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# Regulation and impact of adaptor protein SQSTM1/p62 in the replication cycle of Respiratory Syncytial Virus in Airway Epithelial Cells

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**Regulation and impact of adaptor protein SQSTM1/p62 in the replication cycle of Respiratory Syncytial Virus in Airway Epithelial Cells**

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# Résumé

**Introduction:** le Virus Respiratoire Syncytial humain (RSV) induit un taux élevé de morbidité et de mortalité chez les enfants, les personnes immunodéprimées et les personnes âgées. Il existe un besoin urgent d'un nouveau traitement antiviral et d'un vaccin efficaces. Les cellules épithéliales des voies aériennes (AEC) sont la cible principale de RSV et constituent la première ligne de défense grâce à des mécanismes distincts, qui incluent une réponse antivirale autonome cellulaire. La protéine p62/SQSTM1 a de multiples fonctions cellulaires, y compris la séquestration spécifique de la cargaison ubiquitinée (c'est-à-dire, les protéines/organelles et les bactéries intracellulaires) pour leur clairance par autophagie. Des données publiées ont mis en évidence un rôle important de p62 dans la régulation de plusieurs virus (par exemple, le virus de la grippe et la dengue), favorisant ou restreignant sa réplication en fonction du virus. L'objectif de notre étude est de déterminer le rôle de p62 dans la régulation du cycle infectieux de RSV. **Méthodes et résultats:** L'analyse de l'expression de p62 dans les cellules A549 a montré que p62 est induit et phosphorylé au début de l'infection par RSV. Il est ensuite dégradé plus tardivement durant l'infection. La déplétion des niveaux de p62 a diminué l'accumulation intracellulaire des protéines virales, tandis que la relâche des virions infectieux a été augmentée. De plus, nous avons observé que la réplication de recRSV-GFP est diminuée dans des cellules exprimant de façon stable la protéine associée aux microtubules 1A/1B, chaîne légère 3 (LC3). LC3 recrute p62 et ses cargaisons à l'autophagosome pour qu'ils soient dégradés par autophagie. Des études sont actuellement en cours pour déterminer les mécanismes moléculaires, dépendant de p62, impliqués dans la régulation de la réplication de RSV. **Conclusion:** nos résultats mettent en évidence un rôle clé de p62 dans la réplication et la propagation de RSV. Ces études aideront à définir si p62 pourrait représenter une cible thérapeutique potentielle pour lutter contre l'infection à RSV.

**Mots-clés** : p62/SQSTM1, Virus Respiratoire Syncytial humain, RSV, cellules épithéliales des voies aériennes, réponse antivirale, UPS, autophagie, réplication virale.

# Abstract

**Introduction:** Human respiratory syncytial virus (RSV) causes a high rate of morbidity and mortality worldwide in children, immunocompromised and elderly people. There is an urgent need for effective antiviral treatments and vaccines for RSV. Airway epithelial cells (AECs) are the primary target of RSV and constitute the first line of defense through distinct mechanisms, including intrinsic antiviral responses. The p62/SQSTM1 protein has multiple cellular functions including cell signaling and sequestration of specific ubiquitinated cargo (*i.e.* proteins/organelles and intracellular bacteria) for autophagic degradation. The replication of several viruses has been shown to be sensitive to p62 levels. The goal of our study is to investigate the role of p62 in the regulation of RSV replication. **Methods and Results:** Analysis of p62 expression in A549 cells showed that p62 is induced and phosphorylated during early stages of RSV infection, followed by degradation at later times. P62 silencing diminished the intracellular accumulation of viral proteins, while causing increased release of infectious virions. Additionally, we observed that the stable expression of Microtubule-associated protein 1A/1B-light chain 3 (LC3), which recruits p62 and its cargos to the autophagosome for autophagy degradation, reduces recRSV-GFP replication. Studies are currently undertaken to determine the molecular mechanisms involved in p62-dependent regulation of RSV replication. **Conclusion:** Our results highlight a key role of p62 in the replication of RSV. These studies will help to define whether p62 might represent a potential therapeutic target to fight RSV infection.

**Keywords**: p62/SQSTM1, Respiratory syncytial virus, RSV, Airway epithelial cells, antiviral response, UPS, autophagy, viral replication.

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# Abbreviations

| | |
|---|---|
| AECs: | Airway epithelial cells |
| AP-1: | Activator protein 1 |
| APKCs: | Atypical protein kinases C |
| ATCC: | American Type Culture Collection |
| ATF6: | Activating transcription factor 6 |
| Atg: | Autophagy-related proteins |
| ATP: | Adenosine-5'-triphosphate |
| BGEM: | Bronchial Epithelial Cell Growth Medium |
| BIP: | Binding immunoglobulin protein |
| Cdc42: | Cell division control protein 42 homolog |
| CK2: | Casein kinase II |
| CCL3: | Chemokine (C-C motif) ligand 3 |
| CGAs: | Cyclic GMP-AMP synthase |
| CHIKV: | Chikungunya virus |
| CXCL10: | C-X-C motif chemokine 10 |
| DCs: | Dendritic cells |
| DENV: | Dengue virus |
| DNA: | Deoxyribonucleic acid |
| dsDNA: | Double-stranded DNA |
| dsRNA: | Double-stranded RNA |
| DUBs: | Deubiquitinating enzymes |
| E1: | Ubiquitin activating enzyme |
| E2: | Ubiquitin conjugating enzyme |
| E3: | Ubiquitin ligase |
| EGF: | Epidermal growth factor receptor |
| eIF2α: | Eukaryotic initiation factor 2-alpha |
| ER: | Endoplasmic reticulum |
| F: | Fusion protein |
| $F_0$: | Fusion precursor |

| | |
|---|---|
| FI-RSV: | Formalin-inactivated RSV vaccine |
| G: | Glycoprotein |
| GFP: | Green fluorescent protein |
| GRP78: | 78 kDa glucose-regulated protein |
| HSV-1: | Herpes simplex virus type 1 |
| HI-FBS: | Heat-inactivated fetal bovine serum |
| IFN: | Interferon |
| IKKε: | Inhibitor-κB kinase ε |
| IRE1: | Inositol-requiring enzyme 1 |
| IRF3: | Interferon regulatory factor 3 |
| IL: | Interleukin |
| JEV: | Japanese encephalitis virus |
| K: | Lysine |
| Keap1: | Kelch-like ECH-associated protein 1 |
| KIR: | Keap1-interacting region |
| L: | Large polymerase |
| L-Glu: | L-Glutamine |
| Le: | Leader |
| LC3: | Microtubule-associated protein 1A/1B-light chain 3 |
| LIR: | LC3-interacting region |
| LPS: | Lipopolysaccharide |
| M: | Matrix protein |
| M2-1: | Alternate reading frame 1 protein |
| M2-2: | Alternate reading frame 2 protein |
| MOI: | Multiplicity of infection |
| mRNA: | Messenger ribonucleic acid |
| MAVS: | Mitochondrial antiviral-signaling protein |
| MCP-1: | Monocyte chemotactic protein-1 |
| MDA5: | Melanoma differentiation-associated protein 5 |
| MIP-1α: | Macrophage inflammatory protein-1α |
| mTORC1: | Mammalian target of rapamycin complex 1 |

| | |
|---|---|
| N: | Nucleoprotein |
| NADPH: | Nicotinamide Adenine Dinucleotide Phosphate Hydrogen |
| NBR1: | Neighbor of BRCA1 gene 1 |
| NDP52: | Nuclear dot protein 52 kDa |
| NF-κB: | Nuclear factor-κB |
| NK: | Natural killer |
| NLRs: | Nucleotide-binding oligomerization domain containing like receptors |
| NOX: | NADPH oxidase |
| Nrf2: | Nuclear factor erythroid 2-related factor 2 |
| NS1: | Non-structural protein 1 |
| NS2: | Non-structural protein 2 |
| ORF: | Open reading frame |
| P: | Phosphoprotein |
| PAK1: | p21-activated kinase 1 |
| PAMPs: | Pathogen-associated molecular patterns |
| PB1: | Phox1 and Bem1p domain |
| PERK: | PKR like ER kinase |
| PFU: | Plaque-forming unit |
| Rab-11-FIP: | Rab11 family interacting protein |
| RecRSV-GFP: | Recombinant RSV virus expressing GFP protein |
| RIP1: | Receptor-interacting protein 1 |
| PKR: | Protein kinase R |
| PI3KC3–C1: | Phosphatidylinositol 3-kinase catalytic subunit type 3 complex 1 |
| RIG-I: | Retinoic acid-inducible gene I |
| RLRs: | RIG-I like receptors |
| RNF26: | Ring finger protein 26 |
| PRRs: | Pattern recognition receptors |
| RNA: | Ribonucleic acid |
| RNAi: | RNA interference |
| RNP: | Ribonucleoprotein |
| RSV: | Human respiratory syncytial virus |

| | |
|---|---|
| Ser403: | Serine 403 |
| SH: | Small hydrophobic protein |
| SIN: | Sindbis virus |
| ssRNA: | Single-stranded RNA |
| SQSTM1: | Sequestosome 1 |
| STAT2: | Signal transducer and activator of transcription 2 |
| STING: | Stimulator of interferon genes |
| T6BP: | TRAF-interacting protein with forkhead-associated domain |
| TANK: | TRAF family member associated NF-κB activator |
| TB: | TRAF6 binding region |
| TBK1: | TANK-binding kinase 1 |
| TLRs: | Toll-like receptors |
| TNF-α: | Tumor necrosis factor α |
| Tr: | Trailer sequence |
| TRAF6: | Tumor necrosis factor receptor-associated factor 6 |
| TrC: | Tr complement region |
| TRIF: | TIR-domain-containing adapter-inducing interferon-β |
| TUBEs: | Tandem Ubiquitin Binding Entities |
| Ub: | Ubiquitin |
| UBA: | Ubiquitin associated domain |
| ULK1: | Unc-51-like protein kinase 1 |
| UPS: | Ubiquitin proteasome system |
| WCE: | Whole cell extracts |
| ZZ: | ZZ-type zinc finger region |

