 PFU/ml, significantly higher than NDV control (1.95×10^8 PFU/ml). The NDV titer in NAM post-treated embryos was calculated as 0.9×10^8 PFU/ml (Fig. 4.7D).

4.2.5 Interaction study of SIRT7 and NDV M protein

Considering that NDV M protein exclusively translocate to the nucleus and is implicated in the upregulation of SIRT7, we investigated its interaction with NDV M protein using molecular docking and co-immunoprecipitation assay. The molecular docking study revealed that SIRT7 protein does not interact with NDV M protein as

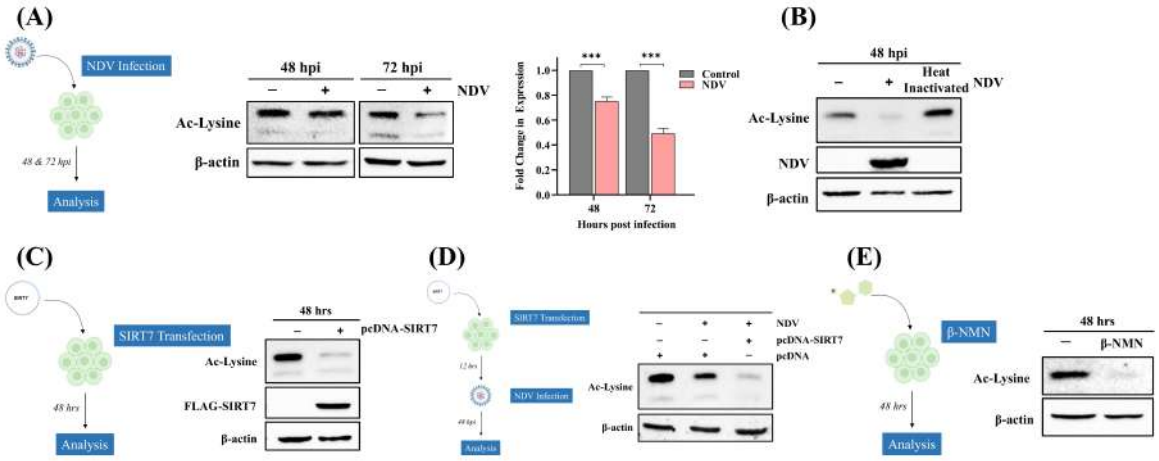


Figure 4.6: SIRT7 activity involvement in NDV infection. The workflow of the experiment designed for acetylated proteins levels evaluation. Immunoblot analysis of acetylated-lysine (representing acetylated-proteins) and β-actin in cells after 48 and 72 hrs post-NDV infection **(A)**. Graph shows the fold change in expression instigated by NDV in acetylated lysine **(A)**. Immunoblot image comparing the acetylated-lysine levels in NDV-infected cells with heat-inactivated NDV-infected cells **(B)**. The workflow of the experiment designed for acetylated proteins levels evaluation post SIRT7 overexpression **(C)**. Immunoblot image showing the effect of SIRT7 overexpression on acetylated-lysine after 48 hrs **(C)**. The workflow of the experiment designed for acetylated proteins levels evaluation in NDV infected and SIRT7-overexpressed cells **(D)**. Immunoblot image showing the levels of acetylated proteins after subjecting the cells with both NDV infection and SIRT7 overexpression **(D)**. The workflow of the experiment designed for acetylated proteins levels evaluation after β-NMN treatment **(E)**. Immunoblot image showing the activity of SIRT7 on acetylated proteins after β-NMN treatment **(E)**.

the docked model evinced only 2 salt bridges, 5 hydrogen bonds, and 235 non-bonded contacts, similar to bovine serum albumin (BSA) protein negative control. The docked models and their interactive residues list are shown in (Fig. 4.8A-E). The results were further confirmed using co-IP assay by transfecting pcDNA-M.GFP and pcDNA-SIRT7. The GFP-tagged M protein was not detected in IP obtained with an anti-FLAG antibody (Fig. 4.8F).

4.2.6 NAD^+ and NAM estimation in NDV-infected embryos

Considering the involvement of NAD^+-dependent SIRT7, the NAD^+ concentration in NDV-infected DF-1 cells was compared with control cells to study the NAD^+ metabolism dynamics. Firstly, the known concentrations of NAD^+ and NAM were injected into the HPLC column to determine the peak retention time; the chromatograms are shown in Fig. A.3A and B. The NAD^+ and NAM peaks were obtained around 16.5

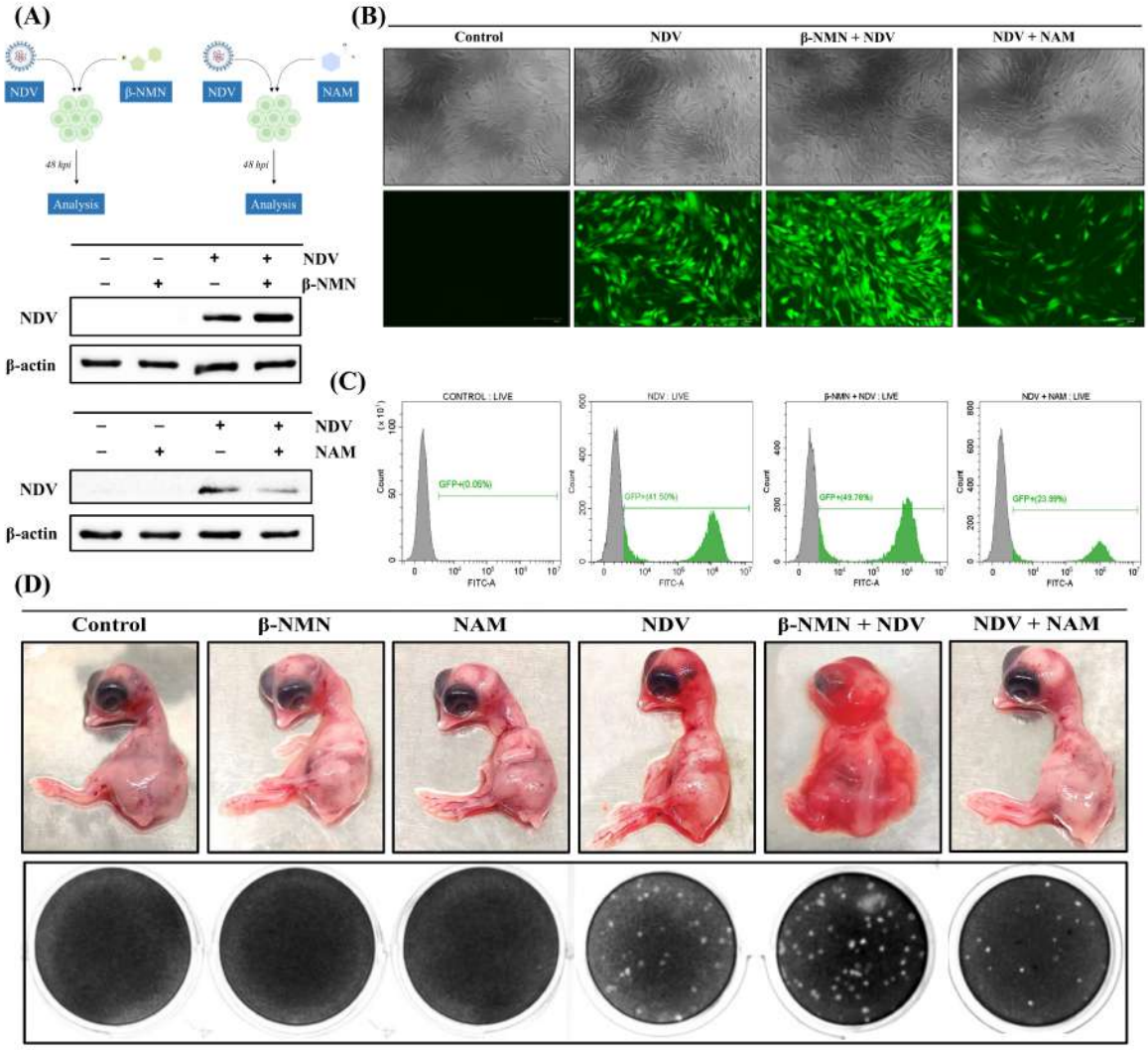
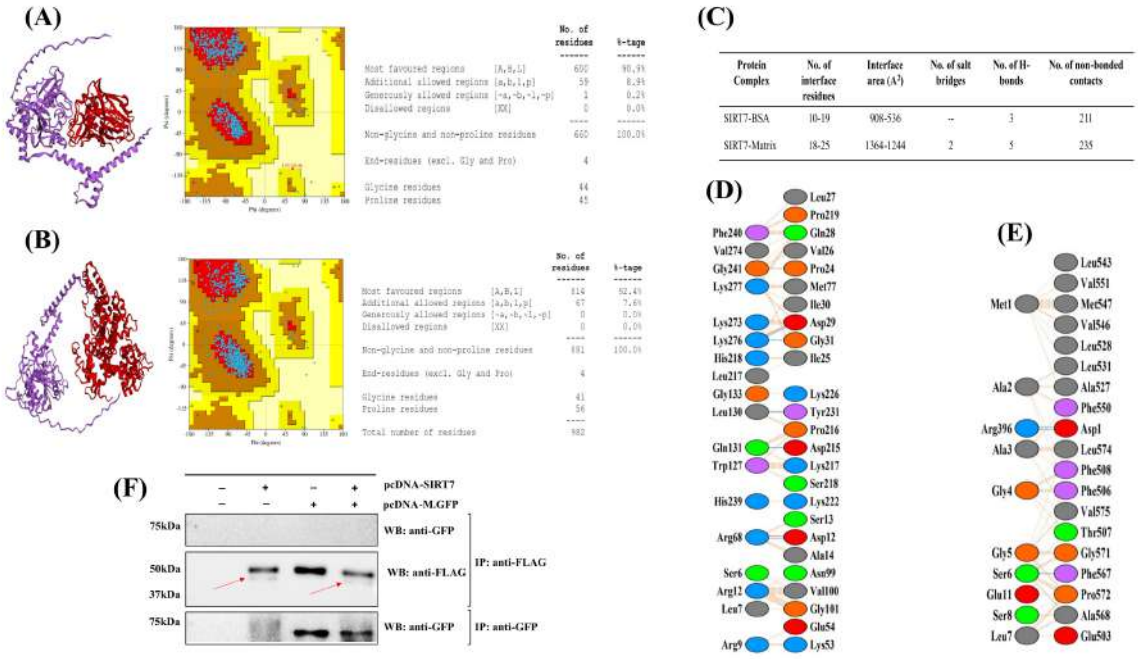


Figure 4.7: *In vitro* and *ovo* SIRT7 activity assessment. The workflow of the experiment designed for SIRT7 activity in NDV infected cells **(A)**. Immunoblot analysis of NDV and β-actin in cells with and without β-NMN treatment **(A)**. Immunoblot analysis of NDV and β-actin in cells with and without NAM treatment **(A)**. Impact of β-NMN and NAM treatment on GFP expressing rNDV using microscopy imaging and flow cytometric analysis **(B and C)**. Hemorrhages on chicken embryo's body after 48 hrs of NDV inoculation in the absence and presence of β-NMN and NAM, respectively. Quantification image of NDV titer in allantoic fluid from embryonated eggs using plaque assay **(D)**.

and 18.4 min. Next, the DF-1 cell samples were distributed in two halves, with only one half supplemented with NAD^+ to point the specific NAD^+ peak out of multiple peaks; the respective chromatograms are shown in Fig. A.3C and D. The DF-1 cells data showed a gradual decrease in the NAD^+ concentrations in infected cells, with a minimal difference of around 11% at early time intervals and reaching a 60% reduction post 72 hrs



Protein Complex	No. of interface residues	Interface area (Å²)	No. of salt bridges	No. of H-bonds	No. of non-bonded contacts
SIRT7-BSA	10-19	908-536	--	3	211
SIRT7-Matrix	18-25	1364-1244	2	5	235

Figure 4.8: Interaction study of SIRT7 and NDV M protein. The 3-D binding complex of SIRT7 (violet) and BSA (red) protein, along with the interacting residues **(A and D)**. The 3-D binding complex of SIRT7 (violet) and M (red) protein, along with the interacting residues **(B and E)**. Data representing the interface statistics of the binding complex of SIRT7-BSA and SIRT7-M **(C)**. Immunoblot of co-IP assay identifying the interaction between SIRT7 and NDV M protein **(F)**.

of infection (Fig. 4.9A). The chromatograms of uninfected and NDV-infected cells are shown in (Fig. 4.10A-F). Afterward, the results were validated by estimating the NAD^+ concentration along with NAM in NDV-infected embryo tissues. *In ovo* study, results showed 20% lower NAD^+ levels in the tissues, whereas NAM levels were estimated to be 130% compared to control embryos (Fig. 4.9B and C).

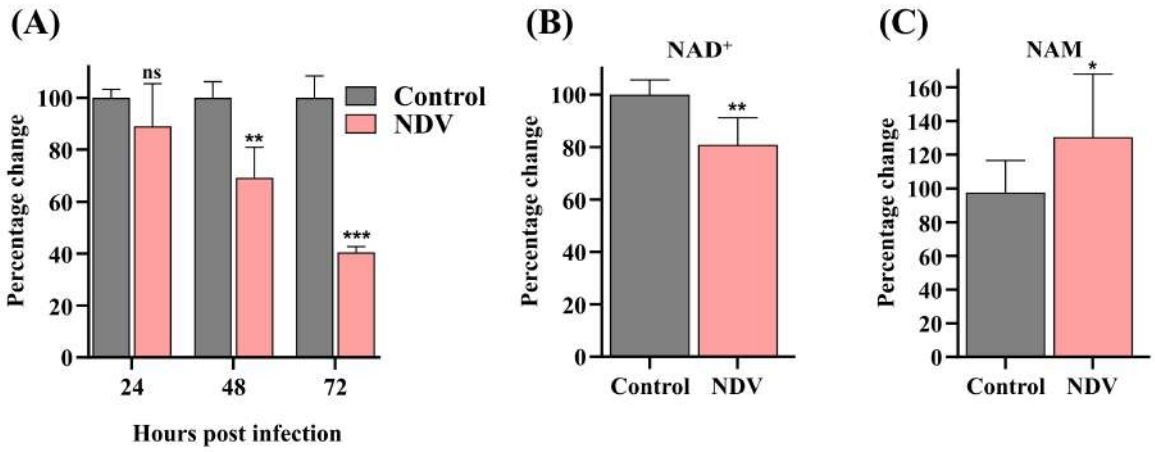


Figure 4.9: NAD$^+$ and NAM estimation in NDV-infected cells and embryo tissues. Graph representing the concentration variation of NAD$^+$ in control and NDV-infected cells at different time points **(A)**. Graph showing the variation in NAD$^+$ concentration in NDV-infected embryo tissues **(B)**. Graph showing the variation in NAM concentration in NDV-infected embryo tissues **(C)**.

