Investigations into the Significance of Nrf2 Signalling and Ubiquitination of Proteins in Respiratory Syncytial Virus Infection.

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Master in Philosophy (MPhil)

By

Dorota Alicja Chudek

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Acknowledgements

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ABSTRACT:

Introduction and Objectives:

Respiratory Syncytial Virus (RSV) is the commonest cause of bronchiolitis in infants. This negative strand RNA virus is known to infect and replicate in the airway epithelium. RSV infection induces elevated levels of reactive oxidative species and subsequent oxidative stress injury in the lungs. Nrf2, a transcription factor that regulates antioxidant protein expression, has an important role in preventing pulmonary oxidative damage. Sulforaphane is a potent, naturally occurring inducer of NRF2 found in vegetables such as broccoli. In this study we sought to determine whether Nrf2 induction by sulforaphane might reduce RSV replication in airway epithelial cells. We also selected six proteins including MAVS, DDX21, RPS10, prohibitin, annexin A1, HMGB1 from proteomics defining changes in their level of ubiquitination following RSV infection. Our aim was to determine which proteins change their level of polyubiquitination following the infection. This could help identify new biochemical pathways involved in the host defence or viral replication and new targets for potential therapeutic intervention.

Method:

BEAS2B cells were infected with RSV at MOI of 1 following pre-treatment with sulforaphane. Samples were harvested at time points 24 and 48 hours and analysed by Western Blotting for NrF2 and RSV. In addition, RT-qPCR was carried out for RSV quantification using an RSV N primer. A549 cells were infected with various concentrations of RSV (1:4-4:1). Samples were harvested at time point of 4 and 24 hours and analysed by Western Blotting using antibodies to ubiquitin and target proteins selected from ubiquitination proteomics data . Immunoprecipitation was used to confirm ubiquitination of these proteins and immunohistology to confirm their cellular localisation. Proteasome activity was inhibited using MG132 a specific, potent, reversible, and cell-permeable proteasome inhibitor.

Results:

Sulforaphane induced Nrf2 production in BEAS2Bs in a dose dependent manner. Results do not show that RSV replication is reduced in airway epithelial cells pre-treated with Sulforaphane. However, preliminary data suggests that virus might have degrading effect on Nrf2. Western blots demonstrate changes in expression of target proteins and their ubiquitination following RSV infection and proteasome inhibition. Breakdown products of DDX21 and MAVS were detected in RSV infected samples.

Conclusions:

RSV infection changes expression of target proteins in A549 cells and might have influence on their ubiquitination, however, most probably it does not affect expression of Sulforaphane induced Nrf2. DDX21 and Nrf2 are likely to be degraded by the virus but results have to be confirmed in further experiments.

Abbreviations:

%- Percentage

°C- Degree Celsius

µl- Microlitre

AOE- Antioxidant enzyme

BCA- Bicinchoninic acid assay

BSA- Bovine Serum Albumin

CCA - Chimpanzee Coryza Agent

COPD- chronic obstructive pulmonary disease

CO_2- Carbon dioxide

DAMP - Damage-Associated molecular Patterns

DMEM- Dulbecco's Modified Eagles Medium DMEM

DNA - Deoxyribonucleic Acid

DTT- Dithiothreitol

DUBs- De-ubiquitinising enzymes

EDTA- Ethylenediaminetetraacetic acid

FCS- Foetal Calf Serum

hMPV- Human Metapneumovirus

IFN – Interferon

KSHV- Kaposi's sarcoma-associated herpesvirus

kDa- Kilodalton

L- Litre

L protein- Large Protein

LRT - Lower Respiratory Tract

mL- Mililitre

mM- Milimolar

M protein- Matrix protein

Maf- Musculoaponeurotic fibrosarcoma

MVB- Multivesicular body pathway

MOI- Multiplicity of Infection

MW- Molecular Weight

N protein- Nucleocapsid protein

NF-κB - Nuclear Factor Kapper B NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells)

NK - Natural Killer Cells

NRF2- Nuclear factor (erythroid-derived 2)-like 2

NS1- Non Structured Protein 1

NS2- Non Structured Protein 2

ORF1- Open reading frame

PAMP- Pathogen associated molecular patterns

PBS- Phosphate Bovine Serum

PCR - Polymerase Chain Reaction

PFU- Plaque Forming Units

P protein – Phosphor protein

PVDF- Polyvinylidene fluoride

PreF- Pre-fusion form

PostF- Post- fusion form

qPCR- quantitative PCR

RNP- Ribonucleoprotein complex

RNA - Ribonucleic Acid

ROS- Reactive oxygen species

RSV - Respiratory Syncytial Virus

SDS- Sodium dodecyl sulphate

SH- small hydrophobic protein

SOD- Superoxide dismutase

TBS-T- Tris-Buffered Saline and Tween 20

TLR- Toll- like receptors

UBL- Ubiquitin like domain

UBA- Ubiquitin associated domains

URT- Upper Respiratory Tract

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1. Introduction

1.1. Respiratory Syncytial Virus

Respiratory Syncytial Virus (RSV) is one of the leading causes of respiratory disease in infants and is a major threat for child health worldwide, regardless of socioeconomic status. It also contributes to an increase in mortality and hospitalisation rates in the elderly and immunosupressed. [1] The virus causes bronchiolitis, lower respiratory conditions characterised by dry coryza, cough and fever.[2] In addition to the consequences of its acute symptoms, it has been linked to asthma and recurrent wheeze in later life. All these aspects make the disease a great burden on both society and the NHS.[2]

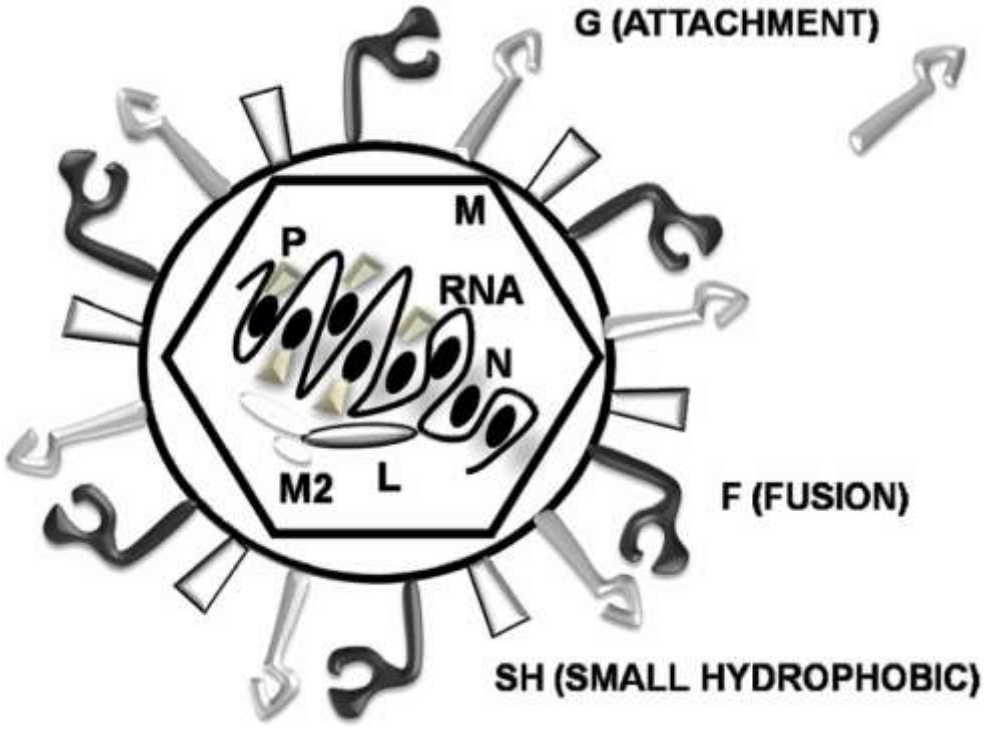
1.1.1. Virology- overview of the virus structure

RSV is a double stranded, enveloped RNA virus from the Paramyxoviridae family. Although mainly veterinary viruses, there are two human viruses in this family- RSV and metapneumovirus.[3] Its genome consists of 15,222 nucleotides and encodes for 11 proteins. Nine of those proteins are structured virion components and 2 are non- structured proteins 1 and 2 (NS1, NS2) responsible for opposing the host innate immune response[4]. Two proteins located on the surface of the virus- proteins F and G play crucial roles in viral infection. Protein G is responsible for attaching to the host cell by

targeting ciliated airway epithelium and protein F for fusing viral and cellular membranes and allowing entry into the cell. Protein F also gives the virus its name as it stimulates production of syncytia enabling direct cell to cell spread.[5] Thanks to antigenic determinants of these two proteins, the host's body produces neutralising antibodies.[6] Protein F exists in two forms, pre- and post-fusion. The pre-fusion form (PreF) is the main target for the development of antiviral drugs due to its superiority in inducing neutralising antibodies in comparison to its post-fusion form (PostF).[7] Proteins G and F are two out of three integral membrane proteins inserted in a lipid envelope surrounding the virus. The third protein is the small hydrophobic (SH) protein but its role is currently unknown.[8] The RSV genome is protected by helical nucleocapsid which also provides a replication template.[9] A mature RSV particle consists of ribonucleoprotein (RNP) complex created by viral RNA (vRNA), the nucleocapsid (N) protein, the phosphor (P) protein and the large (L) protein interacting with one another. The P protein is an essential component of polymerase complex providing clearance and chain elongation during transcription. The L protein is responsible for RNA synthesis stimulation, encoding RNA polymerase, as well as RNA transcription and replication.[10] These proteins are essential for minimal functional polymerase activity; however, matrix proteins also

contribute to its efficiency. These proteins are called M2-1, M2-2 and M and all are required for transcription. M2-1 is a transcription factor and M2-2 is a regulatory factor responsible for balance between replication and transcription. M2 mRNA consists of two open reading frames (ORF1 and ORF2) which overlap. ORF1 promotes chain elongation during transcription and optimizes mRNA production. ORF2 plays a role in accumulation of genomic and antigenomic RNA[11]. M protein, located on the viral envelope, is a matrix protein which enables interaction of plasma membrane and ribonucleoprotein complexes (RNPs) during virion synthesis. As mentioned earlier, NS1 and NS2, which are not part of the mature virion structure, are secreted proteins responsible for antagonising the interferon system. They increase the severity of disease by blocking production of type I interferon (IFN) and causing rapid replication of the virus. NS1 has a greater IFN inhibiting effect in comparison to NS2 but both work synergistically.[12] A schematic illustration of RSV particle is presented in Figure 1.1.

Figure 1.1. Schematic illustration of RSV particle.



A single particle of RSV showing its envelope and negative single RNA strand. 3 surface proteins (G,F,SH) are shown outside the virus particle and protein M (matrix), L (polymerase), N (nucleocapsid) and P (phosphoprotein), as well as two transcription and termination factors M2-1 and M2-2 inside the cell. Non-structural proteins NS1 and NS2 are not shown in the picture.(4)

1.1.2. History and discovery of RSV

RSV was first described in 1957 as “Chimpanzee Coryza Agent” by Blount et al, following an outbreak of disease in research purpose kept group of Chimpanzees.[13] Symptoms of bronchiolitis however, had been described earlier in 1857 by Eberle but at that time, the cause of the disease was unknown.[14] It was the lack of bacteria detected in the infected sample almost 100 years later (in 1955), which made Adams think that the outbreaks of respiratory disease in

infants characterised by cyanosis, cough and dyspnoea might have a viral cause.[15],[16] In 1957, Chanock et al discovered that “Chimpanzee Coryza Agent” cannot be structurally distinguished from the virus causing bronchiolitis symptoms in infants.[17] The name RSV came from ‘syncytia’, or pseudo large cells, which were observed in infected human epithelial cells. The same group of scientists identified the main characteristics of RSV, including its link to bronchiolitis and pneumonia, its seasonability in winter months and its propensity to infect young infants.[18]

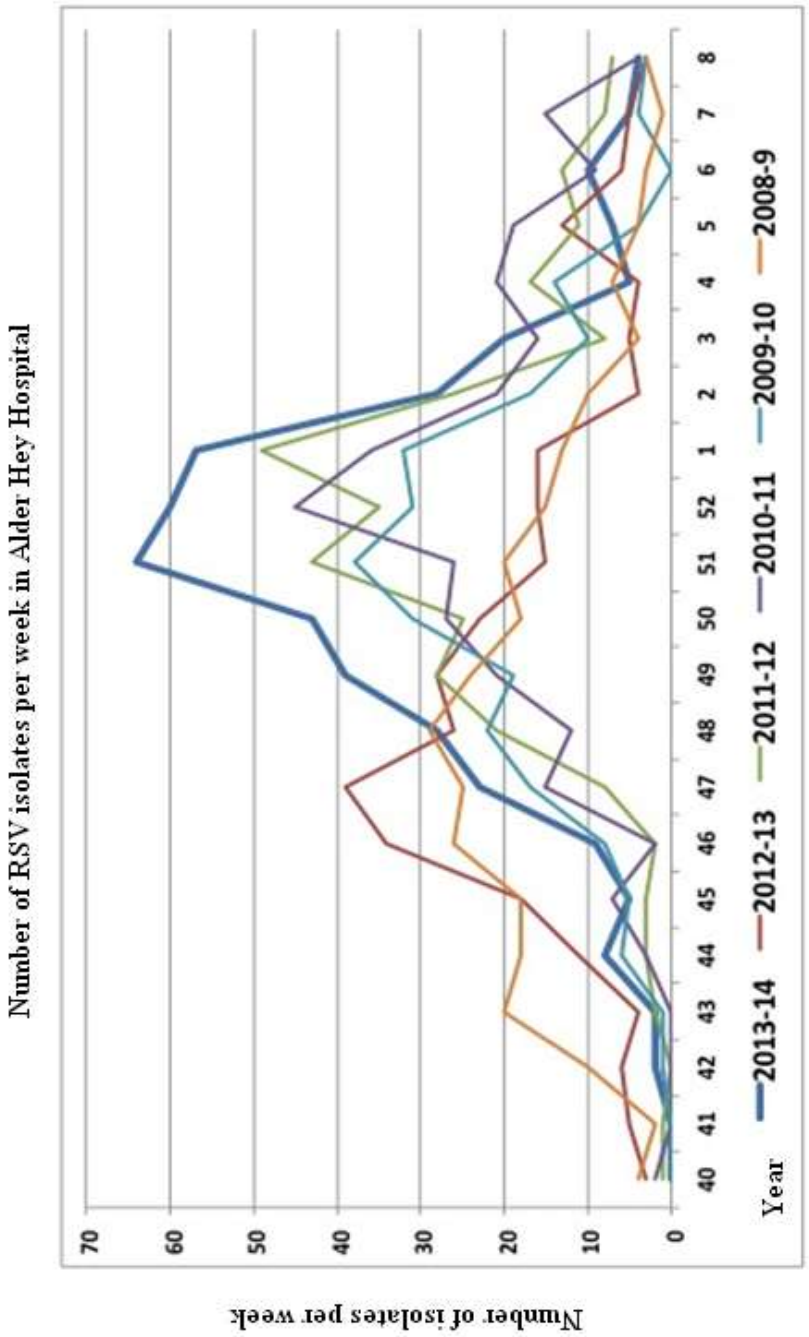
1.1.3. Epidemiology

RSV infections are very common. Every winter the virus causes outbreaks of bronchiolitis in children under the age of 1. The majority of cases in the Northern Hemisphere are recorded between November and April[19] (Figure 1.2.). Almost all children will have had a RSV infection before the age of 5, with 70% of children being exposed to the virus in the first 12 months of life.[20] Incidence peaks during 3rd and 4th month of life.[21] There are two major genetic subgroups, A and B which co-circulate, and their predominance varies by year and geographic location.[22] Worldwide, RSV is estimated to cause over 30 million lower respiratory tract infections each year which contribute to more than 3 million hospitalisations. This makes it the most common cause of hospital admissions in

children under 5 years of age.[23] Overall, 2-3% of children under 1 who are infected with RSV get admitted to hospital.[24] (Figure 1.3. presents hospital admission rates from bronchiolitis in years 2010-2011 in the UK.) In developing countries, bronchiolitis has been reported as the second commonest cause of death during the first 12 months of life after malaria.[25] In the United Kingdom, mortality rates from bronchiolitis have decreased from 21.47 in 1979 to 1.82 per 100,00 live-births in 2000.[26] Re-infection rates of the virus are also vey high, with 74-83% in the 2nd year and 46-65% in the 3rd year of life reported in literature.[27] This data shows that human immunity provides insufficient protection from the virus and highlights the importance of treatment development.

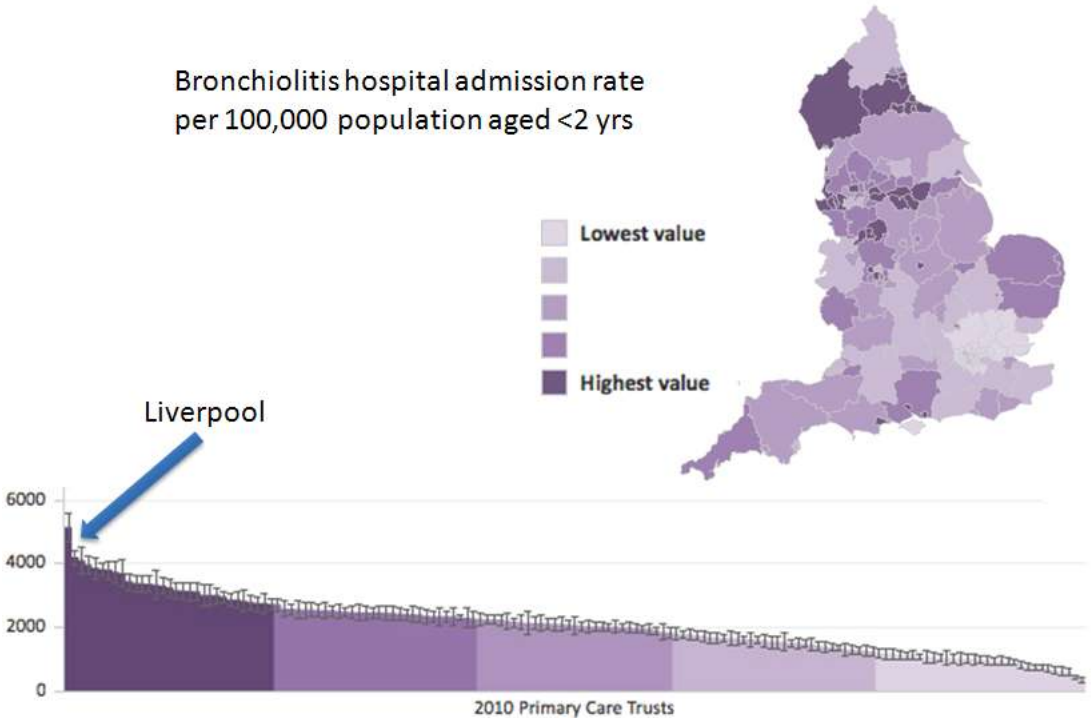
Clinical research data from both the USA and Britain report high rates of Intensive Care Unit admissions and need for mechanical ventilation in children with bronchiolitis.[28] A study conducted in 5 London Paediatric Intensive Care Units showed that the average length of assisted ventilation needed by a child with bronchiolitis was 4.4 days and the average length of hospital stay was 15.9 days.[29] RSV infection is an enormous burden not only on a patient's health but also on the economy. The predicted annual cost of treating RSV infection is 600-750 million USD.[30]

Figure 1.2. Graph presenting number of RSV isolates per week, recorded in Alder Hey Hospital shows seasonality of the disease.



Graph shows changes in the number of RSV isolates per week reported in Alder Hey Hospital between years 2008-2014. Axis x shows week of a calendar year and axis y number of isolates reported per week. Image was kindly provided by Dr Brian Flanagan.

Figure 1.3. Hospital admission rates for bronchiolitis in the UK in 2010/2011.



Images showing differences in hospital admission rates for bronchiolitis in the UK in 2010-2011 in Primary Care Trusts. The values are per 100,000 population aged <2 years. Number of admissions in Liverpool is one of the highest in the country. Data from National Child and Maternal Health Intelligence Network Website (www.chimat.org.uk).

1.1.4. Clinical features and presentation

Presentation of bronchiolitis varies depending on the age of the patient and the severity of symptoms. Mild RSV infection usually results in mild upper respiratory tract (URT) symptoms and does not require medical intervention. However, more severe disease causes significant lower respiratory tract (LRT) symptoms which manifest as bronchiolitis or pneumonia and often require hospital admission. Children with LRT symptoms usually present with cough (98%), fever (75%), rhinorrhoea, wheezing (65-78%), increased work of breathing (73-95%) and sometimes hypoxia.[31] Symptoms of more severe disease include: grunting, nasal flaring, subcostal and intercostal recession. Older children usually present with URT symptoms like cough, coryza, rhinorrhoea and conjunctivitis.[32] Predicting severity based on symptoms might be misleading because children can appear disproportionally ill/well.[33] However, factors associated with more severe disease in full term children include age <60 days, male sex, increased respiratory rate, increased work of breathing, lower socioeconomic status or poor oral intake.[34]

1.1.5. Risk factors and prognosis

Even though all infants are susceptible to RSV infection and bronchiolitis, the following risk factors make the chance of morbidity much higher: pre-existing chronic lung disease (eg bronchopulmonary dysplasia), current weight of less than 5 kg, existing cyanotic heart disease, immune compromise (eg severe combined immunodeficiency), *in utero* exposure to tobacco smoke, low socioeconomic status, neuromuscular disease and premature birth- before 35 weeks of gestation. Atopy or family history of atopy have also been reported to be associated with more severe forms of disease.[35]

The majority of patients with RSV infection recover uneventfully and significant disease does not recur. However, 40% of children hospitalised with bronchiolitis have significantly more wheezy episodes during the first 5 years of life than age matched controls, with 10% continuing to have wheezy episodes past the age of 5.[36] A study by Blanken et al, in which healthy pre term infants were prophylactically treated with palivizumab, showed decrease in number of wheezy days during the first year of life in comparison to

control group and proved that RSV infection plays significant role in the pathogenesis of recurrent wheeze during the first year of life.[37]

1.1.6. Management and prevention

Despite the importance of the disease, a lack of in-depth knowledge about the pathogenesis has resulted in inadequate treatment and vaccination options available. In groups of ex-preterm neonates at the highest risk of severe disease, palivizumab (a monoclonal antibody) is administered. However, it is only effective as prophylaxis not as a therapeutic. Use of the antibody has not been extended to the general population. The process of vaccine development has been significantly prolonged due to safety concerns, biological barriers and practical problems. Extensive research in infected people and animal models has not yet led to commercially available effective antivirals or vaccines, with the exception of palivizumab for immunoprophylaxis in selected high-risk children. Due to the lack of knowledge about the virus' intermediate host or animal reservoir, it is argued that if the vaccine was administered before the first RSV infection, virus ecology could drastically change and stop the ability of RSV to continually re-infect humans.[38]

Currently in the majority of cases, RSV bronchiolitis treatment is supportive, consisting of close monitoring of the clinical symptoms and if necessary IV fluids and oxygen.[39] As a result of overcautious

attitudes and a lack of set treatment protocol, patients often undergo unnecessary treatment with antibiotics, steroids or inhaled bronchodilators despite a lack of evidence-based data about their effectiveness. Bronchodilators (eg nebulised salbutamol) have been reported to cause modest short term improvement, however, a definitive benefit with acute symptoms has not been demonstrated.[40] Anti-inflammatory medication, such as systemic and nebulised corticosteroids, have demonstrated no benefits as bronchiolitis treatment.[41]

1.2. Pathogenesis

RSV replicates in the nasopharynx, where epithelial cells are the primary line of defence, causing URT symptoms.

1.2.1. Viral infection and cytotoxicity

After 2-8 days of incubation, the virus infiltrates the small bronchiolar epithelium causing LRT symptoms. Viral shedding usually lasts 3-8 days but in some cases can extend up to four weeks.[42] In cases which progress to LRT, pathological changes develop including oedema, enhanced mucus production and ultimately necrosis and regeneration of the airway lining. These changes result in small airway obstruction, air trapping and increased airway resistance.[43] In turn this can lead to hypoxia and respiratory failure.[44]

Due to RSV pathogenesis being not fully understood, information available in the literature is not abundant and remains controversial. The impact of the host response is reduced because the virus targets superficial epithelial cells. Lung injury is exacerbated both by the direct cytotoxic effect of the virus and inflammatory responses elicited against the virus.[45] Some studies show that it is only after epithelial cells have released inflammatory mediators that apoptosis occurs.[46] RSV has been reported to cause ciliary damage not long after infection, as well as delayed cell death, even weeks after infection.[47] Numerous studies suggest that damage caused by RSV is to a great extent immune response mediated.[48] Continuous stimulation of the immune system caused by persistent viral infection, may cause chronic inflammation or changes in the expression of immunoregulatory molecules, which may explain why the clinical symptoms persist long after the acute viral infection has resolved.[49]

1.2.2. Immune response to RSV infection

The human immune system can be split into two parts- innate and adaptive immunity. Both of those components work synergistically to recognize and remove unwanted matter from the organism and minimise the damage during the immune response.[50] The first line

defence is the innate system, which is always present and helps in the induction of the adaptive system. RSV infects and replicates in airway epithelial cells. These host cells express toll- like receptors (TLR), sensors detecting pathogen specific structural motifs. They recognize virus pathogen associated molecular patterns (PAMP) and initiate expression of cytokines, soluble protein mediators which regulate the immune response. [51] One of the receptors (TLR-4), binds to the RSV F protein (Section 1.1.1.) and together with CD14 starts NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) mediated cytokine production.[52] The cytokines produced during TLR-4 engagement initiate neutrophil and natural killer (NK) cell migration into the lungs, where they may be themselves further stimulated by virus or surrounding cytokines.[53] Notably neutrophils, although apparently needed to control RSV infection, have also been suggested to damage airway tissue.[54]

Adaptive immunity which includes T cell-mediated immunity and antibody production by B cells is characterised by immunological memory and tolerance to the body's own tissues.[55] Studies conducted on animal models have found that RSV infection considerably changes host innate immunity, which in turn leads to impairment of an efficient adaptive immunity reaction to infection.[56]

The two systems are highly integrated and comprised of both specialised cells and humoral factors.[57]

One example of cell's response to viral infection is the interferon (IFN) pathway. Interferons are a group of cytokines of a pleiotropic type named after their property of 'interfering' with viral replication.[58] Studies on murine models stress the importance of reduced IFN expression in cases with increased RSV spread.[59] IFNs can be split into two groups. Type I can be expressed by the majority of cells and consists of many IFN- α forms and one IFN-β form. During infection with virus, this type of IFN is expressed rapidly in response to viral RNA or DNA recognition. Cells at or around the site of infection are activated via IFN receptors on the cell surface leading to inhibition of viral replication through production of endonuclease which destroys viral DNA/RNA and inhibits translation. Type I IFN also up regulates major histocompatibility complex (MHC) class I production, which increases the chance of cytotoxic lymphocytes identifying an infected cell and increases NK cell activity which up-regulates the production of proteins such as inflammatory chemokines.[60] A study by Spann et al suggests that expression of IFNs is a very early host reaction to RSV, and that a major method of RSV inhibiting innate immunity is by inhibiting IFN secretion.[61]

Type II IFN group consists only of IFN-γ and it is expressed by macrophages, Natural Killer T and Natural Killer cells. IFN-γ is recognized as a part of immune response but the exact part it plays is unknown.[62]

Another molecule of importance in viral infection is Nrf2, a transcription factor which has been recognized as an essential regulator of cellular oxidative stress response caused by viral infection. Its role is described in greater detail in Section 1.3.1.

1.3. NRF2

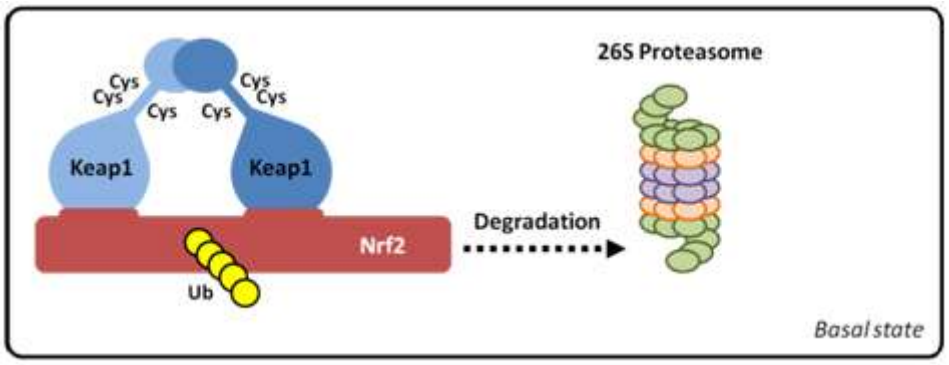
Nrf2 also known as NF-E2-related factor-2, is a transcription factor, profusely expressed in macrophages.[63] It has been recently recognised as one of the main cellular defence mechanisms against environmental toxins and carcinogens. Its main role is to stimulate oxidant response and initiate transcription of genes, which protect the organism from oxidative stress effects and results in the re-establishment of homeostasis.[64]

1.3.1. Molecule overview

Emerging studies suggest that Nrf2 has a major part in the pathogenesis of various types of cancer, chronic lung disease and host defenses against viral infection of the respiratory system.[65] In a healthy cell, Nrf2 is suppressed by Keap1 protein (Kelch-like

erythroid cell-derived protein with CNC homology ECH-associated protein 1)[66], dependent on another protein- Cullin 3 (Cul3). This Keap1-Nrf2 pathway is the main method in which cells regulate protective responses to internal and external stresses resulting from reactive oxygen species (ROS).[67] Keap1 is a substrate adaptor which keeps Nrf2 in the cytoplasm and helps Cul3 ubiquitinate Nrf2 when the cell is in redox homeostasis. Ubiquitinated Nrf2 is transported to proteasome for Cul3-ubiquitin mediated degradation[68] (Figure1.4.).

Figure 1.4. Degradation of Nrf2 in healthy cells.