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I enjoyed a lot living in Montréal and being a student at the Université de Montréal these years. Looking back in time I feel blessed to have learnt from different people and cultures. I will always have very good memories from each of the persons that I met throughout this journey.

# 1. Introduction

# 1.1 Respiratory syncytial virus

## 1.1.1 RSV relevance and physiopathology

Human respiratory syncytial virus (RSV) is a worldwide viral agent associated with the development of pathologies ranging from mild upper respiratory tract illness, like otitis media and rhinitis, to severe lower respiratory tract infections like bronchiolitis and pneumonia (Collins & Melero, 2011).

RSV commonly infects individuals of all ages without severe medical complications. However, in immunocompromised and transplant patients, children under two years of age and the elderly, RSV causes high rates of morbidity and mortality. The main risk factors for severe RSV infection include: preterm birth, low birth weight, congenital or acquired immunodeficiencies and cardiopulmonary disorders (Borchers, Chang, Gershwin, & Gershwin, 2013) although healthy infants, without presenting identified risk factors, can also develop severe RSV disease (Hall et al., 2009; Hervas et al., 2012). Different studies demonstrate that at 2 years of age the majority of children will have experienced infection with this virus at least one time (Dawson-Caswell & Muncie, 2011; Glezen, Taber, Frank, & Kasel, 1986). Severe RSV infection during the first years of life is associated with a susceptibility to develop airway hyperreactivity and pulmonary diseases like asthma (Knudson & Varga, 2015). In addition, RSV has been associated with increased cases of encephalopathy (Morichi et al., 2011).

The histopathological features of severe RSV disease include epithelial cells sloughing and loss of cilia, disruption of the bronchiolar and alveolar epithelium, mucosal and submucosal edema, airway obstruction and immune cells infiltration and inflammation (Pickles & DeVincenzo, 2015). As a consequence, the infected individuals can present airway constriction, rapid or labored respirations, and wheezing (Hall et al., 2009). RSV pathology is complex and has been associated with the ability of this virus to modulate or evade the host immune response (Schmidt & Varga, 2017). This is reflected in the positive association between the excessive activation of the immune system and the pathological effects of the infection (Johnson, Gonzales, Olson, Wright, & Graham, 2007) although a poor immune response can also lead to an uncontrolled infection and pathological outcomes (Welliver, Reed, & Welliver, 2008).

Accumulating evidence indicates that the immune system is unable to provide efficient life-long protection against RSV infection (Falsey et al., 1999).

Different strategies have been developed since the isolation of RSV to fight the infection (Table 1). In the 1960's a formalin-inactivated RSV (FI-RSV) vaccine trial was administered in infants with disastrous results, as immunization with this vaccine led to an enhanced disease after natural infection resulting in the hospitalization of 80% of the vaccinated infants and two deaths (Derscheid et al., 2013; Kapikian, Mitchell, Chanock, Shvedoff, & Stewart, 1969). Since then increased efforts for developing an effective and safe vaccine have been made without success. At present, prophylactic treatment to reduce severe RSV disease is restricted to passive immunization with Palivizumab, which are costly short-term monoclonal antibodies (Table 1); however drug cost restricts the treatment to only infants with high risk to develop severe disease (Robinson, Le Saux, Canadian Paediatric Society, & Immunization, 2015). The treatment for acute RSV disease worldwide is mainly supportive medical care (Table 1). Despite many scientific advances related to the interaction between RSV and the host, to date there is no effective antiviral treatment or vaccine to prevent RSV infection, hence finding a novel therapeutic against this virus is still a priority worldwide.

**Table 1.** Strategies, approaches and treatments for severe RSV disease.

| | |
|---|---|
| **Vaccines** (Anderson et al., 2013; Neuzil, 2016) | ▪ FI-RSV vaccine led to enhanced RSV immunopathology after natural infection.<br>▪ A wide range of promising vaccine strategies have been formulated, including virus-like articles, inactivated vaccines and DNA and viral vectors; however none has proved sufficient efficacy and safety.<br>▪ The immunopathology effects associated with the infection and the suboptimal immunization induced by RSV challenge the development of an efficient and safe vaccine.<br>▪ The highest priority target population are infants that present an immature immune system.<br>▪ There are currently 60 RSV vaccine candidates in development, including live attenuated and particle based-vaccines, this last one already in phase 3.<br>▪ At present, there is no vaccine licenced for human use. |
| **Prophylactic treatment with Palivizumab** (Adams et al., 2010; Robinson et al., 2015) | ▪ Treatment consists in administration of humanized monoclonal antibody against the fusion (F) protein of RSV.<br>▪ High cost: five doses of Palivizumab for an infant with a mean weight of 5 kg cost approximately $5,600 Canadian dollars.<br>▪ Monthly administration is required for effective immunization.<br>▪ Reduces the rate of hospitalizations.<br>▪ Restricted to high risk infants to develop severe RSV disease.<br>▪ Many infants develop severe RSV disease in absence of identifiable risk factors.<br>▪ No benefit is provided during active infection.<br>▪ Palivizumab-resistant RSV viruses have been isolated. |
| **Supportive medical care** (Borchers et al., 2013; Hall et al., 2009; Nair et al., 2010) | ▪ Severe RSV disease causes high rates of hospitalizations worldwide, which are translated in elevated costs of healthcare.<br>▪ Medical care consists in clearing of nasal obstruction, hydration by administration of oral and intravenous fluids, administration of supplemental oxygen and mechanical ventilation.<br>▪ Hospitalization lasts from less than 1 day to 26 days. |

## 1.1.2 RSV classification, structure and organization

The *Paramyxoviridae* family is constituted of enveloped viruses with non-segmented negative strand RNA genomes. This family is divided into two subfamilies: the *Paramyxovirinae* and the *Pneumovirinae*. The *Paramyxovirinae* subfamily includes viruses like mumps virus, measles virus and parainfluenza viruses, while the *Pneumovirinae* subfamily includes RSV and metapneumovirus. Many of these members are pathogenic for humans and animals (Palgen, Jurgens, Moscona, Porotto, & Palermo, 2015). RSV stands out from the other members of this family as it shows a relatively divergent structural complexity in terms of a greater number of genes and proteins (Collins, Fearns, & Graham, 2013).

There is only one serotype of RSV and two circulating strains, A and B, the A-strain being the most prevalent and slightly more pathogenic (Borchers et al., 2013; Hall et al., 2009; Meng, Stobart, Hotard, & Moore, 2014).

As other members of the *Paramyxoviridae* family, the particles of RSV are pleomorphic and found in two different shapes: spherical from 150-250nm and filamentous up to 10µm long, the latter being the predominant one (Bachi & Howe, 1973; Liljeroos, Krzyzaniak, Helenius, & Butcher, 2013). RSV has a non-segmented negative strand RNA genome with a length of 15.2 kb, which contains 10 genes arranged sequentially. The genes are transcribed in a sequential manner in 10 individual mRNAs (Fig. 1). In total, the RSV genome encodes 11 separate viral proteins. Each mRNA encodes a single protein, except for the M2 mRNA that has two overlapping open reading frames (ORF) and encodes two proteins whose expression depends on the stage of infection: the alternative reading frame 1 (M2-1) and 2 (M2-2), the first one is required for transcription and the second for viral replication (Collins et al., 2013).

- The non-structural protein 1 (NS1) and 2 (NS2), which are not found in any other members of the *Paramyxoviridae* family, are small proteins that are not part of the viral particle, but are involved in modulating the host response (Swedan, Musiyenko, & Barik, 2009).
- The nucleoprotein (N), the phosphoprotein (P), M2-1 protein and the large polymerase (L) encapsidate the genomic RNA to form the ribonucleoprotein (RNP) complex.