4.3 Discussion

SIRT7 is an NAD$^+$-dependent deacetylase located in the nucleus that regulates gene expression under oxidative stress conditions [175, 418, 455]. In this study, we observed that it is substantially upregulated in NDV-infected cells post 48 hrs of infection, complimenting the transcriptional profile results. However, there is a decrease in the protein levels at 72 hrs, which could also be observed at the mRNA level. We suspect NDV utilizes SIRT7 protein in the initial phases to halt the host defense system. However, as the infection progresses, NDV no longer needs to restrain host defense actively, resulting in reduced SIRT7 expression. Additionally, there is an imbalance in the protein homeostasis network due to extensive viral protein production. As a countermeasure, the cells have been observed to activate several proteolytic pathways, degrading the excess proteins. While considering the influence of SIRT7 on NDV protein production, we also found the viral titer to be significantly higher in SIRT7-overexpressed cells. Furthermore, on overexpressing the NDV M protein in cells, we observed a significant increase in the SIRT1 and SIRT7 protein levels. However, the observed increase was not comparable to the NDV infection as the moderate levels of NDV M protein produced exogenously failed to match the stress stimulation and substantial presence of viral proteins during the infection. DMF and PDTC treatments showed the association of SIRT7 with oxidative

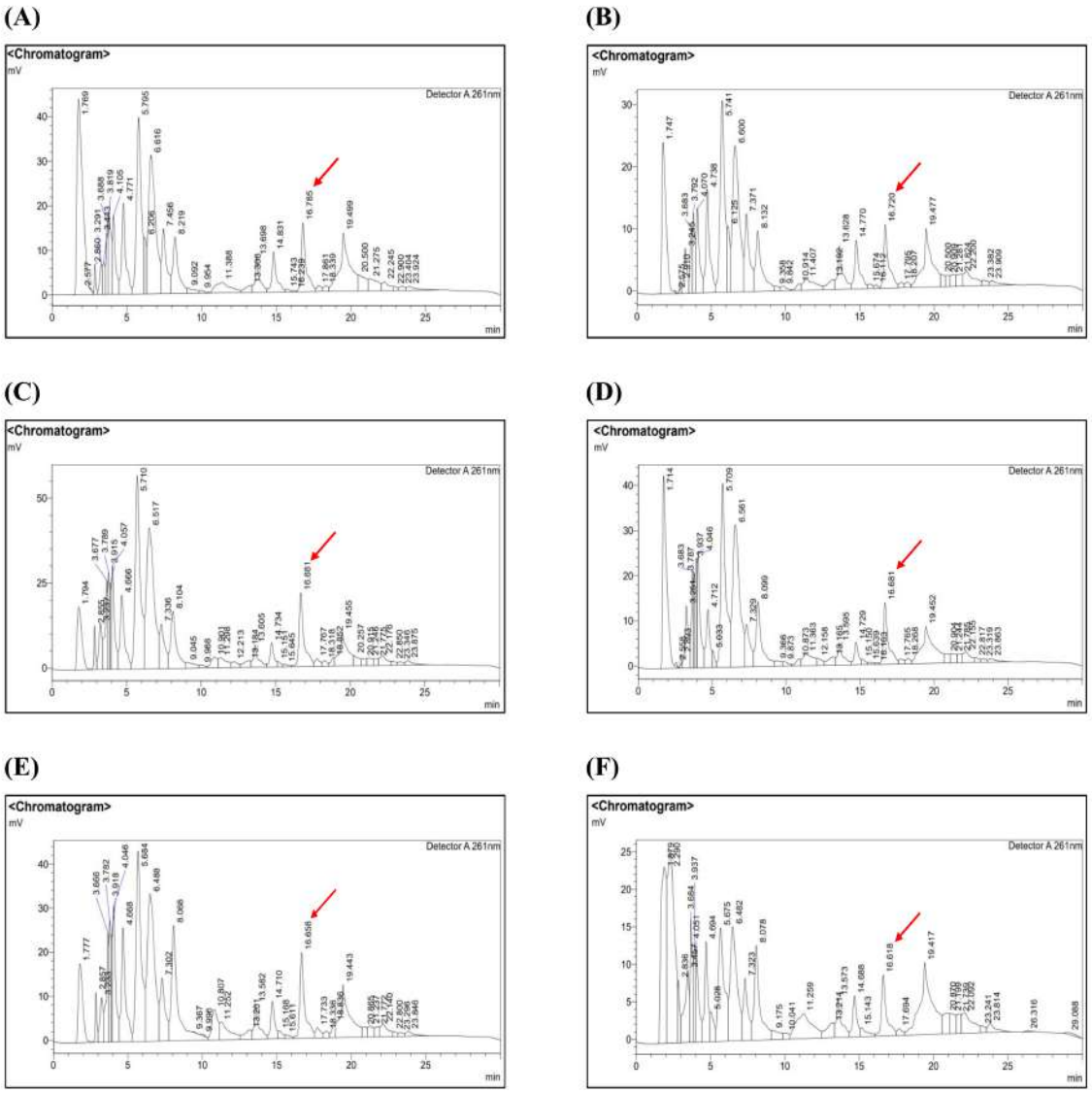


Figure 4.10: HPLC chromatograms of NAD⁺ Chromatograms obtained from control and NDV-infected DF-1 cell samples, respectively at 24 **(A and B)**, 48 **(C and D)**, and 72 **(E and F)** hrs, highlighting the NAD^+ peaks.

stress response; therefore, it is possible that expression enhancements shown here may be directed by NDV-induced stress response to control its activity. Generally, the antiviral proteins are responsible for onsetting the antiviral response to perturb the infection. SIRT7 induced during virus infection may halt the progression of the antiviral response, enabling the virus to replicate effectively. NDV infection in SIRT7-overexpressed cells manifested increased viral protein production, and SIRT7-depleted cells exhibited lower viral protein production in the control groups, thus confirming the hypothesis. These

results collectively conveyed that SIRT7 expressed in cells due to ROS accumulation has a substantial role in NDV replication, and the ROS scavenging consequentially decreased the virus yield. The subsequent experiments addressed the deacetylating activity of SIRT7 on cellular proteins.

Posttranslational modifications regulate the cellular proteome by prompting structural changes in proteins, thus affecting their functionality [251]. Histone modifications, mainly acetylation and deacetylation, function as a specific transcription regulator governing cellular response [14]. Adding and removing acetyl functional groups to lysine residues can change the chromatin architecture and affect the chromatin's open-and-closed state to regulate gene expression [152, 154, 388]. SIRTs, well-characterized class III HDACs that depend on NAD^+, can also influence multiple proteins other than histones [26, 204]. In the present study, SIRT7-influenced levels of acetylated proteins were determined in NDV-infected cells. Utilizing the anti-ac-lysine antibody, we targeted the detection of acetylated-lysine residues on proteins. While our initial anticipation involved observing a smear across the spectrum, our results from infected DF-1 cells showed only distinct bands primarily positioned around 20 kDa, presumably correspond to histone proteins, which constitute a substantial proportion of the acetylated proteins within the cells. The temporal study reveals the variations in the global acetylation profile due to NDV infection, which were further validated with SIRT7 overexpression showing complete abrogation. Correspondingly, the heat-inactivation experiment suggests that the observed changes in acetylation levels were indeed affected by NDV replication in DF-1 cells. These observations of differential deacetylated proteins in infected cells provided information about NDV's induced impediment of host protein production to adjourn the cellular defense mechanisms. The data we are reporting herein unveil the importance of SIRT7-driven deacetylation for viral pathogenesis.

Furthermore, mechanistic studies were also performed with SIRT7 activity modulators. NAD^+-consuming enzymes, including SIRT and poly-ADP-ribose polymerase (PARP) protein families, could metabolize NAD^+ to produce 2'-O-Acetyl-ADP-ribose and NAM [23, 57] (Fig. 4.5). NAD^+ being a rate-limiting co-substrate for SIRT enzymes, its restoration and scarcity could help control their deacetylation activity [61, 452]. Predominantly, NAD^+ is synthesized through NMN [36, 329]; thus, promoting NAD^+ biosynthesis by NMN administration can effectively enhance SIRT7 activity [447], while NAM treatment can suppress the activity by compromising NAD^+ levels via feedback inhibition [40]. However, β-NMN and NAM could also modulate the activity of other

SIRT proteins. In our study, we only focused on exploring the role of SIRT7 in NDV pathogenesis, as it exhibited the most pronounced modulation. Given that these compounds will also influence the activity of other SIRTs within the cell, however, in the conditions where SIRT7 expression is most prominent, its activity will undergo the most significant regulation. Moreover, there are numerous studies in which researchers have been using these compounds to specifically address SIRT7-related biology [447, 474]. The β-NMN supplementation exhibited reduced acetylated proteins, which implied that NAD^+-boosted concentrations induce SIRT7 activity. Moreover, we found that the NDV protein production was significantly increased when we treated the cells with β-NMN before infection. In contrast, NAM treatment negatively affects virulence, suggesting that NAD^+ biosynthesis can control the SIRT7-mediated deacetylation of cellular proteins during the infection. These findings were further supported by the *in ovo* study, which aligns with those of cell culture results. Altogether, these observations suggest that the β-NMN supplementation not only restores normal NAD^+ levels but also increases the virulence of NDV in the host. As could be seen from the results, NDV infection could overall lead to high SIRT7 activity, which suggests that NAD^+ concentration may deplete post-infection due to an increased demand for deacetylating host proteins. The concentration of NAD^+ was determined using an HPLC system to evaluate this possibility. This method is based on the UV-Vis absorbance detection method, providing accurate, reliable, and reproducible results of NAD^+ measurement, which enables us to analyze various pathophysiological changes in NAD^+ levels *in vitro* and *vivo* [451]. The depletion of NAD^+ levels with time in infected cells clearly supports the presumptions made and emphasizes the importance of NAD^+ decrease as a common trigger of virus-associated cytopathic effects. Consistent with these findings, embryo tissues also provide compelling support for NAD^+ scarcity while showing boosted levels of NAM, which might result from SIRT7-driven NAD^+ metabolism to deacetylate the proteins and generate NAM as a by-product. Although numerous cellular pathways depend on NAD^+ metabolism, it has been previously reported that SIRT proteins are responsible for about one-third of the total NAD^+ consumption in cells [13, 95]. However, the ratio is expected to change upon its overexpression. The release of NAM is closely correlated to the deacetylation reaction, as it is exclusively released within the cell during the utilization of NAD^+ for this specific reaction. Moreover, if the NAM concentration does not increase, it implies that other cellular processes, independent of SIRT7-mediated response, are utilizing NAD^+. Our results showed an increase in the concentration of NAM; hence, we concluded

the probable utilization of NAD^+ by SIRT proteins in infected cells, which is in line with our hypothesis.

4.4 Conclusion

Taken together, the data obtained in the study suggest NDV-induced oxidative stress drives the production of SIRT7 in DF-1 cells, leading to the deacetylation of host proteins. Using SIRT7-mediated deacetylation could be a crucial underlying strategy NDV employs to thrive in its host. The notable result of our study confirms that SIRT7, once activated in DF-1 cells due to mitochondrial damage and ROS accumulation, supports virus infection. Our findings suggest that while the SIRT7 involvement in the oxidative stress response may not represent the primary pathway, it certainly plays a vital role in the NDV pathogenesis. It helped us better understand the virus's strategies to generate its numerous copies while perturbing the host cell's standard functionality and opening up new possibilities for infection interventions. Lastly, evaluating the drugs and molecules that can modulate SIRT activity will be essential in developing an effective antiviral strategy to control the disease.

CHAPTER 5

ACTIVATION OF ATE1 ENZYME BY NDV INFECTION

An article based on this chapter is published as follows:

Arginyltransferase 1 (ATE1)-mediated proteasomal degradation of viral haemagglutinin protein: A unique host defense mechanism.
Shokeen K, Baroi MK, Chahar M, Das D, Saini HM, Kumar S. **J Gen Virol.** (2024)

Abstract

The extensive protein production in virus-infected cells can disrupt protein homeostasis and activate various proteolytic pathways. These pathways utilize PTMs to drive the ubiquitin-mediated proteasomal degradation of surplus proteins. Protein arginylation is the least explored PTM facilitated by ATE1 enzyme. Several studies have provided evidence supporting its importance in multiple physiological processes, including aging, stress, nerve regeneration, actin formation, and embryo development. However, its function in viral pathogenesis is still unexplored. The present work utilizes NDV as a model to establish the role of the ATE1 enzyme and its activity in pathogenesis. Our data indicate a rise in the levels of N-arginylated cellular proteins in the infected cells. Here, we also explore the HN protein of NDV as a presumable target for arginylation. The data indicates that the administration of Arg amplifies the arginylation process, resulting in reduced stability of the HN protein. ATE1 enzyme activity inhibition and

gene expression knockdown studies were also conducted to analyze modulation in the HN protein levels, which further substantiated the findings. Moreover, we also observed the ubiquitin addition to the HN protein, indicating the engagement of proteasomal degradation machinery. Lastly, we concluded that the enhanced levels of the ATE1 enzyme could transfer the Arg residue to the N-terminal of the HN protein, ultimately driving its proteasomal degradation.