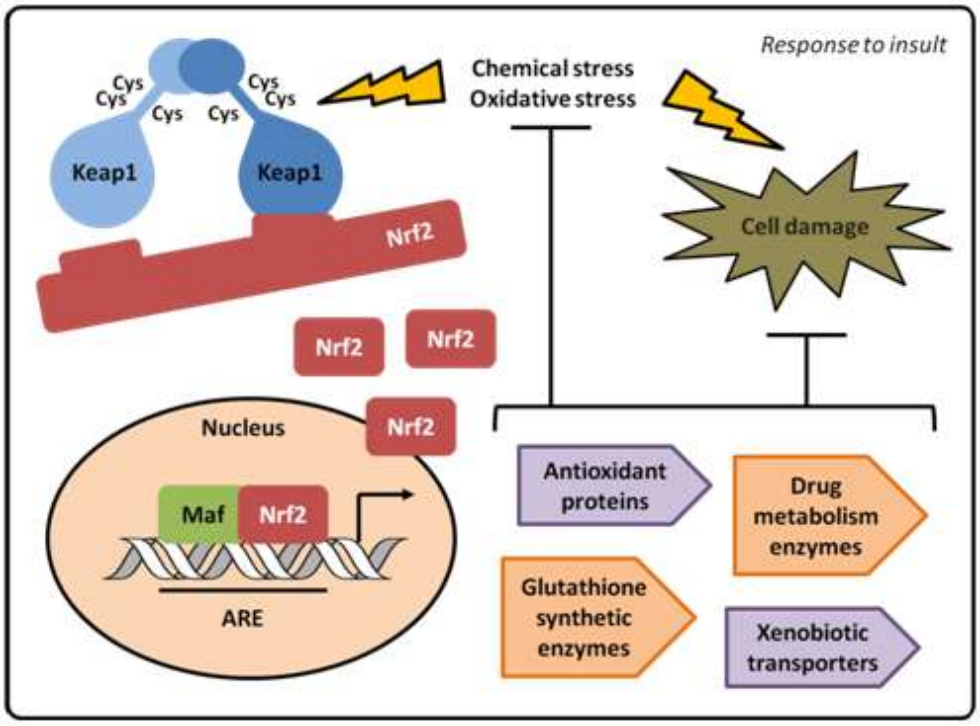
In a non-infected cell Nrf2 is kept in the cytoplasm by Keap1 and Cullin3. Keap1, a substrate adaptor, helps Cull3 degrade Nrf2 by ubiquitination. Nrf2 is marked with ubiquitin and degraded and recycled in the proteasome. This process happens very quickly as Nrf2 half life is only 20 minutes.[69]

Keap1 has multiple cysteine residues which can be regulated in vitro by various oxidants. If a cell undergoes oxidative stress, Nrf2 is released from the complex by a change in Keap1 cysteine disulfide bonds, undergoes phosphorylation and is translocated to the nucleus.[70] Three of the cysteine residues, C151, C273 and C288

have been reported to alter the structure of Keap1 which results in nuclear translocation of Nrf2 and target gene expression.[71] Even though the precise method of cysteine modification in Keap1, which activates Nrf2, is not fully understood, there are two proposed models explaining this process. The first one is the “hinge and latch” model which suggests that Keap1 modification in thiol residues of Keap1 blocks the interaction with Nrf2. This results in Nrf2 lysine residue misalignment and inability to polyubiquitnate the transcription factor. In the second model on the other hand, thiol modification results in Cul3 dissociating from Keap1. In both models, modified by inducer and bound to Nrf2 Keap1, is inactive. Newly formed Nrf2 proteins bypass Keap1 and are translocated into the nucleus where together with the small Maf proteins, attach themselves to antioxidant response element (ARE) and induce expression of Nrf2 target genes[72] (Figure 1.5.).

Agents which regulate Keap1-Nrf2 pathway have been of recent interest as therapeutic targets for treatment of oxidative stress results.

Figure 1.5. Disruption of Keap1- Cul3 ubiquitination system in infected cells.



Under oxidative stress, the Keap1-Cul3 ubiqutiination system is disrupted. Nrf2 is released from the complex by a change in Keap1 cysteine disulfide bonds and translocated to nucleus. Nrf2 is not degraded anymore so it binds to a DNA promoter (Maf) in the nucleus and starts transcription of antioxidative genes and their proteins.[73]

1.3.2. Target genes

The protective mechanism of Nrf2 relies on inducing transcription of genes which reduce lung injury caused by oxidative stress. A number of genes have already been identified but modern technical advances have allowed definition of the transcriptional changes induced following Nrf2 induction and provided further data about direct target genes of Nrf2.[74] These genes include: 1. Intracellular redox-balancing proteins involved in heme and iron metabolism like heme oxygenase-1 (HMOX-1) or glutathione metabolism- glutamate cysteine ligase (GCL). 2. Phase II detoxifying enzymes involved in drug metabolism like NAD(P)H quinine oxidoreductase-1 (NQO1) 3. Transporters (multidrug resistance-associated proteins, MRPs)[75] as well as transcription factors, metabolic enzymes and antioxidants[76]. Antioxidant response element (ARE) is necessary for Nrf2 binding and gene induction and is a specific DNA sequence located on the promoter region of Nrf2 target genes. There are many other Nrf2 downstream genes which are responsible for other cellular processes like cell growth and death, inflammatory response, DNA repair and ubiquitin- mediated degradation pathway (Section 1.4.3.).[77] Nrf2 downstream genes are heterogenous in nature, which shows the importance of their role in detoxification and survival of cells.[78]

1.3.3. Existing evidence of Nrf2 importance

Over 200 diseases have been reported to cause oxidative stress in cells.[79] These include Chronic Obstructive Pulmonary Disease, asthma, various types of cancer and neurological diseases including multiple sclerosis and Alzheimer's, cardiovascular and metabolic disorders such as diabetes, vision disorders and ageing. In this project the focus is on infection with RSV, however, other viruses such as Humman Immunodeficiency Virus (HIV), Hepatitis B (HepB) and C (HepC) have been reported to stimulate reactive oxygen species (ROS) both in vitro and in vivo.[80] One of the characteristics of Nrf2, which demonstrates its importance, is its polymorphism. Various studies report its numerous gene variants and haplotypes appearing in different diseases. For example Arisawa et al described an Nrf2 gene promoter polymorphism and its relationship with Helicobacter infection in chronic gastritis.[81] Another paper by Cordova et al describes a particular genotype of Nrf2 (-653G/A) which plays an important role in nephritis during childhood-onset systemic lupus erythematosis (SLE).[82] Different haplotypes in the promoter region of Nrf2 have also been found in COPD by Hua et al.[83] Most important for this MPhil project, however, is the study by Cho et al stating that Nrf2 has antiviral properties in a murine model

of RSV. In their experiments, mice deficient in Nrf2 suffered from much more severe RSV induced disease in comparison to control mice. The severity was assessed on the basis of higher viral titers, augmented inflammation, enhanced mucus production and epithelial injury. It stresses the importance of Nrf2 mediated cellular antioxidant mechanism in pulmonary anti-RSV activity.[84]

The versatile role of NRF-2 in protecting different systems in the human body is shown in Figure 1.6.

Figure 1.6. Nrf2 as a multi organ protector in the body.[85]

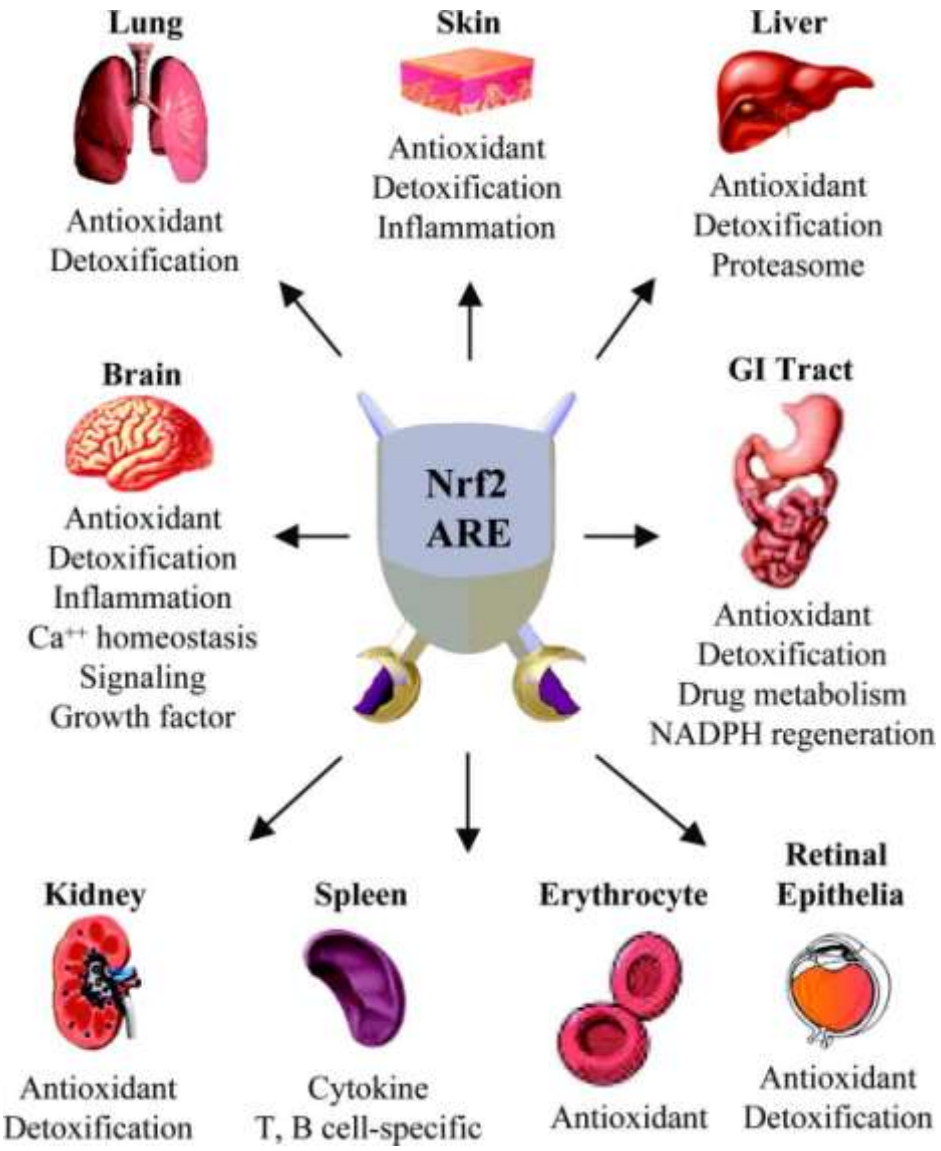


Diagram presents various organs and cell types protected by Nrf2 which increases ARE-driven detoxification and antioxidant genes transcription. Nrf2 is a crucial component of antioxidant pathways in respiratory and nervous system, skin, liver, gastrointestinal system, kidney, spleen, erythrocytes and retinal epithelia.

1.3.4. Nrf2- ARE pathway

In a normal cell, a part of aerobic metabolism is the production of reactive oxygen species (ROS) via respiration and oxidation to create energy. When the level of ROS is elevated (for example as a consequence of infection or exposure to toxins, environmental pollutant or radiation) harmful changes caused by oxidation occur in a cell. Respiratory viruses such as RSV, human metapneumovirus (hMPV) or influenza, stimulate production of ROS and decrease antioxidant enzyme (AOE) efficiency resulting in oxidative injury due to unbalance oxidative-antioxidants status. Nrf2 controls production of AOE by binding to antioxidant responsive element (ARE) in AOE gene promoters. When the cell is exposed to majority of pro-oxidant stimuli, Nrf2 is induced and AOE expression upregulated. During viral infections however, AOE expression is inhibited, Nrf2 nuclear localisation is reduced and transcription of ARE-dependent genes inhibited. For that reason, agents inducing Nrf2 or antioxidant mimetics are a possible therapeutic means of treating harmful effects of respiratory viral infections.[86] Nrf2-Are pathway has been examined in numerous studies, in which pulmonary disorders resulted from various antioxidant and inflammatory agents. These experiments were conducted on mice and showed that animals deficient in Nrf2,

in which the ARE-driven antioxidant expression is suppressed, have exacerbated lung inflammation and injury in comparison to control animals.[87]

It has been previously reported that Nrf2 expression is significantly reduced in RSV infection which might be a potential mechanism for reducing gene expression of AOE. This can be caused by a range of factors like reduced transcription or increased mRNA degradation. [88]

1.3.5. Nrf2 as a clinical drug target

A large number of studies proving how important Nrf2 is in protecting the human body against an array of diseases, lead to a huge interest in developing Nrf2 based therapies. Since pathogenesis of viral-associated lung disease including RSV infection is so strongly related to oxidative stress, agents with potential to regulate antioxidative pathways seem like a rational therapeutic approach to these diseases.[89] Antioxidants are known to quench free radicals, which decreases oxidative damage and enables cells to function normally. Komaravelli et al. tested two therapeutic approaches: Superoxide dismutase (SOD) mimetics, which decrease oxidative damage by interacting with free radicals and Nrf2 inducers which regulate AOE gene expression. A number of compounds, of both synthetic and natural origins, have been reported as stimulating Nrf2-ARE

influenced transcription. They can be broadly divided into two groups: Triterpenoids and isothiocyanates.[90] Triterpenoids originate from oleanolic acid, which itself has been reported to have antioxidative properties.[91] Isothiocyanates include Sulforaphane, mainly found in cruciferous vegetables like broccoli. It has been reported to change a number of cysteine residues in Keap1 by releasing Nrf2, which results in elevated nuclear localisation of Nrf2 and ARE transcription.[92] Kesic et al. showed increased levels of Nrf2 in epithelial cells treated with Sulforaphane before Influenza infection, which contributed to reduction in viral replication. [93] In a different study, mice treated with sulforaphane were shown to have reduced numbers of neutrophils and eosinophils after infection.[94] These findings imply that this compound has a big potential for regulating viral induced oxidative disease process.[95]

Nrf2 is known to be differentially ubiquitinated and the ubiquitinated form rapidly degraded by the proteasome to inhibit Nrf2 activity. Differential regulation of protein activity in a manner similar to Nrf2 occurs for many proteins but has never been studied in relation to viral infection. This type of modification could lead to both activation or inactivation of a protein and also translocation and movement of it in a cell.[96] In this thesis I first examined differential ubiquitination of Nrf2 in response to viral infection and then expanded this work to a

number of candidate genes identified by proteomics. These molecules are introduced at the beginning of Chapter 4.

1.4. Ubiquitin

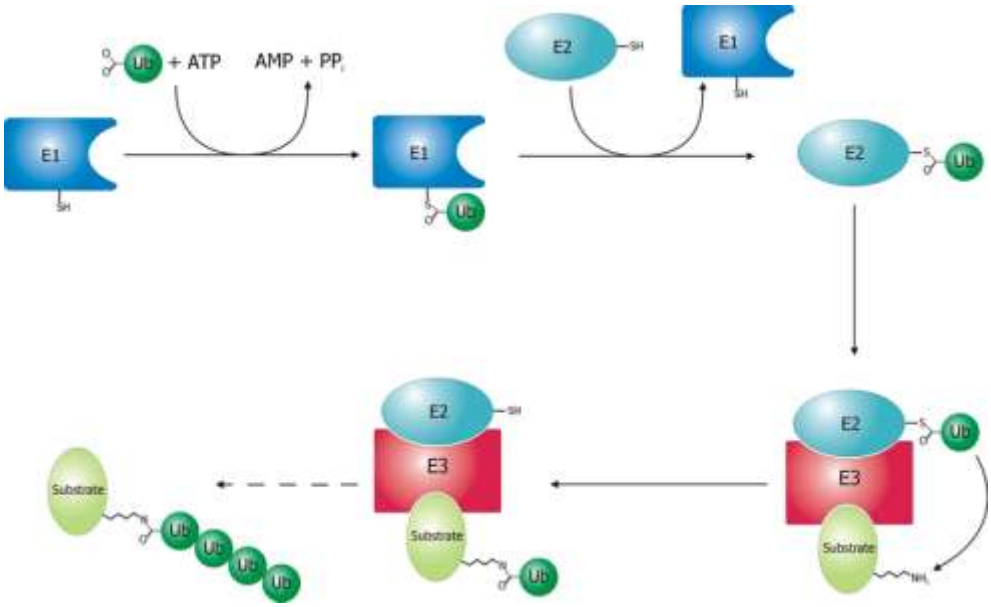
Ubiquitin (Ub) is a highly conserved protein, consisting of 76 amino acids. In a cell, Ub is linked to target proteins by covalent bonds, in a process called ubiquitination. Its name comes from its ubiquitous nature, as it is found in all eukaryotic organisms. [97] Ubiquitination is one of the best described post-translational alterations which controls protein expression and function.[98]

1.4.1. Ubiquitination process

The process is based on an enzymatic cascade. The first enzyme in the cascade is E1 which hydrolyses ATP, activates ubiquitin and transfers it to a cysteine of the second enzyme E2- a ub-conjugating enzyme. The final enzyme is E3 which creates an isopeptide bond between ubiquitin's carboxyl terminus and target protein.[99] E2 and E3 most often determine substrate selection. So far there are only a few E1 enzymes known in mammals, about 30 E2 enzymes and hundreds of E3 enzymes. The final product of the first stage of ubiquitination is the mono- ubiquitinated protein. Every ubiquitin molecule has a specific lysine which can be used to initiate attachment of consecutive ubiquitin molecules. The effect of this

process is a target protein with polyubiquitinated chain. Damaged or misfolded proteins are marked by ubiquitin and are transported to proteasome and destroyed in the ub-proteasome system (UPS).[100] Other ubiquitinated proteins (eg. transmembrane proteins) are transported to a lysosyme via the multivesicular body pathway (MVB).[101] UPS protein degradation is a key process in DNA damage repair, cell cycle regulation, cell development and immune system function.[102] It has also been reported that ubiquitin takes part in protein function and protein interaction with the help of specific hydrolazes. These structures have similar function as kinases and phosphatases in the phosphorylation process. The whole process is very versatile and can be reversed, influencing various properties of proteins, not only their stability.[103]

Figure 1.7. Schematic picture of ubiquitination process.



Schematic diagram of the ubiquitination system. Created by Roger B. Dodd. The first enzyme in the cascade is E1 which hydrolyses ATP, activates ubiquitin and transfers it to a cysteine of the second enzyme E2. E2 is a ub-conjugating enzyme. The final enzyme is E3 which creates an isopeptide bond between ubiquitin's carboxyl terminus and target protein, [104]

1.4.2. Molecule overview and interaction with viruses

The genes encoding for this protein are grouped in tandem repeats, due to high demands for transcription of this protein in all cellular processes. Ubiquitin is a very versatile protein thanks to its seven lysines and extra residues, used by Ub ligases to create different kinds of Ubiquitin chains on target proteins. This results in interactions with different downstream factors.[105] An example is the well known K-58 based linkage which results in proteasome-

mediated protein degradation or K-63 based control of protein endocytosis, as well as enzyme activity.[106]

Being obligated intracellular parasites, viruses have to overcome host cellular machineries at every stage of their life cycle including entry into the cell, replication and genome transcription, protein synthesis etc. up until release from the infected cell. Knowing how important ubiquitination is in those cellular processes, it is expected that ubiquitin and proteins affected by it, must play a part in viral life cycle and pathogenesis.[107]

The first report of viruses being capable of using the UPS for their own benefit was the Scheffner et al. study of small DNA tumour viruses and their ability to modulate cell cycle.[108] Since then, a number of other studies have proven that other viral families take advantage of ubiquitin conjugating system in their life cycle.[109] From this perspective it became obvious that studies involving experiments with proteasome inhibition are crucial, as such treatment not only inhibits the UPS but also removes the free ubiquitin from the cell which would influence all cellular pathways involving ubiquitin. Proteasome inhibitors have been reported to inhibit many human viruses like herpesviruses, poxviruses, adenoviruses, influenza viruses, retroviruses, coronaviruses, paramyxoviruses, picornaviruses and rotaviruses.[110] It has been shown in studies on

herpes simplex virus, influenza virus and adenoviruses, that ubiquitin modulates the first stage of viral replication- entry to the cell and viral capsid presentation to the target cell. [111][112] Other stages of viral life cycle such as gene expression in Epstein-Barr[113] virus or latency (property which enables the virus to cause lifelong infection process in host organism) in Kaposi's sarcoma-associated herpes virus (KSHV) are also affected by blocking the proteasome.[114]

Viruses can also take over the ubiquitin conjugating system to modulate host innate immunity signalling. They stop the induction of Type I IFN by binding to its receptor.[115] Viruses can challenge cellular ub-conjugating system by adjusting substrate specificity of ubiquitin ligases, changing which proteins are marked for degradation. Some viruses , especially large DNA viruses like poxvirus, are able to encode their own ubiquitinating enzymes, eg KSHV encodes two E3 Ub ligases.[116]

1.4.3. UPS- Ubiquitin Proteasome System

Ubiquitin Proteasome Pathways are a crucial method of protein catabolism. Proteins destined for degradation by the proteasome are marked with ubiquitin in the ubiquitination process but it is not fully understood how proteins are targeted by the proteasome. In order to be recognised by a proteasome cap, a protein of interest must have

a chain of at least 4 ubiquitins attached to it, so it must be polyubiquitinated.[117] Ubiquitin receptor proteins contain N-terminal ubiquitin like domain (UBL) and one or multiple ubiquitin associated domains (UBA). Proteasome caps recognise UBL domains and UBA is bound to ubiquitin by three-helix bundles.[118] Because of the large number of cellular processes that UPS regulates, failing of the system may result in various diseases. These processes include: antigen processing, apoptosis, cellular cycle and division, organelles biogenesis, transcription and repair of DNA, development and differentiation, inflammation and immune response, response to stress and extracellular modulators and most importantly for this thesis- viral infection.[119] The process can be divided into two steps: conjugation- attaching of the ubiqutin molecule, and degradation by the 26s proteasome consisting of the catalytic 20s core and the 19s regulator.[120] Recently it has become apparent that ubiquitination also plays a major role in DNA repair and endocytosis. These newly discovered roles are dictated by the type of ubiqutin chain linkage, as well as number of ubiquitin molecules attached – mono versus polyubiquitinated proteins. Linkage of covalent bonds between ubiquitin and target protein can also be reversed in a process called de-ubiquitination or de-conjugation by de-ubiquitinising enzymes (DUBs) which makes the whole conjugation/de-conjugation system

very dynamic.[121] The process has been recognised as crucial and Avram Hershko, Aaron Ciechanover and Irwin Rose who first discovered this were awarded the Nobel Prize for Chemistry in 2004.

1.5. Aims and Objectives

The work throughout my MPhil project was divided between two main objectives. The first set of experiments aimed to determine whether Nrf2 induction by sulforaphane might reduce replication of RSV in airway epithelial cells. Detailed description of these experiments together with results and discussion are included in Chapter 3.

As Nrf2 is known to be differentially ubiquitinated, I first examined differential ubiquitination of Nrf2 in response to viral infection and then expanded this work to proteins selected from the proteomics including DDX21, MAVS, HMGB1, prohibitin, Annexin A1 and RPS10. These experiments aimed to validate data generated in the proteomics about whether these proteins change their level of ubiquitination following RSV infection, as well as answer the questions whether those changes in ubiquitination might result in protein turnover by the proteasome as part of host cell defence or viral manipulation of cellular process to aid viral replication. Detailed description of these experiments and their results together with discussion are included in Chapter 4.

2. Methodology

2.1. Cell Culture

Cell types used during the project:

- A549 cells (adenocarcinoma derived human alveolar basal epithelial cells) normally responsible for substance diffusion in the alveolar epithelium of the lungs and often used in RSV infection model.[122]

- BEAS-2B (non-tumorigenic human bronchial epithelial cells)[123]

- Hep2 cells (human epithelial type 2 cells) believed to come from human laryngeal carcinoma, associated with various autoimmune conditions.[124]

Cells of each type were grown in Dulbecco's Modified Eagles Medium (DMEM, Sigma), supplemented with 10% Foetal Calf Serum (FCS, Sigma), L-glutamine 200mM (Sigma), Penicillin 10,000units/ml (Sigma) and Streptomycin 10mg/ml (Sigma). Cells were grown in an incubator with 5% carbon dioxide (CO_2) at 37°C. Every 2-3 days, dead cells were washed away with Phosphate Bovine Serum (PBS). Viable cells were harvested from the bottom of the flask and sub-cultured with fresh media at a concentration of $1x10^6$cells/ml with 15ml of fresh media in T75 flasks. All cell lines used in this study were mycoplasma free

2.1.1. Seeding the cells.

For each experiment stock cells were seeded as follows:

Media was removed from T75 flasks of cells grown in an incubator. Cell monolayers were washed once with 5ml of PBS. 3ml of 0.25% trypsin 0.02% Ethylene-diamine-tetraacetic acid (EDTA, Sigma) was then added to flasks, which were then incubated at 37°C for 4-5 minutes. Flasks were tapped to allow cells to detach from the bottom of the container. 7ml of FCS supplemented media (L- Glutamine, Streptomycin + Penicillin) was subsequently added to neutralise and deactivate trypsin. Cells were then centrifuged at 1600rpm at room temperature for 10 minutes and the supernatant removed and discarded.

The pellet was resuspended in 1ml of supplemented media and the number of cells measured using a haemocytometer (Section 2.1.2). Depending on the specific experimental conditions used, cells were diluted with supplemented media and pipetted into each flask or well with a correct amount of media.

2.1.2. Counting the cells

Cells were counted using a haemocytometer as follows. Cell monolayers were washed once with PBS and scraped with a cell scraper. Contents of the dish were transferred into a universal

centrifuge tube and the dish washed twice with PBS, to make sure that the maximal number of cells from the dish was in the tube. Cells were spun down in a centrifuge for 10 minutes at 1600rpm at room temperature and the supernatant then discarded. Cell pellets were next resuspended in 1ml of supplemented media and 10µl of this solution put on the haemocytometer and viewed under a microscope. Cells in each of the big corner squares consisting of 16 little squares were counted and the number averaged. This gave a number equivalent to the cell count in 10^4/ml.

2.1.3. Harvesting the cells

Depending on the experiment, cells were harvested at 4, 24 or 48 hours, using the following method.

Cells were washed once with PBS. Small amounts of PBS (according to the surface of the dish, eg. 0,5 ml for a well in a 6 well plate, 3 ml for a T75 flask) were added to each flask and cells carefully scraped from the bottom of the dish. The whole surface of the dish was scraped, in order to maximise the number of cells collected. Scraped cells in PBS were transferred into a 10 ml universal centrifuge tube and the dish (flask/well) washed twice with PBS, to maximise the number of cells collected. Tubes were spun in a centrifuge for 10 minutes at room temperature at 1600 rpm. Supernatant was taken off and discarded and the cell pellet resuspended in 1ml of PBS.

According to the experiment and desired number of samples, cells were divided between micro centrifuge tubes in 100µl of solution, and spun again in a microfuge for 10 minutes at 1330 rpm. Supernatant was taken off and discarded, leaving dry pellets which were stored in labelled tubes at -30°C for future use.

2.2. RSV preparation

All virus stock used for the experiments in my project was made using the following method.

2.2.1. RSV propagation

Day one:

Hep 2 cells were seeded at $3x10^4$ cells/cm in 15 ml of supplemented media in a T75 flask and incubated at 37°C in 5% humidity for 24 hours (or longer if not 50% confluent after 24 hours).

Day Two:

Once 50% cell confluence was reached, media was removed and cells were washed twice with 5ml PBS. 500µg of RSV stock was placed in 3ml of serum-free media and added to the flask with cells. Cells were then incubated on a rocker for two hours at 37°C to make sure that virus particles spread evenly over the whole flask surface.

After two hours, 13ml of supplemented media was added to the flask, which was then left overnight in the incubator at 37°C.

Day Three:

Flasks were inspected under the microscope and media changed. Flasks were again stored in the incubator at 37°C overnight.

Day Four:

Forty-eight hours post-infection, cells were harvested as described above using a cell scraper. These steps were carried out rapidly with the samples kept on ice to ensure that the virus maintained its integrity and did not degrade.

In a cooled centrifuge at 4°C, harvested cells were spun down at 1600rpm for 10 minutes in 50ml tubes. Supernatant was taken off and placed in separate tubes on ice. 2ml of the removed supernatant was used to resuspend the cell pellet and mixed by vortex. Solution was evenly split into two micro centrifuge tubes tubes and kept on ice. Cells were then lysed using a 25 gauge needle and 1ml syringe for ten passes, to burst cells open and release RSV. Next, 500µl of solution was transferred to four pre-labelled cryovials and snap frozen in liquid nitrogen. Vials were then stored at -70°C.

2.2.2. RSV Plaque Assay

In order to establish the number of virus Plaque Forming Units (PFU) per ml of solution, the following method was used for each batch of virus in this project.

Day One:

Using 27 wells of a 96-well flat bottomed plate, 2 x 10^4/ml A549 cells were seeded per well and grown in supplemented DMEM media in an incubator for 48hrs at 37°C.

Day Three:

After 48 hours, serial dilution of RSV was prepared using the following method.

Micro centrifuge tubes tubes were placed on ice to prevent virus degradation. 500µl of serum free DMEM media was placed in one tube and 250µl in seven others. A water bath was warmed to 37°C. A vial of RSV was removed from the freezer, and snap thawed in water. In order to achieve 1:100 dilution in the first tube, 5µl of virus solution was added to the media and mixed well. 250µl out of this solution was then transferred to the second tube and doubling dilutions prepared until the last, eighth tube.

Cell monolayers in each well were washed once with PBS, and 50µl of each dilution of virus added to wells in triplicate going horizontally. Plates were incubated at 37°C. After two hours, 100µl of supplemented media was added to each well and plate put in the incubator until the next morning.

Day Four:

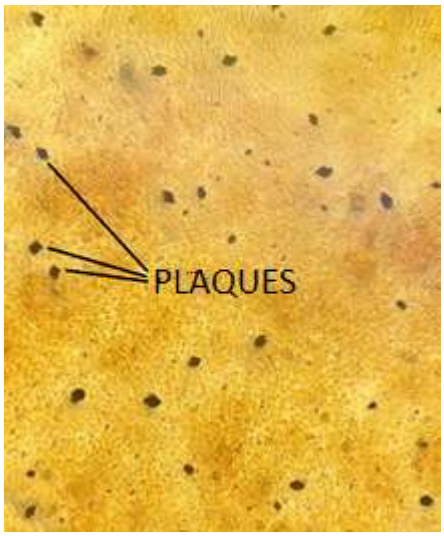
Each well was washed once with 100µl of PBS and cells fixed for 20 minutes at room temperature with 100µl of 100% methanol containing 2% hydrogen peroxidase. Using a multi-channel pipette, cells were washed gently with 100µl of PBS per well. Pipetting the solution directly onto the cell monolayer was avoided, in order not to disrupt it.

After 20 minutes, 100µl of goat anti-RSV antibody (Bio-rad), diluted 1/200 with PBS/1% Bovine Serum Albumin (BSA), was added to each well and incubated at room temperature for one hour. Each well was then washed twice with 100µl PBS/1% BSA. 100µl of extravidin peroxidise (2mg/ml, Sigma-Aldrich) diluted 1/500 with PBS was subsequently added and left for 30 minutes at room temperature. Cells were washed twice with 100µl PBS/1% BSA. A Sigma-Fast Red Tablet (TR/Naphthol AS-MX) was diluted in 5 mls of PBS. 50µl

of this solution was then added to each well for approximately 10 minutes to stain plaques.

After the plaques appeared, PBS was added in order to stop reaction and the plaques were counted. Dilutions which produced around 100-200 plaques per well were selected and each replicate was counted to estimate the average value. In order to decrease the possibility of counting error, plaques in dilutions above and below were also counted and the number averaged. The whole process was repeated by two people separately and the values compared and averaged. The RSV plaque assay is shown in Figure 2.1.