- The matrix (M) protein is present on the inner face of the envelope and stabilizes the RNP complex with the viral envelope (Collins & Melero, 2011).
- The M2-2 protein is involved in the viral replication, but is not part of the mature viral particle (Borchers et al., 2013).
- The viral envelope is derived from the host cellular membrane and contains three anchored viral glycoproteins: the glycoprotein (G), the fusion protein (F) and the small hydrophobic protein (SH). The RSV G protein is highly glycosylated and defines the strain A and B of the virus (McLellan, Ray, & Peeples, 2013). In addition to the G protein, a truncated soluble form is produced and secreted by the infected cells. The F protein is initially synthesized as an inactive precursor protein ($F_0$). To become active, the $F_0$ protein is proteolytically cleaved by a furin-like protease at two sites, 109 and 136 amino acids (aa), to remove a polypeptide chain of 27 aa and form two subunits, F2 (N-terminal) and F1 (C-terminal). These F2 and F1 subunits are covalently linked by two disulfide bonds (Gonzalez-Reyes et al., 2001; Zimmer, Trotz, & Herrler, 2001). The fusion competent F protein integrated in the virion is formed by three of these F2/F1 linked subunits (McLellan et al., 2013). The G and F proteins are the main targets of neutralizing antibodies.

Different strategies have been developed to study the replication and pathogenesis of many RNA viruses including the use of reverse genetic systems, in which recombinant viruses are engineered by recombinant DNA technology. For RSV this strategy is not widespread because of the nature of its genome (–RNA) that requires the use of additional helper constructs or viruses to initiate virus replication. Additionally, reverse genetic models for RSV, including bacterial artificial chromosome-based, display attenuate infectivity (Hotard et al., 2012). Therefore infections with purified RSV viruses remain the most common strategy to study its pathogenesis and interaction with the host.

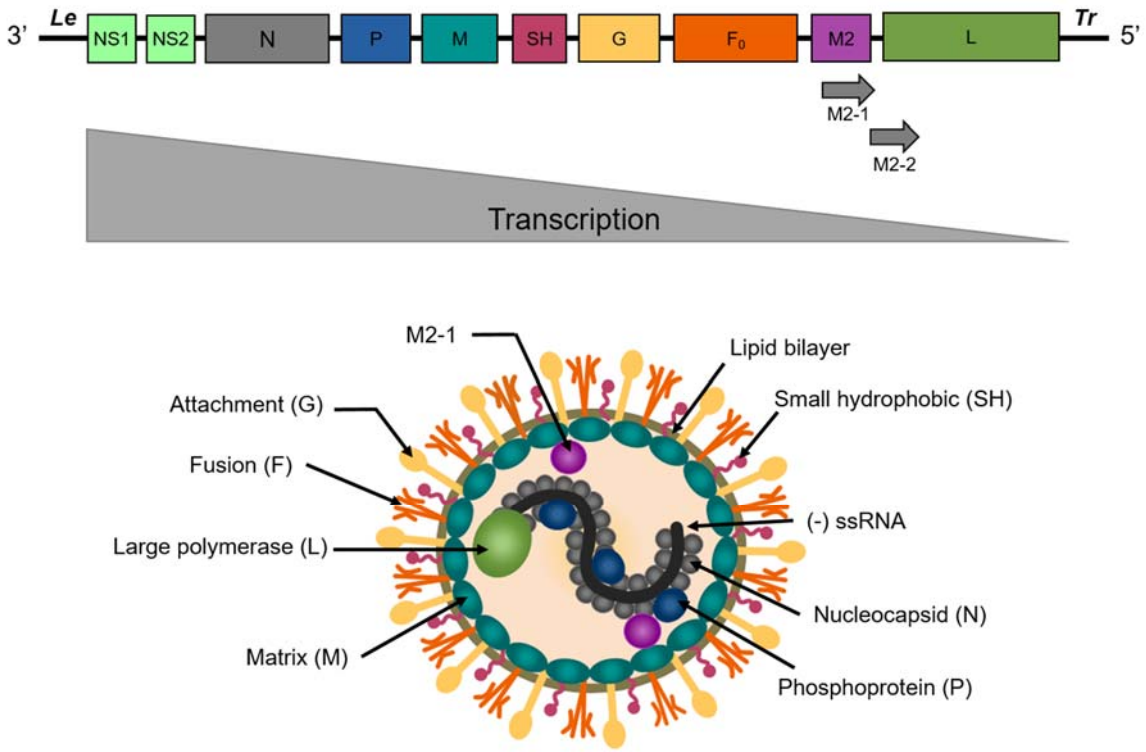


**Figure 1. RSV genome organization and virion structure.** The RSV genome is a non-segmented negative RNA strand that contains a 3' Le promoter region and a 5' Tr sequence. The viral genome encodes 10 genes and 11 proteins (upper panel). The M2 mRNA has two overlapping ORF that encode the M2-1 and M2-2 proteins. The viral mRNAs are transcribed in a polar gradient (from 3' to 5') in which the gene transcription decreases along the gene order. In the lower panel it is shown the organization of the viral proteins in the mature virion. The proteins NS1, NS2 and M2-2 are accessory proteins that do not form part of the viral particle.

Virion structure adapted from http://www.kuleuven.be/rega/mvr/images/RSV-1.jpg

### 1.1.3 RSV infectious cycle

*In vivo* RSV predominantly infects columnar ciliated airway epithelial cells (AECs) (Pickles & DeVincenzo, 2015). Compared to other paramyxoviruses, RSV has the potential to infect myeloid cells, such as dendritic cells (DCs) and macrophages, and produce viral progeny, however, these cells are less permissive to the virus replication and their contribution to the production of infectious particles is minimal (Dakhama, Kaan, & Hegele, 1998; Johnson, Johnson, Corbett, Edwards, & Graham, 2011; Midulla et al., 1989). In AECs, RSV maintains an apical tropism that limits infection to the superficial stratus of the respiratory tract (Brock, Goldenring, & Crowe, 2003).

The replication cycle of RSV involves a series of highly coordinated and organized events that results in the production of a new viral progeny. The different steps of the infectious cycle of RSV will be described below altogether with the molecular interactions between the host and the virus.

#### 1.1.3.1 Attachment and entry

Amongst the RSV envelope glycoproteins, the protein G and F are involved in the entry process of the virus, while the SH protein is dispensable at this step of the viral cycle (Mastrangelo & Hegele, 2013). RSV attaches to AECs by electrostatic interactions of the G protein with the cellular membrane via glycosaminoglycans, particularly heparan sulfate (McLellan et al., 2013). After attachment, the F protein interacts with the cell-surface receptor nucleolin and cholesterol-rich microdomains (San-Juan-Vergara et al., 2012; Tayyari et al., 2011). However, as nucleolin is a ubiquitous protein expressed on the cell surface of many cell types it fails to explain the specific tropism of RSV for the airway epithelium. This suggests that other cellular receptors/factors might be involved in the entry process of RSV (Tayyari et al., 2011). The attachment of RSV triggers the hemifusion of the viral envelope with the cellular membrane and activates multiple cellular factors (*i.e.* EGF receptor, Cdc42, PAK1, etc.) which induce actin rearrangements and membrane blebs to internalize the virus by micropinocytosis (Fig. 2). The virus is transported inside the macropinosomes where it completes its fusion after proteolytic cleavage of the F protein by a furin-like protease (Krzyzaniak, Zumstein, Gerez, Picotti, & Helenius, 2013). The complete fusion of the virus allows the release of the RNP

complex into the cytoplasm after its dissociation from the M protein. This entry process is pH-independent (Kahn, Schnell, Buonocore, & Rose, 1999).

### 1.1.3.2 Replication and transcription

RSV transcription and replication occurs in the cytoplasm immediately after the release of the RNP complex (Ghildyal, Jans, Bardin, & Mills, 2012). The viral genome has a 3' Le promoter region and a 5' extragenic trailer region (Tr) (Fig. 1). The Le region leads the transcription and replication by the L polymerase. During the transcription, the L polymerase sequentially transcribes viral genes into single capped/polyadenylated mRNAs using the P protein as a cofactor and the M2-1 as a transcription processivity factor (transcription anti-terminator) which allows the efficient synthesis of full-length viral mRNAs (Noton, Deflube, Tremaglio, & Fearns, 2012). Single viral mRNAs are further translated by the host ribosomal machinery. Later in the infection cycle and after accumulation of the viral proteins, the M2-2 protein induces transcriptional inhibition to promote replication (Asenjo & Villanueva, 2016). Replication of the viral genome requires the synthesis of an intermediate full-length positive-sense RNA, termed antigenome, which has a 3' Tr complement region (TrC) that contains the promoter for the synthesis of new copies of the negative sense RNA genome (Noton et al., 2012). As the viral antigenome and genome are synthesized they are encapsidated by the N protein. This protects the viral RNA from degradation and sensing by the host pattern recognition receptors (PRRs) preventing activation of the cellular innate immune response (Fearns, Peeples, & Collins, 1997; Groskreutz, Babor, Monick, Varga, & Hunninghake, 2010). The viral RNA synthesis of RSV has been proposed to take place in large, dense cytoplasmic structures known as inclusion bodies (Fig. 2), which also have been shown to play key roles in the cellular immune response by changing the localization of important cellular sensors and host proteins required for the activation of an antiviral state (Lifland et al., 2012).