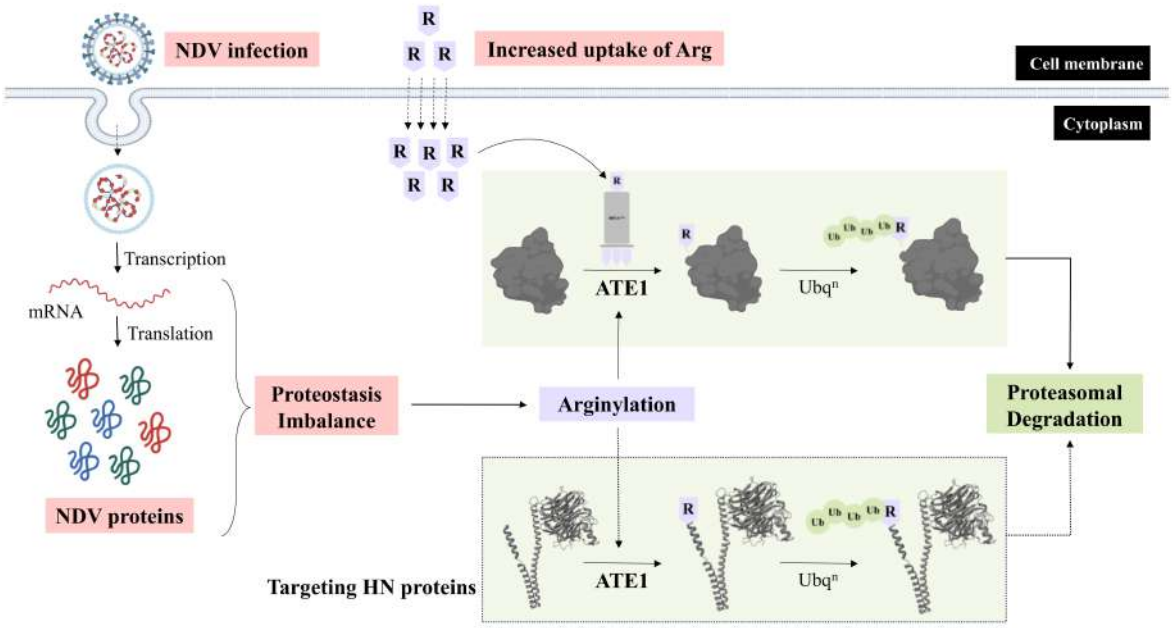


Figure 5.1: Graphical abstract representing the ATE1-mediated arginylation of proteins.

5.1 Introduction

Healthy cells have a complex network of molecular mechanisms that collectively maintain proteome integrity or protein homeostasis [168]. This network continuously monitors the protein life cycle, from synthesis to degradation, for any impairment [167, 427]. Multiple viruses, including influenza A, cytomegalovirus, and SARS-CoV-2, have been shown to disrupt the cellular proteostasis [42, 35, 267]. The activation of proteolytic pathways universally controls the responses of cells to the imbalance caused by the viruses, ensuring the selective degradation of the targeted proteins majorly through the N-degron pathway [102, 118]. The HIV-1 integrase protein has been demonstrated to be a physiological substrate of the N-end rule pathway [291]. In contrast, suppression

of the N-degron pathway during picorna-like virus has also been reported [438]. These findings suggest that the exact part of these proteolytic pathways in viral pathogenesis remains elusive.

The degradation of excess proteins is majorly mediated by UPS [130, 165]. For UPS-mediated degradation, the target proteins must be linked with ubiquitin, which in turn is recognized by the proteasome [298]. This PTM to the target proteins can act as a destabilizing signal, leading to their proteolytic degradation [21, 298]. One less-known regulatory modification is arginylation [194, 195], in which the targeted proteins are added with Arg residue from tRNA either at the N-terminal or side chains having Asp and Glu [25, 173]. The Arg transfer is mediated by the ATE1 enzyme [86]. Firstly, the target proteins are prepared for degradation through an endoproteolytic cleavage of the first residue, methionine, by Met-aminopeptidases [45]. It is then recognized and further bound by the N-recognin family proteins, mediating ubiquitination and proteasome degradation [423, 433]. Several studies have demonstrated the critical role of arginylation in protein degradation, autophagy, and cell migration [29, 187, 382]. The physiological significance of arginylation has also been shown in aging, cardiovascular development, and embryonic development [228, 335, 228]. Recently, SARS-CoV-2-mediated arginylation has unveiled its plausible relationship with viral pathogenesis [259]. Much of the current knowledge about arginylation is related to intracellular metabolic pathways and biological processes; however, its regulation in infectious diseases, especially viruses, remains to be established.

NDV is a highly contagious avian pathogen that causes grievous disease with extreme morbidity and mortality worldwide [142, 414]. The genome encodes six structural proteins: the nucleocapsid (N), the phosphoprotein (P), the matrix (M), the fusion (F), the hemagglutinin-neuraminidase (HN), and the large polymerase (L) [6]. The M and HN proteins have Asp residue in the second position of their N-terminus, suggesting the possibility of exploring them as substrates for arginylation-mediated degradation. Other NDV proteins, such as N, lack the such residues at this position, rendering them suitable negative controls for the study.

The present chapter aims to investigate the impact of viral infection on the activation of the ATE1 enzyme and its subsequent activity using NDV as a model. The research aims to get insight into the crosstalk of arginylation and viral pathogenesis. Additionally, ATE1-mediated viral protein degradation was also studied to decipher the underlying molecular event.

5.2 Results

5.2.1 Function of ATE1 in NDV replication

The expression pattern of the *ATE1* gene in NDV-infected cells at 48 and 72 hrs was determined and compared to mock-infected cells (Fig. 5.2A). Firstly, the relative fold change and protein production of the *NDV N* gene was examined to understand the dynamics of viral replication (Fig. 5.2A and B). The *ATE1* mRNA expression was increased from 1.0 to 1.8-fold and 2.0-fold after 48 and 72 hrs of NDV infection, respectively (Fig. 5.2B). ATE1 protein modulation upon NDV infection and DMF treatment was also studied temporally. A progressive increase in the ATE1 expression was observed in both groups at 48 and 72 hrs (Fig. 5.2B). Flow cytometric analysis further confirmed the overexpression, where more than a 200% increase in ATE1 levels was noted (Fig. 5.2C). The expressions of proteins involved in oxidative stress, SIRT7, Hsp90β, and SQSTM1, were also examined in ATE1 overexpressing cells. The immunoblot showed that ATE1 overexpression exhibited the degradation of these stress factors (Fig. 5.2D and A.4G). The activity of the ATE1 enzyme was inhibited by TA treatment, which resulted in the accumulation of these stress factors (Fig. 5.2E and A.4H).

Next, we sought to determine if exogenously expressed ATE1 influences NDV protein production. The immunoblot showed that the NDV HN and N protein levels were considerably lower in ATE1 overexpressing cells than in the controls (Fig. 5.3A). The plasmid expressing NDV N protein was also transfected both with and without ATE1 overexpression, and the results indicated that ATE1 activity did not impact the levels of N protein (Fig. 5.3B). Additionally, ATE1 overexpressing cells were depleted with ATE1 through siRNA transfection. The mRNA levels showed an almost 95% reduction of ATE1 relative to the siNT control cells, and immunoblot also exhibited significant silencing (Fig. 5.3D). To further validate the role of the ATE1 enzyme in NDV infection, siATE1 transfected cells were infected with NDV (Fig. 5.3C). The results indicated NDV HN levels were significantly higher, while N protein levels showed a minimal difference in ATE1-depleted cells (Fig. 5.3E). Cells were pre- and post-treated with TA to inhibit the activity of the ATE1 enzyme. The addition of TA, irrespective of the time, showed a substantial increment in HN protein levels, while N protein was found to be similar in all groups (Fig. 5.3F). However, the mRNA levels of HN and N genes remained unchanged after the TA treatment (Fig. 5.3F). NDV titer in the respective groups was

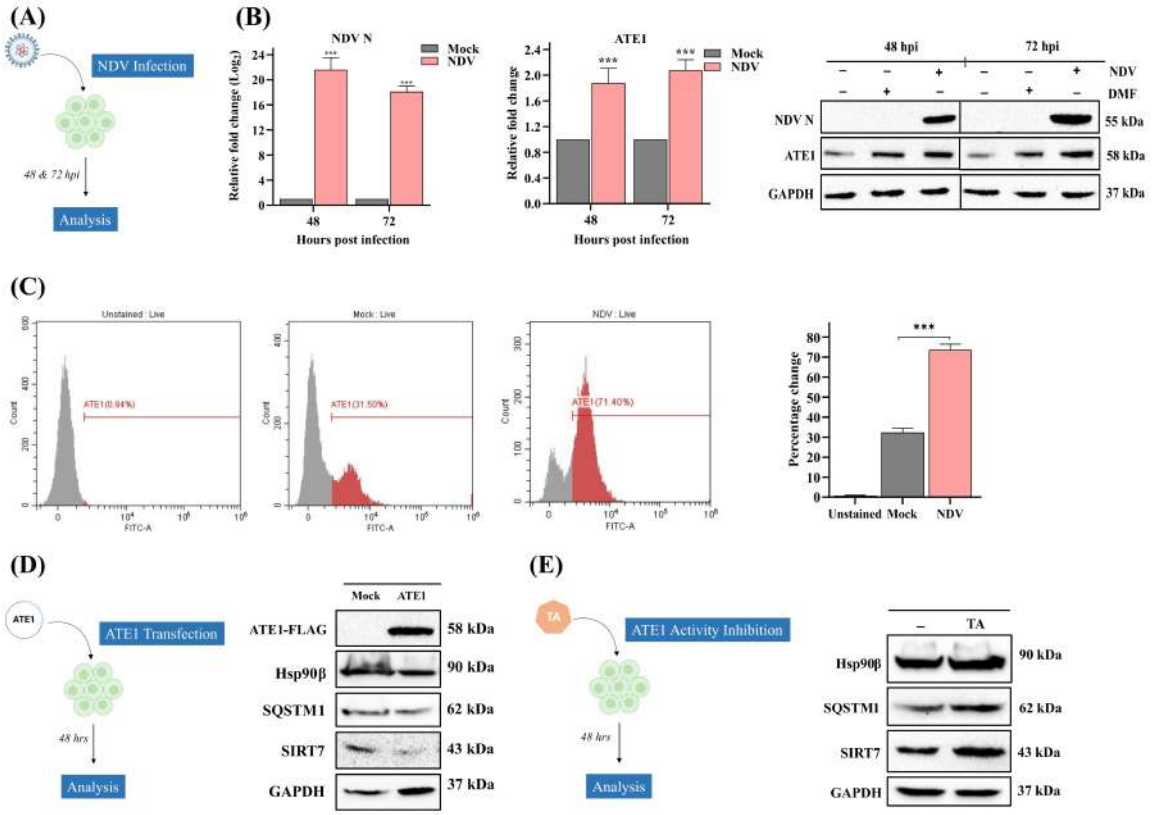


Figure 5.2: Modulation of ATE1 post-NDV infection. The workflow of the experiment designed for ATE1 expression evaluation **(A)**. The graph shows the relative fold change of *ATE1* and *NDV N* gene mRNA in DF-1 cells after 48 and 72 hrs post-infection. Immunoblot analysis of ATE1 and NDV N after 48 and 72 hrs post-NDV infection and DMF treatment **(B)**. Percentage change in ATE1 expression in mock and NDV-infected cells by flow cytometric analysis **(C)**. Immunoblot analysis of SIRT7, Hsp90β, and SQSTM1 post ATE1-overexpression **(D)**. Immunoblot analysis of SIRT7, Hsp90β, and SQSTM1 post-TA treatment **(E)**.

also determined by standard plaque assay. The titers in pre- and post-TA treated cells were calculated to be 0.99x10^5 and 0.95x10^5 PFU/ml, respectively, compared to NDV control (0.26x10^5 PFU/ml) (Fig. 5.3G and A.4A).