Figure 2.1. Microscopic image of RSV plaques formed during the plaque assay protocol.



RSV plaques indicated with lines.

In order to count the PFU per ml of solution, the following formula was used:

Number of plaques x dilution (eg. 100, 200, 400 etc) x 20

The dilutions for each well were as follows:

Dilution 1- 1/100

Dilution 2- 1/200

Dilution 3- 1/400

Dilution 4- 1/800

Dilution 5- 1/1600

Dilution 6- 1/3200

Dilution 7- 1/6400

Dilution 8 1/12800

In order to use the right amount of virus for each experiment, Multiplicity of Infection (MOI) was calculated for each batch. Ratio between the number of cells in a particular dish and the number of RSV particles was calculated by dividing the number of RSV particles over the known cell number in the well/flask.

2.2.3. RSV Infection

BEAS- 2B and A549 cells were seeded in the correct sized plates or flasks as explained in Section 2.2.1. Cells were usually ready to be infected after 48 hours in the incubator when they reached 90% confluence. The correct number of vials was removed from the -70°C freezer and snap thawed in water bath at 37°C. Those steps were carried out promptly in order to avoid RSV degradation. Cells were washed once with PBS and new media added to the flasks. Correct amount of virus for each plate or flask was then diluted in serum free media and added to the dishes with cells.

Depending on experiment, cells were left in the incubator at 37°C until the harvest time (usually 4, 24 or 48 hours).

2.3. BCA protein Assay

The bicinchoninic acid assay (BCA assay) or the Smith's assay was carried out on the samples destined for western blotting, in order to assess protein concentration in each sample and ensure that protein loading on gel was even. Samples were prepared in the following micro plate procedure and protein concentration calculated by a colorimetric scanner.[125] Pierce™ BCA Protein Assay Kit produced by Life Technologies was used in this project.

Samples for the procedure were prepared by adding 100µl of 1% protease inhibitor (Sigma) and 99% protein extraction reagent (CytoBuster™ Protein Extraction Reagent) to each dry pellet taken out of the freezer. A total of eight standards were made by serial dilution of 500µl of Albumin Standard Ampules (2mg/mL, 10 x 1 mL) in each tube, giving the concentrations of 2000, 1000, 500, 250, 125, 62.5, 31.25 and 0. 25µg of each sample, and standard was pipetted into a 96 microplate well in a working range of 20-2000 . 25µl of each sample was added in duplicates horizontally on the plate, diluting the samples as appropriate in sterile distilled water (23µl H^2O+ 2µl sample= 1:12.5 dilution)

Working reagent was made up to 1:50 dilution, 1 part of solution B (green) and 50 parts of solution A (colourless). 200 µl of this mixture was carefully added to each well and plate left for 30 seconds on a plate shaker in room temperature to mix thoroughly. Each plate was then covered and incubated at 37°C for 30 minutes. After 30 minutes, the plate was left on the bench in room temperature to cool down for about 5 minutes and read with a plate reader at or near 562 nm.

The amount of sample loaded on the western blot gel was adjusted accordingly to the least concentrated sample.

2.4. RNA extraction

Extraction and isolation of RNA methodology was carried out as described by the manufacturers of TRIzol® reagent (Life Technologies) and is described below.

2.4.1. Homogenising sample

Culture supernatants were taken off from the cells and stored at -20°C for future experiments. Samples were homogenised using TRIzol® reagent (a monophasic solution made up of phenol and guanidine isothiocyanate)[126].

0,5ml of TRIzol® was used per well containing 5-10x10^6 cells or per dry pellet stored in a freezer. This amount of TRIzol® was used to make sure that there was no DNA contamination within the isolated RNA. Each sample was carefully pipetted up and down a number of times to ensure that cells from the whole surface of the well were taken into the solution. The tubes with dry pellets were mixed using vortex for 2 minutes and each of the samples were placed in an individual, labelled tube.

2.4.2. Phase separation and precipitation

Samples were incubated for five minutes at room temperature, to allow nucleoprotein complexes to completely dissociate. 200µl of chloroform was the added to each sample, which were then mixed thoroughly by vortex for 10 seconds and centrifuged at 13,300rpm for 15 minutes at room temperature.

Samples separated into the following three layers:

1) Bottom pink phenol-chloroform phase
2) Middle interphase
3) Top colourless aqueous phase with the RNA

The top layer was carefully removed using P200 pipette at 45° angle. Removing the middle or bottom layer was avoided, as that would result in DNA contamination of the sample. Contents of each tube were placed in new tubes containing 250µl of isopropanolol with corresponding labels. Samples were then mixed by vortex and placed in -70°C freezer for ten minutes, before being centrifuged at 13,300rpm for fifteen minutes. Putting samples in the centrifuge in the exact same position, allowed for the gel-like pellet formed at the bottom of the tube to be easily identified.

2.4.3. RNA wash

Supernatant was removed from each tube, carefully avoiding touching the pellet with the pipette tip and discarded. 200µl of 70% ethanol was added to each tube and mixed by vortex for fifteen seconds. Samples were microfuged at 13,300rpm for 10 minutes. Supernatant was then removed carefully with the pipette tip and the tubes left open to make sure that the pellets air dry for approximately fifteen minutes. Once the pellets were dry, they were resuspended in 20µl of sterile, nuclease-free water.

2.5. Reverse transcription

Complementary DNA (cDNA) was prepared with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) using random primers. To each 200 µl tube labelled accordingly, the following components were added from the kit:

2µl 10x RT Buffer

1µl 25x dNTP Mix (100 mM)

2µl 10x RT Random Primers

5.2µl sterile, nuclease-free water

0.8µl of Multiscribe Reverse Transcriptase

10µl of the corresponding sample (RNA solution).

The contents of the tubes were reverse transcribed at 37°C for one hour.

2.6. PCR

PCR (Polymerase-chain reaction) is a method used to amplify the desired region of DNA, with the purpose of making multiple copies of that sequence. In this project, PCR was used to assess the presence and amount of RSV in the samples.

2.6.1. Principles of PCR

The process of PCR amplification can be divided into four phases:

1. Baseline - no signal is released during the cycles because the amplification level is too low to be detected by the quantitative PCR (qPCR).
2. Exponential - amplicons are quantified, signal is above the detection level and the product should double exactly every cycle to produce the number of amplicons if the assay is 100% efficient.
3. Linear - the efficiency of amplification is reduced to less than 2 per cycle because the amount of reagents goes down with their use.
4. Plateau - after all the reagents have slowly been used, the reaction ceases and no more products are synthesised.

During the second phase, the threshold level line is set. This line is the point at which fluorescent signal is detected against the background. Cycle number is quantified when the sample signal goes above the line and that gives the result obtained from q PCR, called the Cycle Threshold (Ct).[127]

2.6.2. Reagents and processing of PCR

TaqMan assay kit by Applied Biosystems was used for the qPCR in this project. For every PCR, RSV and the housekeeping gene (L32) TaqMan probes were used. The probes are linear oligonucleotides, which match specific gene sequence located between forward and reverse primers. Each of the probes was diluted in TaqMan gene expression Master Mix, which contains DNA polymerase. It allows copying the strand of the cDNA template after the primers have attached onto it. The PCR plate was prepared when reverse transcription was taking place, using a 96-well plate which fits into the LightCycler 480 machine.

For the Life Technology probes, 1.25µl of readymade probe was mixed with 12.5µl of TaqMan gene expression master mix (Applied Biosystems) per each well. RSV probe was designed on the basis of Dewhurst-Maridor et al paper[128] and ordered from Sigma. For every PCR, it was mixed using three different primers and Nuclease-free water as follows:

RSV AF (forward) 10µl

RSVAR (reverse) 10µl

RSVN-TAQ 40µl

RNA free water 40µl

1.25µl of this mixture was used in the same way as Life Technology probes, with 12.5µl of TaqMan gene expression master mix per each well. 13.75µl of probe mix (1.25 probe and 12.5 master mix) and 11.25µl of diluted cDNA (20µl of cDNA and 180µl of Nuclease free water) was pipetted into each well required for the PCR, making up 25 µl in total. The duplicates of samples were made for each of the probes, so that the values can be averaged during the analysis in order to limit pipetting error. L32 housekeeping gene was used as a control, as RSV infection does not affect its expression in A549 cells. A plate was carefully covered with film and spun down in a microfuge for 15 seconds to ensure all the liquid was at the bottom of the wells. Finally, the plate was placed in the LightCycler 480 Real-Time pCR machine and read with the MxPro software.

2.7. Western blots

Western blotting, also known as immunoblotting, is a widely recognised scientific technique, used to isolate a single protein of interest from a biological sample. Briefly, the technique aims to transfer biological samples from gel onto a membrane by electrophoresis and then detect proteins on the surface of this membrane.

2.7.1. Background and theory

The process can be divided into two steps:

1. Tissue preparation - cells in a sample are broken down by various mechanical and biochemical techniques, such as centrifugation and by using buffers. Protease and phosphatase inhibitors are added to the sample at this stage, to prevent digestion of proteins by enzymes within the sample. This process is carried out at low temperatures (samples are kept on ice), to avoid protein denaturation and degradation.
2. Gel electrophoresis - proteins are separated according to their isoelectric point, molecular weight or electric charge. Samples are then loaded on gel next to a standard, which will allow identifying molecular weight of proteins by creating visible coloured bands. Voltage is applied along the gel and proteins start to migrate through it at different speeds, depending on

their size and weight. These different rates allow for the proteins to be separated into bands within each lane.

The proteins are then moved from the gel on to nitrocellulose/ polyvinyle membrane, to make them accessible for antibody detection by electroblotting. The membrane is next blocked, in order to avoid interaction between membrane and the antibody used for protein detection. Protein in dilute solution attaches to the membrane in all places where target proteins have not attached. When the antibody is added, it attaches to the binding site of the specific target protein only. In order to detect protein, the membrane is probed with modified antibody linked to a reporter enzyme. When exposed to appropriate substrate, the enzyme drives a colorimetric reaction and produces colour. First, the primary antibody binds to protein. Next, the secondary antibody, which has an enzyme visible under chemiluminescence, binds to the primary antibody. The enzyme most commonly used is horseradish peroxidise and produces luminescence in proportion to the amount of protein present.

2.7.2. Protocol

Cells were previously isolated and stored as dry pellets.

The required amount of lysis buffer was prepared and 100µl was added to each dry pellet in an Micro centrifuge tubes tube.

Lysis buffer:

89% Bromophenol blue (Sigma)

10% DTT-(Dithiothreitol) (Sigma)

1% Protease inhibitor (Thermo-Scientific)

Micro centrifuge tubes were boiled in a heating block for 3 minutes at 100°C and mixed by vortex every 60 seconds. Samples were loaded on to the gel, one sample for each numbered well, leaving the first well empty to be loaded with standard (Bio-rad).

The pre-cast gel (Bio-rad) was removed from the storage pouch and the comb taken out gently, not to disrupt the wells. Gel cassette was placed in electrophoresis module and tank filled with Running Buffer.

Running Buffer:

Tris Base 3,03g (Fisher)

Glycine 14,1g (Fisher)

Sodium dodecyl sulphate (SDS) 1g (Bio-rad)

Sterile water- up to 1L

Leads were connected to the tank and voltage was set to 160 volts. Gel was run for approximately 40 minutes, but this time was adjusted according to the size of band of interest.

The gel was then separated from the plates by slicing along the sides of gel cassette and the edges were cut, so that area containing proteins fits onto Polyvinylidene fluoride (PVDF) membrane. The gel was placed on membrane (Trans-Blot Turbo Mini PVDF Transfer Pack, Bio Rad) between two filter papers soaked in transfer buffer (already prepared in a pack). The membrane was carefully rolled between two filter papers, to remove air bubbles and allow an even transfer of proteins from membrane onto gel. A cassette was locked, placed in a semi dry transblot machine and run on mixed Molecular Weight (MW) protocol. After 7 minutes, the cassette was taken out of the machine and blotting sandwich disassembled. PVDF membrane was cut and after discarding the areas not containing transferred proteins, immediately placed in a dish with blocking buffer (TBS-T + 5% milk). These steps were carried out very promptly, to prevent the membrane from drying out.

The membrane was incubated with a blocking buffer for one hour at room temperature, under gentle agitation on orbital shaker. Next, the membrane was washed with TBS-T (Tris-Buffered Saline and Tween 20) on an orbital rocker, at room temperature, twice for 30 seconds, twice for 5 minutes and 1 time for 15 minutes. Primary antibody solution was made using TBS-T and 5% milk. The membrane was then incubated with the solution overnight at -4°C under gentle

agitation. The whole area of the membrane was covered with a solution and the dish covered with lid or parafilm, so that the solution does not evaporate. The names and concentrations of all primary antibodies can be found in Table 1.

The next morning, primary antibody was removed and the membrane washed, in the same way as previously described. The membrane was incubated with secondary antibody solution using TBST and milk, with addition of secondary standard component (Bio-rad) at the concentration of 1:5000 and incubated for 1 hour at room temperature on orbital shaker. The membrane was afterwards washed with TBST using the exact same protocol (2x 30 seconds, 2x5 minutes, 1x 15minutes). After washes, the membrane was carefully transferred onto cling film. Chemiluminescence solution was prepared by adding 400µl of solution A and B into one Micro centrifuge tubes tube and carefully mixing together (Chemiluminescent substrate, Li-Cor). This solution was then pipetted onto the protein side of the membrane, ensuring that it covers membrane evenly and incubated for 5 minutes. Excess chemiluminescence was removed and membrane placed face down on scanner (Li-cor). The blot was scanned using Image Studio Light Version Software and membrane stored in TBST or re-probed with different primary and secondary antibody if needed.

2.8. Immunofluorescence

Protocol for this procedure was based on the technique used by Calo et al to stain for DDX21.[129]

A549 cells were seeded into 24-well plates, containing 12-mm glass coverslips and cultured for 24 hours in supplemented DMEM media. Cells were infected with RSV at multiplicity of infection (MOI) of 1:1 for 4 hours. After that, cover slips were washed once with 1ml of PBS per well and transferred to a new, clean 24-well plate. Cells were fixed in 4% paraformaldehyde for 10 minutes at room temperature and washed three times for 5 minutes with sterile PBS. Cells were then fixed with ice cold methanol (previously placed in -80°C freezer and transferred onto ice) for 2 minutes and washed twice with PBS, each wash lasting 5 minutes. Cells were then permeabilised in PBS containing 0.3% (v/v) Triton X-100 for 5 minutes and blocked in PBT buffer (PBS with 1% BSA, 0.1% Triton X-100(v/v), 0,05% sodium azide (w/v)) overnight at 4°C.

The next morning, coverslips were incubated in PBT with corresponding antibody for 2 hours at room temperature. The concentrations of antibodies were acquired from the products' datasheets and are listed in Table 1 together with products' codes. After the incubation period, cover slips were washed three times for 5 minutes with PBT and incubated with corresponding secondary

antibodies for one hour. The cells were washed 3 times for 5 minutes with PBT and two times for 5 minutes with PBS. Finally, cells were mounted onto glass slides using VECTASHIELD mounting medium with DAPI.

2.8.1. Confocal Microscope

Slides were viewed under confocal microscope. Three pictures of each slide were taken, one using low power lens and two using high power lens with immersion liquid (Type F Immersion liquid, Leica). Colour threshold was first adjusted to corresponding control antibody (anti-mouse for RPS10 and Annexin A1 and anti-rabbit for DDX21) to avoid creating false positives.

2.9. Immunoprecipitation

Immunoprecipitation is a technique used to precipitate antigen out of solution, using antibodies that specifically bind to the proteins of interest. This technique allows the isolation of a single protein out of mixture and requires solid substrate to bind to antibody at some point during the procedure. In this case, magnetic beads were the substrate. The immunoprecipitation of samples used for the western blot analysis was carried out with Dynabeads® Protein G Immunoprecipitation Kit, according to the protocol provided by the manufacturer and involved the following steps:

2.9.1. Antibody binding

A bottle of Dynabeads (ThermoFischer Scientific) was placed on a rotating shaker for 5 minutes to completely resuspend the beads. 50µl of solution was added to the new, labeled Micro centrifuge tubes tubes and put on magnet to remove supernatant. Beads were then resuspended in 200 µl of Antibody Binding and Washing Buffer and the antibody of choice (1.25 µl of antibody per each tube).Tubes were incubated with rotation at room temperature for 10 minutes. Supernatant was removed again by placing tubes on a magnet and the beads-antibody complex washed with 200 µl of Antibody Binding and Washing Buffer.

2.9.2. Antigen immunoprecipitation

Supernatant was removed by placing the tubes on the magnet and 50µl of antigen containing sample was added to the beads-antibody complex and carefully resuspended by pipetting. This solution was incubated for 30 minutes with rotation at room temperature, to allow binding of maximal number of antigen-antibody particles. Supernatant was then removed and put into new tubes, with corresponding labels. Beads-antibody-antigen complex was washed 3 times with 200µl of washing buffer and mixed carefully by pipetting

each time. After that, tubes were either frozen at -30°C with 100 µl of the washing buffer or immediately used for western blot.

Table 1. Names and product codes of antibodies used for Western blots and immunofluoroscopy.

Name of the antibody	Company and product code	Concentration used
Anti- beta actin	Abcam Ab8226	1 in 5000
Goat Anti-Mouse IgG HRP	R&D HAF007	1 in 1000
Anti- Nrf2	Abcam Ab62352	1 in 2000
Human HMGB1	R&D MAB1690	1 in 1000
Anti-MAVS	Abcam Ab25084	1 in 10000
Goat Anti- Rabbit IgG HRP	R&D HAF008	1 in 1000
Anti- Annexin	Abcam Ab118060	1 in 2000
Anti- DDX21	Abcam Ab182156	1 in 5000
Anti- RPS10	Abcam Ab151550	1 in 5000

3. Nrf2 signaling in RSV infection

3.1. Introduction

Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), transcription factor regulating antioxidant protein expression, has an important role in preventing pulmonary oxidative damage. The protective pathway is believed to involve antioxidant response element (ARE)-mediated gene induction. Animal studies, show exacerbated lung inflammation and damage in Nrf2 deficient animals compared to those that express Nrf2. It has been suggested that Nrf2 induction may offer a future treatment approach for RSV disease.[130] Sulforaphane is a potent, naturally occurring inducer of Nrf2 found in vegetables such as broccoli.[131] In this study, I sought to determine whether Nrf2 induction by sulforaphane might reduce RSV replication in airway epithelial cells. (Section 1.1)

The Hypothesis examined was: Induction of Nrf2 in RSV infected airway epithelial cells inhibits RSV replication.

Specific aims which lead to answering the hypothesis were:

1) To determine what is the best antibody to detect Nrf2.
2) To determine what is the best cell line to conduct experiments with Nrf2.

3) To determine what is the best concentration of sulforaphane to induce Nrf2.
4) To determine what is the best time to add sulforaphane to samples and its kinetics of expression.
5) To determine whether sulforaphane induces expression of Nrf2
6) To determine whether induction of Nrf2 in bronchial epithelial cells inhibits RSV replication.

3.2. Results

Western blot and Real Time q-PCR analysis results.

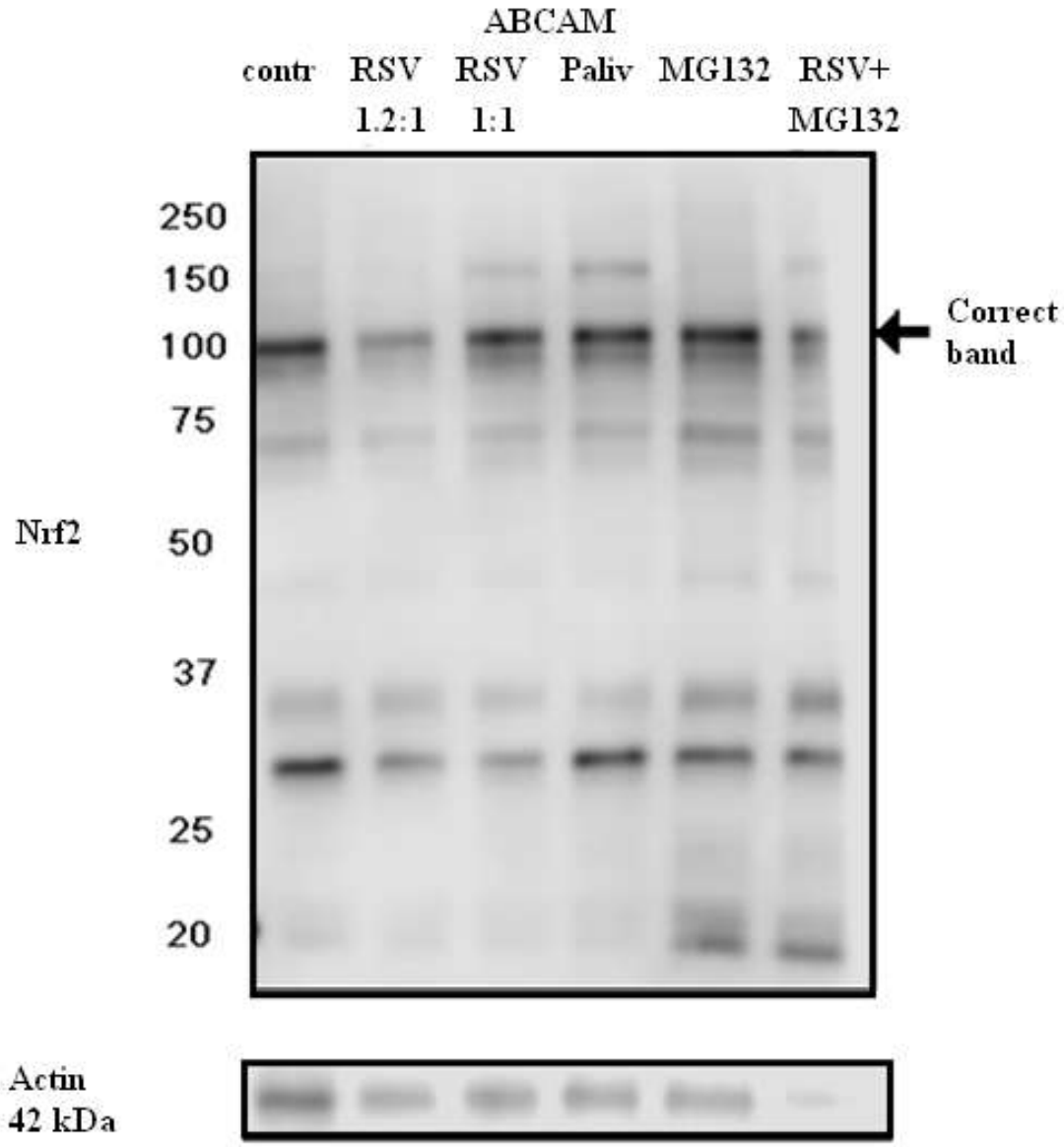
3.2.1. Validation of antibodies.

While choosing reagents for the experiment, it became apparent that there is a wide variety of companies offering different anti-Nrf2 antibodies on the market. It was important to choose one giving most reliable results. On the basis of conversations with colleagues conducting research on Nrf2 and a literature search, 3 different antibodies were chosen for the initial optimisation. Each antibody is produced by a different, widely recognized company (Abcam, R&D, Santa Cruz). The Santa Cruz antibody was kindly provided by Prof Chris Sanderson from the University of Liverpool others were

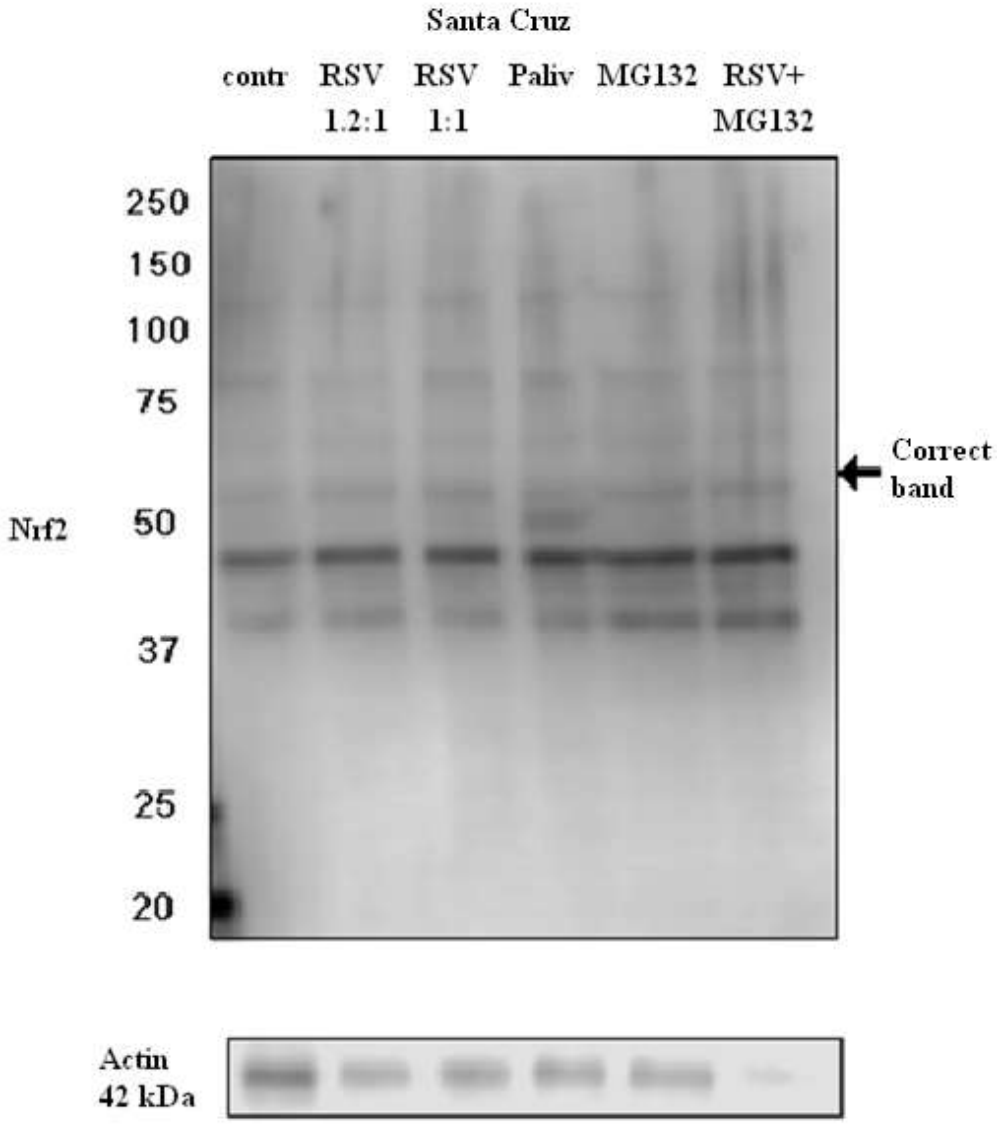
purchased from the supplier. Figure 3.1. shows western blot analysis of the comparison of antibodies.

Figure 3.1. Anti-Nrf2 antibodies comparison on BEAS- 2B cells.

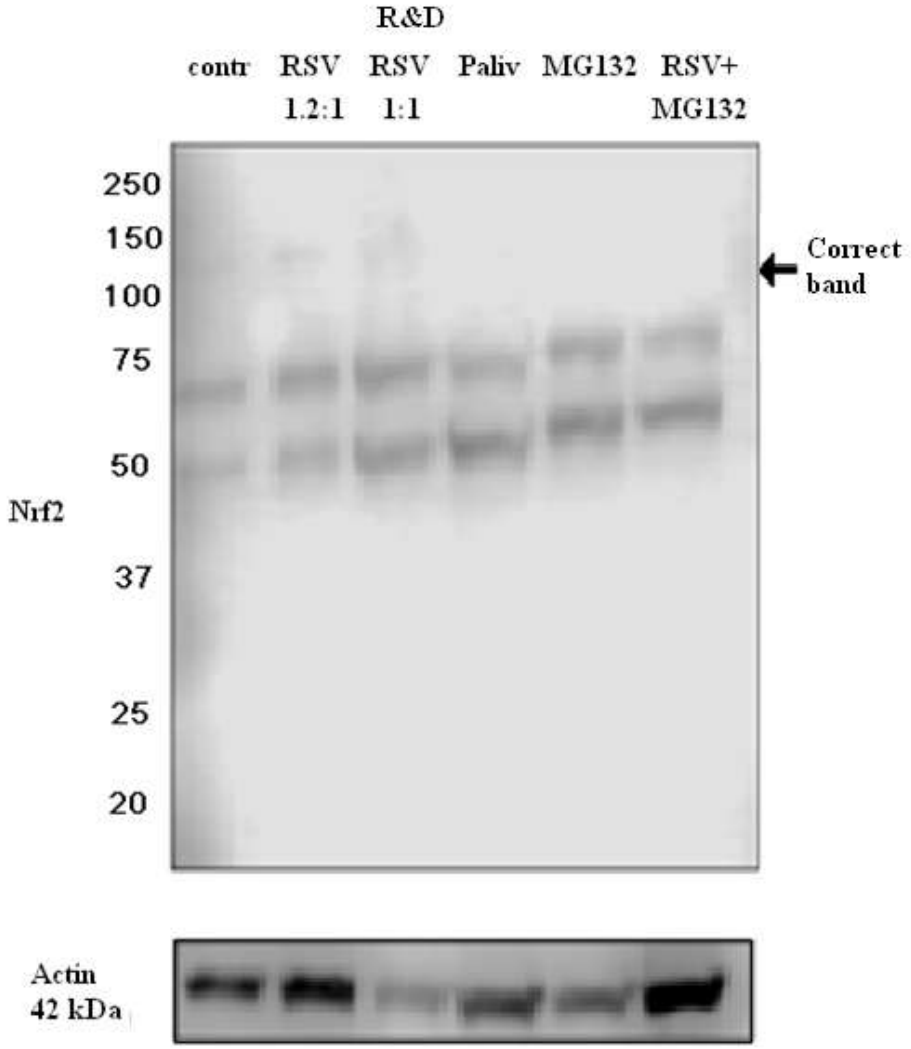
a) Abcam antibody



b) Santa Cruz antibody



c) R&D antibody



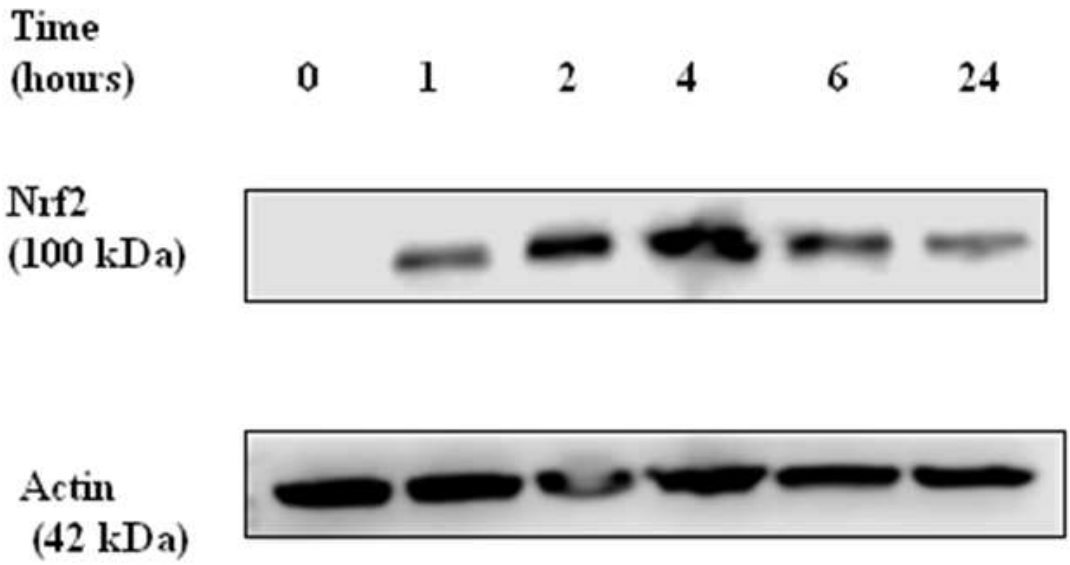
Western blot analysis showing changes in expression and pattern of Nrf2 bands detected by 3 different anti-Nrf2 antibodies produced by a) Abcam b) Santa Cruz and c) R&D. Each antibody was used on the same set of samples at concentration suggested by the manufacturer on the data sheet. The correct band for Nrf2 is marked with an arrow on the first blot (~100 kDa). Actin band of 42kDa was provided as loading control. Molecular weight markers are presented on the left side of blots.