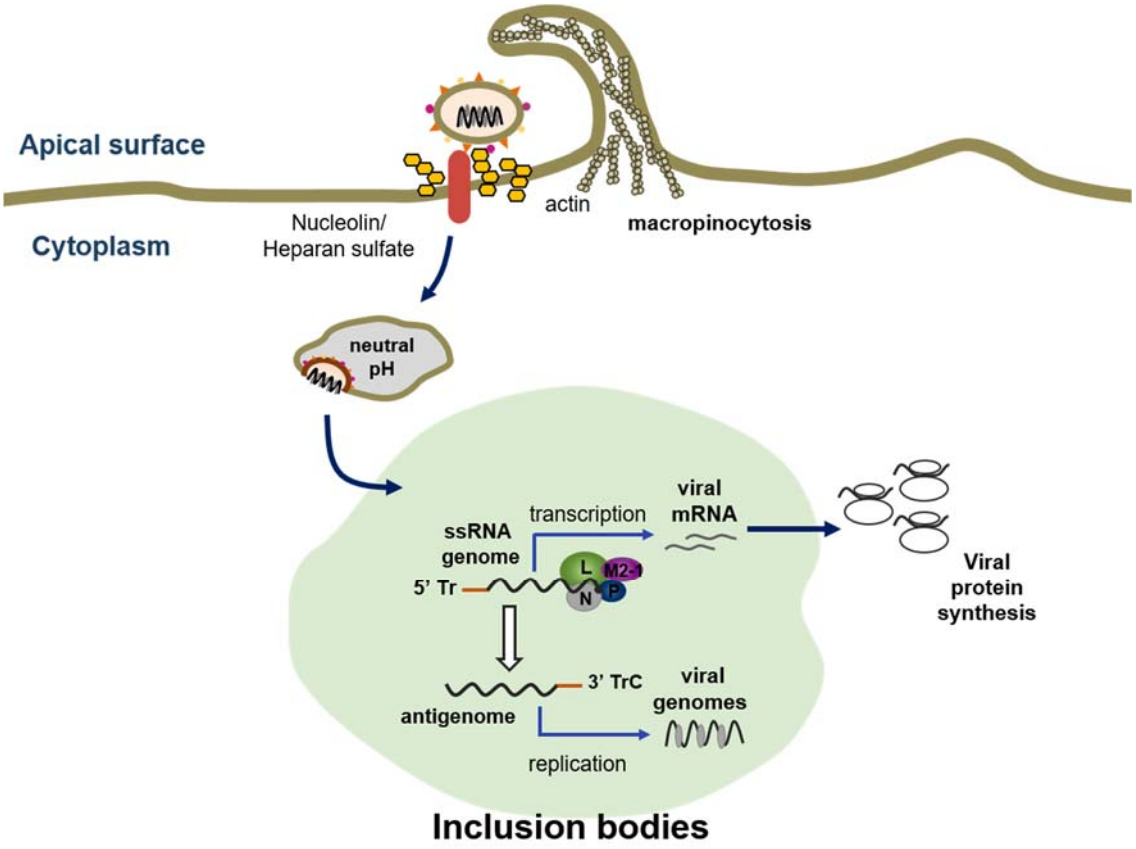


**Figure 2. RSV entry, transcription and replication.** The entry process of RSV requires attachment of the G protein with glycosaminoglycans, particularly heparan sulfate, and the interaction of the F protein with the cellular receptor nucleolin, which triggers diverse molecular mechanisms that induce the entry of RSV by macropinocytosis. Transcription and replication are thought to be carried on in dense cytoplasmic structures termed inclusion bodies. Replication of the viral genome requires the synthesis of an intermediate RNA called antigenome.

Image adapted from Cervantes-Ortiz *et al*., *Viruses*, 2016 (Cervantes-Ortiz, Zamorano Cuervo, & Grandvaux, 2016)

### 1.1.3.3 Protein trafficking

The viral proteins P, N, L, and M2-2 have been shown to accumulate at the IBs where it is suspected that they assemble with newly synthesized genomes to form RNP complexes. Led by the M protein, these complexes transit to the apical membrane of the infected cell (Fig. 3) (Mitra, Baviskar, Duncan-Decocq, Patel, & Oomens, 2012). Meanwhile, the glycoproteins $F_0$, G and SH follow the secretory pathway to undergo post-transcriptional modifications, including glycosylation and proteolytic cleavages to be functionally active. The secretory pathway involves protein transit from the endoplasmic reticulum (ER) to the Golgi apparatus and vesicular transport to the cell surface (Fig. 3) (Brock, Heck, McGraw, & Crowe, 2005). The vesicular transport of the viral glycoproteins to the plasma membrane is thought to be mediated by the apical recycling endosome whose transport is regulated by the Rab11 family interacting protein (Rab-11-FIP) and actin motor proteins like Myosin Vb (Brock et al., 2003). The F protein is required to localize the viral glycoproteins with the rest of the viral structural proteins at the apical membrane of the infected cell, where the new viral particles are assembled (Batonick & Wertz, 2011).

### 1.1.3.4 Assembly and budding

Assembly of fully infectious RSV virions at the plasma membrane requires the coordinated incorporation of the proteins that transit through the cytoplasm (RNP complex and the M protein) with the viral glycoproteins (F, G and SH) that transit from the secretory pathway. To accomplish this, the glycoproteins, are translocated and anchored to the cellular membrane to form the viral envelope (Brock et al., 2003). Deletion of the G and F proteins reduces the incorporation and association of the M proteins with the cellular membrane. Therefore once anchored, the G protein and the F protein probably act as docking sites for the M proteins which get distributed under the cellular membrane to associate the RNP complex with the viral glycoproteins and complete the assembly (Batonick & Wertz, 2011; Ghildyal et al., 2005; Henderson, Murray, & Yeo, 2002). This process occurs within regions enriched by lipid-raft (Brown, Rixon, & Sugrue, 2002). Although the SH protein is incorporated into the mature virion, it is dispensable at this stage (Batonick & Wertz, 2011).

Assembly typically induces conformational changes of the cellular membrane to initiate the budding and scission of the new viral particles (Fig. 3). The molecular mechanisms behind the budding and scission of RSV are largely unknown, but compared to other enveloped RNA viruses, seem to be Vps4-independent but mediated by the Rab11-FIP2 and the actin interacting GTPase RhoA (Bitko, Oldenburg, Garmon, & Barik, 2003; Jeffree et al., 2007; Utley et al., 2008). Two different virion morphologies are observed in RSV: free spherical and cell-associated filamentous, the latest one being the most predominant (Shaikh & Crowe, 2013). It is not clear whether the filamentous represents infectious progeny or sites of assembly, but their loss positively correlates with the decrease of viral infectivity (Mitra et al., 2012). For RSV, it has been reported that a great quantity of the virus progeny remain cell-associated and this may be correlated with an incomplete budding (Collins & Melero, 2011).