5.2.2 Interaction of ATE1 and HN

The three-dimensional model of chicken ATE1 protein was generated. The model considered for the study showed 86.7% residues in most favored regions, 12.1% in additional allowed regions, 0.7% in generously allowed regions, and only 0.5% in the disallowed areas (Fig. 5.4A). Similarly, modeled full-length NDV HN showed 88.8% residues in most favored regions, 10.8% in additional allowed regions, and 0.4% in

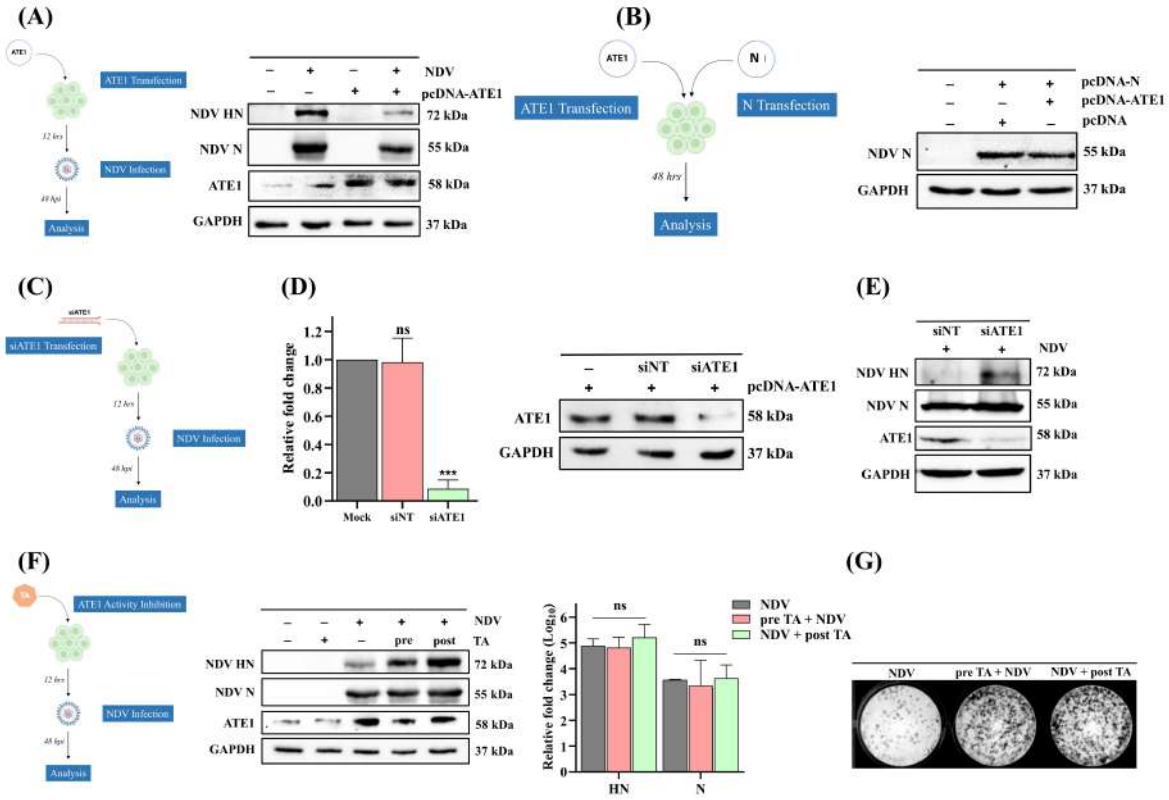


Figure 5.3: Function of ATE1 in NDV replication. The workflow of the experiment designed for ATE1 overexpression before NDV infection **(A)**. Immunoblot shows the production of NDV HN and N protein along with ATE1 after overexpressing ATE1 protein by transfection. Immunoblot showing the expression of NDV N protein with and without ATE1 overexpression **(B)**. The workflow of the experiment designed for ATE1 silencing before NDV infection **(C)**. The graph shows the relative fold change in ATE1 mRNA post siNT and siATE1 transfection in ATE1 overexpressed cells **(D)**. Immunoblot shows the expression of ATE1 protein post siNT and siATE1 transfection in ATE1 overexpressed cells **(D)**. Immunoblot shows the production of NDV HN and N protein in ATE1-depleted cells **(E)**. Immunoblot shows the production of NDV HN and N protein along with ATE1 after pre-and post-treatment with TA **(F)**. The graph shows the relative fold change in *NDV HN* and *N* gene mRNA after pre-and post-treatment with TA **(F)**. Assessment of NDV titer in TA-treated cells using plaque assay **(G)**.

generously allowed regions (Fig. 5.4B). The final models were further used for molecular docking. ATE1 protein was docked with β-actin and ubiquitin as positive controls and GAPDH and NDV N as negative controls. Subsequently, ATE1 interaction was also tested with HN protein. The ATE1-β-actin complex yielded 4 salt bridges, 21 hydrogen bonds, and 203 non-bonded contacts (Fig. 5.4C), while the ATE1-ubiquitin complex displayed 5 salt bridges, 16 hydrogen bonds, and 171 non-bonded contacts (Fig. 5.4C). The ATE1-GAPDH complex displayed only 1 salt bridge, 4 hydrogen bonds, and 113 non-bonded

contacts (Fig. 5.4C), whereas the ATE1-N complex had 1 salt bridge, 15 hydrogen bonds, and 176 non-bonded contacts (Fig. 5.4C). Subsequently, ATE1 interacted with NDV HN with 4 salt bridges, 13 hydrogen bonds, and 173 non-bonded contacts (Fig. 5.4C). All the interacting residues for every model are mentioned in Table 5.1, and common resides among different groups were also highlighted. Interestingly, the docked complexes of ATE1 with β-actin, ubiquitin, and HN yielded the same four binding regions, i.e., 80-85, 303-308, 440-443, and 482-484 amino acids, thus creating a single binding pocket (Fig. 5.5A). However, ATE1 complexes with GAPDH and N did not present the binding pocket with positive controls. The hydrogen bonds between the ATE1 binding pocket residues and HN protein residues were also outlined, and the average distance between these residues was determined to be less than 3.2 Å(Fig. 5.5A).

Table 5.1: Data representing the amino acid residues of ATE1 protein involved in interacting with different proteins.

Protein	Residues
β-actin	**A80, L81, F83, Q84, T85**, S86, K87, K94, **Y303, G304, F306**, I324, L325, P326, Y327, C328, V329, S330, **L440, K442, R443**.
Ubiquitin	V43, **A80, L81, R82, F83, Q84**, T85, K90, **G304, F306, H307, Q308**, I322, D323, I324, L325, P326, Y327, C328, **H441, K442, R443**, R455, **F482, R483, S484**.
NDV HN	V43, Q44, Y46, Q47, D48, D51, **L81, R82, Q84**, D287, S288, P289, E291, N297, P299, C301, G302, **G304, S305, F306, H307, Q308**, R438, V439, **L440, H441, K442, R443**, R455, **F482, S484**.
GAPDH	Y303, D323, I324, L325, P326, Y327, C328, V329, E353, F356, Q359, L360, F369, Y370, Y371, M372, K381.
NDV N	K217, K221, D287, N297, E300, C301, G302, Y303, **G304**, D323, I324, L325, V329, R352, E353, F356, Q359, L360, K363, D366, L367, C368, F369, Y370, Y371, M372, K381, R383, Y384.

Moreover, we performed MD simulations of ATE1-β-actin, ATE1-GAPDH, and ATE1-HN complexes for 100 ns timescale to validate their interaction by assessing their stability and binding strength. Parameters, including RMSD, RMSF, SASA, and Rg, were studied to serve the purpose. Firstly, RMSD plots were analyzed to study the structural stability of the bound complex. The ATE1-β-actin complex showed a slight increase in RMSD value from 0.3 to 0.7 nm and then started stabilizing after 5 ns and

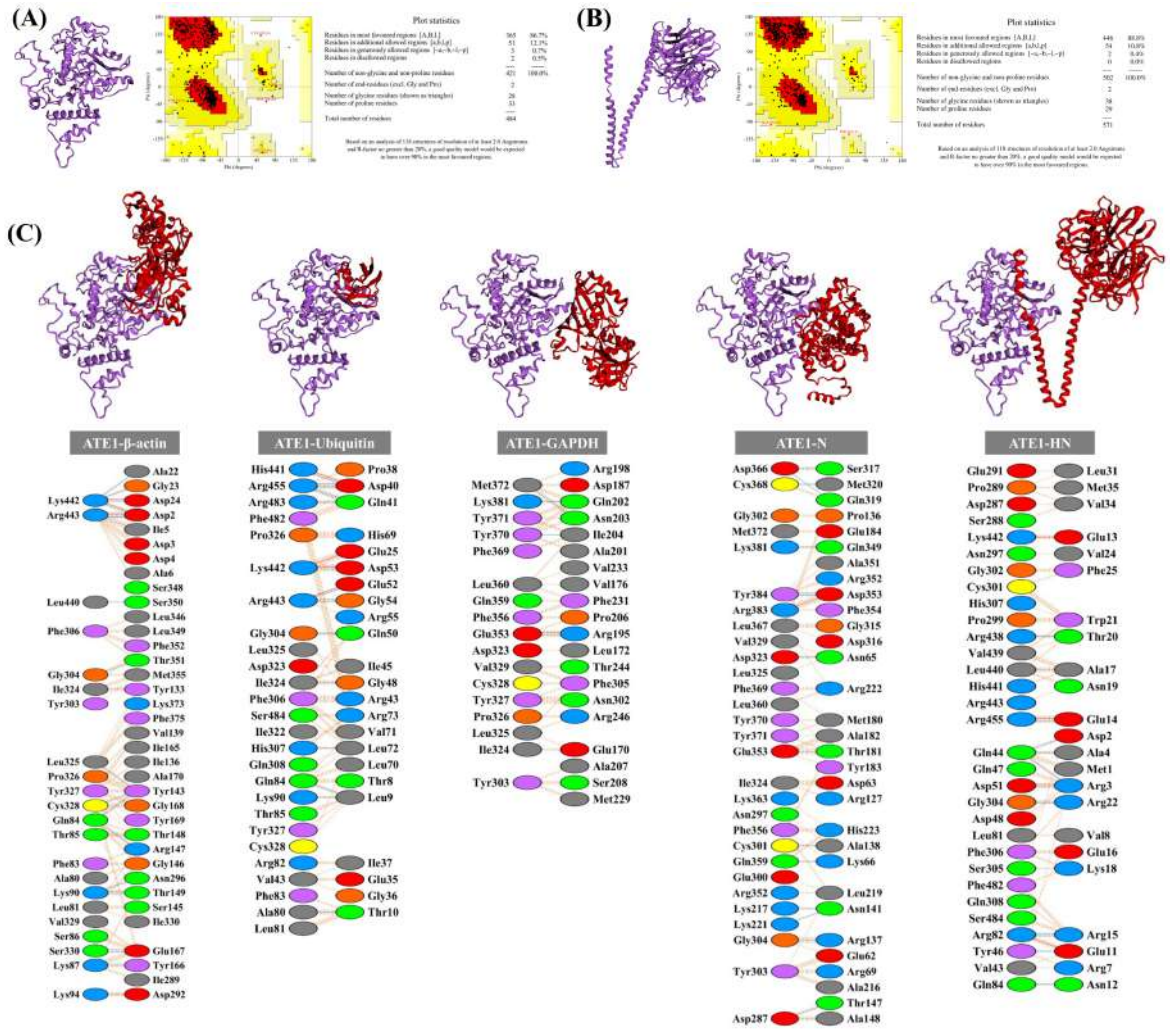
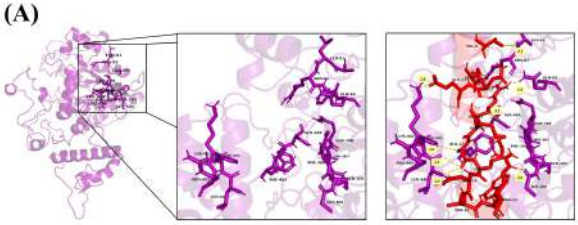


Figure 5.4: Molecular docking of ATE1 and HN protein. The 3-D models of ATE1 and HN protein with its analyzed Ramachandran plot and plot statistics **(A)(B)**. 3D-binding complex of ATE1 (violet) with β-actin (red), ubiquitin (red), GAPDH (red), N (red) and HN (red) **(C)**. The interacting residues of ATE1-β-actin, ATE1-ubiquitin, ATE1-GAPDH, ATE1-N, and ATE1-HN docked complexes **(C)**. Table representing the interface statistics of the binding complex of ATE1 with β-actin, ubiquitin, GAPDH, N and HN protein **(E)**. The 3-D model of the ATE1 protein highlights the empty binding pocket, with its interaction with HN protein residues (red) and the hydrogen bonds **(D)**.

kept its equilibrium until the end with minor fluctuations (Fig. 5.6A). Similarly, the ATE1-GAPDH complex started stabilizing after 10 ns and maintained its strength until the end of the simulation (Fig. 5.6B). The ATE1-HN complex also attained steadiness at 40 ns and held it with only minor changes (Fig. 5.6C). The average RMSD values obtained for the complexes are 0.76, 0.92, and 1.47 nm, respectively. RMSF data was plotted to assess the flexibility and rigidity of the complexes. Here, we marked (red) the

(A)



(B)

Protein Complex	No. of interface residues	Interface area (A^2)	No. of salt bridges	No. of H-bonds	No. of non-bonded contacts
β-actin	22-36	1568-1310	4	21	203
Ubiquitin	26-23	1270-1168	5	16	171
NDV HN	31-23	1273-1322	4	13	173
GAPDH	17-20	1006-975	1	4	113
NDV N	29-31	1557-1522	1	15	176

Figure 5.5: Molecular docking of ATE1 and HN protein. The 3-D model of the ATE1 protein highlights the empty binding pocket, with its interaction with HN protein residues (red) and the hydrogen bonds **(A)**. Table representing the interface statistics of the binding complex of ATE1 with β-actin, ubiquitin, GAPDH, N and HN protein **(B)**.

ATE1 protein's c-alpha atoms of the interacting residues obtained from the docking study to infer the fluctuations of binding pockets. The marked residues of the ATE1-β-actin complex were observed to have lower RMSF values of around 0.2 nm (Fig. 5.6A). Similarly, the ATE1-HN complex's marked residues also exhibited similar values around 0.5 nm on the graph (Fig. 5.6C). However, the ATE1-GAPDH complex did not have identical interacting residues (Fig. 5.6B). Further analysis was conducted on the SASA values to estimate the solvent behavior of the bound complexes. The ATE1-β-actin complex gradually decreased from around 480 to 450 nm2 with minor volatility throughout the simulation (Fig. 5.6A), whereas the ATE1-GAPDH complex initially measured 450 nm2 and subsequently exhibited significant fluctuations. An increase from 430 to 450 nm2 was observed at 60 ns, followed by a decrease to approximately 430 nm2 at the end (Fig. 5.6B). Moreover, the ATE1-HN complex started with a 570 nm2 value and then steadily decreased to 520 nm2 at 100 ns (Fig. 5.6C). Lastly, we calculated Rg values to interpret the compactness and rigidity of the complexes. The ATE1-β-actin complex revealed strength stability and maintained values between 2.8 and 3.0 until the end (Fig. 5.6A). Contrarily, the ATE1-GAPDH complex showed significant volatility and did not attain considerable strength until the end, showing a steep increase and decrease in the values (Fig. 5.6B). The ATE1-HN complex exhibited minor fluctuations throughout the simulation, ranging from 3.3 to 2.7 nm (Fig. 5.6C).