Results show different pattern of detected Nrf2 bands for each antibody. Santa Cruz and Abcam antibody detected multiple additional bands most probably due to non-specific binding. R&D antibody detected bands at the level of ~60/70 kDa, which are not recognizable as Nrf2 bands. [132] Based on literature search and opinion of experts in the field of Nrf2 research, antibody selected for the future experiments was rabbit monoclonal Anti-Nrf2 abcam (ab62353) antibody which detected a desired band of 100kDa. (Research papers further discussed in Section 3.3).

3.2.3. Time course

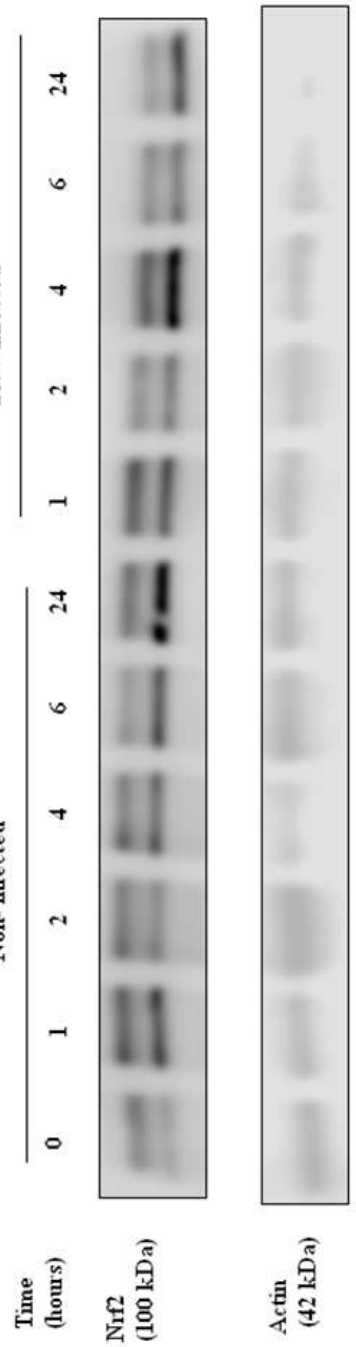
In order to establish the optimal time for Nrf2 induction by sulforaphane, time course experiment was carried out on RSV infected and non-infected BEAS 2B cells. Both experimental conditions were used to show whether time needed to induce Nrf2 expression by sulforaphane changes with infection. Based on the results from previous experiments, cells were incubated with 5nM of sulforaphane. Time course results on non-infected cells are shown in Figure 3.2 and on both conditions in Figure 3.3.

Figure 3.2. Induction of Nrf2 by sulforaphane in non-infected BEAS 2-B cells. Time course experiment.

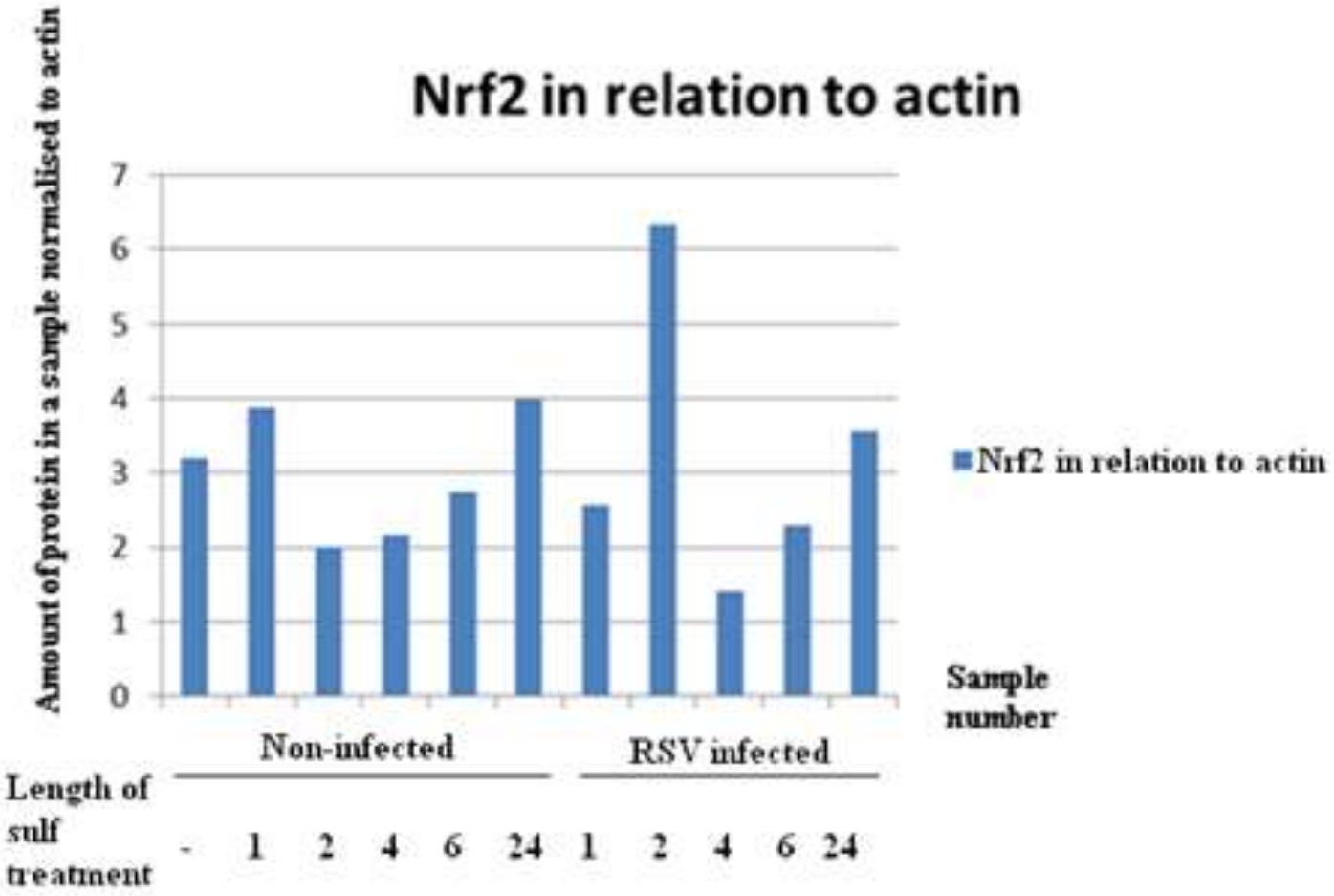


Western blot analysis of changes in expression of Nrf2 by BEAS 2-B cells after stimulation with 5 nM of Sulforaphane for various amounts of time, ranging from 0 to 24 hours. Expression of Nrf2 was analysed with rabbit monoclonal Anti-Nrf2 abcam (ab62353) antibody. Actin band of 42 kDa provided loading control.

Figure 3.3. Induction of Nrf2 by sulforaphane in non-infected and RSV infected BEAS 2-B cells. Time course experiment.



Western blot analysis of changes in Nrf2 expression by BEAS-2B cells with and without infection by RSV, after stimulation with 5 nM of sulforaphane for different amounts of time, ranging from 0 to 24 hours. Cells were infected with 1:1 MOI of virus at 0 hours. Expression of Nrf2 was analysed with rabbit monoclonal Anti-Nrf2 abcam

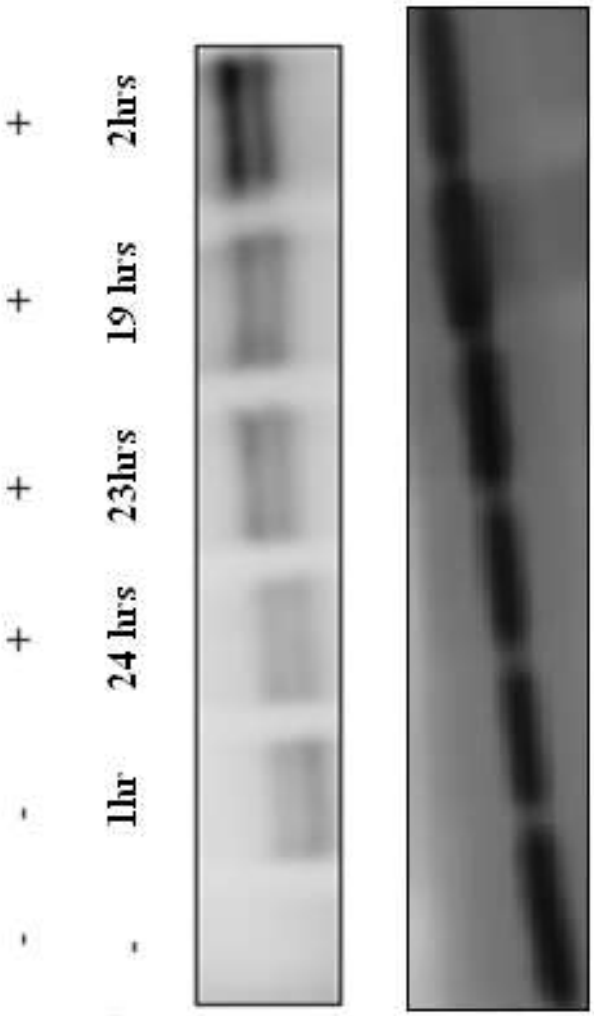


Densitometric analysis of this western blot bands intensities was performed using Li-cor Imgae Studio Lite Versio 3.1. software. Nrf2 bands were normalised to loading control actin band and are presented in the graph above. Cells were infected with 1:1 MOI of RSV at 0 hours and treated with 5 nM of sulforaphane for different amounts of time, ranging from 0 to 24 hours. Cells were infected with 1:1 MOI of virus at 0 hours.

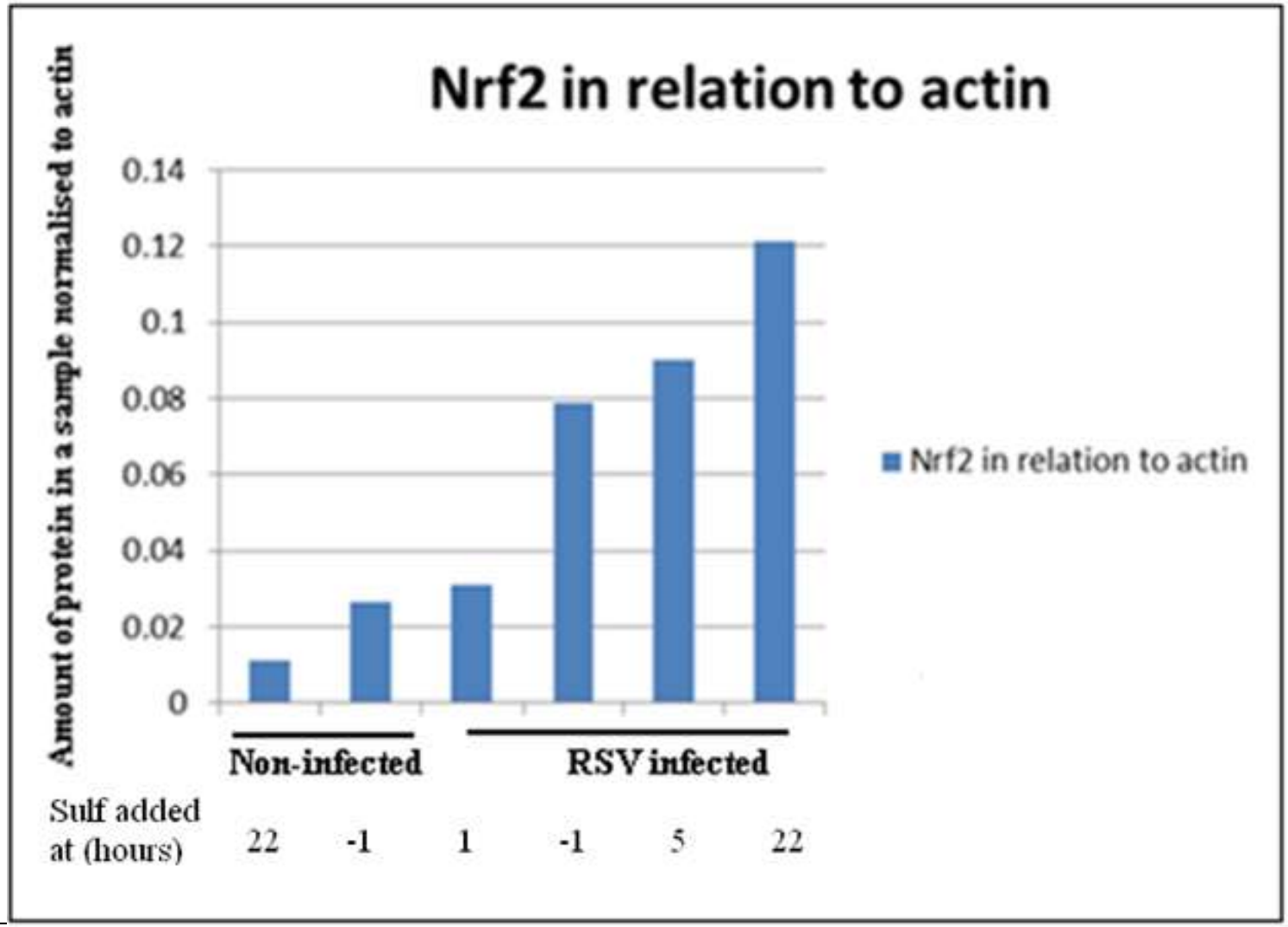
Results from Figure 3.2. show highest expression of Nrf2 in sample stimulated with sulforaphane for 4 hours (non-infected cells). In Figure 3.3., the most enhanced bands are apparent in samples stimulated for 1 and 4 hours. Densitometry results show highest expression of Nrf2 in samples incubated with sulforaphane for 2 hours for infected cells and 1 hour for non-infected cells. 4 hours was chosen as the time for maximal induction in further experiments.

An important finding in Figure 3.3., is that the amount of Nrf2 in infected cells does not necessary increase in comparison to control. As it was not known at what time point Nrf2 expression may affect RSV replication further experiments, included stimulating cells with sulforaphane before, as well as after RSV infection. Results are shown in Figure 3.4.

Figure 3.4. Expression of Nrf2 by BEAS-2B cells with sulforaphane stimulation pre and post RSV infection.



Western blot analysis showing changes in the amount of Nrf2 expressed by BEAS-2B cells infected with RSV and treated with sulforaphane pre and post infection for various durations, ranging from 1hour to 24hours. Expression of Nrf2 was analysed with rabbit monoclonal Anti-Nrf2 abcam (ab62353) antibody. Histone band of 15 kDa provided loading control .



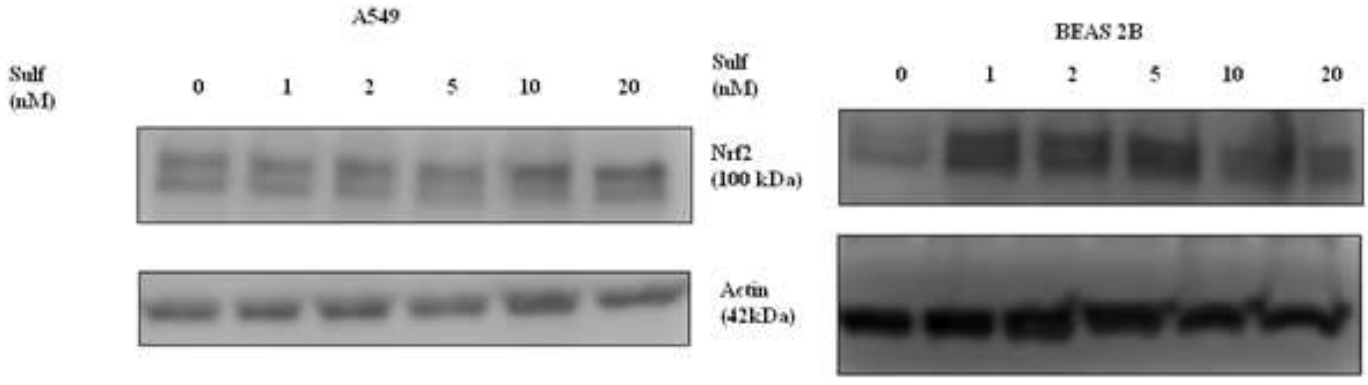
Densitometric analysis of this western blot bands intensities was performed using Li-cor Imgae Studio Lite Versio 3.1. software. Nrf2 bands were normalised to loading control histone band and are presented in the graph above. Cells were infected with 1:1 MOI of RSV at 0 hours and treated with 5 nM of sulforaphane for different amounts of time. Cells were infected with 1:1 MOI of virus at 0 hours.

In samples treated with sulforaphane, increase in Nrf2 expression is apparent. The biggest visible increase is 22 hours post infection, 2 hours before sample collection. Densitometric analysis confirms these results. This time point was used in further experiments.

3.2.2. Choice of the cell line

It was important for experiments to be optimised. Initial choice of cell lines and reagents, as well as their concentration and time of exposure etc. were based on literature search and then optimised in this study. Firstly, A549 and BEAS-2B cell lines were compared to assess induction of Nrf2 with increasing doses of sulforaphane. Figure 3.5. shows this comparison.

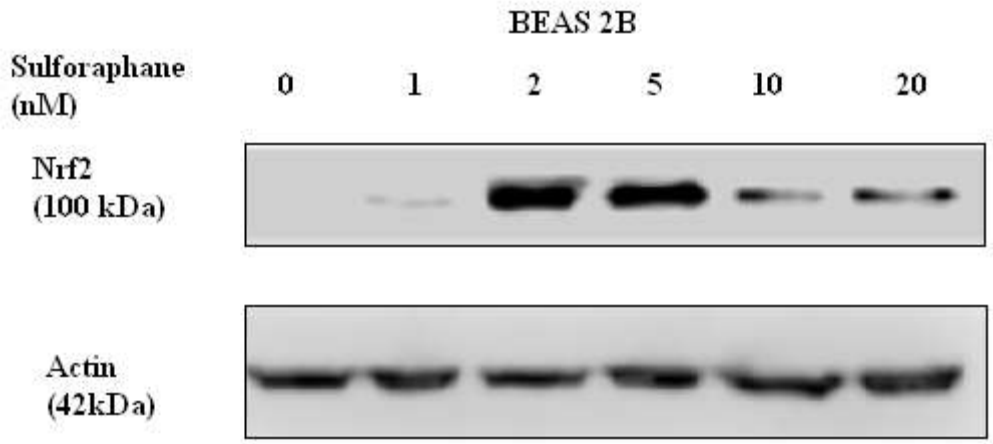
Figure 3.5. Comparison of Nrf2 induction by sulforaphane in A549 and BEAS-2B cell lines.



Western blot analysis showing changes in expression of Nrf2 in A549 and BEAS2B cells, with and without treatment with sulforaphane. Cells were treated with increasing doses of sulforaphane ranging from 0 to 20 nM for 4 hours. Expression of Nrf2 was analysed with rabbit monoclonal anti-Nrf2 abcam (ab62353) antibody. Actin band of 42 kDa provided loading control.

Results show continuous expression of Nrf2 in A549 cells, despite the change of experimental conditions. In BEAS-2B cell line, expression of Nrf2 is induced by sulforaphane in comparison to control sample. BEAS-2B was decided to be the cell line of choice for further experiments and would allow a comparison between cells with and without NRF-2 induction by sulforaphane. This experiment was next repeated on BEAS-2B cells to choose optimal concentration of sulforaphane. Results are presented in Figure 3.6.

Figure 3.6. Sulforaphane dose response on BEAS-2B cell line.



Western blot analysis showing changes in the amount of Nrf2 expressed by BEAS-2B cells after stimulation with different doses of sulforaphane, ranging from 0 to 20 nM for 4 hours. Expression of Nrf2 was analysed with rabbit monoclonal Anti-Nrf2 abcam (ab62353) antibody. Actin band of 42 kDa provided loading control.

The strongest signal was detected in samples treated with sulforaphane at concentrations between 2 and 5nM. Concentration of sulforaphane chosen for further experiments was decided to be 5nM.

3.2.4. The effect of Nrf2 induction by sulforaphane on RSV expression.

BEAS-2B cells were infected with 1:1 concentration of RSV and treated with 5nM of sulforaphane pre and post infection at different time points. Amount of Nrf2 was measured by western blot analysis. Sulforaphane was added to samples 4 hours prior to viral infection. All cells were harvested at 24 hours, excluding controls harvested at 0 hours (just before RSV infection of remaining samples),to confirm induction of NRF-2. Figure 3.7. includes a diagram presenting steps of experiment. Figure 3.8. demonstrates results of this experiment on a western blot.

Figure 3.7. Design of the experiment.

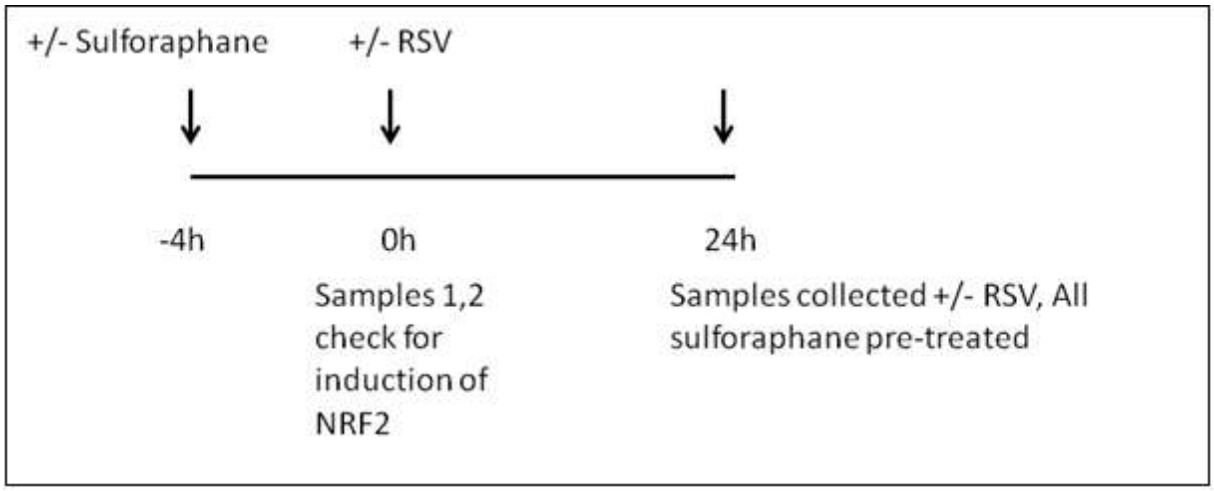
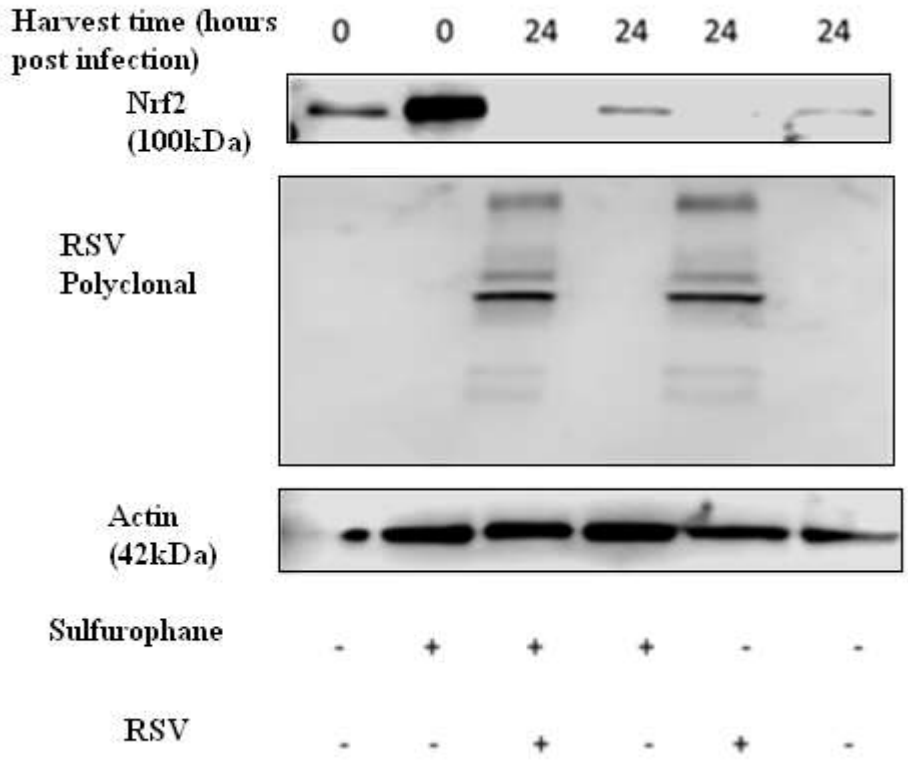


Diagram showing steps of the experiment in chronological manner. Cells were infected 4 hours prior to RSV infection at 0hrs. Cells were infected with 1:1 MOI of virus and harvested at 24 hours, excluding non-infected controls harvested at 0 hours.

Figure 3.8. Induction of Nrf2 expression by BEAS-2B cells using sulforaphane (1).

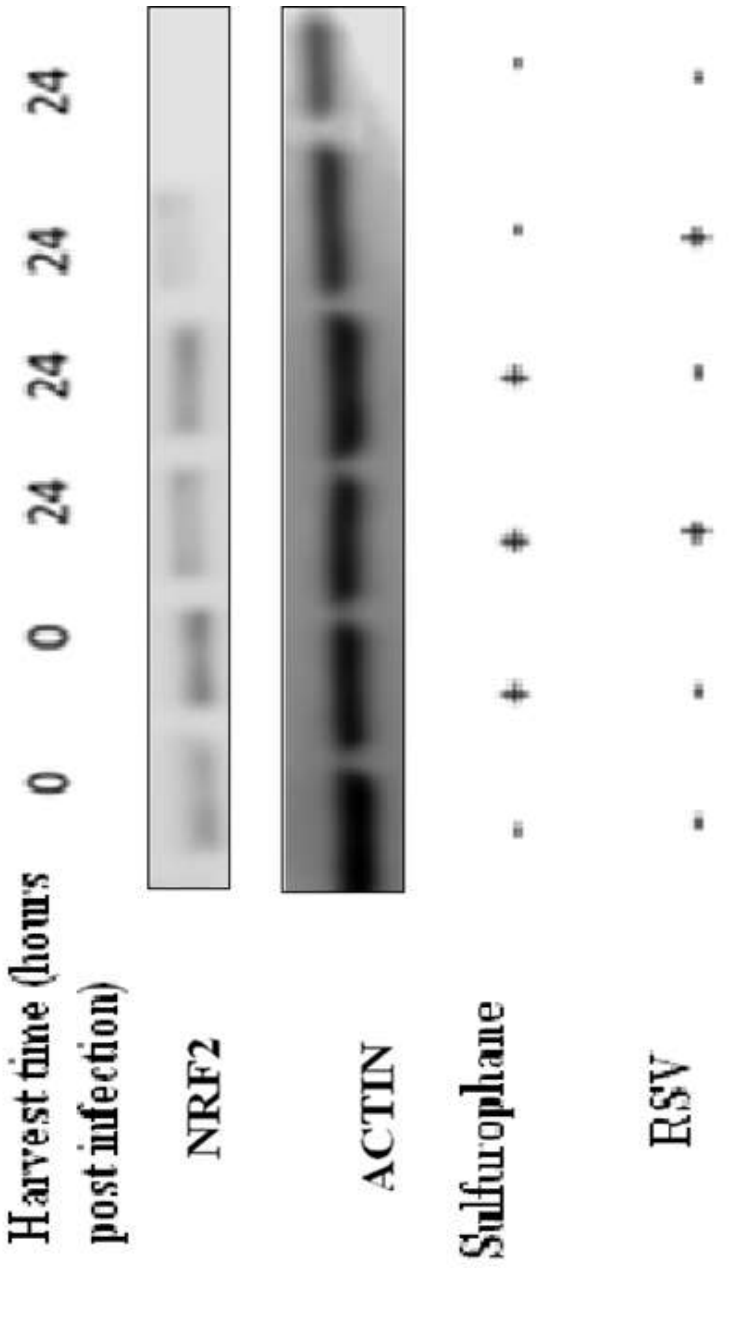


Western blot analysis showing changes in the expression of Nrf2 and amount of RSV in BEAS-2B cells treated with sulforaphane. Cells were treated with 5nM of sulforaphane 4 hours prior to infection and infected with 1:1 MOI at 0 hours. Samples were harvested at 24 hours, excluding non-infected samples harvested at 0 hours. Expression of Nrf2 was analysed with rabbit monoclonal anti-Nrf2 abcam (ab62353) antibody. Expression of RSV was analysed with polyclonal anti-RSV antibody (7950-0104 Bio-rad). Actin band of 42 kDa provided loading control.