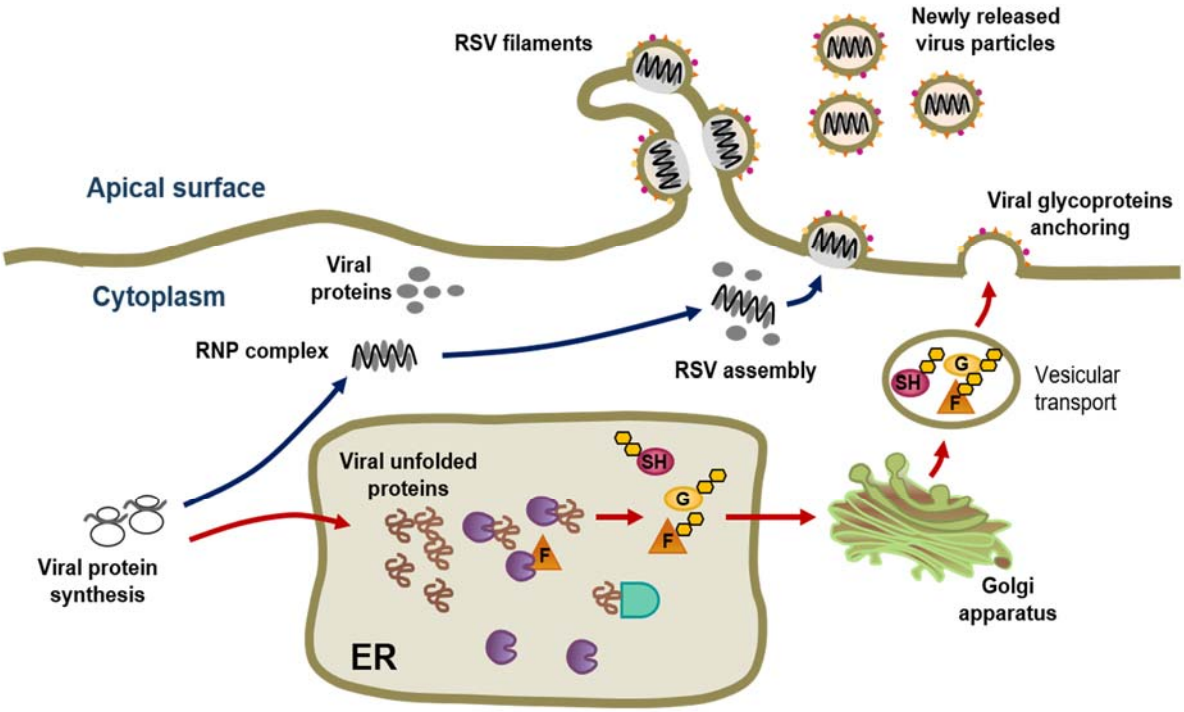


**Figure 3. RSV protein trafficking, assembly and budding.** The viral proteins follow two different routes for trafficking: cytoplasmic (blue arrows) and the secretory pathway (red arrows). The viral glycoproteins are transported to the apical cellular membrane by vesicular transport from the Golgi apparatus and are anchored to the cellular membrane to form the viral envelope. The RNP complex and the rest of the structural viral proteins transit from the cytoplasm to the cellular membrane where they encounter the anchored glycoproteins for assembly, budding and release. Two different RSV virion morphologies are formed: round-shape which is released and filamentous which remains cell-associated.

Image adapted from Cervantes-Ortiz *et al*., *Viruses*, 2016 (Cervantes-Ortiz et al., 2016)

## 1.2 The host immune response against RSV

AECs are the main permissible target of RSV infection but also play a central role in the defense and restriction of this virus. These cells are the first defense barrier against different pathogens and display a broad repertoire of self-defence mechanisms by which they directly restrict viral replication. This intrinsic protection is termed "cell-autonomous immunity" (Randow, MacMicking, & James, 2013). In addition, AECs mount an antiviral state that shapes both the innate and adaptive host immune responses to facilitate the clearance of the virus (Vareille, Kieninger, Edwards, & Regamey, 2011). By contrast, RSV has evolved different strategies to evade the host defenses and hijack the cellular machinery to promote its replication (Schmidt & Varga, 2017).

In the subsequent sections, the detailed interaction between the host immune response and RSV will be described.

### 1.2.1 AECs and the cell autonomous immune response to RSV

AECs sense highly conserved pathogen-associated molecular patterns (PAMPs), displayed by viruses to trigger an early antiviral response to alert surrounding uninfected cells and to recruit and activate immune cells at the site of infection (Vareille et al., 2011).

The sensing of infectious viruses is mediated by a broad repertoire of PRRs, which include the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), the Toll-like receptors (TLRs), and the nucleotide-binding oligomerization domain containing like receptors (NLRs) (Akira, 2009). RSV sensing is mediated via several PRRs (Marr, Turvey, & Grandvaux, 2013). However, in AECs cytosolic RLRs receptors, RIG-I and melanoma differentiation-associated protein 5 (MDA5), are the main players in the detection of single-stranded RNA (ssRNA) viruses. RIG-I recognizes RSV genomic RNA (Liu et al., 2007), this recognition induces the interaction between RIG-I and the mitochondrial antiviral-signaling protein (MAVS) to activate multiple signalling cascades and ultimately activates the transcription factors activator protein 1 (AP-1), nuclear factor-κB (NF-κB) and interferon (IFN) regulatory factor 3 (IRF3) (Fig. 4) (Dey, Liu, Garofalo, & Casola, 2011; Yoboua, Martel, Duval, Mukawera, & Grandvaux, 2010). MDA5 generally recognizes long double-stranded RNA (dsRNA) (Yoneyama, Onomoto, Jogi,

Akaboshi, & Fujita, 2015). The specific mechanism by which MDA5 sense RSV is not clear, however, this sensor sustains the activation of IRF3 and activates NF-κB by a non-canonical pathway (Grandvaux et al., 2014; Yoboua et al., 2010). Activation of AP-1, NF-κB and IRF3 induces their nuclear translocation and binding to gene promoters to induce the expression of many pro-inflammatory cytokines and chemokines, including the macrophage inflammatory protein-1α (MIP-1α)/ Chemokine (C-C motif) ligand 3 (CCL3), the monocyte chemotactic protein-1 (MCP-1), RANTES, C-X-C motif chemokine 10 (CXCL10), interleukin 8 (IL-8), IL-1β, IL-6, tumor necrosis factor α (TNF-α) and type I and type III IFN (Bueno et al., 2011; McNamara, Flanagan, Hart, & Smyth, 2005; Yoon, Kim, Lee, & Lee, 2007). These molecules help to recruit and activate specialized immune cells to clear the virus (Durbin, Kotenko, & Durbin, 2013). The production of type I IFN (α/β) is crucial for the induction of an antiviral state by triggering the transcription of several IFN-stimulated genes that interfere with the replication of the virus (Durbin et al., 2013; Goritzka et al., 2014). Activation of IRF3, and therefore the production of type I IFN, largely depends on the downstream constitutively expressed signaling molecule TANK-binding kinase 1 (TBK1). TBK1 shares a lot of structural and functional characteristics with the Inhibitor-κB kinase ε (IKKε), including its localization, architecture and interacting partners (Nakatsu et al., 2014), however, in non-hematopoietic cells IKKε is no constitutively expressed and rather inducible (Hiscott, 2007). MAVS interacts with TBK1 and IKKε to induce their autophosphorylation on Ser172 and enhance their kinase activity required to phosphorylate IRF3 (Fitzgerald et al., 2003). This phosphorylation allows IRF3 dimerization, nuclear translocation and DNA-binding to induce the transcription of type I IFN (Fig. 5) (Liu et al., 2015). Despite the similitudes between TBK1 and IKKε, studies have demonstrated that these kinases have also not redundant functions and regulate different subsets of interferon-responsive antiviral genes (Indukuri et al., 2006; Nakatsu et al., 2014).

The RSV NS1 and NS2 proteins inhibit the production of type I IFN by the degradation of the signal transducer and activator of transcription 2 (STAT2) by the proteasome (Elliott et al., 2007). These viral proteins also interact with MAVS and RIG-I to inhibit the activation of IRF3 and the production of IFN (Boyapalle et al., 2012; Ling, Tran, & Teng, 2009).

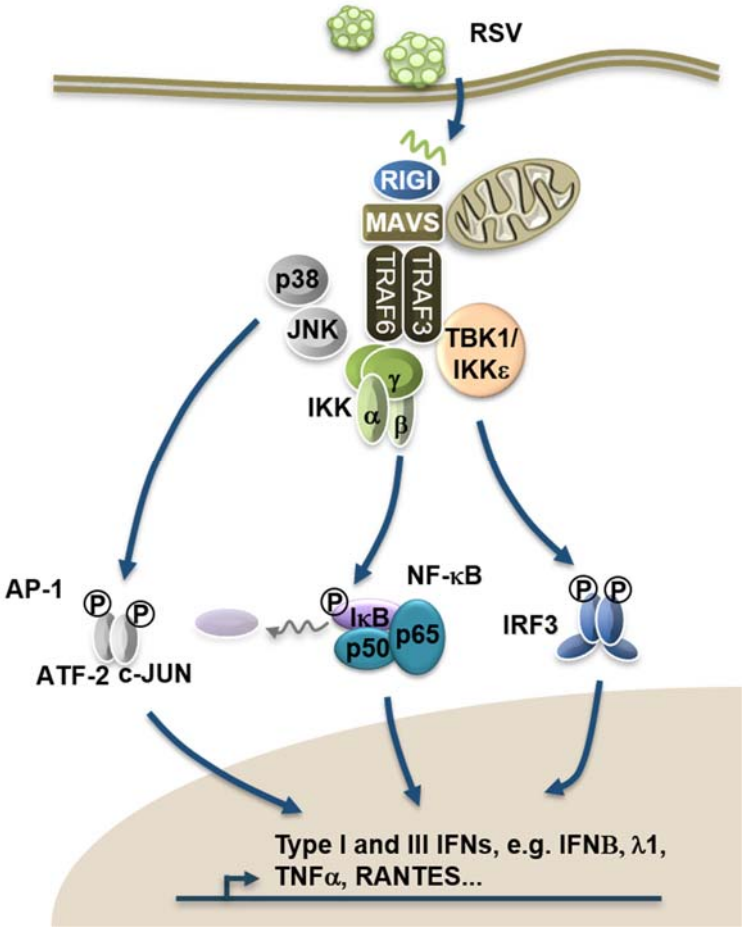


**Figure 4. Signaling pathways induced by RIG-I after recognition of RSV genomic RNA in AECs.** Upon infection AECs sense RNA products from RSV by a broad repertoire of PRRs, including the cytosolic receptor RIG-I. The sensing of RSV allows the signaling transduction through MAVS and the downstream activation of transcription factors like AP-1, NF-κB and IRF3. Their activation allow their nuclear translocation and the binding to cellular promotors to regulate the expression of functional molecules like chemokines and pro-inflammatory cytokines, and the expression of antiviral restriction factors.