To substantiate the ATE1 and HN protein interaction *in vitro*, a Co-IP assay was carried out with lysates prepared from NDV-infected and ATE1-overexpressed 293T cells using HN and ATE1 antibody-directed pull-down. The HN protein was not detected in IP obtained with an ATE1 antibody; ATE1 was not detected when pulled down with an HN antibody (Fig. A.4C).

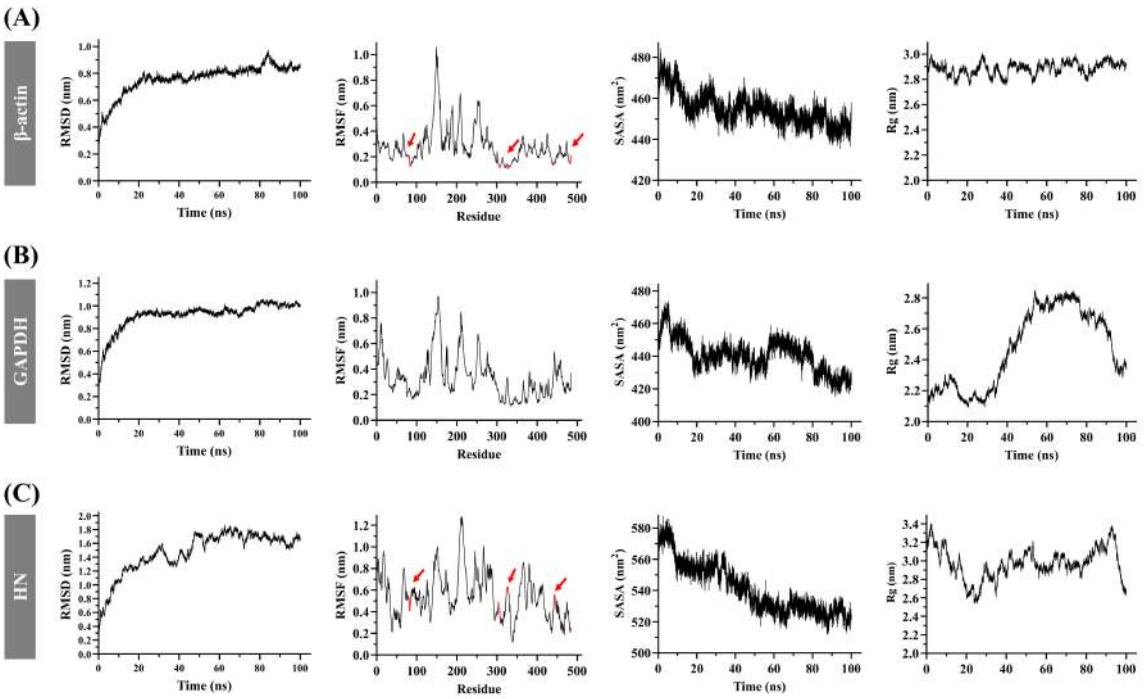


Figure 5.6: Involvement of L-arginine in NDV infection. Graph representing the concentration variation of Arg in the supernatant of NDV-infected, ATE1-overexpressed, TA-treated, and ATE1-depleted cells, compared to control cells **(A)**. The workflow of the experiment designed for Arg treatment in NDV-infected cells. Immunoblot analysis of NDV HN and N after Arg treatment. The graph shows the fold change in the expression of NDV HN and N proteins **(B)**. The workflow of the experiment designed for NDV inoculation and Arg administration in chicken embryonated eggs. Hemorrhages on chicken embryo's body after 48 hrs of NDV inoculation after Arg administration **(C)**. Quantification image of NDV titer in allantoic fluid using plaque assay **(D)**. Immunoblot analysis of NDV HN and N in allantoic fluid after Arg **(D)** treatment.

5.2.3 Involvement of Arg in NDV infection

Firstly, we quantified the Arg utilization rate of NDV-infected, ATE1-overexpressed, TA-treated, and ATE1-depleted cells and compared them with control cells. The results indicated 10% and 30% lower Arg in the infected and ATE1-overexpressed cells' supernatant, respectively. Furthermore, accumulation of Arg in the supernatant of TA-treated and ATE1-depleted cells was observed. (Fig. 5.7A). Moreover, we also administered the infected cells to varying concentrations of Arg. The immunoblot showed a drastic 60% reduction of HN protein following the treatment; conversely, the N protein levels throughout remained unaffected (Fig. 5.7B). Additionally, cells were treated with Gly, and the results yielded no variation in the HN and N protein levels (Fig. A.4B). Further, to assess the Arg-specific effects *in ovo*, chicken embryonated eggs were first inoculated with NDV

and subsequently treated with 5 mg Arg. The Arg-administered NDV-inoculated chicken embryos showed reduced hemorrhagic lesions. The NDV titer in the allantoic fluid from eggs was also quantified using plaque assay (Fig. 5.7C). The titer from Arg-treated embryos was $4.1x10^6$ PFU/ml, while the titer in NDV-inoculated embryos was calculated as $7.7x10^6$ PFU/ml. The immunoblot image results from the collected allantoic fluid further supported the initial results (Fig. 5.7D).

5.2.4 Post-translational modification of cellular and HN protein

Considering the heightened ATE1 expression in infected cells, we analyzed globally N-arginylated proteins in cells. The immunoblot showed a notable increase in the proteins exhibiting N-Arg modification after 48, 60, and 72 hrs (Fig. 5.8B). ATE1 overexpressing cells also displayed a similar rise in the N-arginylated proteins (Fig. 5.8C). The observations were further validated by TA treatment, which revealed a notable drop (Fig. 5.8D), while Arg administration confirmed its enhancements of ATE1 activity (Fig. 5.8E).

Given the relationship between ATE1 enzyme activation and cellular protein stability, we explored the degradation mechanism. As protein degradation is primarily controlled by ubiquitin-mediated proteasomal degradation, we first studied the status of global ubiquitination of cellular proteins in infected cells. We detected polyubiquitinated protein's lower levels in infected cells compared to control (Fig. 5.8F). Moreover, the proteasome inhibitor BTZ treatment reversed the degradation of ubiquitinated proteins (Fig. 5.8G). We also examined the ATE1 overexpressing cells and observed a notable reduction in ubiquitinated protein levels 24 hrs after transfection, contrasting with the higher levels observed after 12 hrs (Fig. 5.8H). Later, the proteasome inhibitor MG132 treatment reverted the ATE1-specific effects by displaying the accumulation of ubiquitinated proteins (Fig. 5.8I).

Furthermore, to confirm if HN is arginylated, we produced rabbit anti-arginylated-HN polyclonal antibody using a synthetic peptide (Fig. A.4D-F). We pulled HN protein from infected and Arg-treated cells, and detected by the raised antibody. The results validated the incorporation of Arg to HN protein, moreover, cells treated with Arg exhibited elevated levels of arginylated-HN proteins (Fig. 5.9B). Subsequently, we also pulled ubiquitin and HN protein from infected cells. The results unequivocally confirmed their linkage, as evidenced by an HN-specific band in the pulled-down samples of ubiquitin and ubiquitin-specific bands in the pulled-down samples of HN (Fig. 5.9C and D).

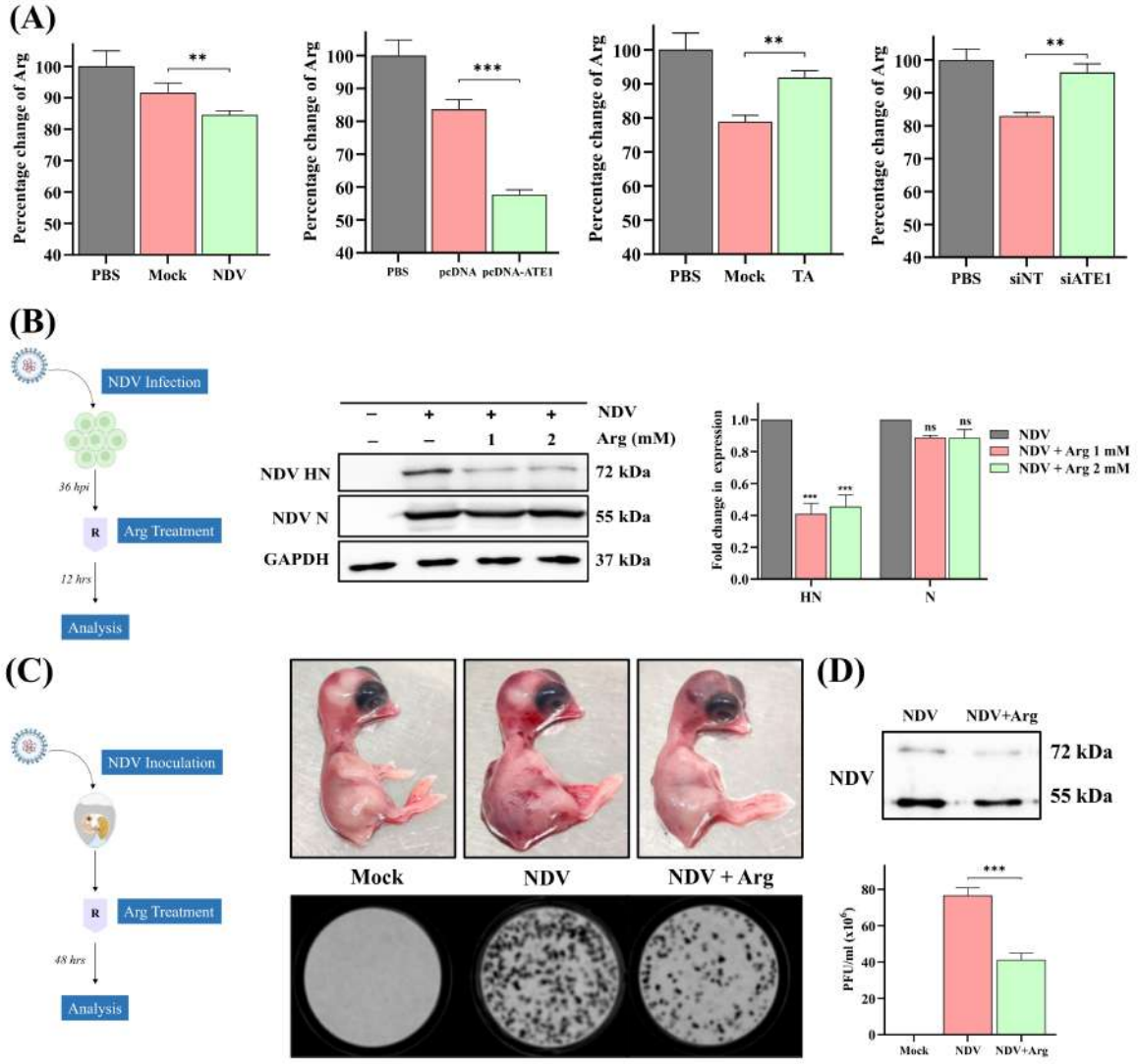


Figure 5.7: Involvement of Arg in NDV infection. Graph representing the Arg concentration variation in supernatant of NDV-infected, ATE1-overexpressed, TA-treated, and ATE1-depleted cells **(A)**. The workflow of the experiment designed for Arg treatment in NDV-infected cells. Immunoblot analysis of NDV HN and N after Arg treatment. The graph shows the fold change in the expression of NDV HN and N proteins **(B)**. The workflow of the experiment designed for NDV inoculation and Arg administration in embryonated eggs. Hemorrhages on embryo's body after 48 hrs of NDV inoculation **(C)**. Quantification image of NDV titer in allantoic fluid using plaque assay **(D)**. Immunoblot analysis of NDV HN and N in allantoic fluid **(D)**

5.2.5 Stability assessment of HN protein

The effects of ATE1 activity on the stability of HN protein were studied by CHX treatment, an inhibitor of protein biosynthesis (Fig. 5.10A). The immunoblot revealed a substantial reduction (~90%) of HN protein after inhibiting cellular translation; more-

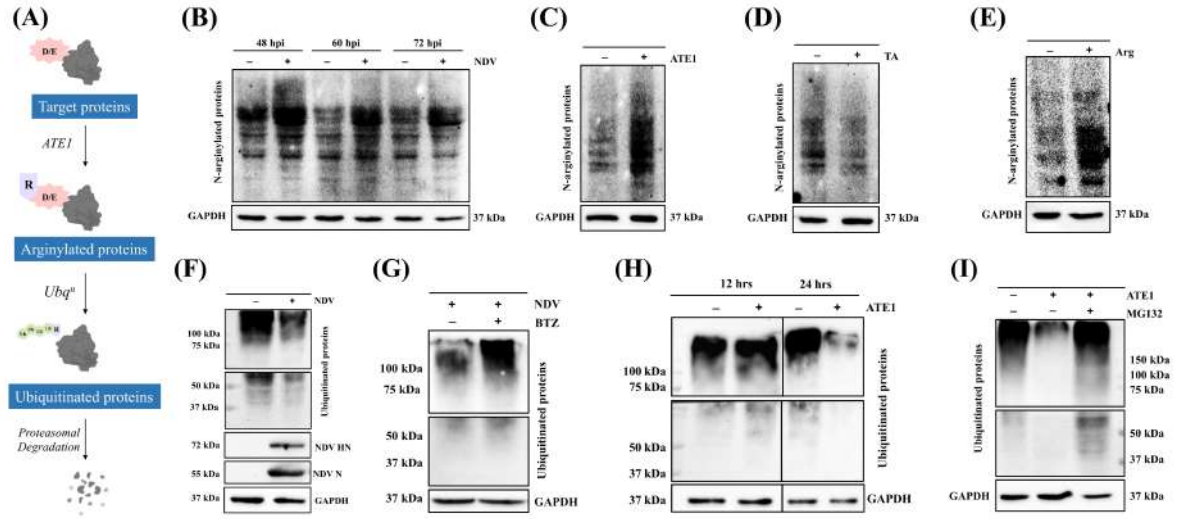


Figure 5.8: Posttranslational modifications of cellular proteins. Schematic representations of the proteasomal degradation of cellular proteins through arginylation **(A)**. Immunoblot analysis of N-arginylated proteins in DF-1 cells after 48, 60, and 72 hrs post-infection **(B)**. Immunoblot analysis of N-arginylated proteins in ATE1 overexpressing DF-1 cells **(C)**. Immunoblot analysis of N-arginylated proteins in TA-treated cells **(D)**. Immunoblot analysis of N-arginylated proteins in Arg-treated cells **(E)**. Immunoblot analysis of global ubiquitinated proteins in DF-1 cells after 48 hrs post-NDV infection **(F)**. Immunoblot analysis of global ubiquitinated proteins in infected cells with and without proteasomal degradation inhibitor, BTZ treatment **(G)**. Immunoblot analysis of global ubiquitinated proteins in ATE1 overexpressing cells after 12 and 24 hrs of transfection **(H)**. Immunoblot analysis of global ubiquitinated proteins in ATE1 overexpressing cells with and without proteasomal degradation inhibitor, MG132 treatment **(I)**.

over, there was a reduction of about 65% in Arg-treated cells. The N protein levels remained largely unaffected by Arg treatment, while around 10% reduction was observed in CHX-treated cells (Fig. 5.10A). ATE1 overexpressing and NDV-infected cells were also incubated with CHX to validate the results (Fig. 5.10B). In our observations, ATE1 overexpressing cells displayed approximately 30% HN protein. CHX-treated cells showed 45% HN protein levels, while CHX-treated cells with ATE1 overexpression exhibited only 15% HN protein compared to cells solely infected with NDV (Fig. 5.10B). In addition, we examined the mechanism of ATE1-mediated HN degradation by blocking the cellular proteasome using BTZ and measuring the HN protein levels (Fig. 5.10C). The results showed that HN protein levels were predominantly restored by around 30% in the presence of BTZ (Fig. 5.10C), confirming the involvement of ubiquitination.