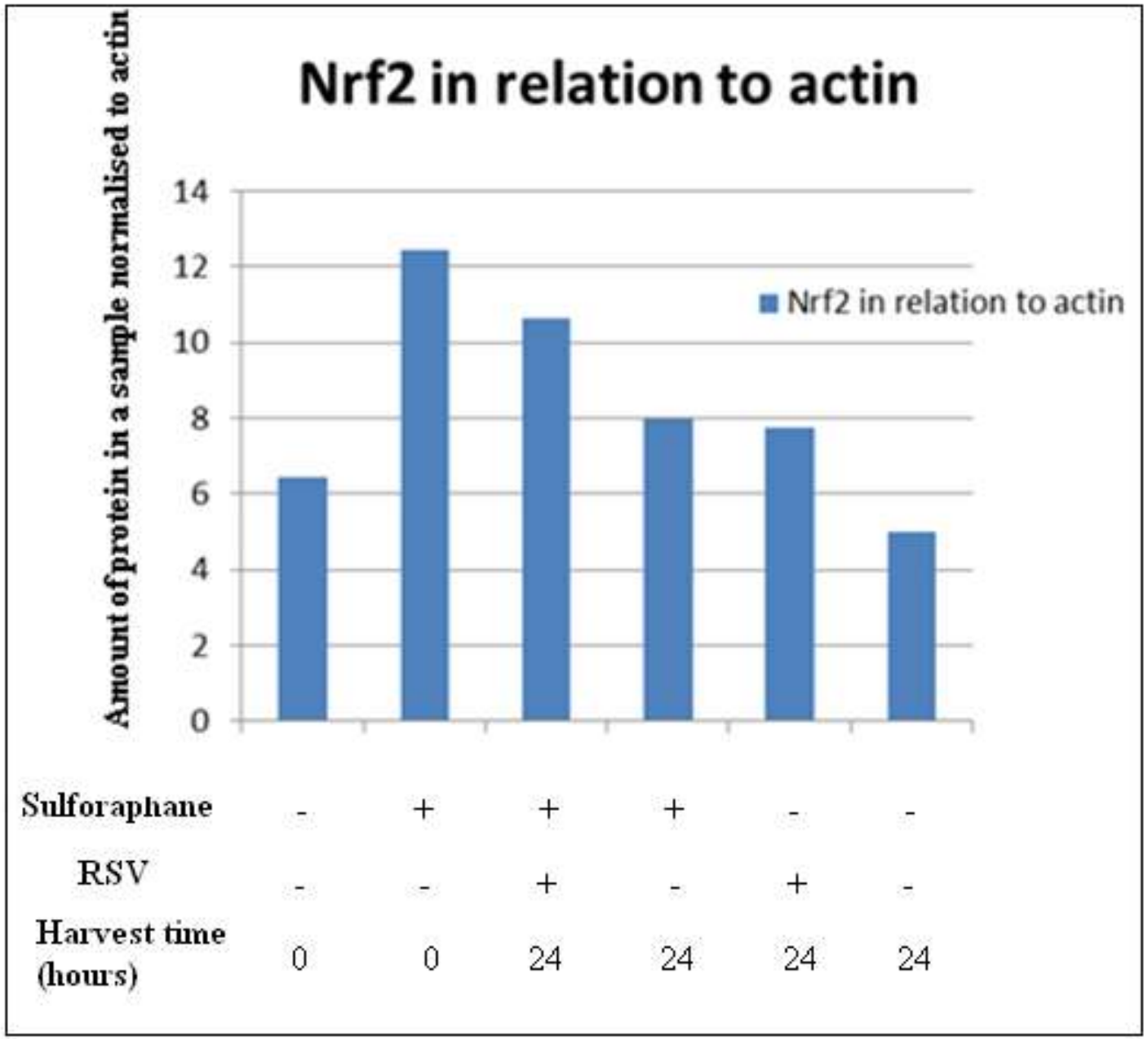
Western blot analysis confirmed that samples infected with RSV contain virus and that there is no contamination of control samples. No difference in RSV protein expression was observed between sulforaphane treated and non-treated samples. There is no change in the darkness of Nrf2 band between non-infected samples

harvested at 24 hours. The strongest signal is detected in a non-infected sample treated with sulforaphane. Nrf2 band disappears in samples infected with RSV. Experiment was repeated to determine if this would be a consistent finding or if any pattern emerges. The results are shown in Figure 3.9.

Figure 3.9. Induction of Nrf2 expression by BEAS-2B cells using sulforaphane (2).



Western blot analysis showing changes in expression of Nrf2 and amount of RSV in BEAS-2B cells treated with sulforaphane. Cells were treated with 5nM of sulforaphane 4 hours prior to infection and infected with 1:1 MOI at 0 hours. Samples were harvested at 24 hours, excluding non-infected samples harvested at 0 hours. Expression of Nrf2 was analysed with rabbit monoclonal anti-Nrf2 abcam (ab62353) antibody. Expression of RSV was analysed with polyclonal anti-RSV antibody (7950-0104 Bio-rad). Actin band of 42 kDa provided loading control.

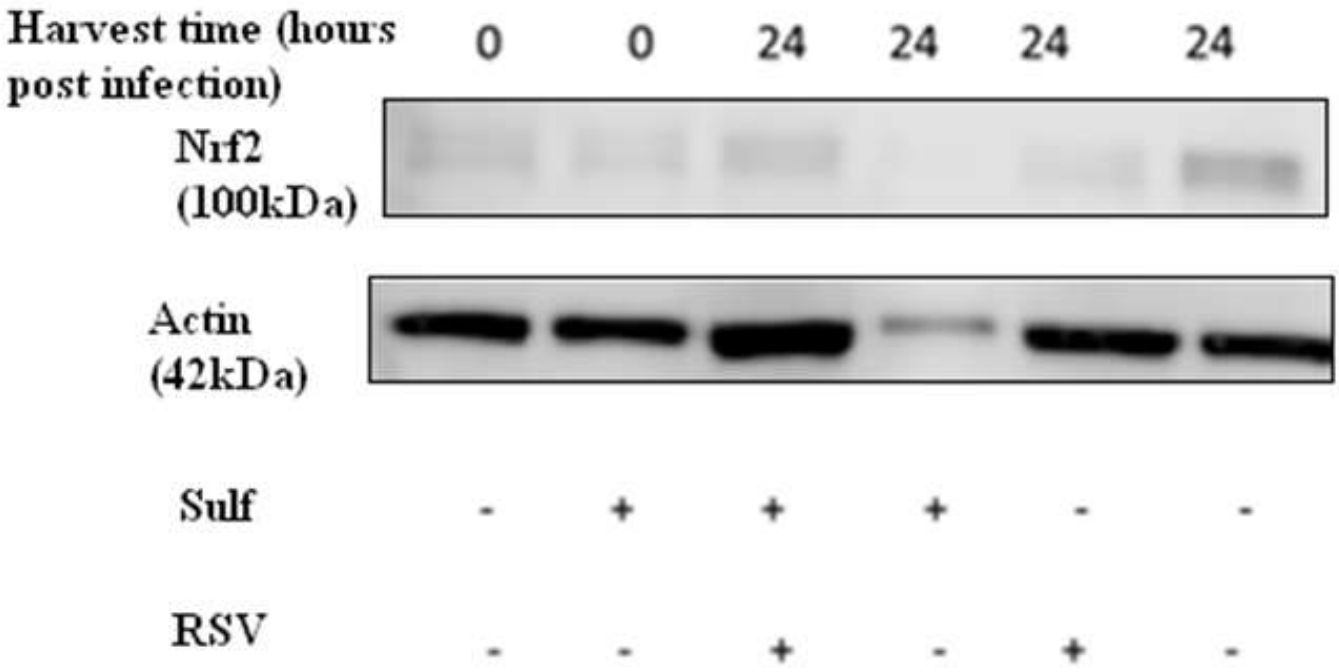


Densitometric analysis of this western blot bands intensities was performed using Li-cor Imgae Studio Lite Versio 3.1. software. Nrf2 bands were normalised to loading control actin band and are presented in the graph above. Cells were treated with 5nM of sulforaphane 4 hours prior to infection and infected with 1:1 MOI at 0 hours. Samples were harvested at 24 hours, excluding non-infected samples harvested at 0 hours. Expression of Nrf2 was analysed with rabbit monoclonal anti-Nrf2 abcam (ab62353) antibody.

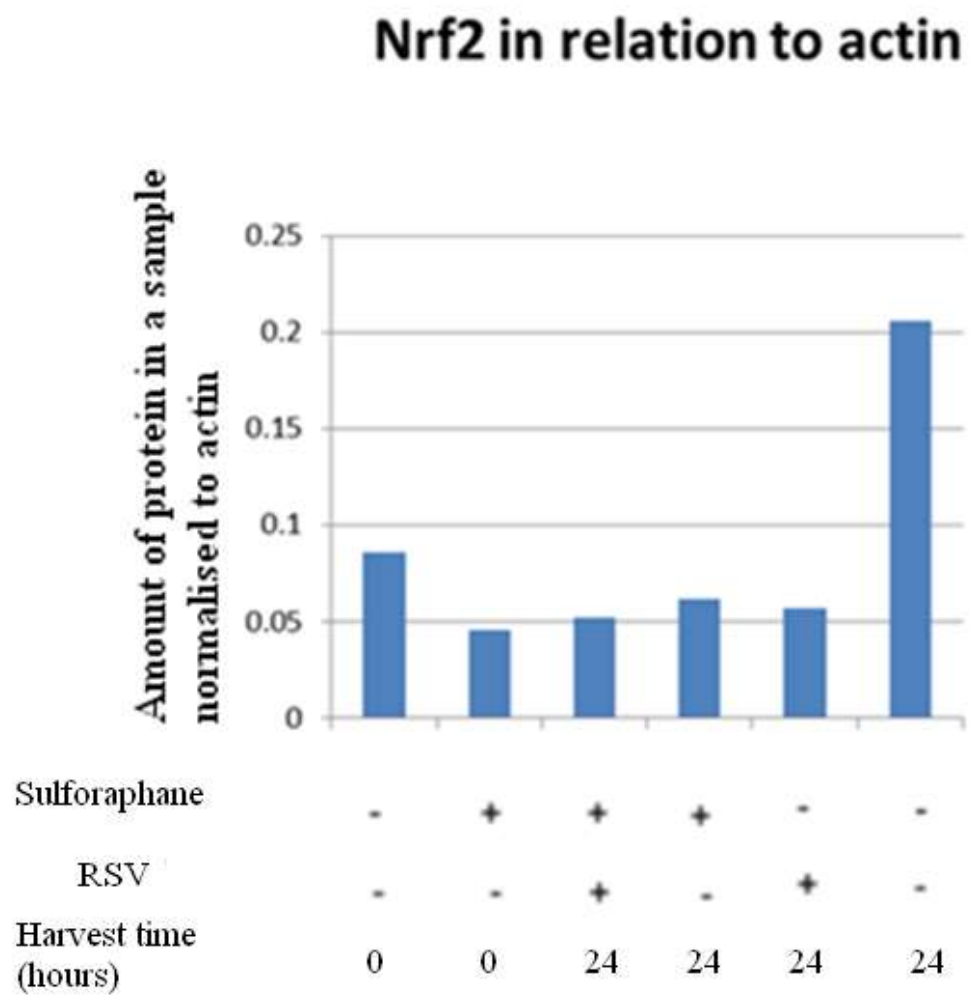
Antibody detects the correct size of Nrf2 band. Signal is stronger in non-infected samples treated with sulforaphane in comparison to control without treatment and weaker in infected sample without treatment in comparison to infected control, as previously. In the 24 hours control sample, signal from Nrf2 band decreases significantly. Densitometric analysis confirms these results.

Experiment was repeated once again. Results are shown in Figure 3.10.

Figure 3.10. Induction of Nrf2 expression by BEAS-2B cells using sulforaphane (3).



Western blot analysis showing changes in the expression of Nrf2 and amount of RSV in BEAS-2B cells treated with sulforaphane. Cells were treated with 5nM of sulforaphane 4 hours prior to infection and infected with 1:1 MOI at 0 hours. Samples were harvested at 24 hours, excluding non-infected samples harvested at 0 hours. Expression of Nrf2 was analysed with rabbit monoclonal anti-Nrf2 abcam (ab62353) antibody. Expression of RSV was analysed with polyclonal anti-RSV antibody (7950-0104 Bio-rad). Actin band of 42 kDa provided loading control.



Densitometric analysis of this western blot bands intensities was performed using Li-cor Imgae Studio Lite Versio 3.1. software. Nrf2 bands were normalised to loading control actin band and are presented in the graph above. Cells were treated with 5nM of sulforaphane 4 hours prior to infection and infected with 1:1 MOI at 0 hours. Samples were harvested at 24 hours, excluding non-infected samples harvested at 0 hours.

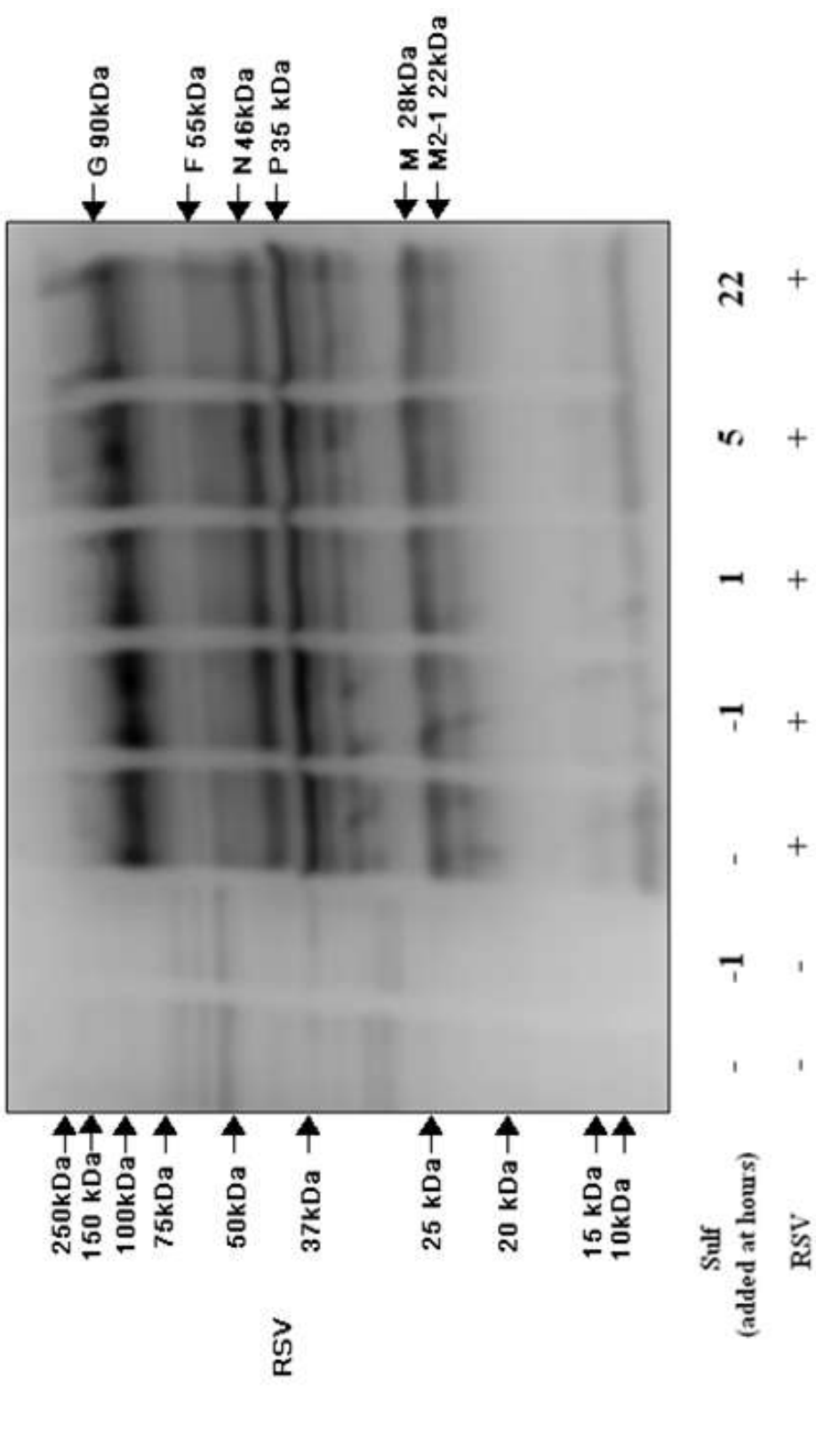
Signal from Nrf2 band is weak and it is difficult to notice any difference between the samples. Results are different to two previous blots: there is no increase in Nrf2 in sulforaphane treated samples or decrease in infected samples and no pattern is emerging.

Densitometric analysis shows increase in Nrf2 in non-infected

sample without treatment, harvested at 24 hours which is difficult to explain.

To examine if induced sulforaphane affected RSV protein expression an experiment with Nrf2 induction with sulforaphane pre and post infection was repeated and western blot probed with anti-RSV antibody. The results are shown in Figure 3.11.

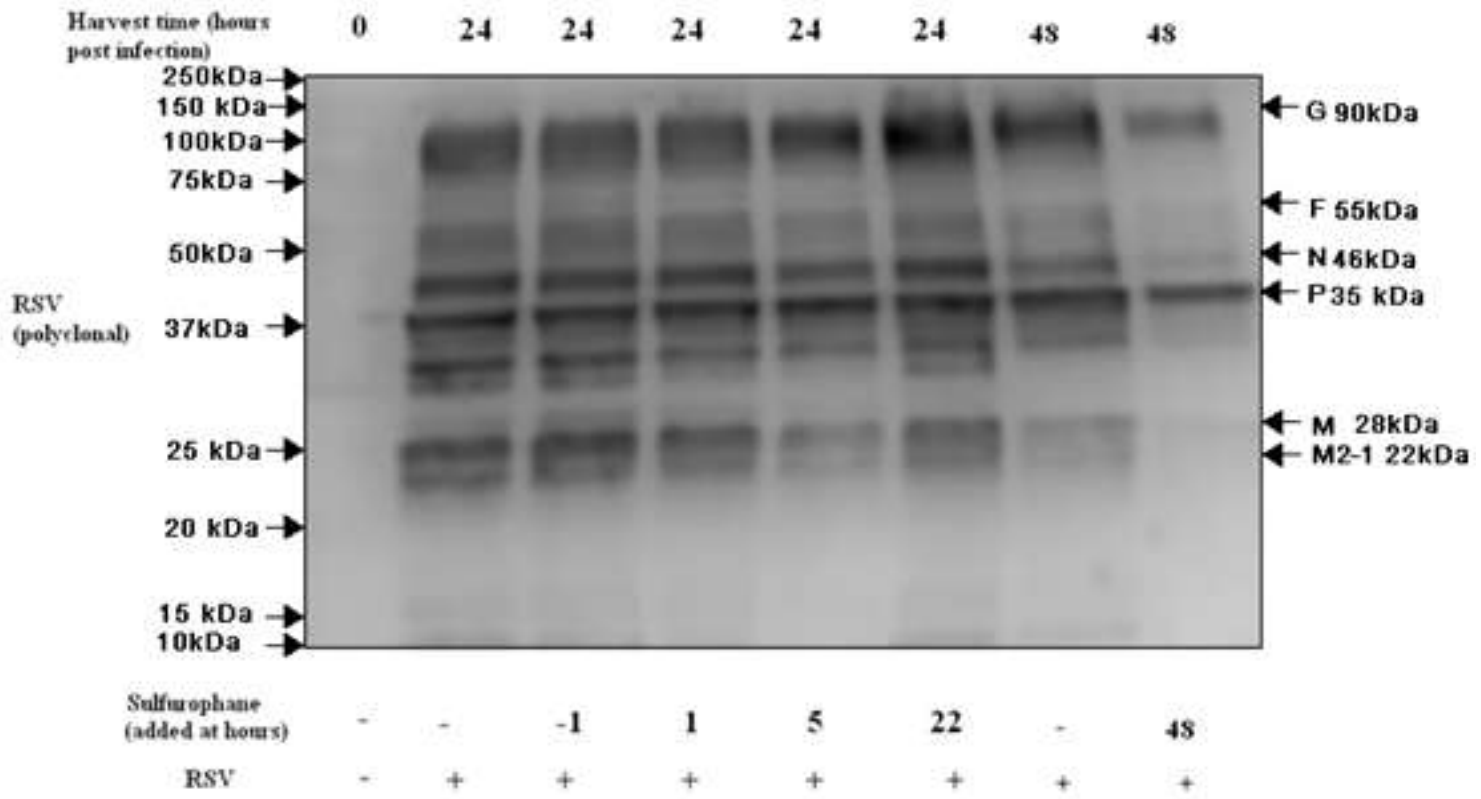
Figure 3.11. Suppression of RSV replication by sulforaphane in BEAS-2B cells (1).



Western blot analysis showing changes in the amount of RSV in BEAS-2B cells treated with sulforaphane. Cells were treated with 5nM of sulforaphane at various times prior and post infection with 1:1 MOI. Time 0 hours is the infection time. Times of treatment with sulforaphane are -1 hour, 1hour, 5 hours, 22 hours. Samples were harvested at 24 hours, excluding non-infected samples harvested at 0 hours. Expression of RSV was analysed with polyclonal anti-RSV antibody (7950-0104 Bio-rad). There is no loading control available for this blot. Bands representing RSV proteins are marked on the right with corresponding molecular weights. From top to bottom: Protein G-90kDa, F-55kDa, N-46kDa, P- 35kDa, M- 28kDa, M2-1- 22kDa.

Signal from infected samples is stronger in comparison to non-infected controls. Difference in the amount of RSV or pattern of bands between the infected samples is not apparent. In order to see whether this result can be reproducible, it was decided to repeat this experiment with two additional samples, both harvested at 48 hours and one of them treated with sulforaphane for 2 hours. Results are shown in Figure 3.12.

Figure 3.12. Suppression of RSV replication by sulforaphane in BEAS-2B cells (2).

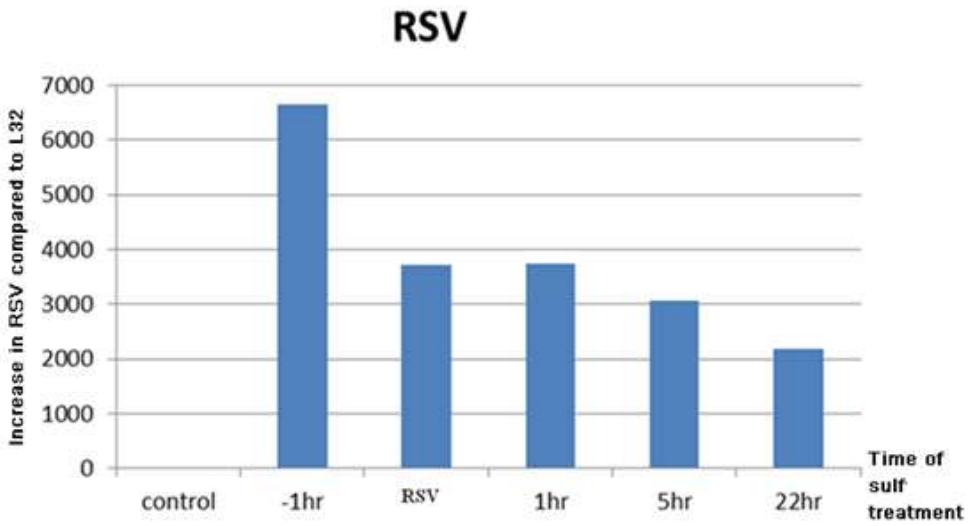


Western blot analysis showing changes in the amount of RSV in BEAS-2B cells treated with sulforaphane. Cells were treated with 5nM of sulforaphane at various times prior and post infection with 1:1 MOI. Time 0 hours is the infection time. Times of treatment with sulforaphane are -1 hour, 1hour, 5 hours, 22 hours. Samples were harvested at 24 hours and 48 hours, excluding the non-infected sample harvested at 0 hours. Expression of RSV was analysed with polyclonal anti-RSV antibody (7950-0104 Bio-rad). There is no loading control available for this blot. Bands representing RSV proteins are marked on the right with corresponding molecular weights. From top to bottom: Protein G- 90kDa, F-55kDa, N-46kDa, P- 35kDa, M- 28kDa, M2-1- 22kDa.

Results of this western blot analysis, similarly to previous experiments do not show a difference between samples apart from the last sample (treated with sulforaphane for 2 hours and harvested 48 hours post infection), where bands are weaker. This result however is likely to be a result of uneven protein loading (Further discussed in section 2.3.).

The amount of virus in samples was next analysed with Real Time PCR. Figure 3.13 shows PCR results of samples corresponding to the western blot in Figure 3.11.

Figure 3.13. RSV expression in comparison to L32 in BEAS-2B cells treated with sulforaphane corresponding to western blot in Figure 3.13.

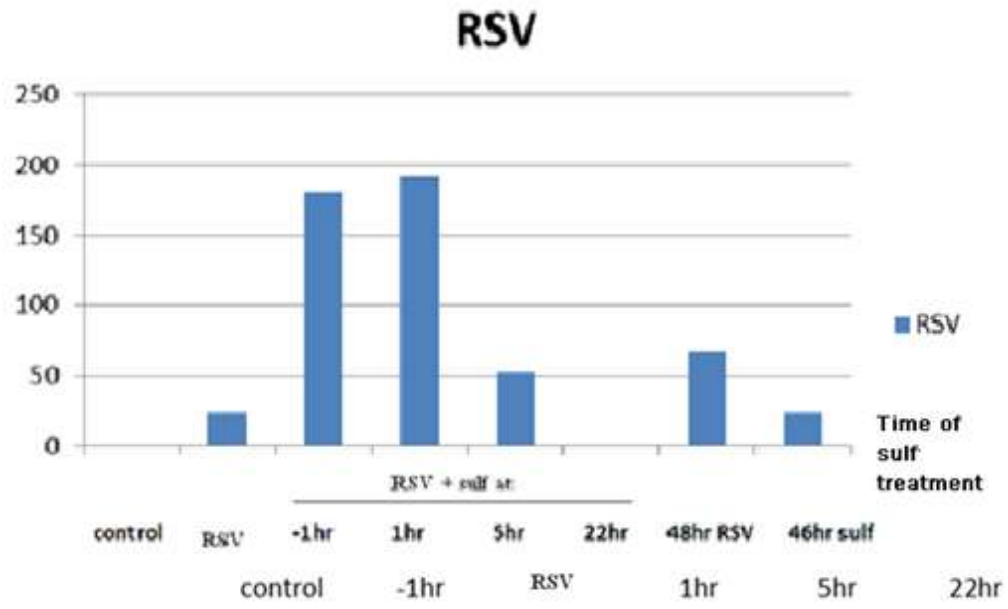


PCR analysis of the increase of RSV in comparison to housekeeping gene (L32) in BEAS-2B cells treated with 5nM of sulforaphane pre and post infection for various amount of time, ranging from 1hr to 22hours. Cells infected with 1:1 MOI of RSV and treated with RSV for various amounts of time.

No RSV signal was detected in negative control. The amount of RSV decreased in samples treated with RSV at 5 and 22 hours after sulforaphane treatment and increased in sample treated with sulforaphane before infection in comparison to infected control without treatment.

The second set of samples, corresponding to western blot in Figure 3.12 was also analysed by PCR. Results are shown in Figure 3.14.

Figure 3.14. Percentage RSV expression in comparison to L32 in BEAS-2B cells treated with sulforaphane corresponding to western blot in Figure 3.14.

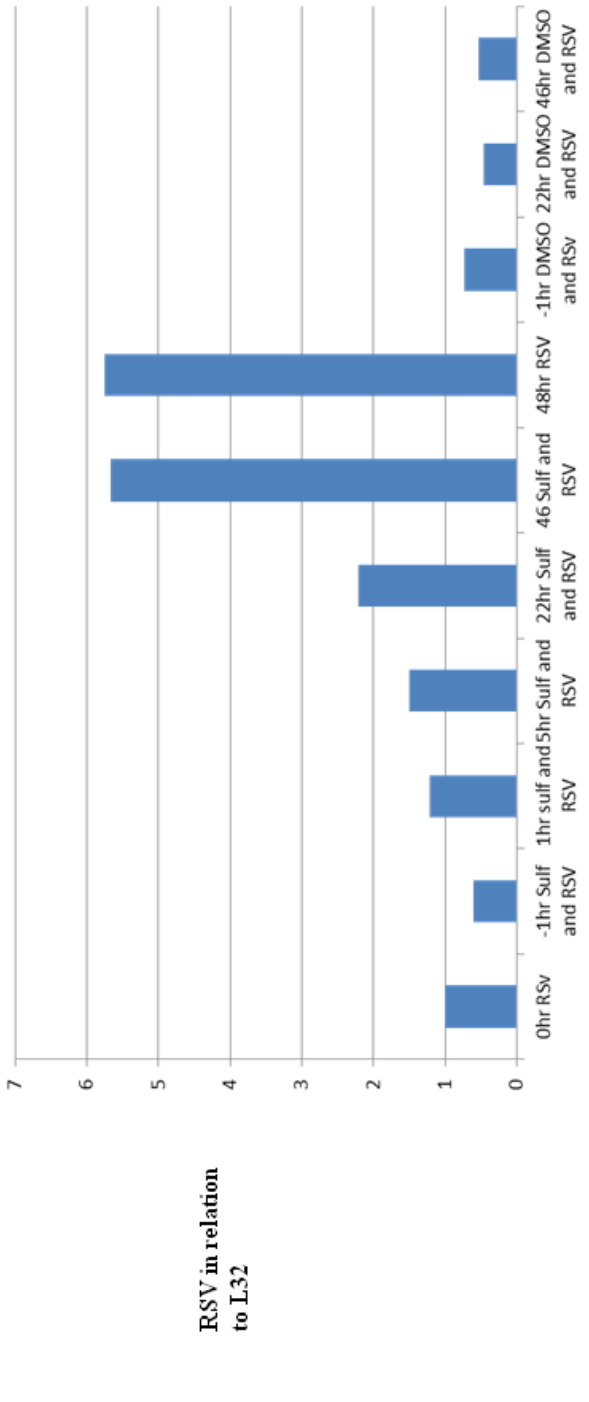


Percentage increase of RSV in comparison to housekeeping gene (L32) in BEAS-2B cells treated with 5nM of sulforaphane pre and post infection for various amount of time, ranging from 1hr to 22hours.

Control sample is negative for virus. There is an increase in the amount of virus with sulforaphane treatment in almost all samples in comparison to non-treated infected sample. Results from experiments conducted do not follow a pattern and are difficult to explain in any other way than error while conducting the experiment or analysing samples.

Experiment was modified and additional control samples with Dimethyl Sulfoxide (DMSO) were added to see whether this reagent used to dissolve sulforaphane has influenced results. DMSO is a commercial solvent, used in experiments as a carrier of sulforaphane due to its cell permeability- the ability to cross cell membrane and allow sulforaphane to enter the cell. Experiment with 3 additional conditions was conducted- cells treated with DMSO at -1hour, 22 hours and 46 hours. The first two samples were collected at 24 hours and the last one at 48 hours. Results of PCR performed on samples from this experiment are shown in Figure 3.15.

Figure 3.15. Percentage RSV expression in comparison to L32 in BEAS-2B cells treated with sulforaphane and DMSO.