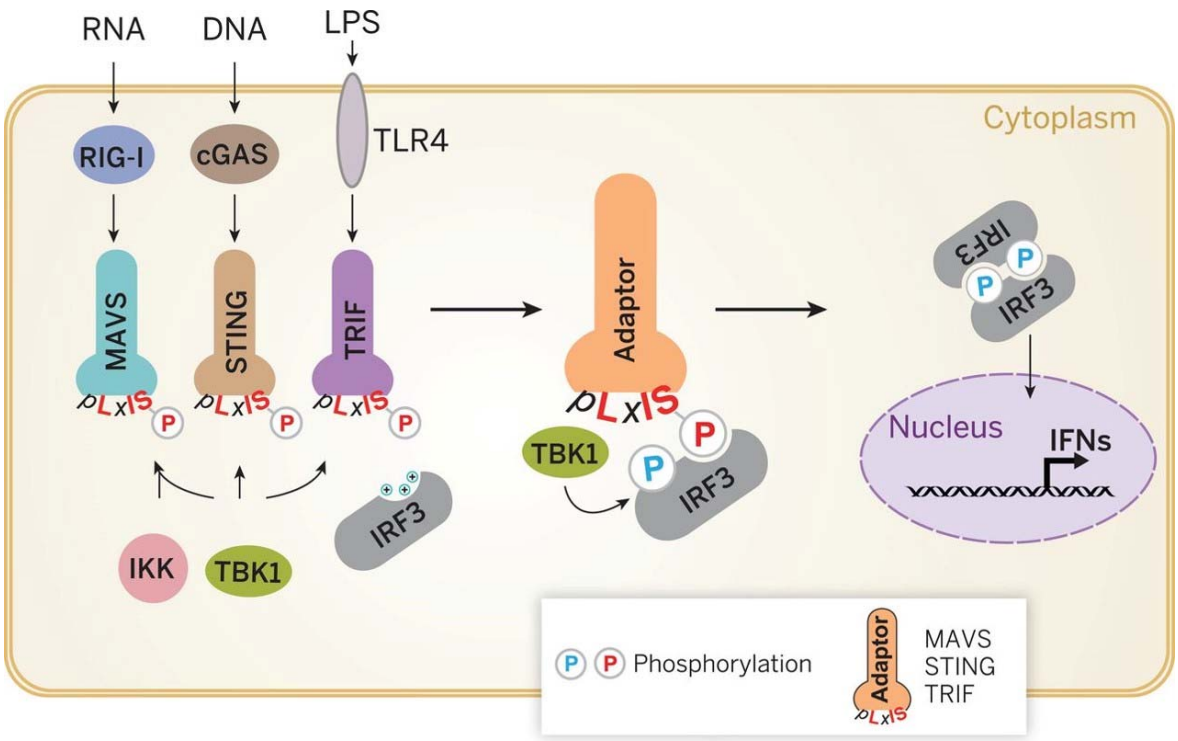


**Figure 5. Activation of the TBK1-IRF3 pathway for the production of IFNs.** The activation of the different PRRs like RIG-I, cGAs and TLR4 by viral RNA, foreign DNA and bacterial lipopolysaccharide (LPS) respectively, induces its interaction with multiple signaling adaptors including MAVS, STING and TRIF. These adaptors mediate downstream activation of the kinase TBK1 which induces IRF3 phosphorylation and dimerization. IRF3 homodimer is subsequently translocated to the nucleus to induce the production of IFNs.

Image from Liu *et al*., *Science*, 2015 (Liu et al., 2015)

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Alternatively to the production of different functional molecules that shape the innate and adaptive immune responses, AECs display many mechanisms to directly counteract the replication of the virus. These mechanisms rely in a broad repertoire of cellular factors, mainly constitutively expressed in the cells, with the potential to target different steps of the viral cycle to early cope with the infection (Yan & Chen, 2012). This intrinsic immunity is highly conserved among all the living organisms and relies in mechanisms like translational inhibition, intracellular transport, apoptosis, and cell disposal pathways, *i.e.* autophagy and proteasome degradation (Randow et al., 2013). The relevance of this response is also reflected by the capacity of many viruses to display different strategies to block, evade, or modulate their activity.

Few cellular factors with the potential to modulate RSV infection have been characterized in AECs and many of them are antagonized by this virus. Among them the protein kinase R (PKR) and the inositol-requiring enzyme 1 (IRE1). PKR recognizes dsRNA and induces translational inhibition by phosphorylation of the eukaryotic initiation factor 2-alpha (eIF2α) (Dalet, Gatti, & Pierre, 2015). Although PKR is activated during RSV infection, its activity is antagonized by the RSV N protein and hence it does not impact the virus replication (Groskreutz et al., 2010). In addition, RSV has been shown to induce an ER stress response probably associated to the high traffic of viral proteins through the secretory pathway, which is reflected in the expression of many ER-associated stress genes, including the folding chaperones immunoglobulin protein (BIP, also known as GRP78) and calreticulin. These folding chaperones are required to reduce the amount of unfolding proteins accumulated in the lumen of the ER (Bitko & Barik, 2001; Hassan et al., 2014). In addition, during ER stress the cells activate three signaling pathways regulated by the ER-resident transmembrane proteins: activating transcription factor 6 (ATF6), PKR like ER kinase (PERK) and the ER-stress sensor (IRE1). Under homeostatic conditions these mediators are bound to BiP preventing their activation, however, under ER stress, due to competitive binding to increase levels of misfolded proteins, BiP is released from these mediators activating them. RSV induces a non-canonical ER response led by the activation of IRE1 and ATF6, but not the PERK pathway. Additionally, IRE1 was shown to decrease RSV replication early in the infection, although the mechanism behind this effect has not been characterized (Hassan et al., 2014).

### 1.2.2 The innate and adaptive immune responses to RSV

Production of different chemokines and pro-inflammatory cytokines by AECs stimulates the recruitment of different specialized immune cells, including macrophages, DCs, natural killer (NK) cells, neutrophils, eosinophils, and T lymphocytes, and modulates their activation (Lay, Bueno, Galvez, Riedel, & Kalergis, 2016). These immune cells aid the clearance of RSV, however, during acute infection, there is evidence correlating their recruitment and activity with detrimental inflammation, small airway obstruction and exacerbation of disease (Johansson, 2016; McNamara et al., 2005). This has been supported by different pathological reports from infants with fatal RSV disease where it has been detected infiltrates of neutrophils, monocytes, NK cells and T cells into the small airways (Geerdink, Pillay, Meyaard, & Bont, 2015; Johnson et al., 2007). How these cells contribute to RSV pathology and how their defensive mechanisms turn into detrimental is not well understood, but it has been partially attributed to alterations and deficiencies of the host immune system (Johansson, 2016). As an example, it is reported that in infants RSV infects macrophages and DCs preventing their maturation and capacity to present antigens to T and B cells (Ruckwardt, Malloy, Morabito, & Graham, 2014), while in immunocompetent adults this does not occur (Jones, Morton, Hobson, Evans, & Everard, 2006).

The adaptive immune response to RSV infection is mediated by the production of neutralizing antibodies and the T cell responses. It has been shown that adults naturally infected with RSV produce high quantities of protecting neutralizing antibodies (Falsey et al., 1999), but their protection is short lasting (Falsey, Singh, & Walsh, 2006). This has been associated with the high incidence of RSV reinfection observed in all-age individuals. In infants under 5 years of age, the production of neutralizing antibodies is even less favorable because their humoral response is under development while neonates almost entirely dependents on the adoptive transfer of maternal antibodies. The titer of maternal antibodies positively correlates with the decrease of RSV disease severity, however the protection conferred is also not long-lasting (Ochola et al., 2009).

As mentioned before, RSV produces a soluble G protein that is secreted to a high concentration compared with the full-length G protein. The soluble G protein is targeted by the host neutralizing antibodies to reduce their availability and neutralization capacity as a

mechanism of viral evasion (Bukreyev et al., 2008). Despite these complications, passive immunization with neutralizing antibodies has been so far the most efficient prophylactic strategy to protect infants from RSV infection (Wegzyn et al., 2014).