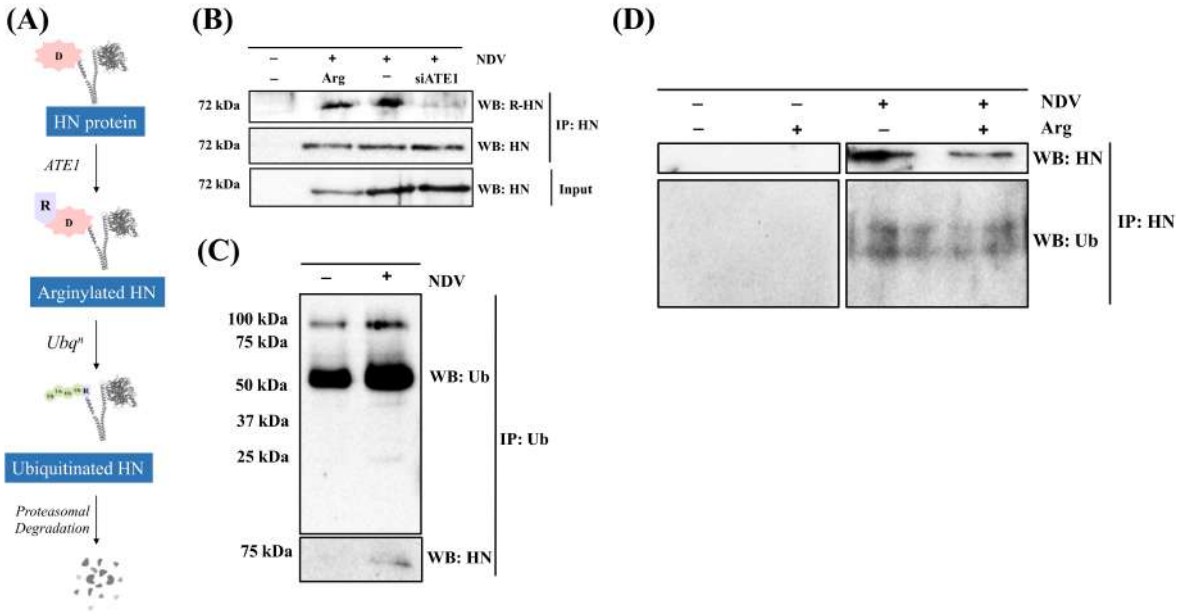


Figure 5.9: Posttranslational modifications of HN protein. Schematic representations of the proteasomal degradation of HN protein through arginylation **(A)**. Immunoblot analysis of arginylated-HN protein after co-IP assay in NDV-infected, Arg-treated, and ATE1-depleted cells **(B)**. Immunoblot of co-IP assay identifying the linkage of ubiquitin to HN protein **(C)(D)**.

5.3 Discussion

The cellular proteostasis-maintaining pathways are majorly regulated by PTMs on the target proteins, functioning as a degradation signal [398, 408]. These additional residues drive the selective degradation of proteins by marking them for N-recognin ubiquitin receptors (UBRs), thereby recruiting the proteosomes [21]. Protein arginylation is one such proteolytic pathway in place to achieve proteome maintenance [431]. The addition of Arg changes the surface property of the targeted protein, resulting in either its activity regulation or ubiquitination-mediated degradation [123, 355]. Viruses have been actively reported to offset cellular homeostasis, thus activating stress response [310, 331]. At the same time, DMF has been widely accepted as an inducer of the oxidative stress response [148]. Additionally, arginylation has been observed to play a significant role in controlling the oxygen-sensing pathways [286]. In the present study, we observed elevated levels of ATE1 enzyme in NDV-infected and DMF-treated cells, suggesting the prominent activation of the proteolytic pathways to restore protein homeostasis. Our previous studies reported that NDV instigates ER and oxidative stress, ultimately activating stress response within cells. We also reported the upregulation of

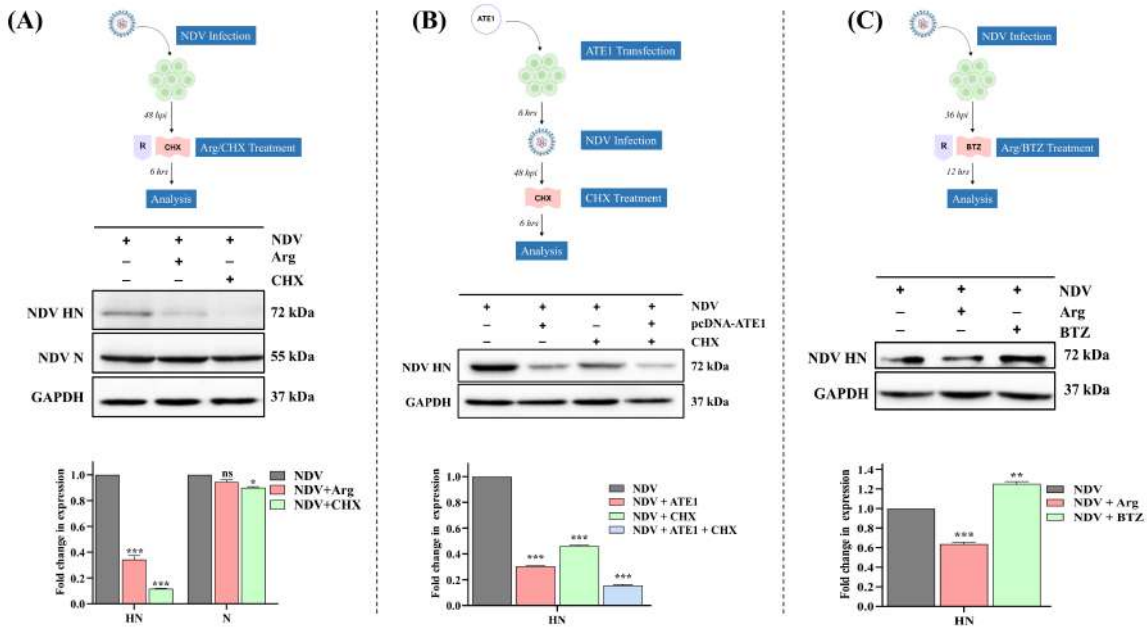


Figure 5.10: Stability assessment of HN protein. The workflow of the experiment designed involving Arg and CHX treatment. Immunoblot analysis of NDV HN and N proteins in Arg and CHX treated cells, with relative change in expression graph **(A)**. The workflow of the experiment designed involving CHX treatment and ATE1 overexpression. Immunoblot analysis of NDV HN protein post-CHX treatment in ATE1 overexpressed cells, with the relative change in expression graph **(B)**. The workflow of the experiment designed involving Arg and BTZ treatment. Immunoblot analysis of NDV HN protein in Arg and BTZ treated cells, with relative change in expression graph **(C)**.

several stress factors, including Hsp90β and SIRT7 due to oxidative stress [375, 374]. We analyzed these proteins post ATE1-overexpression and its activity inhibition. The degradation of stress factors in ATE1-overexpressing cells confirmed its involvement in proteolysis during oxidative stress to prevent the proteostatic imbalance. Consequently, we concluded that the ATE1 activation may be associated with the NDV-instigated oxidative stress response, thus helping the cell in maintaining the proteome. Additionally, heightened ATE1 expression in DMF-treated cells confirms its association in cells with aberrant proteostasis. We evaluated the function of the ATE1 enzyme in infection by its overexpression and knockdown system, and the results revealed that the enzyme had detrimental effects on NDV HN and N proteins. The data indicates the potential to exploit arginylation-driven proteolytic pathways to target the virus replication cycle. HN, one of six essential proteins of NDV, functions as a viral envelope component, thus allowing the virus to attach and bind to host cell receptors [6, 142]. Among all the NDV

proteins, only the HN and M proteins have the specific amino acid residue (Asp) at their N-terminal required for Arg addition, making them probable candidates for arginylation.

Next, we employed TA as an ATE1 inhibitor, as described previously [356], and the treatment resulted in the restoration of only HN protein levels. Moreover, the N protein lacks the specific residues on its N-terminal, rendering it unaffected by ATE1 activity. This distinction was clearly noticeable in the TA treatment experiments. However, N protein levels were decreased in overexpression experiments, suggesting the engagement of additional cellular proteolytic pathways, which could be influenced by ATE1 expression in cells. Furthermore, N protein levels remained unaltered when N and ATE1 were exogenously expressed together. Additionally, TA treatment yielded a higher NDV titer, implying that the ATE1 enzyme might be a restricting factor for its replication to a certain extent in basal conditions. Based on our initial observations, this study is the first to report that the viral proteins become a substrate for posttranslational arginylation, ultimately leading to their degradation.

Moving ahead, we utilized molecular docking to study the interaction between ATE1 and the HN protein. Upon analyzing the docked complex of ATE1-HN, we observed interactions similar to the positive groups, implying their potential binding. The docking studies convey that the ATE1 protein employs small identical amino acid regions when interacting with its target proteins. We also conducted MD simulations to provide insights into the structural and dynamic characteristics of binding interactions of the protein complexes. RMSD plot measures the deviation of atom positions compared to a reference set of atom positions, thus reflecting the structural stability of the protein complex. The results depicted the lower RMSD values for all three complexes, implying their stability. RMSF plot measures the average deviation of the amino acids over time from a reference position. The amino acids from the binding pockets of ATE1 exhibited similar RMSF values in β-actin and HN complex, highlighting their restricted movements. This indicates that the critical residues at the probable binding pockets are more ordered than other protein regions. SASA value measures the surface area of the protein, specifying the enlargement of protein volume, where low fluctuations are expected throughout the simulation. ATE1-HN complex showed a continual decline in the values, similar to the ATE1-β-actin complex. Meanwhile, the ATE1-GAPDH complex showed a sporadic increase in the values, suggesting the disappearance of existing interactions. A rise in SASA values typically associated with the protein expansion indicates the complex's instability. Rg measures the distance from the center of mass of the protein to both of its

termini, thus helping assess protein structure compactness. ATE1 complex with β-actin and HN showed lower fluctuations than the GAPDH complex, which failed to achieve stability. Our analysis considering these parameters helped us decisively infer that the ATE1-HN complex is relatively stable and compact during the simulation run. However, no interaction between ATE1 and HN protein was observed when pulled down using ATE1 and HN-specific antibodies. It could be attributed to the possibility that ATE1 binds to its target protein (in our case, HN protein) for such a brief duration that the complex is difficult to capture through pull-down assays. So far, among numerous target proteins, only one protein named ligand of Ate1 (liat1), a non-arginylation substrate, has been shown to interact with the ATE1 [47].

A recent study has described that the arginylation depends upon free Arg and arginyl-tRNA, along with the functional activity of Arg–tRNA synthetase (RARS) enzyme to produce arginyl-tRNA. The free Arg, a rate-limiting factor, has been emphasized as crucial for facilitating the arginylation reaction [18]. We conducted an Arg uptake assay to establish the part of free Arg in NDV infection. The observed higher uptake of Arg by infected cells strongly suggested the occurrence of active arginylation reactions in the cells. Subsequent Arg treatment experiments solely revealed HN protein degradation, thereby confirming that Arg influences ATE1's exclusive activity in targeting the HN protein. The *in ovo* study further supported the Arg-exclusive impact, affecting the NDV titer, thus aligning with those of *in vitro* findings. Several studies have shown Arg's positive effects on embryo hatching rate, survival rate, post-hatch growth performance, heat shock proteins, and improved muscle growth [143, 393]. As these studies did not delve into the molecular aspects underlying the observed effects, our results could offer a plausible explanation by suggesting the importance of arginylation in chicken embryos.