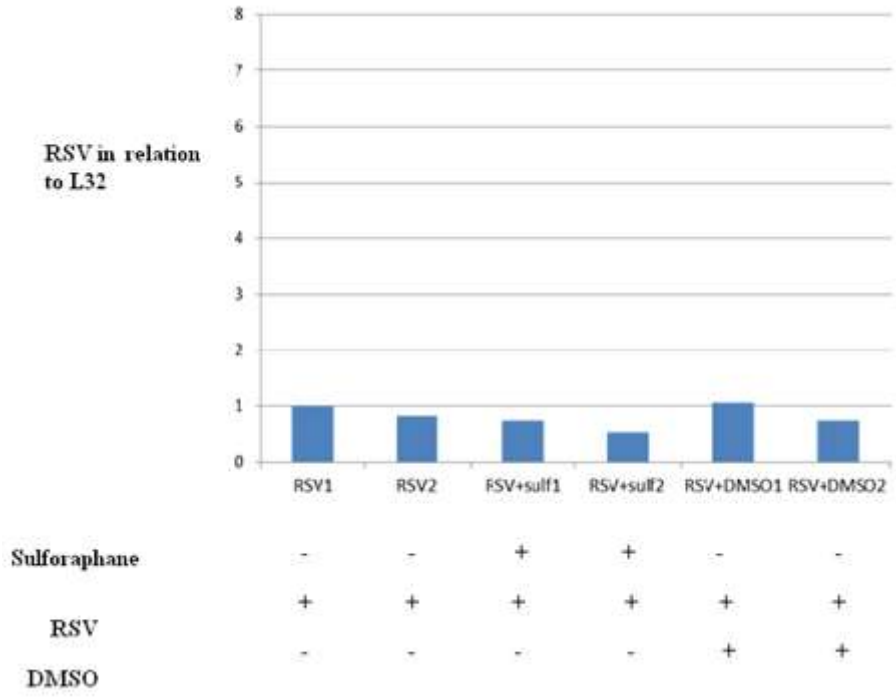


PCR analysis showing increase of RSV in comparison to housekeeping gene (L32) in BEAS-2B cells treated with 5nM of sulforaphane and DMSO pre and post infection for various amount of time, ranging from 1hr to 22hours.

Results show decrease in RSV in samples treated with DMSO and increase in those treated with sulforaphane in comparison to control sample.

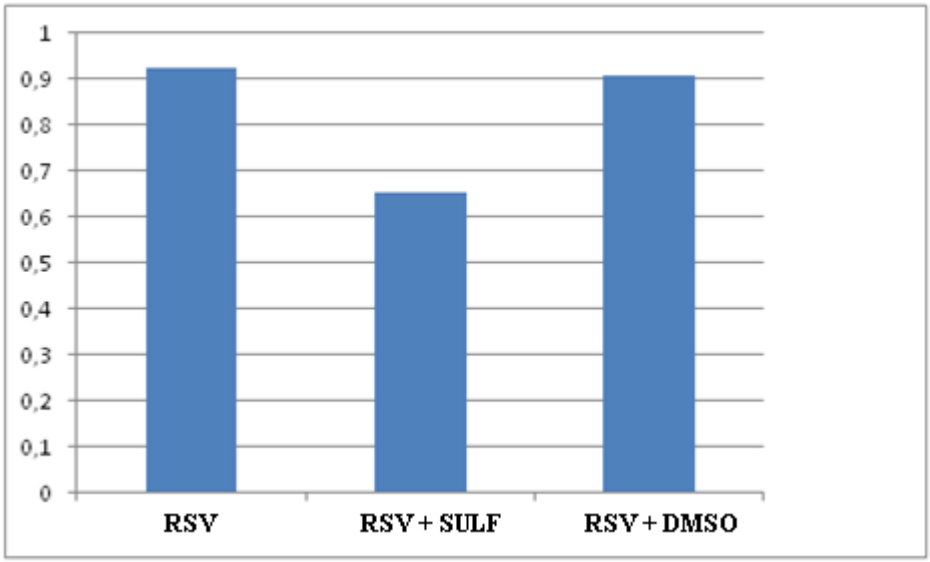
As no pattern of results emerged, It was decided to take a step back and design a simpler experiment, which could demonstrate relationship between the reagents on a basic level. New experiment consisted of only three conditions: cells were infected with RSV and treated with Sulforaphane or DMSO. Results are shown in Figure 3.16.

Figure 3.16. Expression of RSV in BEAS-2B cells treated with sulforaphane and DMSO.



Changes in the amount of RSV detected by PCR in BEAS-2B cells treated with sulforaphane or DMSO. Each condition in duplicate. Outliers were excluded

3.17 Expression of RSV in BEAS-2B cells treated with sulforaphane and DMSO after calculating the average from duplicates.



Changes in the amount of RSV detected by PCR in BEAS-2B cells treated with sulforaphane or DMSO. Values from duplicates of each conditions were averaged after excluding outliers.

Table 2. Table presenting data exported from the PCR spreadsheet after analysis, corresponding to PCR results from Figure 3. 16.

	Sample Name	Detector	Ct	Ct (duplicate)
1	RSV1	PPIA	21.7051	21.5946
1	RSV2	PPIA	20.0895	20.1206
1	RSV3	PPIA	35.2495	36.0177
1	RSV+sulf1	PPIA	21.92	21.9835
1	RSV+sulf2	PPIA	32.408	32.8241
1	RSV+Sulf3	PPIA	20.3151	20.4382
1	RSV+DMSO1	PPIA	28.7124	28.6368
1	RSV+DMSO2	PPIA	21.5269	21.6592
1	RSV+DMSO3	PPIA	29.6093	29.7256
1	RSV1	RSV A2	21.5776	21.6884
1	RSV2	RSV A2	20.3287	20.4059
1	RSV3	RSV A2	32.2829	33.4199
1	RSV+sulf1	RSV A2	22.3861	22.2982
1	RSV+sulf2	RSV A2	33.45	33.5543
1	RSV+Sulf3	RSV A2	20.6452	20.7361
1	RSV+DMSO1	RSV A2	28.6407	28.5598
1	RSV+DMSO2	RSV A2	22.0635	21.9107
1	RSV+DMSO3	RSV A2	29.9743	29.7656

The amount of RSV in cells was calculated as follows:

1. *Average Ct value for each sample and each probe was calculated from duplicates.*
2. *The average ct value for the house keeping gene L32 minus the average ct value for RSV.*
3. *2 to the power of n, where n = value determined in step 2.*
4. *100 divided by n, where n= the value calculated in step 3.*

Statistical analysis of the results showed that difference between RSV and RSV with DMSO samples is not statistically significant with a two-tailed P value of 0.93. The mean of RSV minus RSV and DMSO equals 0.02 with a 95% confidence interval from -0.72 to 0.75. Difference between RSV and RSV with Sulforaphane samples is not statistically significant either with two-tailed P value of 0.2. the mean of RSV minus RSV Sulf equals 0.27 with 95% confidence interval from -0.34 to 0.88. Data from t-tests is presented in Table 3.

Table 3. Unpaired t-test data.

a) RSV and RSV+DMSO comparison

Group	RSV	RSVDMSO
Mean	0.92	0.91
SD	0.13	0.21
SEM	0.10	0.15
N	2	2

P value and statistical significance: The two-tailed P value equals 0.2. By conventional criteria, this difference is considered to be not statistically significant.

Confidence interval: The mean of RSV minus RSVDMSO equals 0.27
95% confidence interval of this difference: From -0.34 to 0.88

Intermediate values used in calculations: t = 1.9, df = 2, standard error of difference = 0.14

b) RSV and RSV+SULF comparison

Group	RSV	RSV sulf
Mean	0.92	0.65
SD	0.13	0.16
SEM	0.01	0.11
N	2	2

P value and statistical significance: The two-tailed P value equals 0.2. By conventional criteria, this difference is considered to be not statistically significant.
Confidence interval: The mean of RSV minus RSV sulf equals 0.27
95% confidence interval of this difference: From -0.34 to 0.88
Intermediate values used in calculations: t = 1.9, df = 2, standard error of difference = 0.14

3.3. Discussion

The first step in optimisation of experiment was finding the right antibody. True molecular weight of Nrf2 has been causing a lot of controversy since its discovery over a decade ago. Very comprehensive report by Lau et al stresses the recent raise of interest in Nrf2 in various research environments.**Error! Bookmark not defined.** However, it also explains that a large portion of research projects, report the apparent wrong molecular weight of Nrf2 due to confusion about the migratory pattern of the molecule. This review provides evidence that the predicted molecular weight of Nrf2 is ~95-110 kDa not ~55-64 kDa based on its 2-kb open reading frame, which a large portion of available research projects report. These authors present data based on chemical activation, vector driven mammalian expression and recombinant protein expression. This report and conversations with experienced colleagues conducting research involving Nrf2 helped with selecting the right antibody (Figure 3.1). Abcam antibody was the only one detecting ~100 kDa band. Looking at the blot, it becomes clear that there is a lot of other, non-specific bands detected by all antibodies. This is a common problem with Nrf2 western blots and makes this laboratory method technically challenging. The next step in experiment optimisation was finding the right cell line. Two different airway epithelial cell lines

were compared and results are shown in Figure 3.2. BEAS-2B cells show sulforaphane induced expression of Nrf2, A549 cells express Nrf2 continually despite change of experimental conditions. In order to be able to show Nrf2 induction by sulforaphane, BEAS-2B was the cell line of choice for all experiments involving Nrf2 and sulforaphane, which is consistent with literature.[133] Optimal concentration of sulforaphane was examined in a dose response experiment presented in Figure 3.3. The strongest expression of Nrf2 was seen in samples treated with 2 and 5 nM of sulforaphane. Five nM was chosen as the optimal concentration of sulforaphane for further experiments, as it has also been previously reported in successful induction of Nrf2[134]. The final step of optimisation was choosing the most efficient time of Nrf2 induction by sulforaphane. Time courses were conducted on both infected and non-infected cells to see whether time for optimal induction changes with infection. Results of a time course experiment on non-infected cells are included in Figure 3.4. and on both infected and non-infected in Figure 3.5. and show the strongest expression of Nrf2 at 1 and 4 hours. Because of possible inconsistency of densitometry results discussed in Section 4.3, the time chosen for maximal induction for future experiments was 4 hours, as it's been previously reported in literature.[135]

Interesting observation from this blot, is that the amount of Nrf2 does not necessary increase in RSV infection, which has previously been reported in literature.[136] Antibody also detected two bands which is surprising and might be explained by the use of different percentage gel for this particular blot. Experiment was modified and additional time of sulforaphane stimulation was added before viral infection. It might have been the case, that there is a difference in Nrf2 expression, depending on whether cells were exposed to sulforaphane before infection. Results are presented in figure 3.6. and show that Nrf2 expression is stimulated by sulforaphane. The highest induction was achieved with treatment for two hours (at 22 hours time point) and this time point was added to some of the later experiments as it is likely to show changes. However, the most important observation for this experiment is that Nrf2 is induced in all samples with different treatment times which creates good starting point for further experiments, as Nrf2 could be key for affecting RSV replication at any of these time points.

It is important to note that results from blots 3.4, 3.5. and 3.6. are inconsistent and it is difficult to see a pattern emerging.

After optimisation and gaining all necessary information to conduct the experiment, sulforaphane was added to samples for four hours prior to viral infection and cells were harvested at 24 hours, excluding

controls harvested at 0 hours. Flow diagram of the experiment is presented in Figure 3.7 and results in Figure 3.8. Cells have been successfully infected with virus and there is no contamination of controls. Signal of Nrf2 band is stronger in sample with added sulforaphane which confirms Nrf2 induction. There is no change in RSV protein expression between samples with and without sulforaphane treatment which is a crucial finding for the hypothesis and indicates that these results do not support the idea that RSV expression is influenced by sulforaphane. Nrf2 band disappears in samples infected with RSV. This could mean that RSV down-regulates Nrf2 expression. It could be a case of virus infection leading to transcription factor degradation and not Nrf2 inhibiting replication of virus in a cell as previously thought. There is not much information available about the effect of RSV infection on Nrf2 expression, however, a paper by Komaravelli et al. published in January 2015, after we concluded Nrf2 experiments, states that Nrf2 undergoes deacetylation-proteasomal degradation in RSV infection which supports our findings.[137]

Experiment was repeated to see if similar results are obtained or if a pattern emerges. Results are shown in Figure 3.9. Nrf2 band gives stronger signal in non-infected samples treated with sulforaphane which confirms Nrf2 induction. Signal is weaker in infected sample

without treatment like in the previous blot which could support the idea of Nrf2 degradation by RSV. Densitometry was performed on this blot and confirmed results. Figure 3.10. shows results of repeated experiment but these results differ from the ones obtained before. There is no Nrf2 induction by sulforaphane and the amount of Nrf2 does not decrease with infection like previously. Samples do not show changes apart from increase in Nrf2 in control sample harvested at 24 hours (not treated and not infected). This result is unexpected and no pattern emerged which might be explained by an error while conducting the experiment or analysing the results and highlights the need to repeat the experiment. To examine if induced sulforaphane affected RSV protein expression, samples from experiment with induction of Nrf2 before and after infection was analysed on western blot with anti-RSV antibody.

Results shown in figure 3.11 confirm successful infection of samples and lack of contamination of control. The polyclonal antibody detects multiple bands which correspond to RSV proteins of the following molecular weights: Protein G-90kDa, Protein F- 55 kDa, Protein N-46kDa, Protein P- 35kDa, Protein M- 28 kDa and M2-1 22kDa.[138] Unfortunately, there is no difference in the amount of RSV protein between samples, which is not what we were hoping to see according to our main hypothesis. This experiment was repeated

adding two more control samples. Results, presented in figure 3.12. do not show a difference between samples apart from the last one (treated with sulforaphane for 2 hours and harvested 48 hours post infection) where the band is weaker. This however, could be caused by uneven protein loading as there is no loading control for blots in Figures 3.11 and 3.12 due to technical problems with anti-actin antibody at the time. This is also why densitometry could not have been carried out, however, the lack of change in RSV expression between samples is indicated by western blot. The decrease in protein amount in the last sample can also possibly be explained by sulforaphane being cytotoxic after this amount of time as it has been previously reported to cause reduced cell viability.[139] This however, is only a speculation and experiments should be conducted once again with actin control and densitometry to see if that change is reproducible.

Western blot analysis of samples did not support the hypothesis that RSV expression changes with Nrf2 induction, however, this method only looks at proteins in samples. To see whether the hypothesis can be supported using a different laboratory method, samples were analysed by PCR, which is a technique quantifying viral RNA expression. It could have been the case that RNA was present in the sample but the protein not. Plaque assay would have been the best

method for looking at viable virus however this technique is also the most difficult one. This could be the next step in this project, once more laboratory experience is obtained.

First set of samples analysed with PCR (Figure 3.13) correlates to western blot in Figure 3.11. Negative control confirms no contamination and successful infection of other samples. Results show decreased signal of RSV in samples treated with sulforaphane at 5 and 22 hours and increase in sample treated with sulforaphane before viral infection. Even though decrease in RSV with sulforaphane treatment is what we wanted to achieve, increase in the sample treated prior to infection is surprising and difficult to explain which makes the set of results less reliable due to possible error. In order to see if results are reproducible or whether there is a pattern emerging, the second set of samples from western blot 3.12 was also analysed by PCR (Figure 3.14). Results show successful infection and lack of contamination of controls but again increase in viral RNA in almost all samples is difficult to interpret and any differences can most probably be explained by intra-experimental variation while either conducting experiment or analysing samples.

Experiment was modified and additional control samples with DMSO were added. It was suspected that DMSO, which is a carrier of sulforaphane, could influence results (Figure 3.16). There is

decrease in RSV in samples treated with DMSO and increase in those treated with sulforaphane. Increase in RSV is inversely proportional to the length of exposure to sulforaphane. This could suggest that the amount of RSV increasing with time in samples treated with sulforaphane could be the virus replicating in cells and sulforaphane having no effect on it. It was decided to take a step back and design a simpler experiment which could demonstrate relationship between reagents used in the experiment in a more straight forward manner. Results of this experiment are shown in Figure 3.16 and 3.17. Figure 3.16 presents values from duplicates and figure 3.17 averaged values. Statistical analysis with a non-paired t-test revealed that results are not statistically significant.

Overall, Nrf2 induction probably does not influence RSV expression as majority of western blots for RSV and RSV PCR did not show change, however, it is also obvious that there was a lot of technical difficulties due to lack of previous laboratory and research experience and other technical problems. If it was not for time limitation of the degree, experiments should be conducted once again with loading controls and densitometry. Bigger N number created by repeating experiments would enable statistical analysis of quantifiable results. DMSO influence on results should be further examined by creating more experimental conditions with this reagent.

Experiments based on plaque assay should be conducted once more laboratory experience is gained. Another alternative experimental approach could be looking at different viral RNA, as only one gene was checked for during PCR analysis. As Nrf2 is known to change cellular location in infection,[140] immunofluorescence could bring interesting results, especially if all: nucleus, Nrf2 and RSV are labelled with different colours.

An idea that emerged from a portion of results was that RSV might have a destructive effect on Nrf2 (Figure 3.9). This is a potential for further experiments, especially that Nrf2 has been reported to be degraded by RSV by a different research group[141] but because this result was not reproduced in more experiments and at the time there was no literature available about this interaction, it was decided to conclude the Nrf2 study at this point.

4. Changes in the ubiquitination of proteins during RSV infection

4.1 Introduction:

Proteomics is the study of the proteome, a set of proteins produced and modified by an organism or cell which determine its structure and function. [142] Mass spectrometry, a proteomics related technology can be used to provide information about the structure and function of a large number of proteins expressed in one sample. By analysing samples taken under different conditions proteomics, using mass spectrometry, allows insight into how protein expression changes under a given set of experimental conditions.[143] Data used as the starting point for this project was generated by Dr Angela Fonceca, Dr Brian Flanagan and Dr Paul McNamara working with Prof Rob Beynon and Dr Debra Simpson from the Liverpool proteomics group. Their experiments defined the changes in the level of ubiquitination of proteins following RSV infection and identified 62 differentially ubiquitinated proteins when comparing uninfected and RSV infected A549 airway epithelial cells. Six of those proteins (described in Section 1.4.4.) were selected for further validation and study. These six proteins were selected because their level of ubiquitination either increased or decreased and showed the greatest

level of change following RSV infection (DDX21) or because they were known to be involved in interferon signalling after viral infection (MAVS).

In the experiments we used MG132 which is a specific, cell permeable, reversible and potent proteasome inhibitor.[144] If a protein is ubiquitinated and broken down by the proteasome, we would expect the amount of the ubiquitinated form to increase following MG132 treatment. Determining which proteins change their level of poly-ubiquitination following the infection could help identify new biochemical pathways involved in the host defence or viral replication, and new targets for potential therapeutic intervention. Table 4. presents proteomics data involving six proteins of interest chosen for this project.

Table 4. Proteomics data including 6 proteins of interest used in the experiment during the project.

Name of the protein	Molecular Weight (kDa)	Heavy/Light chain ratio	Light/Heavy chain ratio	More/Less of ubiquitinated form in infected cells
Prohibitin	29.8	0.657	1.52	More
Ribosomal Protein S10	18.9	1.508	0.66	Less
MAVS	40.4	0.418	2.39	More
DDX21	87.3	0.477	2.10	More
HMGB1	24.9	2.036	0.49	Less
Annexin A1	0.49	1.96	0.49	Less

Table presents data from the proteomics experiments on 6 proteins studied further in this project (Prohibitin, RPS10, MAVS, DDX21, HMGB1, Annexin A1). Heavy chain represents non-infected cells which have been labelled with a stable isotope Carbon13 by growing them in C13 supplemented media, whereas light chain- the infected ones grown in standard C12 supplemented media. Heavy over light chain ratio, or light over heavy is the crucial data point. It is expressed in both ways in the table. If ratio is below 1 for light over heavy chain, it means more of the protein was ubiquitinated in non-infected cells. If value is over 1, more of the protein is ubiquitinated in light fraction (infected cells). Samples were harvested at 4 hours post infection. Table also includes molecular weight of each protein.

These experiments aim to show if, following viral infection, host proteins may be differentially ubiquitinated either as part of the hosts cells defence response to infection or as a result of viral induced

changes in cellular metabolism. These differences in ubiquitination level could lead to an increase or decrease in protein breakdown or turnover via the proteasome (Ubiquitin Proteasome System explained in Section 1.4.3.) either as part of host cell defence or viral manipulation of normal cellular processes to favour its reproduction.

The hypothesis examined was that viral infection of airway epithelial cells leads to changes in ubiquitination of normal cellular proteins. These changes represent either RSV, manipulation of normal cellular proteins to support replication or host cell defence to infection.

This was examined firstly by treating infected and non-infected cells with MG132 to inhibit proteasome activity and examining if protein expression changed. Secondly, differential ubiquitination was also examined using immunoprecipitation with either antibodies against individual proteins to purify them followed by western blotting with antibody against ubiquitin to determine how much of this fraction was ubiquitinated. Alternatively, immunoprecipitation with anti-ubiquitin followed by western blot with a protein specific antibody to determine how much of specific protein was to be found within the ubiquitin fraction. Thirdly, as differential ubiquitination can lead to a change in cellular localisation, immunofluorescence was used to examine protein localisation in both infected and non-infected cells.

Potentially differentiated molecules studied in this project include DDX21, an RNA helicase, a member of the DexD/H-box helicases family. All RNA helicases, are highly conserved enzymes which change secondary RNA structures and ribonucleoprotein complexes by the use of ATP energy during RNA metabolism. Even though the exact mechanism of this process is not known, DDX21 is essential in pre-rRNA processing. It has been shown that DDX21 plays a major part in regulating transcriptional and post transcriptional steps of ribosome biogenesis, and has a crucial part in coordinating transcriptional programs across different nuclear compartments. DDX21 has been proposed as a host restriction factor which modifies expression of the influenza A gene by down regulating viral RNA formation, and as a result viral protein production at early stages of infection. No further information about interaction of DDX21 with virus has been published.

Another protein MAVS is an intermediary protein, essential in the interferon signalling pathways triggered by viral infection as it activates transcription factors responsible for regulation of IFN-β signalling and plays a role in anti viral immunity. There are a number of transcript variants encoding different isoforms of the gene. Diseases associated with MAVS include viral infections such as Hepatitis C and Influenza A. Its peroxisomal and mitochondrial

forms, act simultaneously to create antiviral states in a cell. During infection with a virus, peroxisomal MAVS initiates IFN-independent production of defence factors which protect cells in the short-term. The mitochondrial type of MAVS initiates IFN-dependent signalling pathway with delayed kinetics. This in turn intensifies and stabilises the defense against a virus. It has been suggested that MAVS is likely to protect cells from apoptosis and that Lys-48-linked polyubiquitination of MAVS leads to its proteasomal degradation.

HMGB1 is a DNA binding protein associated with chromatin, composed of 215 aminoacids and of a molecular weight of 24 kDa. It plays a significant role in inflammation. Macrophages and monocytes secrete HMGB1 which acts as a as a cytokine like mediator. It has been reported to promote viral growth and enhance viral polymerase activity.[145] It undergoes ubiquitination at Lys12, 43, 50, 112, 114, 128 and 157. The role of HMGB1 in the host response to respiratory viral infection has not been further studied.

Annexin A1, also known as lipocortin1, has been identified as an important therapeutic target in treatment of inflammation with glucocorticoids due to its inhibitory effect on phospholipase A2. Their main mechanism of action is through increasing the synthesis and function of Annexin A1, which downregulates phospholipase A2 blocking eicosanoid production and suppressing a number of

leukocyte inflammatory events (Epithelial adhesion, emigration, chemotaxis, phagocytosis). These processes lead not only to suppression of the immune system, but also to two main products of inflammation- prostaglandins and leukotrienes. It undergoes ubiquitination at Lys58, 166, 274. It has been suggested that Annexin negatively regulates viral RNA replication but its role in viral infections has not been studied in depth.[146]

Ribosomal protein S10 (RPS10) is involved in several cellular pathways, one of which include viral RNA transcription and replication and ctivation of the mRNA reported in Influenza A infection. The knowledge about this process is however fragmentary. RPS10 undergoes ubiquitination at Lys38, 47, 59, 107, 138, 139.

Prohibitin encoded by the PHB gene is involved in negative regulation of transcription from RNA polymerase II promoter. Prohibitin undergoes ubiquitination at Lys128. The influence of prohibitin on controlled viral replication has not been previously studied.

4.2. Results

The aim of this series of experiments was to firstly validate the suggested changes in ubiquitination observed in the proteomics and to determine if these potentially key proteins which control cell's

response to RSV or allow viral replication, change in their level of expression or ubiquitination after RSV infection and proteasome inhibition.

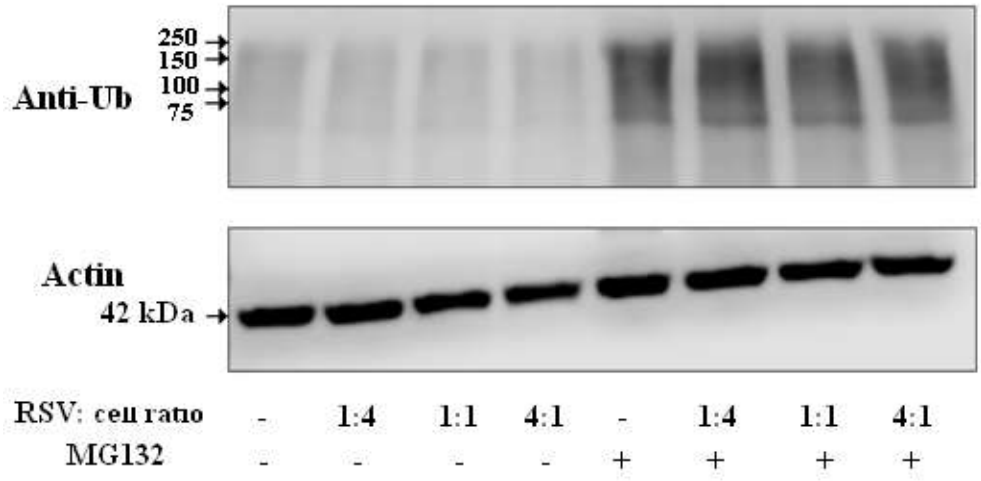
4.2.1. Changes in protein expression in A549 cells after RSV infection and proteasome inhibition.

Firstly, samples were run on a blot probed with anti-ubiquitin antibody. It was to determine if there are ubiquitinated proteins in samples and whether the amount of these changes with RSV infection and proteasome inhibition.

4.2.1.1. Influence of RSV infection on the expression of ubiquitin in A549 bronchial epithelial cells with and without proteasome inhibition.

A549 cells were infected with RSV at three different concentrations and after two hours treated with MG132 for another two hours. Results of a western blot with samples probed with FK2 anti-ubiquitin antibody are shown in Figure 4.1.

Figure 4.1. Level of ubiquitin in A549 cells infected with increasing concentrations of RSV and treated with proteasome inhibitor (MG132).



Western blot analysis showing changes in the amount of ubiquitinated proteins in A549 cells after RSV infection and proteasome inhibition. Cells were infected with 3 different concentrations of virus (MOI= 1:4; 1:1; 4:1). After 2 hours, 4 samples were treated with 10mM of MG132 in DMSO for another 2 hours. Protein ubiquitination was analysed with species independent, mono- and polyubiquitinated conjugates monoclonal Anti-FK2 antibody agains ubiquitin (Enzo). Actin band of 42 kDa provided loading control.

Antibody detects correct seized, high molecular weight, smeared band reported in other research studies, consistent with detection of multiple ubiquitinated proteins. Detected ubiquitin bands are enhanced in samples treated with MG132 consistent with inhibition of the proteasome. No differences were observed between samples treated with different MOIs of virus and the level of ubiquitin in each sample. There is also no overall visible difference in the total amount of ubiquitinated proteins between infected and non-infected samples whether treated with MG132 or not. However, some proteins could

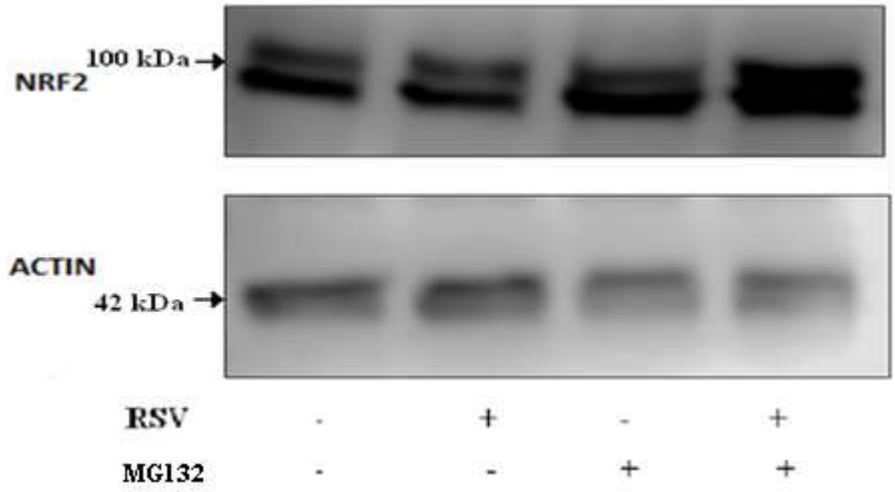
be more and some less ubiquitinated, as individual changes in protein ubiquitination will not be evident.

It was decided that the most optimal concentration of virus to further examine changes in Ubiquitination would be 1:1 ratio of virus particle to cell.

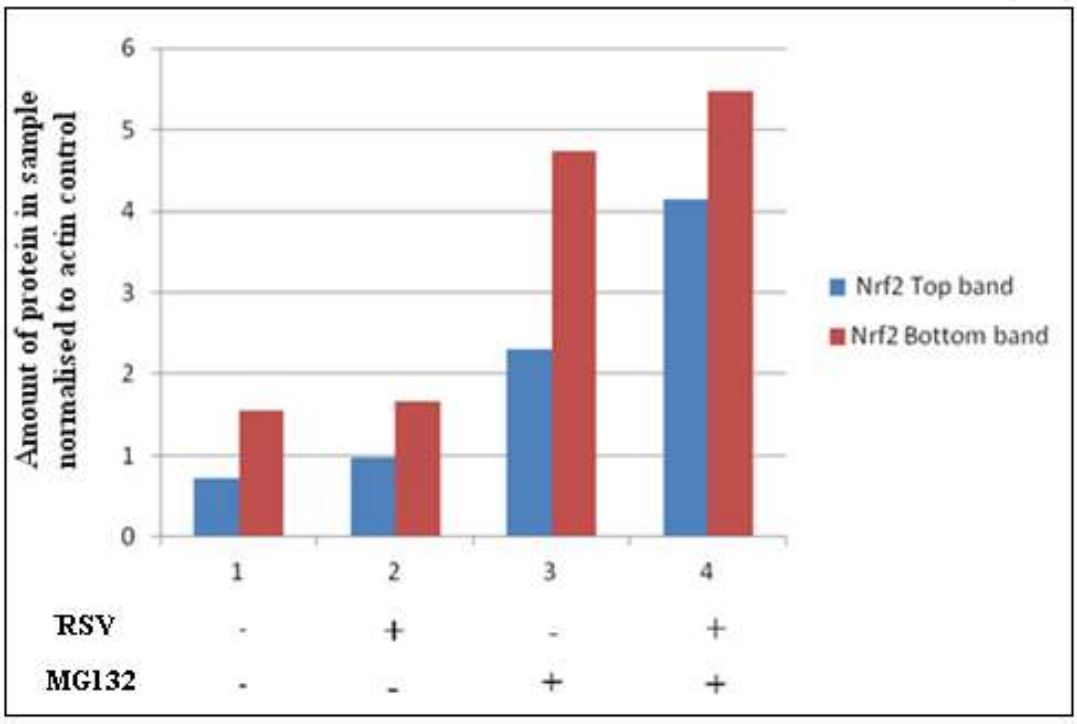
4.2.1.2 Influence of RSV infection on the expression of Nrf2 in bronchial epithelial cells with proteasome inhibition.