T cells are also important for the control of RSV infection in adults (Falsey & Walsh, 2000), but in RSV-infected infants these cells display many features that reflect a deficient response and an immunologic imbalance that are associated with detrimental outcomes. Among the different findings, it has been shown that in infants with severe RSV-bronchiolitis, memory T cells fail in conferring protection against future infections and display low proliferation responses measured by an *in vitro* proliferation assay (Bont et al., 2002). It has also been reported that in infants RSV induces transient apoptosis of $CD8^+$ and $CD4^+$ T cells and this correlates with disease severity (Roe et al., 2004). In addition, in RSV-associated bronchiolitis it has been observed a marked imbalance between the type 1 and type 2 T helper immune responses that results in the modification of the immunological environment of the respiratory tract and the propensity of inflammation (Pinto, Arredondo, Bono, Gaggero, & Diaz, 2006).

Although the adaptive immunity to RSV infection plays a protective effect in the restriction of this virus in healthy adults, the main groups of risk to develop severe RSV infection have either an immature immune system (*i.e.* neonates and infants under 5 years of age) or a reduced immune response (*i.e.* elderly and immunocompromised patients) that decrease their capacity to effectively fight RSV infection.

## 1.3 Sequestosome 1 (SQSTM1)/p62 cellular factor

The Sequestosome 1 (SQSTM1)/p62, from here on referred to as p62, is a cellular protein conserved among metazoans that plays several roles in homeostatic and stress-inducible conditions (Katsuragi, Ichimura, & Komatsu, 2015). This protein has been broadly studied in the context of cancer, neurodegenerative diseases and age-associated pathologies, and in recent years in the context of a cell-autonomous immune response against intracellular pathogens, including both bacteria and viruses.

### 1.3.1 P62 structure and function

P62 is a multifunctional protein with a molecular weight of 62kDa that is localized in the cytoplasm and in the nucleus. P62 acts as a signaling hub in pathways involving redox control and cytokines production (Ichimura et al., 2013; Kim & Ozato, 2009). It is also involved in regulating protein homeostasis (proteostasis) and vesicular transport, and acts as a cargo receptor for ubiquitinated substrates to mediate their degradation by macroautophagy (from here on just referred to as autophagy) or by the proteasome (Calderilla-Barbosa et al., 2014; Milan et al., 2015). The different functions of p62 are mediated by its multiple protein-protein interaction domains: the Phox1 and Bem1p (PB1) domain, the ZZ-type zinc finger (ZZ) region, the tumor necrosis factor receptor-associated factor 6 (TRAF6) binding (TB) domain, the microtubule-associated protein 1A/1B-light chain 3 (LC3)-interacting region (LIR), the Keap1-interacting region (KIR), and the ubiquitin (Ub)-associated (UBA) domain (Fig. 6).

The ZZ region of p62 has multiple binding partners including the receptor-interacting protein 1 (RIP1) by which p62 induces the activation of NF-κB (Sanz, Sanchez, Lallena, Diaz-Meco, & Moscat, 1999). In addition, p62-ZZ interacts with arginylated ER chaperones, including BIP, calreticulin and protein disulfide isomerase to recruit misfolded proteins for their degradation by autophagy as part of a defense mechanism induced by the presence of cytosolic double-stranded DNA (dsDNA) (Cha-Molstad et al., 2015; Cha-Molstad et al., 2016).

The TB domain of p62 interacts with TRAF6 to induce the activation of the NF-κB pathway and the production of several cytokines in a cell-dependent manner (Schimmack et al., 2017; Wooten et al., 2005).

The KIR region of p62 binds the kelch-like ECH-associated protein 1 (Keap1) which is responsible of targeting the nuclear factor erythroid 2-related factor 2 (Nrf2) for its degradation. Under homeostatic conditions Nrf2 is constitutively targeted by Keap1 and degraded via the Ub-proteasome pathway. While under oxidative stress p62 binds to Keap1 in a competitive manner inhibiting its interaction with Nrf2 and the degradation of the latter. Cytoplasmic accumulation of Nrf2 induces its nuclear translocation and binding to gene promoters to generate an antioxidant response (Jiang et al., 2015). In addition, translocation of Nrf2 to the nucleus induces *p62* gene expression imposing a positive feedback loop (Jain et al., 2010). The phosphorylation of p62 on serine 349 (Ser349) by the mammalian target of rapamycin complex 1 (mTORC1) kinase increases the binding affinity with Keap1 and as consequence Nrf2 activation (Ichimura et al., 2013).

The C-terminal UBA domain of p62 allows the sequestration of ubiquitinated cargo for degradation mainly by autophagy in cooperation with the PB1 and LIR domains. P62 also mediates the degradation of cargos by the proteasome, but in less extent (Myeku & Figueiredo-Pereira, 2011). The binding affinity of the UBA-p62 domain for ubiquitinated cargos is regulated by phosphorylation on serine 403 (Ser403) (Matsumoto, Wada, Okuno, Kurosawa, & Nukina, 2011). Additionally, this domain has been reported to be necessary to target specific vesicles for its retention to the ER membrane and therefore restrain their fast transport and release into the cell's periphery (Jongsma et al., 2016).

The N-terminal PB1 domain binds signaling molecules containing the same domain such as the atypical protein kinases C (aPKCs) and the polarity protein 4 (Duran et al., 2004; Moscat, Diaz-Meco, & Wooten, 2009). In addition, the PB1 domain mediates p62 oligomerization and heterodimerization via PB1-PB1 interaction. The binding of the PB1 and UBA domain with their respective targets contributes to the formation of p62 aggresome-like structures which are targeted for degradation via autophagy (Bjorkoy et al., 2005). The ability of p62 to oligomerize with substrates is linked to the formation of cytoplasmic inclusion bodies (Watanabe & Tanaka, 2011). The p62-PB1 domain also has been shown to directly bind to inactive proteasomes, although the functional consequences of this interaction have not been described (Myeku & Figueiredo-Pereira, 2011).

The p62-LIR region interacts with the with the autophagosomal membrane lapidated LC3-II form to recruit p62 and its selective cargo for degradation via autophagy (Pankiv et al., 2007).

P62 has been shown to interact with caspase-8 to promote its aggregation and activation to induce apoptosis when late autophagy is blocked (Huang, Okamoto, Yu, & Sinicrope, 2013). Recently p62 has been shown to interact with the motor protein dynein through a previously unidentified site, between 177- 194 aa, to promote adequate dynein function and transport (Calderilla-Barbosa et al., 2014). Other functions of p62 are involved in regulating mitochondria homeostasis during basal conditions and promoting DNA repair in the nucleus (Hewitt et al., 2016; Seibenhener et al., 2013).

To date, it is not defined if the functions and interactions of p62 with its multiple binding partners, except for the UBA, PB1 and LIR region during autophagy, are mutually exclusive or additive or if one of these functions is primary or secondary.