To assess the ATE1 activity in infected cells, we detected all the N-arginylated proteins. Although arginylation can affect a protein's N-terminus or side chains of acidic residues (Asp or Glu), we only focused on detecting the proteins with N-terminal modification. The intense smear observed throughout the blot indicates an active arginylation process of numerous cellular proteins, corroborating the initial findings from experiments demonstrating the elevated levels of ATE1 expression. These results were further confirmed through additional experiments conducted in ATE1 overexpressing cells or by modulating its activity using TA or Arg treatments. The N-terminus modifications to the cellular proteins act as degradation signals, flagging them for ubiquitin-dependent proteolysis [402, 424]. Numerous proteins, such as β-catenin, RGS4, and RGS5, have al-

ready been reported to undergo degradation via ubiquitin proteolytic pathways [239, 441]. Therefore, we determined the polyubiquitinated protein levels in NDV-infected and ATE1 overexpressed cells. The data indicated the virus-infected cells exhibit reduced levels of ubiquitinated protein, while exogenous ATE1 expression initially leads to an increase in ubiquitinated proteins, followed by their subsequent degradation. It was confirmed further by MG132 and BTZ treatment following infection and ATE1-overexpression, respectively, which halted the ongoing proteasomal degradation of arginylated proteins. The detection of the arginylated form of HN protein substantiates the activity of the ATE1 enzyme on HN protein. Later, the HN protein was also probed for ubiquitin linkage, which confirmed its ubiquitination, thereby indicating that the ATE1 enzyme could negatively stimulate the metabolic stability of HN by promoting its ubiquitination. The presence of the arginylated form of a target protein is considered enough to classify it as a ATE1-mediated arginylation substrate. We believe the detection of R-HN protein and the linkage of ubiquitin to HN provide substantial evidence to support our argument of "HN as substrate for ATE1-mediated degradation". To further potentiate the findings, we suppressed the protein synthesis by CHX and investigated the HN protein stability. The data revealed an active degradation of HN protein within the cells in the absence of cellular protein synthesis. The degradation of existing HN protein molecules implies the persistent role of the ATE1 protein in eliminating its targeted proteins from the cells. Moreover, the HN clearance was more pronounced in the presence of exogenously expressed ATE1 enzyme, substantiating the observations. To confirm the ubiquitin proteolytic mechanism driving the HN protein degradation, we impaired the proteasomal activity in the cells. The results revealed that treatment with BTZ prevented the ATE1-induced HN degradation and restored the protein levels. The data support the involvement of proteasomal machinery in the ATE1-mediated degradation of HN, where the cooperative action of all these enzymes drives the sophisticated machinery to degrade targeted proteins.

5.4 Conclusion

Our findings collectively suggest the modulation of the ATE1 enzyme and its activity during NDV infection, which is closely associated with the activation of proteolytic pathways. Moreover, we have also established the detrimental role of arginylation in HN protein stability. Additionally, the results indicated that the ATE1 enzyme can add Arg

to the N-terminus of the HN protein, ultimately leading to its ubiquitination-mediated proteasomal degradation.

The research field of arginylation is currently in its nascent stages of development. The administration of Arg could serve as a modulator of arginylation, thus offering a simple strategy to help fight viral infections. However, additional studies are required to establish its role as an effective antiviral strategy to control the disease other than Newcastle disease. The current research provides substantial evidence supporting the active involvement of the ATE1 enzyme in NDV pathogenesis, making a considerable advancement in our present comprehension of protein arginylation and its specificity. The outcome of the study could be applied to understand the biology of HN-like proteins, which serve as substrates for the ATE1 enzyme. Considering the importance of proteolytic pathways in combating viral infections, further studies have the potential to unveil new insights into cellular defense strategies while identifying promising therapeutic targets.

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

Viruses are long known to trigger a series of cellular stress responses and modulate the expression of numerous SRPs. These SRPs, synthesized at elevated levels, demonstrate protective effects against various stresses. However, the extent of stress and the activated SRP's contribution to the virus replication remains to be thoroughly understood. Firstly, this thesis confirms the instigation of ER and oxidative stress in NDV-infected cells and successfully establishes the constructive role of oxidative stress in its pathogenesis. The heightened levels of intracellular ROS and the consequent oxidative stressed environment are vital for facilitating effective viral replication. The findings elucidate the molecular mechanisms of crosstalk between oxidative stress and NDV replication, thereby unveiling a comprehensive understanding of why viruses induce stressful conditions despite their prime objective being self-replication and producing new viral particles.

NDV can suppress the expression patterns of certain SRPs, including Nrf2, HO-1, SOD-1, SIRT2, SIRT3, and SIRT5, to sustain the stressed environment while simultaneously elevating the expression of specific SRPs, including SIRT1, SIRT4, SIRT6, and SIRT7, to facilitate its replication cycle. SIRT7 exhibited the most notable modulation among all the family members, with enhanced deacetylation activity in infected cells. Previously, it has been shown to function as a specific transcription regulator governing cellular gene expression. Although its primary role is associated with chromatin's architecture and open-and-closed state, it also deacetylates numerous non-histone proteins, primarily

involved in antiviral response. Comprehending the SIRT7 biology enables us to establish the part of SRPs in NDV replication, as the results collectively imply that leveraging SIRT7-mediated deacetylation of cellular proteins could be a pivotal strategy NDV employs to thrive within its host. It has also opened up new avenues of employing SIRT activity modulators for possible disease intervention.
Next, we also demonstrated the activation of proteostasis-maintaining pathways in NDV-infected cells, as evidenced by the modulation of the ATE1 enzyme and its enhanced activity. Moreover, we illustrated that disruption of protein homeostasis could be sustained through the activation of proteolytic pathways, specifically arginylation. In NDV-infected cells, the active arginylation reaction prompts an increase in the uptake of Arg, necessary for the subsequent charging of Arg-tRNA. The post-translational addition of Arg to the N-terminus of targeted proteins triggers their ubiquitination, followed by proteasomal degradation. Furthermore, the NDV HN protein, with Asp at the 2^{nd} position, could also serve as a substrate for Arg addition by ATE1 enzyme, leading to its degradation and ultimately inhibiting the production of new viral particles. This finding of the thesis furnishes evidence that emphasizes the involvement of the ATE1 enzyme and its activity in viral pathogenesis, marking a significant stride in our present understanding of protein arginylation and its specificity, a research area still in its nascent stages of development [376]. Furthermore, this insight has improved our understanding of the potential of the cell's proteolytic pathways to degrade viral haemagglutinin proteins specifically.

Lastly, the knowledge from the thesis aids in our enhanced comprehension of stress-related and proteolytic pathways during viral pathogenesis, thereby revealing new insights into cell defense strategies while identifying new therapeutic targets. Considering the overall thesis findings, by leveraging the proteins activated implicitly by the virus, we have employed a strategy to degrade viral proteins selectively, essentially **outsmarting the smart virus** approach.

6.1 Future Directions

In the present study, we established the constructive role of SIRT7 in NDV infection cycle. However, the target host proteins of SIRT7-mediated deacetylation remain elusive. Unveiling these targets promises to deepen our understanding of viral strategies

to impede host antiviral response. Our focus has been solely on uncovering the role of SIRT7 protein; moreover, it will be intriguing to explore the function of other SIRT proteins in infection.

Here, we also established the expression and activity of the ATE1 enzyme in NDV-infected cells, highlighting the importance of protein arginylation in viral pathogenesis. This finding suggests that the activation of ATE1 enzyme could also be studied across a range of other viruses. Furthermore, our research could be extended to identify the specific target proteins for arginylation during infection. Lastly, it paves the way for exploring other viral proteins as probable substrates for ATE1-mediated proteasomal degradation.

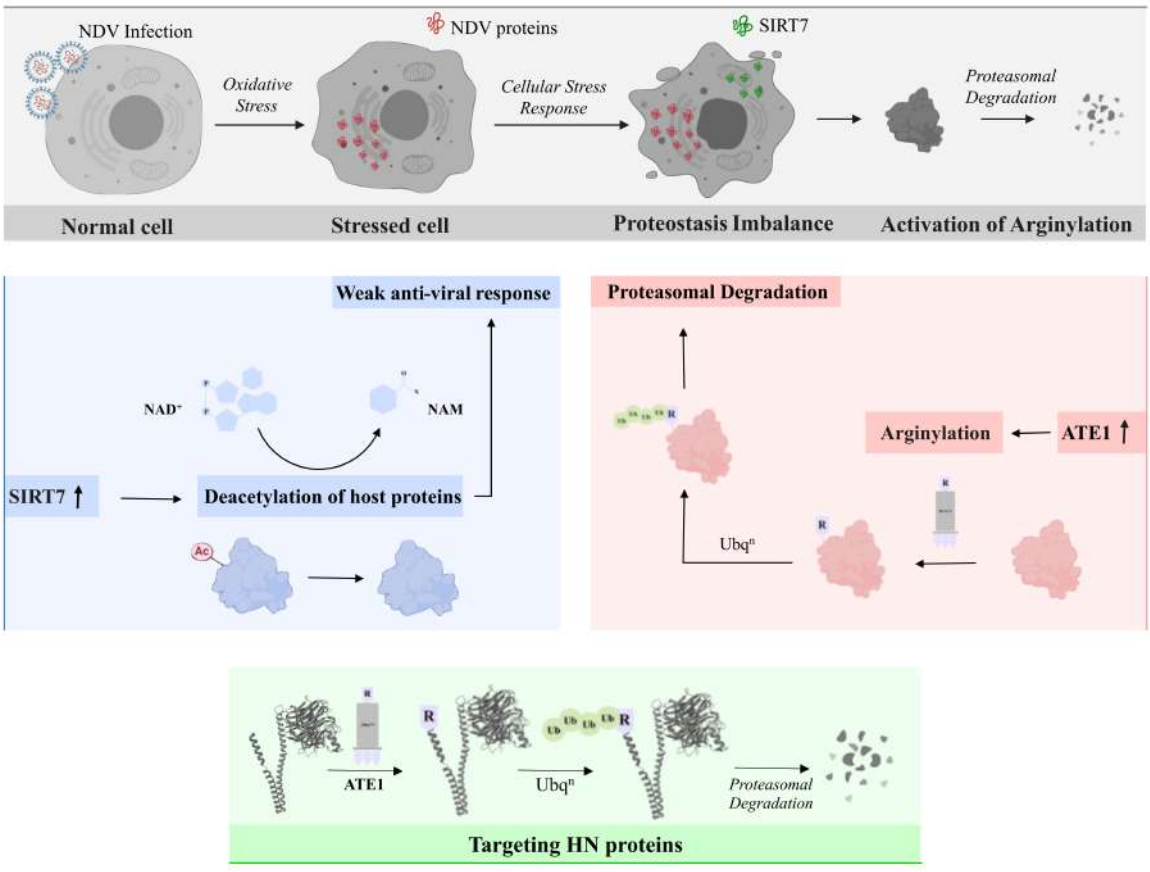


Figure 6.1: Schematic representation of the conclusions from thesis work.

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APPENDIX A

SUPPLEMENTARY INFORMATION

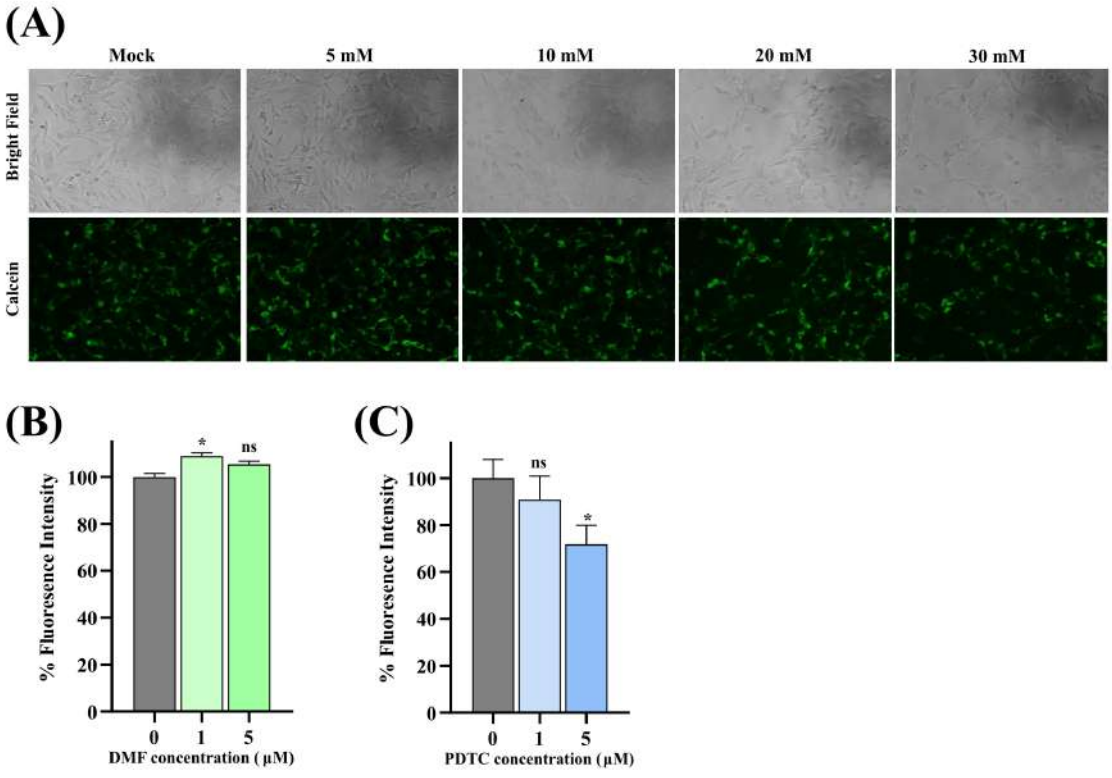

Figure A.1: Cytotoxicity assessment of LiCl, DMF, and PDTC on DF-1 cells. Bright-field and calcein dye stained images of DF-1 cells at different LiCl concentrations after 24 hrs **(A)**. Changes in intracellular ROS concentration in DMF **(B)** and PDTC **(C)** treated cells were determined using DCFH-DA dye by fluoroscopic measurements.