As Nrf2 is known to be ubiquitinated and broken down by the proteasome during RSV infection[147], it was decided to look at this molecule first in order to validate the experimental approach and show that MG132 inhibition was working in the experiments. Results are shown in Figure 4.2

Figure 4.2. Level of Nrf2 in A549 cells infected with RSV and treated with proteasome inhibitor (MG132).



Western blot analysis showing changes in the amount of Nrf2 in A549 cells after RSV infection and proteasome inhibition. Cells were infected with one concentration of virus (MOI 1:1). After two hours, two samples were treated with 10mM of MG132 in DMSO for another two hours. The expression of Nrf2 was analysed with rabbit monoclonal Anti-Nrf2 abcam (ab62353) antibody. Aactin band of 42 kDa provided a loading control. Molecular weight of the detected Nrf2 is ~ 95/105 kDa.



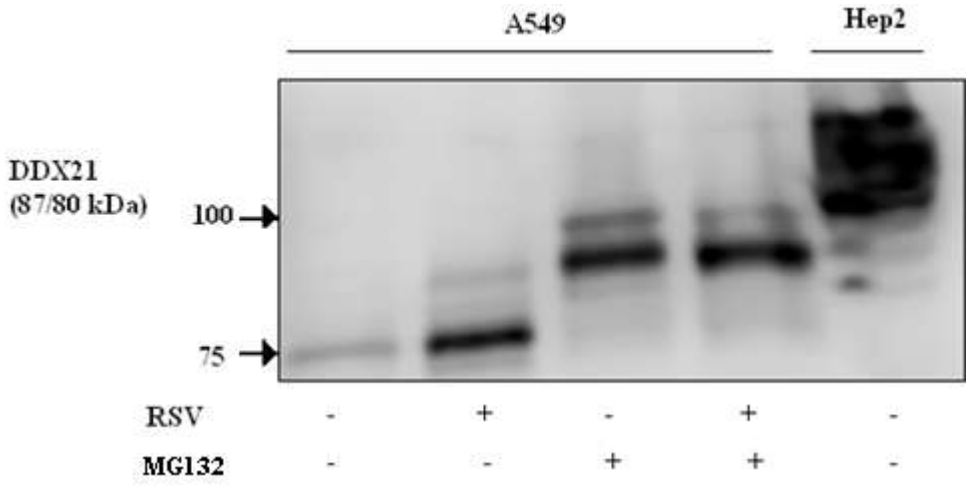
Densitometric analysis of this western blot bands intensities was performed using Li-cor Imgae Studio Lite Versio 3.1. software. Nrf2 bands were normalised to loading control actin band and are presented in the graph above. Samples were infected with 1:1 MOI of RSV and treated with MG132 for 2 hours.

Western blot analysis of this experiment detects two bands for Nrf2. Both bands are in range of ~95-110 kDa which is the correct molecular weight reported in other research studies.[148] Top band in the RSV infected and MG132 treated sample is darker even though the protein loading is lower. No change between the control sample and the infected sample is apparent. As expected, densitometry results show increase in Nrf2 in MG132 treated samples in comparison to control and increase in Nrf2 in infected sample treated with MG132 in comparison to non-infected one.

4.2.1.3. RSV influence on the expression of DDX21 in bronchial epithelial cells with proteasome inhibition.

The Hep2 cell line expresses DDX21constituitivly and can be used as a positive control.[149] Detection of DDX21 in Hep2 cells, would suggest that our reagents are working and that the experiment has been carried out correctly. Figure 4.3 shows western blot analysis of samples which were infected with RSV, treated with MG132 after 2 hours and harvested at 4 hours.

Figure 4.3. Level of DDX21 in A549 cells infected with RSV and treated with proteasome inhibitor.

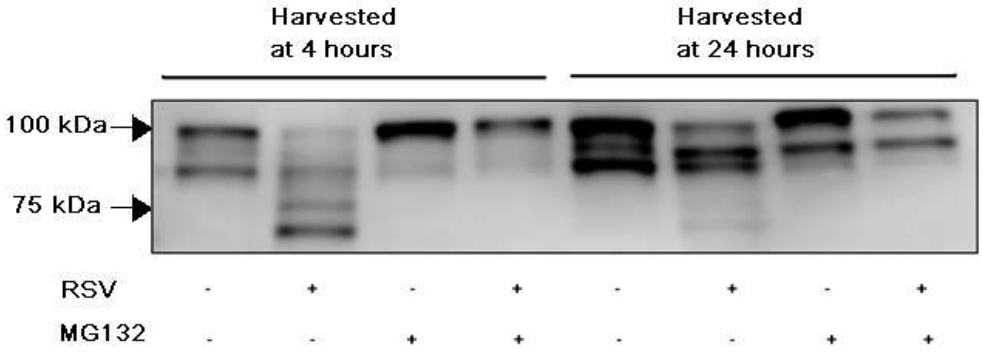


Western blot analysis showing changes in the amount of DDX21 in A549 cells after RSV infection and proteasome inhibition. Cells were infected with one concentration of virus (MOI 1:1). After 2 hours, 2 samples were treated with 10mM of MG132 in DMSO for another 2 hours and harvested 4 hours post infection. Expression of DDx21 was analysed with rabbit monoclonal anti-DDX21 abcam (ab182156) antibody. No loading control is available for this blot. Desired molecular weight detected by antibody is 80/87 kDa.

The result shows a strong band of high molecular weight in Hep2 cells and lighter bands in A549 cells. For A549 cells there is a difference in the pattern of bands between samples. Additional band of lower molecular weight appears in A549 samples without MG132. This band is darker in the infected sample. Samples treated with MG132 show enhanced signal and bands have different higher molecular weight pattern. In comparison to control, there is more of the heavier form of DDX21 in infected sample without proteasome inhibition. To examine the possibility that the lower molecular weight bands seen in both control and infected sample could be breakdown

product of DDX21, experiment was repeated with samples harvested at 4 and 24 hours. Results are shown in Figure 4.4.

Figure 4.4. Level of DDX21 in A549 cells infected with RSV and treated with proteasome inhibitor, harvested at 4 and 24 hours.



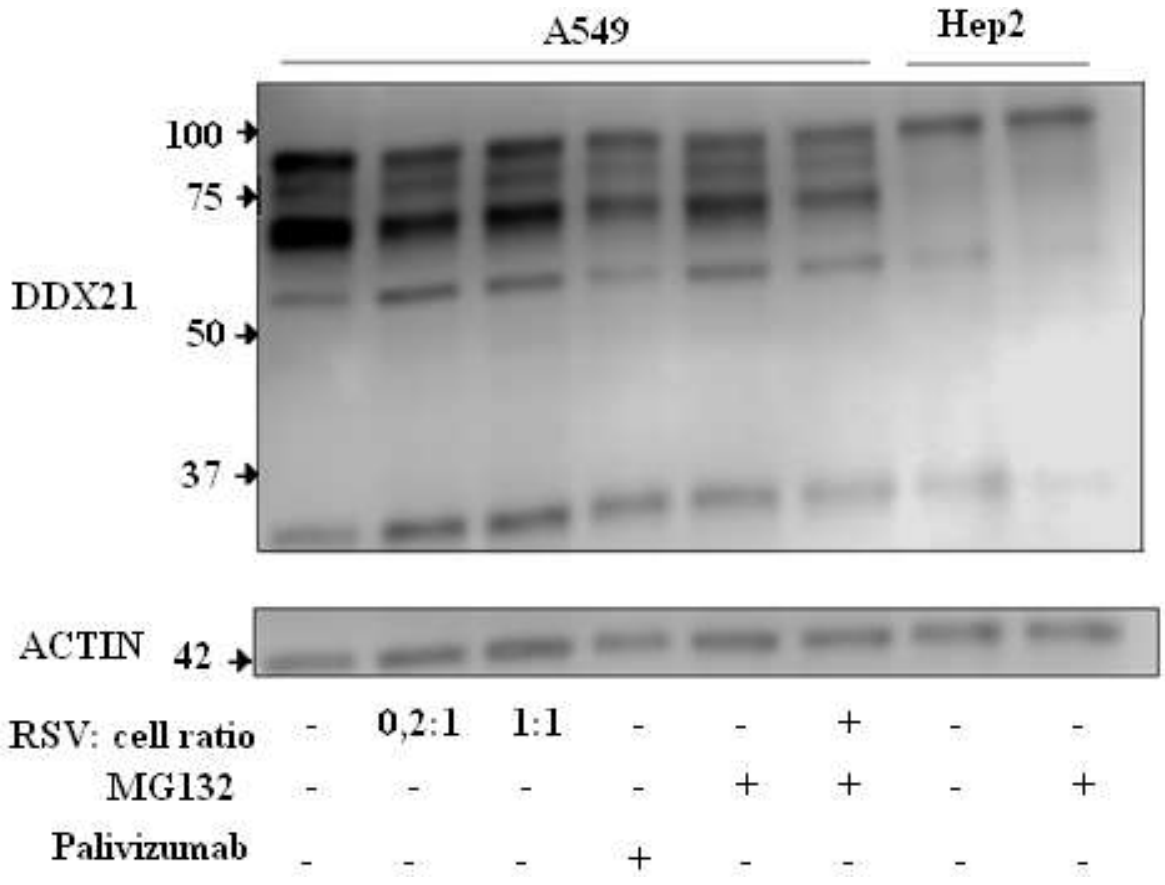
Western blot analysis showing changes in the amount of DDX21 in A549 cells after RSV infection and proteasome inhibition. Cells were infected with one concentration of virus (MOI 1:1). After 2 hours, 4 samples were treated with 10mM of MG132 in DMSO. Two samples for 2 hours and harvested at 4 hours and another to for 22 hours and harvested at 24 hours post infection. Expression of DDX21 was analysed with rabbit monoclonal anti-DDX21 abcam (ab182156) antibody. No loading control is available for this blot. Desired molecular weight detected by antibody is 80/87 kDa.

Results are similar and consistent with western blot in Figure 4.3. The top band is enhanced in samples where proteasome was inhibited and there are multiple lower molecular weight bands in infected samples.

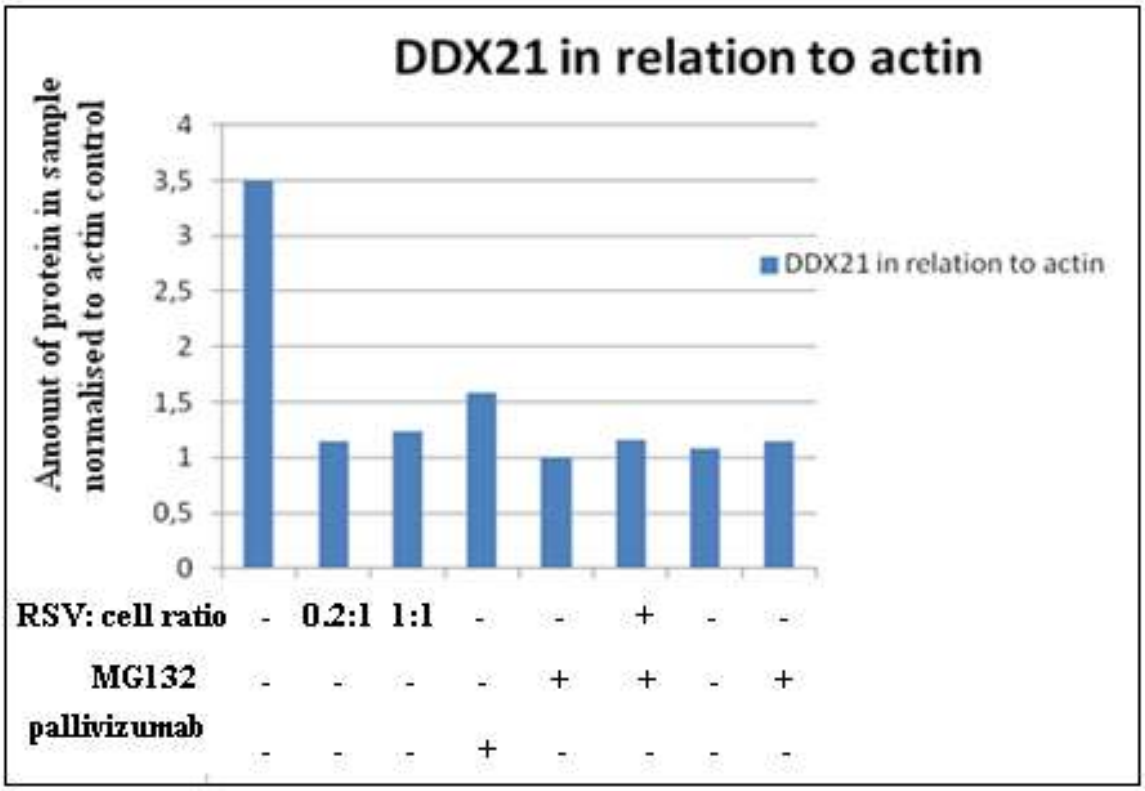
To examine these changes further, design of the experiment was changed by introducing more controls which could explain different patterns of bands. Samples were infected with two concentrations of

virus to look for a dose response. Palivizumab, a monoclonal antibody used prophylactically for infants in the high risk group of RSV infection, was added to one of the samples to remove virus and determine if other molecules present in the RSV preparations could be influencing results. Two Hep2 lysates, one of which was treated with MG132, were added as positive controls. Results are shown in Figure 4.5.

Figure 4.5 Level of DDX21 in A549 cells infected with RSV and treated with proteasome inhibitor and palivizumab.



Western blot analysis showing changes in the amount of DDX21 in Hep2 and A549 cells after RSV infection and proteasome inhibition. Cells were infected with two concentrations of virus (MOI= 0,2:1; 1:1). After 2 hours, 3 samples were treated with 10mM of MG132 in DMSO and one with palivizumab for another 2 hours and harvested 4 hours post infection. Expression of DDX21 was analysed with rabbit monoclonal anti-DDX21 abcam (ab182156) antibody. Actin band of 42 kDa provided loading control. Desired molecular weight detected by antibody is 80/87 kDa.



Densitometric analysis of western blot band intensities was performed using Li-cor Imgae Studio Lite Versio 3.1. software. DDX21 band was normalised to loading control actin band and presented in the graph above. Axis x shows sample number: 1. Non- infected sample without treatment. (A549) 2. Sample infected with 0.2:1 MOI if virus without treatment. (A549) 3. Sample infected with 1:1 MOI of virus without treatment. (A549) 4. Non-infected sample with palivizumab treatment. (A549) 6. Non-infected sample with MG132 treatment. (A549) 7. Infected sample with MG132 treatment. (A549) 7. Non-infected sample without treatment. (Hep2) 8. Non-infected sample with MG132 treatment (Hep2). Axis y shows the amount of protein in each sample.

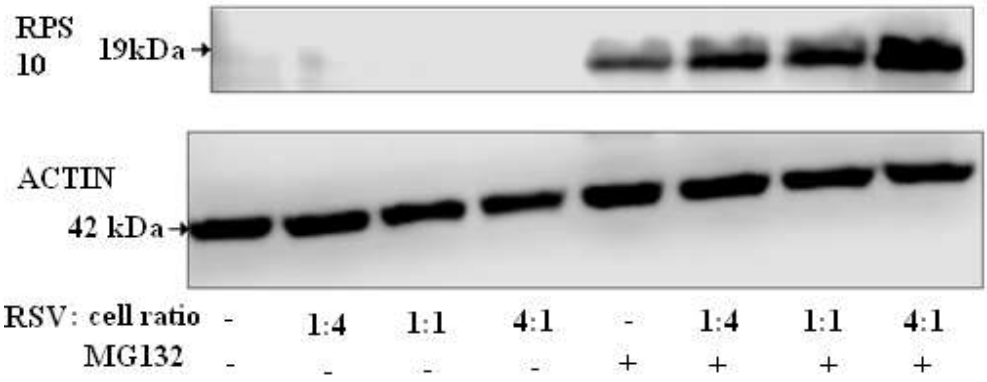
Antibody detects a band of ~87 kDa in all samples. In A549 cells there are additional bands of lower molecular weight. Bands in control sample give stronger signal than in infected samples. Change between the rest of the samples is not apparent. Densitometry results confirm higher level of DDX21 in control sample and no difference between the other samples. These results show a different pattern from that observed in previous blots with no other apparent

differences due to RSV or MG132. It is possible that there is a problem with this result which will be further discussed in Section 4.3.

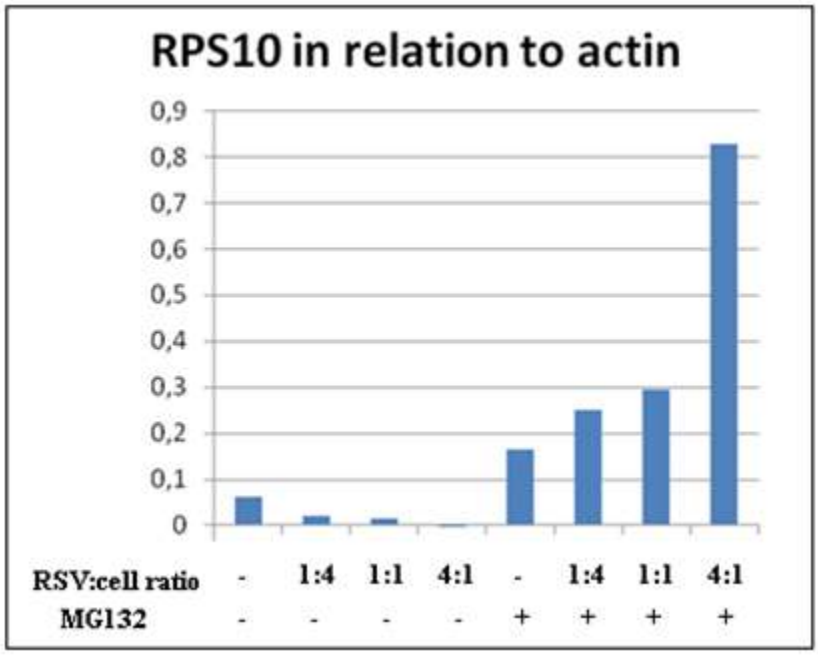
4.2.1.4. Influence of RSV infection on the expression of Ribosomal Protein S10 (RPS10) in bronchial epithelial cells with and without proteasome inhibition.

A549 cells were infected with 3 different concentrations of RSV (cell: virus particle ratio of 4:1, 1:1, 1:4) and treated with 10mM of MG132 for 2 hours. Cell were then harvested at 4 hours and used for western blots and immunohistology.

Figure 4.6. Level of RPS10 in A549 cells infected with increasing concentrations of RSV and treated with proteasome inhibitor (MG132).



Western blot analysis showing changes in the amount of RPS10 in A549 cells after RSV infection and proteasome inhibition. Cells were infected with three different concentrations of virus (MOI 1:4,1:1,4:1). After 2 hours, 4 samples were treated with 10mM of MG132 in DMSO for another 2 hours. Expression of RPS10 was analysed with rabbit monoclonal Anti-RPS10 abcam (ab151550) antibody. Anti-actin band of provided loading control. Molecular weight of detected RPS10 is 19kDa.



Densitometric analysis of western blot band intensities was performed using Li-cor Imgae Studio Lite Versio 3.1. software. RPS10 band was normalised to loading control actin band and presented in the graph above. Axis x shows sample number: Cells were infected with various MOIs of RSV and treated with MG132 for two hours.

As expected, a band of 19 kDa consistent with RPS10 was observed. Enhanced intensity of RPS10 band is apparent in samples treated with MG132. Relation between the amount of virus added to sample and the amount of RSP10 detected is proportional. In samples infected with virus but without MG132 treatment RPS10 levels were almost undetected. Densitometry confirms higher levels of RPS10 in MG132 treated samples, increasing proportionally to the amount of virus added to cells. The amount of RPS10 also decreases in proportional manner to the amount of RSV added to samples without MG132 treatment. The biggest difference is observed in MG132 treated sample, infected with 4:1 MOI of virus.

The rest of studied proteins showed similar results to RPS10, namely successful proteasome inhibition with MG132 but no change with viral infection.

4.2.2. Changes in the expression of proteins in A549 cells after RSV infection and proteasome inhibition, analysis by immunoprecipitation.

In the following set of experiments, antibody against ubiquitin (anti-Ub) was used for detection of proteins ubiquitination. Each protein of interest was also detected by corresponding antibody. Immunoprecipitation of samples aimed to show whether changes seen on blots without immunoprecipitation are related to

ubiquitination and whether the amount of ubiquitinated proteins changes with RSV infection. This technique enabled examination of two properties of one protein at the same time. In this case, identifying the right protein and whether it is ubiquitinated or not. To achieve this, magnetic beads were coated with anti-ubiquitin antibody, which allowed ‘pulling’ all ubiquitinated proteins from the samples. Samples were then loaded onto gel and blot probed with antibody against the protein of interest or the other way round. This should reveal all ubiquitinated proteins in samples.

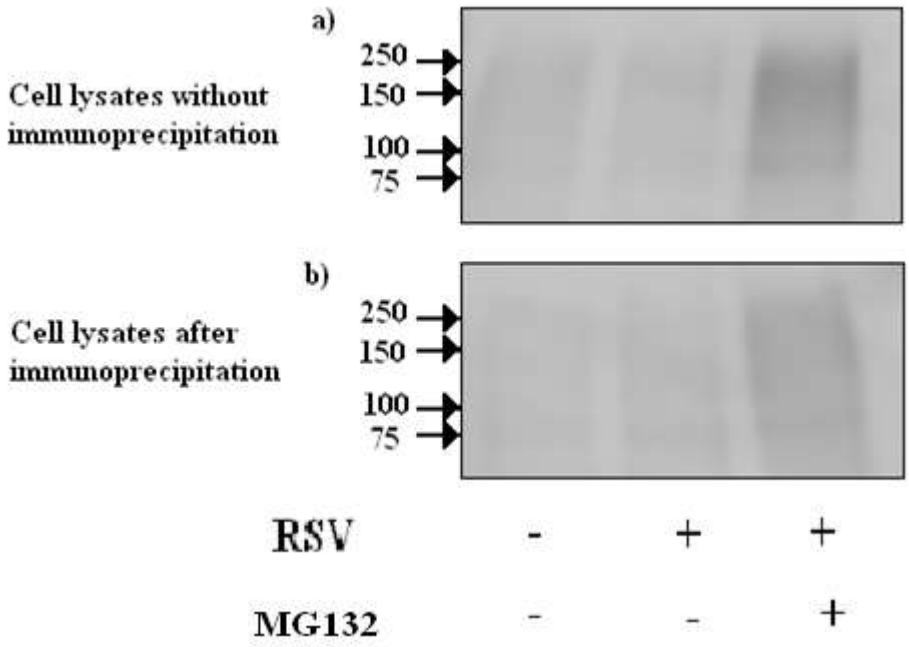
4.2.2.1. RSV influence on the expression of ubiquitin in bronchial epithelial cells subjected to immunoprecipitation.

Magnetic beads were coated with anti-ubiquitin antibody to precipitate ubiquitinated proteins from sample and then presented on a blot probed with the same antibody.

Figure 4.7. RSV influence on the expression of ubiquitinated proteins in A459 cells infected with RSV and treated with MG132.

a) **cell lysates without immunoprecipitation, western blot probed with anti-ubiquitin antibody.**

b) **cell lysates after immunoprecipitation with anti-ubiquitin antibody, western blot probed with the same antibody.**



Western blot analysis showing changes in the amount of ubiquitinated proteins in A549 cells after RSV infection and proteasome inhibition. Cells were infected with 1:1 MOI of RSV. After 2 hours one sample was treated with 10mM of MG132 in DMSO for another two hours.

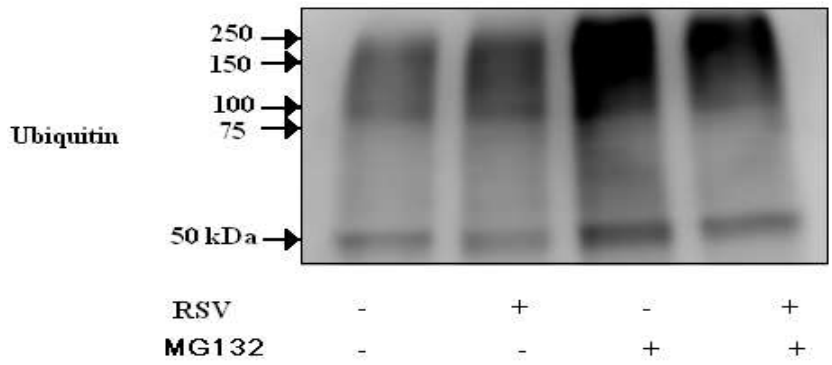
a) Cell lysates were analysed with species independent, mono- and polyubiquitinylated conjugates monoclonal anti-FK2 antibody against ubiquitin.

b) Cell lysates were subjected to immunoprecipitation with species independent, mono- and polyubiquitinylated conjugates monoclonal Anti-FK2 antibody against ubiquitin. Protein ubiquitination was then analysed with the same antibody.

Antibody detects the correct, smeared band of high molecular weight which has previously been reported in other studies and is consistent with that shown in figure 4.1. Ubiquitin band shows enhanced intensity in MG132 treated samples. Difference between infected and non-infected samples is not apparent.

Experiment was repeated, adding one experimental condition and MG132 infected treated sample. Results are shown in figure 4.8.

Figure 4.8. RSV influence on the expression of ubiquitinated protein in A459 cells infected with RSV and treated with MG132 after immunoprecipitation with anti-ubiquitin antibody. Western blot probed with anti-ubiquitin antibody.



Western blot analysis showing changes in the amount of ubiquitinated proteins in A549 cells after RSV infection and proteasome inhibition. Cells were infected with 1:1 MOI of RSV. After two hours two samples were treated with 10 mM of MG132 in DMSO for another two hours. Cell lysates were subjected to immunoprecipitation with species independent, mono- and polyubiquitinylated conjugates monoclonal Anti-FK2 antibody. Protein ubiquitination was analysed with the same antibody.

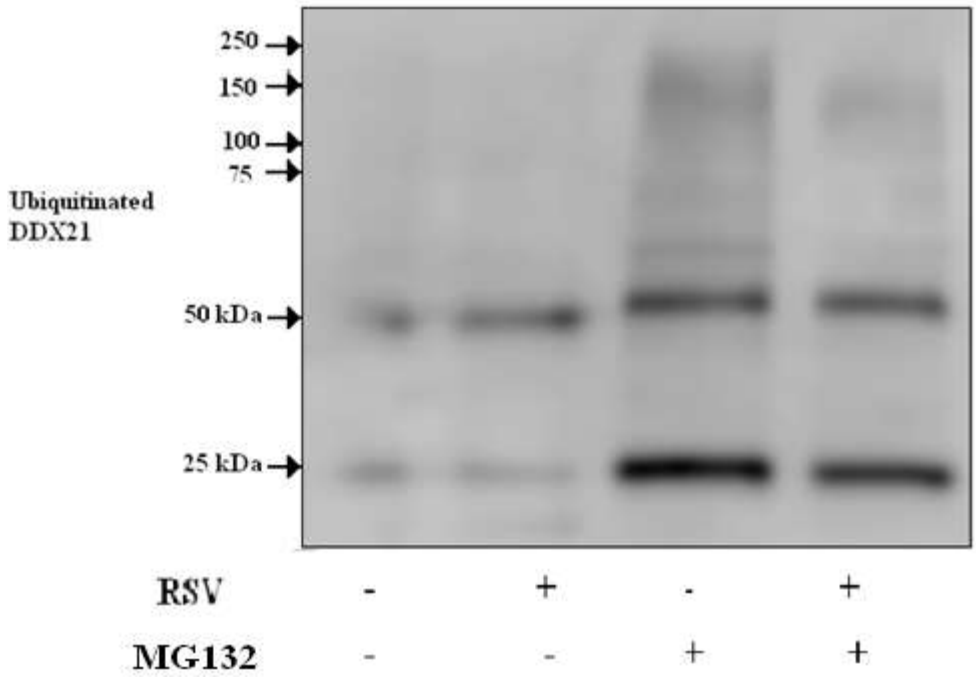
Antibody detects a band consistent with ubiquitin, which is darker in non-infected, MG132 treated sample. There is also additional band at the level of 50 kDa in all samples- possibly heavy chain of the Immunoglbulin used during immunoprecipitation.

4.2.2.2. Influence of RSV infection on expression of DDX21 in bronchial epithelial cells subjected to immunoprecipitation.

In the next blot, magnetic beads were coated with anti-DDX21 antibody and blot probed with anti-ubiquitin antibody. Results are shown in Figure 4.9

Ubiquitin band gives the strongest signal in non-infected sample treated with MG132. There are also two bands detected at the level of 25 and 50 kDa in all samples consistent with immunoglobulin light and heavy chains respectively. (See Figure 4.9)

Figure 4.9. RSV influence on the expression of ubiquitinated DDX21 in A459 cells with proteasome inhibition after immunoprecipitation with anti- DDX21 antibody. Cell lysates immunoprecipitated with anti-DDX21 antibody. Western blot probed with anti-ubiquitin antibody.