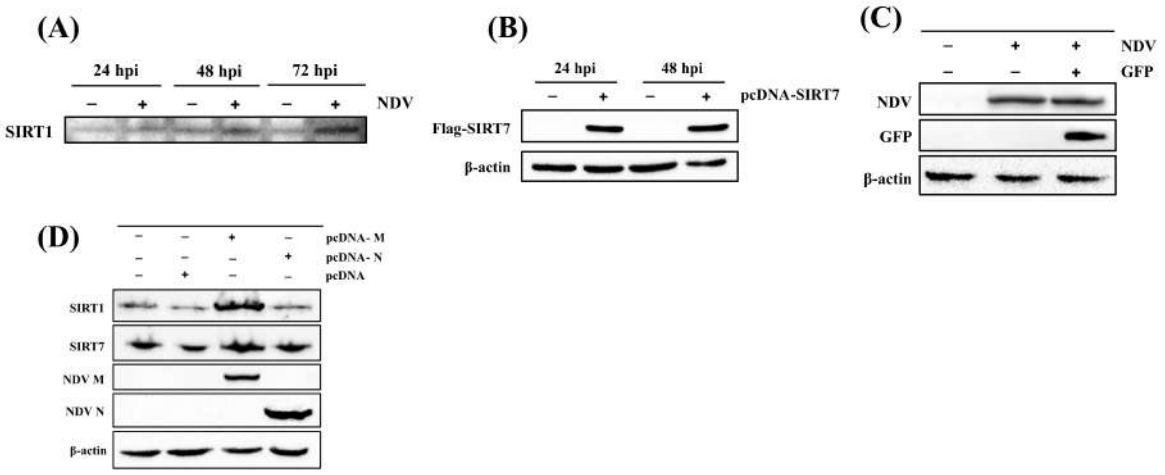

Figure A.2: Immunoblot images of SIRT1, SIRT7, and NDV protein under various conditions. Immunoblot analysis of SIRT1 and β-actin in cells after 24, 48, and 72 hours post-infection with NDV **(A)**. Immunoblot image showing the expression of SIRT7 protein, detected by FLAG antibody, after transfection with a pcDNA-SIRT7 plasmid **(B)**. Immunoblot shows the production of NDV protein along with GFP and β-actin after over-expressing GFP protein by transfection **(C)**. Immunoblot image showing the effect of NDV M and N protein overexpression on SIRT1 and SIRT7 protein **(F)**.

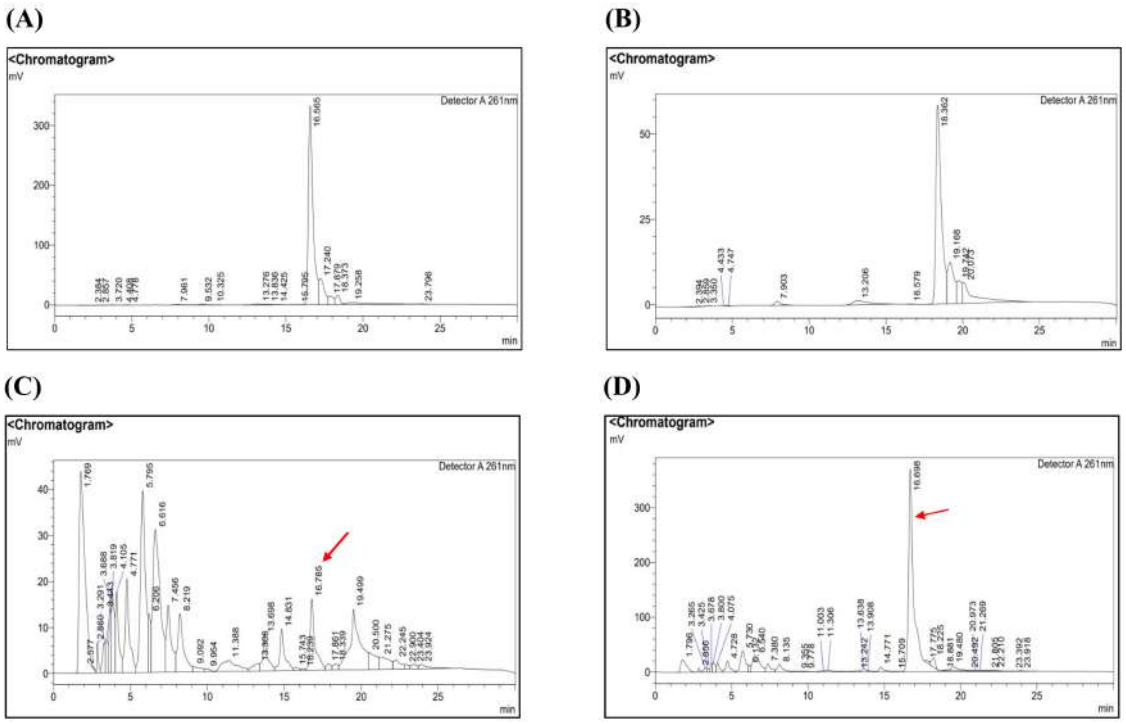

Figure A.3: HPLC chromatograms of NAD^+ and NAM. Chromatograms showing the standard peaks corresponding to NAD^+ and NAM **(A and B)**. Chromatograms obtained from DF-1 cells with and without NAD^+ **(C and D)**.

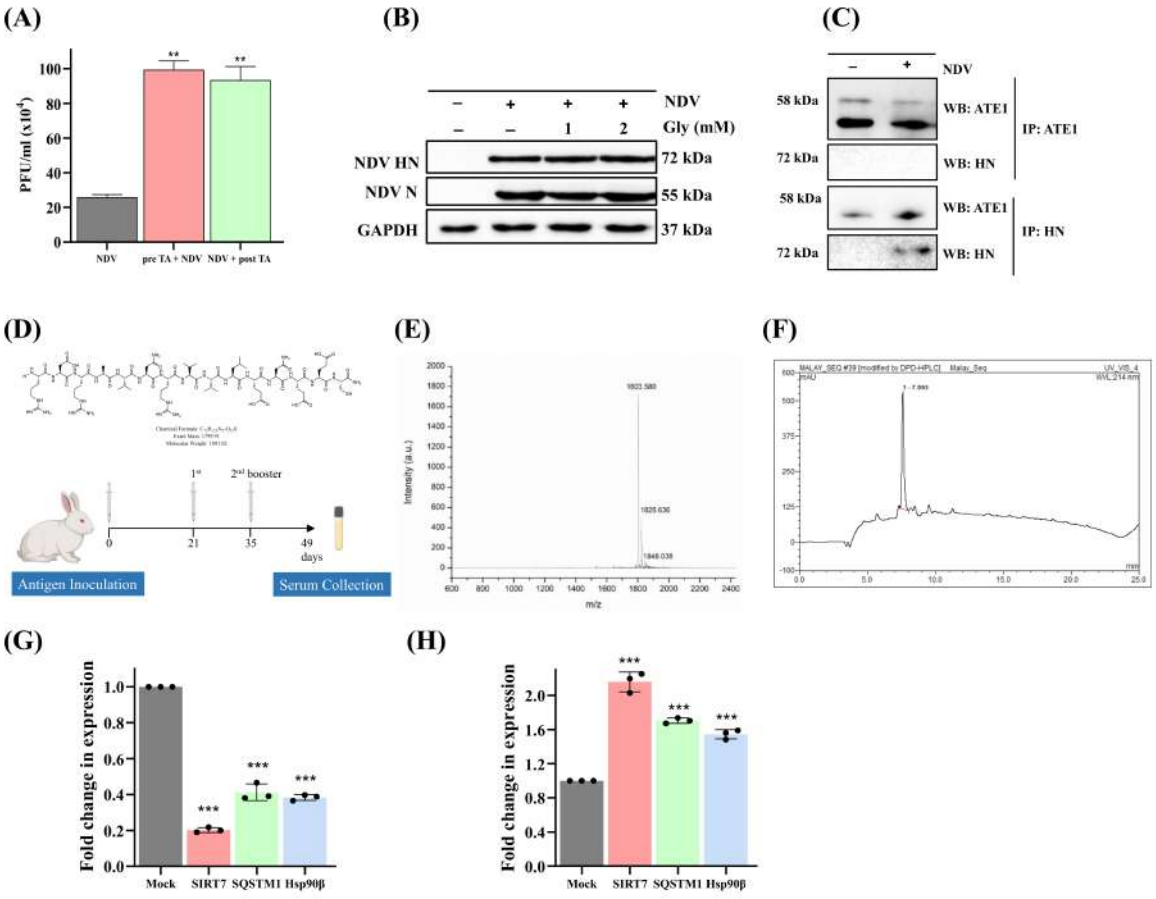

Figure A.4: Quantitative assessment and graphs of fold change in the expressions from ATE1 study. Quantitative assessment of NDV titer in TA-treated cells using plaque assay **(A)**. Immunoblot analysis of NDV HN and N after treatment with Gly (1 and 2 mM) **(B)**. Immunoblot of co-IP assay identifying the interaction between ATE1 and HN protein **(C)**. The amino acid sequence of synthesized arginylated-HN peptide and the workflow of the experiment designed for rabbit immunization using arginylated-HN-KLH conjugate **(D)**. MALDI-TOF peak of arginylated-HN peptide **(E)**. Chromatogram of arginylated-HN peptide at 214 nm wavelength **(F)**. Graph shows the fold change in expression of Hsp90β, SQSTM1 and SIRT7 in ATE1-overexpressing cells **(G)**. Graph shows the fold change in expression of Hsp90β, SQSTM1 and SIRT7 post-TA treatment **(H)**.

APPENDIX B

PUBLICATIONS

Thesis Publications

1. **Shokeen K**, Baroi MK, Chahar M, Das D, Saini HM, Kumar S. *Arginyltransferase 1 (ATE1)-mediated proteasomal degradation of viral haemagglutinin protein: A unique host defense mechanism.* **J Gen Virol.** (2024)

2. **Shokeen K**, Kumar S. *Newcastle disease virus regulates its replication by instigating oxidative stress-driven Sirtuin 7 production.* **J Gen Virol.** (2024)

3. **Shokeen K**, Srivatsan A, Kumar S. *Lithium chloride functions as Newcastle disease virus-induced ER-stress modulator and confers antiviral effect.***Virus Res.** (2021)

Other Publications

1. Baroi MK, **Shokeen K**, Chen X, Kumar S, Scherman O, Das D. *Reconstruction of Fully Functional Protein from its Peptide Fragments.* (2025) 'Under preparation'

2. **Shokeen K**, Kumar S. *Minus strand RNA Viruses.* **The Textbook of General Virology, Chapter 13, Taylor Francis.** (2024)

3. Ghosh P, **Shokeen K**, Mondal S, Kandasamy T, Kumar S, Ghosh SS, Iyer PK. *Amyloid Targeting Red Emitting AIE Dots for Diagnostic and Therapeutic Application Against Alzheimer's Disease.* **ACS Chem Neurosci.** (2023)

4. **Shokeen K**, Chowdhury P, Kumar S. *Recent Development in Detection Systems for Human Viral Pathogens from Clinical Samples with Special Reference to Biosensors.*

Next-Generation Nanobiosensor Devices for Point-Of-Care Diagnostics, Chapter 01, Springer Nature Singapore. (2022)

5. **Shokeen K**, Shambhavi P, Shah M, Kumar S. *Insight towards the effect of the multibasic cleavage site of SARS-CoV-2 spike protein on cellular proteases.* **Virus Res.** (2022)

6. Deka P, Nath MK, Das S, Das BC, Phukan A, Lahkar D, Bora B, **Shokeen K**, Kumar A, Deka P. *A study of risk factors associated with Newcastle disease and molecular characterization of genotype XIII Newcastle disease virus in backyard and commercial poultry in Assam, India.* **Res. Vet. Sci.** (2022)

7. Banerjee G*, **Shokeen K***, Chakraborty N, Agarwal S, Mitra A, Kumar S, Banerjee P. *Modulation of Immune Response in Ebola Virus Disease.* **Curr Opin Pharmacol.** (2021) *equal contribution

8. Patel A, Dey S, **Shokeen K**, Karpinski TM, Shivaprakasam S, Kumar S, Manna D. *Sulfonium-based liposome-encapsulated antibiotics deliver a synergistic antibacterial activity.* **RSC Med. Chem.** (2021)

Awards

1. **Travel Grant Award** by Department of Biotechnology (DBT), Government of India. [*June, 2023*]

2. **Global Scholar Travel Award** by ASV. [*June, 2023*]

3. **Best Oral Presentation Award** for '*Increased metabolization of NAD$^+$ by Sirtuin 7 protein supports Newcastle disease virus infection*', VIROCON. [*November, 2022*]

4. **Sir. F.M. Burnett Memorial Award** by Indian Society for Veterinary Immunology and Biotechnology. [*February, 2022*]

5. **Deepika Phukan Oncology Research Grant Award** of Dr. B. Borooah Cancer Institute. [*April, 2021*]

6. **Best Poster Award** for '*Lithium Chloride reduces Newcastle disease virus-induced ER stress*' Association of Microbiologists of India (AMI). [*November, 2019*]

Conferences/Workshops attended

1. **42nd Annual meeting of American Society for Virology**, ASV, The Classic Center-Georgia. [*June, 2023*]

2. **International Symposium on 'Zoonotic and Transboundary Diseases: Breaking the Chain through Multidisciplinary Approach'**, Indian Association of Veterinary Public Health Specialists (IAVPHS), ICAR Research Complex, Meghalaya. [*December, 2022*]

3. **Biosafety and Biosecurity Procedures**, Research and Development Section, IIT Guwahati. [*June, 2021*]

4. **Flow Cytometry Techniques and Applications**, North East Centre for Biological Sciences and Healthcare Engineering, IIT Guwahati. [*December, 2020*]

5. **Virtual Research Outreach Programme**, India International Science Festival (IISF), IIT Guwahati. [*December, 2020*]

6. **International Symposium on 'Microbial Technologies in Sustainable Development of Energy, Environment, Agriculture and Health'**, AMI, Central University of Haryana. [*November, 2019*]

7. **International Congress of Cell Biology (ICCB)**, CSIR- Centre For Cellular And Molecular Biology (CCMB), Hyderabad. [*January, 2018*]