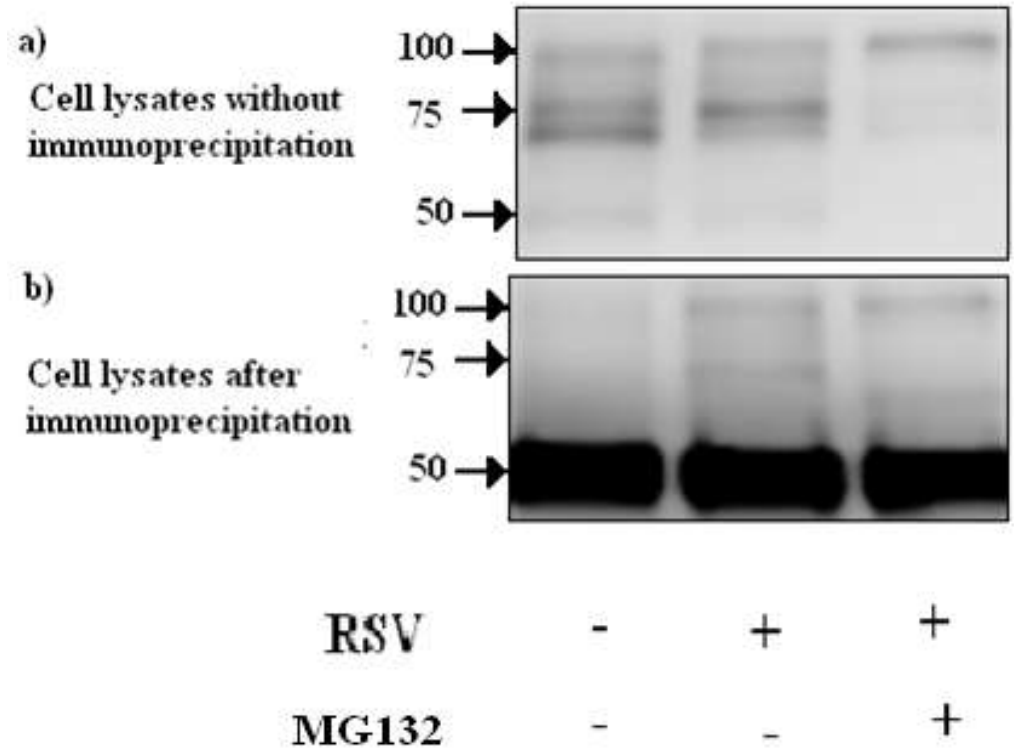


Western blot analysis showing changes in the amount of ubiquitinated DDX21 in A549 cells after RSV infection and proteasome inhibition. Cells were infected with 1:1 MOI of RSV. After 2 hours, 2 samples were treated with 10mM of MG132 in DMSO for another 2 hours. Cell lysates were subjected to immunoprecipitation with rabbit monoclonal anti-DDX21 abcam (ab182156) antibody. Protein ubiquitination was analysed with species independent, mono- and polyubiquitinylated conjugates monoclonal Anti-FK2 antibody against ubiquitin.

Figure 4.10. RSV influence on the expression of ubiquitinated DDX21 in A459 cells with proteasome inhibition after immunoprecipitation with anti-ubiquitin antibody.

a) **cell lysates without immunoprecipitation, western blot probed with anti-DDX21 antibody.**

b) **cell lysates after immunoprecipitation with anti-ubiquitin antibody, western blot probed with anti- DDX21 antibody.**



Western blot analysis showing changes in the amount of ubiquitinated DDX21 in A549 cells after RSV infection and proteasome inhibition. Cells were infected with 1:1 MOI of RSV. After 2 hours, 1 sample was treated with 10mM of MG132 in DMSO for another 2 hours.

a) Cell lysates were analysed with rabbit monoclonal anti-DDX21 abcam (ab182156) antibody.

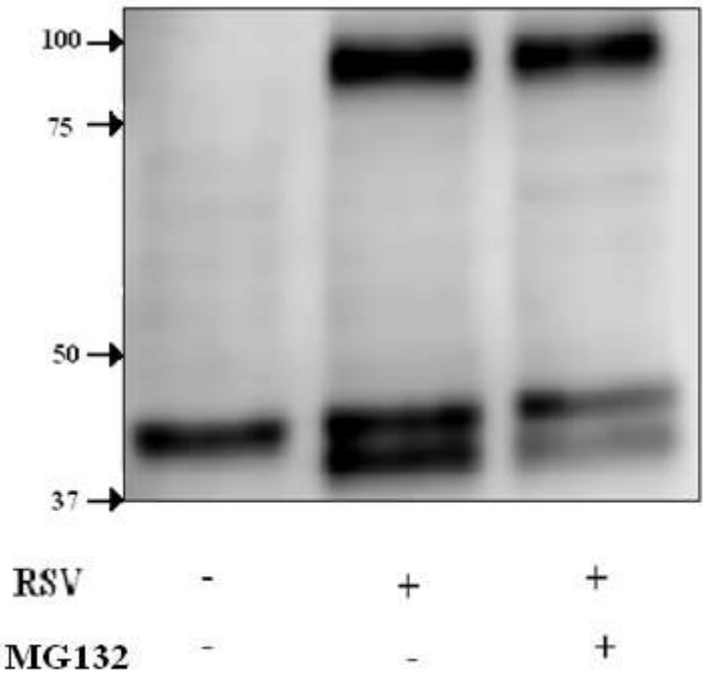
b)Cell lysates were subjected to immunoprecipitation with species independent, mono- and polyubiquitinylated conjugates monoclonal Anti-FK2 antibody against ubiquitin. Protein ubiquitination was then analysed with rabbit monoclonal anti-DDX21 abcam (ab182156) antibody.

Results obtained from this experiment resemble the first DDX21 blot (Figure 4.3). Distribution of bands changes between samples and lower molecular weight bands disappear with MG132 treatment. In immunoprecipitated samples, the strongest signal is detected in infected sample without MG132 treatment. The 50 kDa band is again detected in all samples.

These results led to the next step, to make sure that samples were infected with RSV and that there was no contamination of control. In order to prove it, western blot analysis of the same samples was carried out using anti-RSV antibody. Results are shown in Figure 4.11

Polyclonal RSV antibody detects 3 bands possibly corresponding to 3 RSV proteins. Protein G ~90 kDa, protein F~55 kDa and protein N~46 kDa. 90 kDa band and a lighter bands ~40kDa is detected only in infected samples. 46kDa band is detected in all samples.

Figure 4.11. RSV expression in A459 cells infected with RSV and treated with MG132.



Western blot analysis showing changes in the amount and pattern of bands of RSV in A549 cells after RSV infection and proteasome inhibition. The cells were infected with 1:1 MOI of RSV. After 2 hours, one sample was treated with 10mM of MG132 in DMSO for another 2 hours. Cell lysates were analysed with purified IgG conjugated to Biotin anti-RSV antibody(7950-0004Bio-rad).

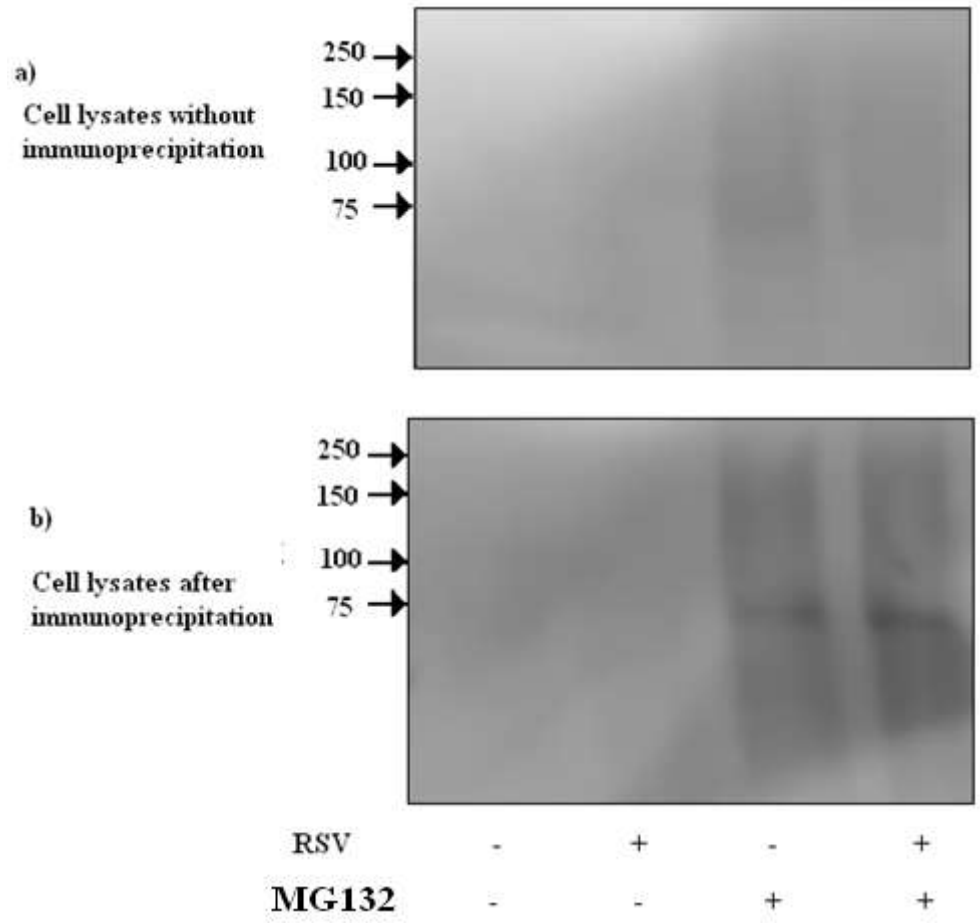
4.2.2.3. RSV influence on the expression of RPS10 in bronchial epithelial cells subjected to immunoprecipitation.

RPS 10 was immunoprecipitated from samples. Western blot was probed with anti-ubiquitin antibody to demonstrates changes in ubiquitinated RSP 10 in the samples. Results are shown in Figure 4.12.

Figure 4.12. RSV influence on the expression of ubiquitinated RPS10 in A459 cells with proteasome inhibition.

a) **Cell lysates without immunoprecipitation, western blot probed with anti-ubiquitin antibody.**

b) **cells lysates immunoprecipitation with anti-RPS10 antibody, western blot probed with anti-ubiquitin antibody.**



Western blot analysis showing changes in the amount of ubiquitinated RPS10 in A549 cells after RSV infection and proteasome inhibition. Cells were infected with 1:1 MOI of RSV. After 2 hours, 2 samples were treated with 10mM of MG132 in DMSO for another 2 hours.

a) Cell lysates were analysed with species independent, mono- and polyubiquitinylated conjugates monoclonal Anti-FK2 antibody against ubiquitin.

b)Cell lysates were subjected to immunoprecipitation with rrabbit monoclonal anti-RPS10 abcam (ab151550) antibody. Protein ubiquitination was analysed with species independent, mono- and polyubiquitinylated conjugates monoclonal anti-FK2 antibody against ubiquitin.

Results of both sets of data show stronger signal in MG132 treated samples. There is no change between infected and non-infected samples.

It the next western blot analysis, the samples were immunoprecipitated with RPS10 antibody and then probed with the same antibody.

Results show a similar pattern to previous blots. RPS10 band is enhanced in samples treated with MG132. Signal detected in infected sample is weaker than in the non-infected one.

The other proteins examined similarly did not show any change with viral infection.

4.3. Discussion

The main objective of the work described in this chapter was to determine whether following infection there is a difference in ubiquitination of proteins selected from the proteomics (DDX21, annexin A1, HMGB1, RPS10, prohibitin and MAVS). The first step was to assess whether there are ubiquitinated proteins in the samples and if so, whether their amount changes following viral infection or proteasome inhibition. To examine this, cells were infected with increasing amounts of RSV and treated with MG132. Samples were then analysed by western blot with anti-ubiquitin antibody. The molecular weight of ubiquitin is 8,5 kDa. Results in Figure 4.1 show, instead of a single band at that level, a series of high molecular weight bands which look like a smear. This can be explained by ubiquitin attaching to proteins and creating a chain, making their molecular weight significantly higher. Bands are enhanced in MG132 treated samples indicating that the proteasome was inhibited successfully and caused accumulation of proteins, normally destroyed by this organelle. There is no change between samples infected with various MOIs of virus and level of ubiquitin. It is also not possible to tell the difference in the total amount of ubiquitinated proteins on the blot, as we see a cumulative effect. As proteomics data suggests, some proteins are more and some less

ubiquitinated, some are up- and some down-regulated. In order to determine changes in ubiquitination of each particular proteins and the optimal MOI of the virus, samples were analysed by western blot technique with antibody against each target protein. In order to validate the experimental approach and show further that proteasome inhibition was working, the first molecule probed for was Nrf2, as it was known to be ubiquitinated and degraded by proteasome. (Section 1.3.1).

Results of this blot (Figure 4.2.) show two bands detected for Nrf2, both in the correct range of ~95-105 kDa. Top band is darker in MG132 treated samples which suggests successful inhibition of proteasome, and validates this experimental approach. A possible explanation of the results is that lighter band is non-ubiquitinated/mono-ubiquitinated form of Nrf2 and heavier band polyubiquitinated one, as only polyubiquitinated Nrf2 is degraded by proteasome. Due to uneven loading of proteins between those samples visible on actin loading control, densitometry was performed. There is no difference between infected and non-infected samples without MG132 treatment. However, when comparing between MG132 treated samples, there is more of the heavier form in infected sample which could show that under viral infection Keap1-Cul3 ubiquitination system is disrupted and leads to accumulation of

Nrf2 in a cell. A link between MG132 treatment and increased Nrf2 level has been reported before.[150]

The first protein from the proteomics analysed by western blot was DDX21. (Figure 4.3) Results show a very thick band in Hep2 cell lysates, used as a positive control for DDX21 expression, in comparison to A549 cell lysates. This thick band could be a result of discrepancy in protein loading between samples as Hep2 cell lysate was prepared separately to A549 lysates. Unfortunately, there is no loading control available for this blot which makes it impossible to perform control normalised densitometry. Interesting finding on this blot is that pattern of DDX21 bands varies between samples. The band of interest (~87 kDa) is enhanced in MG132 treated samples which indicates proteasome inhibition. This band is also darker in the infected sample in comparison to non-infected one. Due to lack of densitometry results, it is impossible to quantify this result and compare to proteomics value. There are additional lower molecular weight bands in infected samples without MG132 treatment, these could be breakdown products from RSV generated degradation of DDX21. This however is only a speculation as, it has only been suggested that DDX21 is degraded by another respiratory virus-Influenza A virus.[151] An additional, heavier band was detected in samples treated with MG132 which could be explained as

accumulation of the polyubiquitinated form of DDX21 normally destroyed by proteasome.

Results from a repeated experiment with additional samples harvested at 24 hours posit infection (Figure 4.4.) are consistent and strongly suggest that DDX21 is normally broken down via the proteosome in A549 cells both with and without RSV. In both infected samples, lower molecular weight bands appear again which supports the theory that RSV infection leads to DDX21 degradation. However, it has to be pointed out that this experiment alone cannot definitively confirm that it is the virus having a degrading effect on the cell, as there is still the possibility that it is the cell not producing or reducing amount of DDX21 whilst infected.

In an attempt to explain the appearance of extra bands and their pattern, this experiment was modified and extra controls were added. One of the samples was treated with palivizumab, a monoclonal antibody used prophylactically for infants in high risk group of RSV bronchiolitis, in order to see if it is the virus or other contaminants in the viral preps that influence the results. Two Hep2 controls were added to see if MG132 has an effect on DDX21 in this cell line. This time, the amount of protein in samples was first measured by BCA assay (Section 2.3) to ensure an even loading of protein in all samples and loading control was provided in a form of actin

band(Figure 4.5). A band of ~87 kDa was detected in all samples which confirms role of Hep2 as positive control. In all A549 samples there are additional lower molecular weight bands present, which have the same pattern in all experimental conditions. There is no difference in the amount of protein between samples, apart from control sample (without infection or treatment), in which the signal is stronger. These findings are confirmed by densitometry. This blot only validates Hep2 cells as control for DDX21 expression but does not show change in expression of DDX21 between different conditions. It is hard to explain the lack of change between samples but it is most probably due to a human error while conducting the experiment or western blot analysis. This points to a need for more repeats of experiments as the control with palivizumab could be very informative. The antibody could not be used in all experiments due to cost and limited availability.

Infected and non-infected cells were analysed using immunohistology to determine whether cellular location of DDX21 changes with infection.(Figure 4.5.). In healthy cells DDX21 is visible as green spherical shapes, contained within what are most probably nucleoli of the cell which is consistent with literature.[152] To confirm this dual staining with a nucleolar protein would have to be performed. In infected cells, DDX21 is more dispersed and its colour

less intense. On closer observation, it could be interpreted that DDX21 escapes the nucleolus and migrates into nucleoplasm during infection. Together with results of western blots this could contribute to the possible explanation that DDX21 is degraded during viral infection, either by viral proteins or the cells own degradative mechanisms. It has been previously reported that in Influenza A virus, DDX21 is counteracted by the viral NS1 Protein.[151] Immunohistology results have a descriptive rather than quantifiable character, which makes it difficult to assess whether the amount of protein actually changes in different conditions. The next step would be to quantify these results but a suitable method has not been found so far and results remain preliminary. Further research would have to be carried out to quantify these results. Potentially, samples could be analysed with immunochemistry where the chromogen intensity can be measured. Chromogen stains appear more intense in fields with more protein, unlike immunofluorescence, in which the brightness of a region is directly proportional to the amount of detected protein.[153] Because it is known that DDX21 inhibits Influenza virus transcription, the next useful addition to immunohistology would be staining with red fluorescent-labelled RSV to visualise change in virus location and determine if any RSV proteins co-localise with DDX21.

The same set of samples was next analysed with anti-RPS10 antibody (Figure 4.7). Obvious accumulation of protein in samples treated with MG132 again indicates successful proteasome inhibition. The more RSV was added to the sample, the less protein there was in samples without MG132 treatment. Reverse relation is apparent in samples with MG132 treatment. The higher the MOI of virus, the more protein is present in the sample because the proteasome which would normally degrade it is inhibited. Immunohistology slides for RPS10 show the protein contained within multiple, small spherical shapes (most probably ribosomes) in cytoplasm of healthy cells. In infected cells, the protein is almost invisible. This correlates with western blot, and densitometry findings which might mean that the protein is down-regulated in RSV infection. RPS10 has been suggested to be involved in viral RNA transcription and replication and results of western blot and immunohistology analysis presented in this thesis, show the potential for an important interaction between this protein and RSV. This could be direct for example by NS1 or 2, or indirect through viral proteins acting on other cellular proteins which influence RPS10 expression and ribosome integrity. It is only an assumption that RSV is inflicting these changes directly and would have to be demonstrated in an additional way, perhaps by co-precipitation. Similarly to Nrf2 and

DDX21, it would be useful to incubate immunohistology slides with a PE stained anti-RSV antibody to show RSV proteins in red and its location in cells with and without infection. This result gives a reason for running more experiments with immunoprecipitation, to see whether it undergoes differential ubiquitination.

The next stage of experiments was immunoprecipitation. For each of the proteins, optimal MOI was chosen on the basis of results from previous blots (Figure 4.1). Immunoprecipitation aimed to show whether changes on the blots are caused by protein ubiquitination and whether they can be linked to virus infection.

The aim of the first two blots was to validate experimental approach and precipitate ubiquitin/ubiquitinated proteins from samples. Figures 4.7 and 4.8 show successful proteasome inhibition and ubiquitin precipitation from samples. A difference between infected and non-infected cells is not apparent on the first blot. On the second one, the amount of ubiquitinated proteins is higher in non-infected samples treated with MG132 in comparison to infected cells with treatment. This could mean that overall the amount of ubiquitinated proteins decreases in RSV infection because of different ratios of ubiquitination for each protein but some would get up and some down regulated. More probably however, this could just be loading differences. This led to the next step which was immunoprecipitation

of target proteins one by one. A finding worth mentioning is an additional band at the level of ~55 kDa in all samples. Most probably it is immunoglobulin band which appears on further immunoprecipitation blots and is a common problem in immunoprecipitated samples in western blotting. It results from using antibodies raised in the same species for immunoprecipitation and western blotting which shows up as heavy and light chain on the level of ~25 and ~55 kDa on the image. Further optimisation of the experiment, such as antibody crosslinking, should be performed in future experiments in order to avoid this problem.

Figure 4.9 shows samples immunoprecipitated with anti- DDX21 antibody and probed with antiubiquitin antibody which should result in detection of ubiquitinated DDX21. Surprisingly, there is more ubiquitinated DDX21 in non-infected MG132 treated sample than in the one with RSV. This result is contrary to what was expected on the basis of proteomics data, however, the ~50 kDa immunoglobulin band which appears on this blot is also more enhanced in this sample. This could mean that protein loading is uneven and darker band in the non-infected MG132 treated sample simply means higher concentration of protein in this sample. The next, very important step

in optimising these experiments would be to find a way to provide loading control for immunoprecipitated samples.

Figure 4.10 shows result of immunoprecipitation performed the other way round. Lysates were immunoprecipitated with anti-ubiqtuitin antibody and blot probed with antibody against DDX21. Retrospectively it would be very helpful if the blot consisted of the same samples as previously, not only 3 samples which is another suggestion for improvement in future experiment. Lower molecular weight bands appear in infected sample again (like in Figure 4.3.) which support the potential explanation of DDX21 being degraded by the virus. Pattern of bands in control samples is slightly different than in Figure 4.3. which makes the results for this protein inconsistent. In order to examine if difference in results is caused by an error during conducting the experiment such as contamination of controls with virus or unsuccessful infection of the RSV samples, anti- RSV was used on the samples. Results from Figure 4.11 show 3 bands detected for virus. These should correspond to three surface protein of RSV ~90 kDa protein G, ~55kDa protein F and ~46kDa protein N. Two of the bands on the blot match proposed molecular weights (Protein G and F) and are present only in infected samples. Third band present in all samples is most likely to be a non-specific band.

This blot would have to be repeated to see if the same pattern of bands appears again.

The same approach was used for RPS10. Samples immunoprecipitated with anti-RPS10 antibody and probed with anti-ubiquitin antibody in Figure 4.12, show successful inhibition of proteasome by accumulation of ubiquitinated RPS10 in samples treated with MG132. There is no difference between infected and non-infect samples. Again, in this case densitometry could give a more detailed, quantifiable results if there was a way of producing loading control. Lack of change on the blot led to the next step which aimed to validate that proteins are truly being precipitated from samples. To prove this, samples were precipitated with antibody against RPS10 and the same antibody used for western blot. This time infected, MG132 treated sample showed less RPS10 than non-infected control. This data correlates to the proteomics data but to draw conclusions and preferably quantify it, a loading control would have to be produced and densitometric analysis conducted. It would also be helpful to turn the experiment around, like it was done with DDX21 and precipitate samples with anti-ubiquitin antibody and then analyse on a western blot with anti-RPS10 antibody.

5. Final discussion:

In this study I have investigated the significance of Nrf2 signaling and ubiquitination of proteins in RSV infection.

The first part of the project aimed to determine whether sulforaphane might reduce RSV replication in airway epithelial cells. As outlined in the introduction, sulforaphane or related compounds are found in foodstuffs and may form a potential therapeutic approach.

Optimising experiment which aimed to answer the question, created smaller specific objectives which had to be achieved in order for the main experiment to be reliable. All these objectives including finding the correct antibody for detecting Nrf2, identifying appropriate cell line, concentration of sulforaphane and time of treatment were met.

Majority of experiments have also proven that sulforaphane induces expression of Nrf2 in BEAS-2B bronchial epithelial cells, which is in agreement with available literature.[154] However, there was no change in RSV protein expression between samples with and without sulforaphane treatment which is a crucial finding for the study and indicates that these results do not support the idea that RSV expression is influenced by sulforaphane. Results of many of these experiments were not consistent and often did not follow a pattern which partly can be blamed on technical difficulties due to lack of previous laboratory experience and temporary problems with

reagents but mainly means that it is likely that the hypothesis was wrong. Data generated during this research does not support the role *in vitro* for Nrf2 in suppressing viral replication in epithelial cells. Viral replication was measured by western blot and real time PCR for the N gene mRNA. It would be helpful to look at different analysing techniques like plaque assay to further confirm that functional viral levels are not reduced. This is one of the suggestions for future work. One of the potential reason why my experiments do not show the changespreviously reported, could be that, *in vivo* experiments using an animal model may give different results because the antiviral activity of other cells such as macrophages or neutrophils may be affected by NRF2 knockdown.[155] In my experiments I have only looked at *in vitro* conditions using the BEAS 2B human bronchial epithelial cell line.

Results of Nrf2 experiments suggest a new idea, that Nrf2 might be degraded by virus. (Figure 3.9.) Decrease or complete removal of Nrf2 from samples infected with virus suggest that RSV could inhibit Nrf2 expression or have a degrading effect on it. This however, is only a hypothesis and would have to be further examined, however, Komaravelli et al study[141] has recently confirmed that RSV infection down-regulates antioxidant enzyme expression by triggering deacetylation-proteasomal degradation of Nrf2.[156] Similar result has

been reported in a different research group, which states that Nrf2 expression was reduced in RSV infection as a potential mechanism for reducing gene expression of AOE.[157] Even though our hypothesis has not been proved, this finding could still help in developing therapeutic agents against RSV or explaining why currently an effective treatment does not exist.

The aim of the second part of the study was to validate data from a series of proteomics experiements and show if selected proteins change their level of expression or are differentially ubiquitinated following RSV infection. This could allow the identification of novel protein targets for development of new therapuetics. Some of the proteins, such as prohibitin, Annexin A1, RPS10, HMGB1or MAVS did not show any change between samples in the experiments. DDX21 has been described as virus recognition protein in Influenza studies and as an essential component of ribosomes biogenesis.[151] Virus might be trying to degrade the protein in order to stop it from down regulating viral RNA and be more successful in early stages of infection process and also disable cell recognition mechanism so that viral RNA cannot be detected.

Almost all experiments confirmed inhibition of the proteasome and if the project did not have to be concluded due to approaching deadline of the degree and more immunoprecipitation experiments were

conducted, majority of proteins shows high potential of proving that they are differentially ubiquitinated in RSV infection. The aims therefore have been met partially. The knowledge about proteins chosen for this study is fragmentary and some of the post-translational modifications such as ubiquitination have not been studied at all.

This research is the first one to show evidence that DDX21 could undergo ubiquitination and gives a basis for designing experiments which could prove this process. Western blots also suggest that DDX21 is degraded by RSV which has previously been reported for Influenza A but not for RSV.

5.1. Limitations

The main limitation of this project was the restricted time to conduct experiments. More time would allow repeating each of the experiments at least 3 times to be able to assess if results are reproducible and if any patterns are emerging. It would also allow for the results to be quantified and statistical analysis to be conducted to see whether the change in results is statistically significant.

Due to the lack of previous laboratory experience, many of conducted experiments, especially at the beginning of the year contained errors. Considering the large variety of laboratory techniques including cell culture, virus preparation, BCA assay,

western blots, PCR and immunofluorescence used during 12 months of this project, these mistakes were treated as a part of learning experience and were unavoidable. Another consequence of no prior lab exposure is the lack of loading control as well as poor quality of some of the blots which made interpreting and quantifying results very challenging and incomparable. Technical difficulties which slowed down conducting experiments but could not have been avoided included issues like contamination of samples with mycoplasma for one of the immunofluorescence staining attempts. Each of the laboratory techniques used during this project has its own limitations, however, some of them proved to be especially challenging eg. use of western blotting for detecting Nrf2. Even though the methodology has been described many times in other research, optimisation of my experiments consumed a lot of time and many technical problems have been encountered on the way, such as antibody not detecting desired bands or detecting multiple non-specific bands. The issue of differences in reporting Nrf2 molecular weight has been thorough described in Lau et al report and discussed previously in Section 3.3. The discrepancy between presented results in appearance of bands has been cause by use of different percentage gels in western blot. On lower percentage gel (12%) Nrf2 band is „separated" and appears as double band

whereas on higher percentage gel (15%) it looks like antibody detects only one band. In the future, all experiments involving the same molecule should be conducted using exactly the same reagents so that results are comparable.

Densitometry is another technique which often appeared to be inconsistent with corresponding western blot like in Figure 4.9. This highlights the pitfalls of technique and why sometimes it is difficult to make western blot results quantifiable. Most of western blots have imperfections, eg one side of the blot has a higher background than the other or there are scratches on the blot which are picked up by the software on top of target protein bands. Densitometry is a way of confirming what can be seen on the blot with a naked eye but on worse quality blots results can be skewed because of the background noise.

The main limitation to the PCR assay used in this project, is that it only measures N gene expression and as such is not able to detect any change in expression of other viral RNA or protein molecules. It might be the case that the change would have been detected for a different viral RNA.

It is also important to note here, that any affect seen in the results could be directly done by RSV or indirectly by the cell responding to virus. It is an assumption that it is RSV that is causing these changes

and to be confident that they are truly caused by virus they need to be demonstrated by other techniques eg co-precipitation.

Another limitation is the fact that all experiments were conducted in vitro and in order to confirm results interaction between molecules and virus should be studied in vivo. It is possible that in a living organisms other factors would influence examined processes.

The experiments show that DDX21 is degraded in response to RSV infection. As DDX21 is involved in ribosome assembly at the nucleolus, degradation of this molecule would almost certainly affect the structure and function of ribosomes, changing the ability of the cell to make proteins. It is not clear if this is a response of a host cell to viral infection which might in turn limit translation of viral mRNA to protein and virus replication, or alternatively the virus causing breakdown of DDX21 as part of its programme to manipulate the cell's production of protein to favour viral replication. This could be tested at least in part by overexpressing DDX21 within the cell and seeing how this influences viral replication. In the respect to viral Influenza it is known that early in infection DDX21 limits viral RNA expression as part of the cell's defence to viral infection.[158] It remains possible that this is also the case in RSV infection but as DDX21 is degraded it seems more likely that RSV itself is causing a breakdown

of DDX21. It might be useful to use siRNA to reduce DDX21 expression and determine how this influences RSV replication.

5.3. Future work

All experiments which showed evidence of changes should be repeated at least 3 times in order to prove that results are reproducible and allow statistical analysis. It would be also beneficial to add more time points and controls to the experiments eg. Samples treated with Palivizumab. A reliable method of quantifying western blots and immunohistology should be found and used for statistical analysis.

All proteins of interest which showed changes in expression following viral infection should be studied by immunofluorescence including triple color staining for nucleus, target protein and RSV to examine interactions between proteins and virus and determine whether infection influences protein expression and localisation in a cell.

Results also show that MAVS and DDX21 could potentially be degraded by RSV in infected cells. Although we cannot take the initial results alone, they are promising and should be further examined by more western blots and other laboratory techniques. Seeing these differences suggests that these proteins have a role in either defending cell from infection or in viral replication within the

cell. As MAVS is essential in IFN pathway due to its role in activating IFN 1 signaling production it would be beneficial for virus to degrade it and stop its production of interferon which is one of the main ways the cell protects itself against the virus. Inhibiting MAVS would be important at multiple stages of viral infection as it has been shown that the molecule initiates production of defence factors which protects the cell in short term as well as stabilizes the defense against the virus with delayed kinetics[159].

The fact that, this research is the first one which could have suggested ubiquitination of DDX21 and its degradation by RSV is very promising and should be further examined. It also validates the approach of proteomics as a method of identifying proteins which change in ubiquitination after RSV infection, at least in A549 cells. Another suggestion for future work could be more experiments with immunoprecipitation reactions to prove it is differentially and gene complementation studies.

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