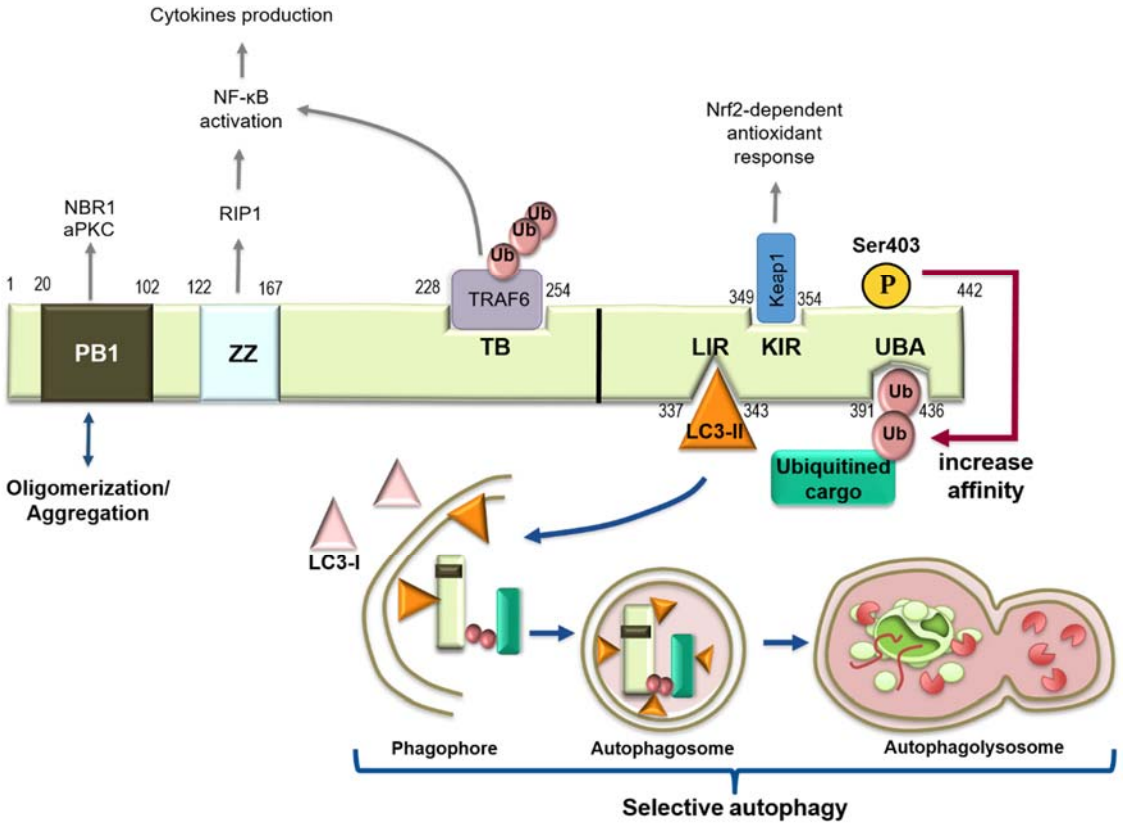


**Figure 6. SQSM1/p62 structure and functions.** P62 is a scaffold protein with different interaction domains: The N-terminal PB1 domain by which it interacts with other PB1 domains to form aggregates, the ZZ region and the TB domain which are involved in the production of pro-inflammatory cytokines, the KIR region that promotes a cellular antioxidative response, the C-terminal UBA domain that targets ubiquitinated cargos after phosphorylation on Ser403, and the LIR region by which p62 interacts with the LC3-II form to mediate selective autophagy of different cargos, like proteins/organelles and intracellular pathogens.

### 1.3.2 P62 cargo adaptor for selective autophagy

P62 was the first Ub-binding cargo receptor identified for mediating selective autophagy (Bjorkoy et al., 2005). To date a total of five receptors that mediate selective autophagy have been described: neighbor of BRCA1 gene 1 (NBR1), nuclear dot protein 52 kDa (NDP52), TRAF-interacting protein with forkhead-associated domain (T6BP), the Optineurin, and SQSTM1/p62 (Bjorkoy et al., 2005; Kirkin et al., 2009; Thurston, Ryzhakov, Bloor, von Muhlinen, & Randow, 2009; Wild et al., 2011). These receptors have some domains in common, including the PB1 domain and the UBA domain that selectively recruit cargo, and the LIR region by which these receptors and their cargos are sequestered into double-membrane vesicles called autophagosomes (Kim, Kwon, & Song, 2016). The cargo potential of these adaptors during selective autophagy are context-dependent and can be independent on, or cooperative with, other adaptor receptors. In a cooperative manner, these adaptors recruit ubiquitinated cargos and form heterodimers by interaction of their N-terminal PB1 domains that contribute to the formation of aggregate-like structures which are targeted via autophagy for degradation (Lamark, Kirkin, Dikic, & Johansen, 2009).

P62 is a receptor that targets a broad repertoire of cellular components and intracellular pathogens for their degradation by autophagy, including damaged-mitochondria (mitophagy), peroxisomes (pexophagy), protein aggregates (aggrephagy), ribosomal proteins (ribophagy), intracellular bacteria (xenophagy) and viral components (virophagy) (Bjorkoy et al., 2005; Ding et al., 2010; Orvedahl et al., 2010; Pilli et al., 2012; Ponpuak et al., 2010; Yamashita, Abe, Tatemichi, & Fujiki, 2014; Zheng et al., 2009).

The p62-UBA domain has the potential to recognize lysine 48 (K48) and K63 mono, di and poly-Ub chains. However, p62 has higher affinity for cargos conjugated with K63-poly-Ub chains (Long et al., 2008; Rui et al., 2015). It has been reported that p62 can also recruit cargo via Ub-independent manner by its PB1 domain to deliver them for degradation by autophagy (Watanabe & Tanaka, 2011). The cargo function of UBA-p62 is negatively regulated by an autoinhibitory mechanism that is based on the formation of p62 dimers via UBA interaction, which restricts the recruitment of ubiquitinated cargo (Isogai et al., 2011). While the recruitment of poly-Ub cargo is increased by the phosphorylation of p62 on Ser403 localized within the UBA domain (Matsumoto et al., 2011). Although it is unknown if p62 needs to be in a

monomeric state to be phosphorylated on Ser403 or if this posttranslational modification facilitates dimer to monomer transition at the UBA domain, thereby promoting Ub binding.

The Unc-51-like protein kinase 1 (ULK1) phosphorylates p62 on the serine 407 destabilizing the formation of the UBA-dimer (Lim et al., 2015). Two different kinases have the potential to directly phosphorylate p62 on Ser403: the casein kinase II (CK2) and TBK1 (Matsumoto et al., 2011; Pilli et al., 2012). ULK1 also has the potential to directly phosphorylate p62 on Ser403 while associated with stress-inducible molecule Sestrin2 (Ro et al., 2014). However, among these kinases only TBK1 has been shown to mediate the phosphorylation of p62 on Ser403 in a context of cellular self-defence against intracellular pathogens (Pilli et al., 2012).

Phosphorylation of p62 on Ser403 promotes the sequestration of ubiquitinated cargos by the UBA-domain, while p62 via PB1-PB1 interaction forms homo or heterodimers that promote the packaging of the ubiquitinated cargos. The engagement of the UBA and PB1-p62 domains contribute to the formation of aggregate-like structures that are targeted for degradation via autophagy (Bjorkoy et al., 2005; Katsuragi et al., 2015). Degradation of p62 and its cargos is mainly mediated via autophagy, and in less extent via the Ub-proteasome system (UPS). In autophagy-lysosomal pathway, p62 interacts with the autophagosomal membrane conjugated form LC3-II. This interaction allows the selective recruitment of p62 and its cargo or p62 aggregate-like structures within an autophagosome, which subsequently is fused with the lysosome to degrade its content (Johansen & Lamark, 2011). In this degradation pathway termed “selective autophagy”, p62 confers selectivity and acts itself as a substrate for degradation. During proteasome degradation, it is not fully known how p62 delivers its cargos to the proteasome and under which conditions, but it might implicate the p62-PB1 domain (Lippai & Low, 2014; Myeku & Figueiredo-Pereira, 2011).

### 1.3.3 P62 and the self-autonomous immune response against intracellular pathogens

P62 has been associated with the control of intracellular pathogens, such as bacteria and viruses. In the context of bacterial infections, p62 has been shown to restrict the replication of different bacteria, including *Mycobacterium tuberculosis* and *Salmonella typhimurium*, by recruiting them via Ub-dependent manner and target them for degradation by autophagy (Pilli et al., 2012; Zheng et al., 2009). In addition, p62 targets cellular proteins for autophagy to produce antimicrobial peptides. In this process cytosolic proteins are proteolytically processed inside autophagosomes after fusion with the lysosome to produce products capable of killing bacteria (Ponpuak et al., 2010).

Recently, the role of p62 in the regulation of the viral cycle of different viruses has started to be elucidated. In the context of virus infections, the function of p62 varies according to the type of virus where p62 can act as an antiviral factor to reduce virus replication, but can also act as a proviral factor to promote different steps of the viral cycle. In the case of Dengue virus (DENV) infection, p62 has been reported to restrict viral replication, but in the course of the infection it is specifically degraded via the proteasome, probably as a mechanism of viral evasion to block its activity (Metz et al., 2015). In Sindbis virus (SIN) infected- neurons, p62 recruits viral capsids for their degradation via autophagy to reduce their toxic accumulation which is associated with the induction of cell death. This mechanism does not affect the viral replication, but protects the central nervous system from tissue damage which is key in the pathogenesis of SIN-lethal infection (Orvedahl et al., 2010). Conversely, p62 aids replication of the Japanese encephalitis virus (JEV) after viral internalization by an unknown mechanism (Tasaki, Nukuzuma, & Takegami, 2016). Another study has shown the potential of coxsackievirus B3 to cleave p62 by its viral protease 2A to abrogate the induction of the NF-κB pathway and the selective cargo function of p62 during autophagy (Shi et al., 2013).

## 1.4 The UPS and selective autophagy

Cells have two major degradation pathways/systems, the UPS and autophagy (Cohen-Kaplan, Livneh, Avni, Cohen-Rosenzweig, & Ciechanover, 2016). Both systems are highly conserved among all eukaryotes and play central roles in the maintenance of normal protein turnover, cellular signaling and protein quality control. These systems are in charge of removing misfolded proteins, protein aggregates and damaged organelles that can be harmful for the cells. In addition, these systems contribute to mediate cellular immune responses and under stress conditions are essential to generate resources to sustain the functionality of essential cellular processes and promote survival (Deretic, 2011; Kammerl & Meiners, 2016).

Although for many decades the UPS and autophagy were considered completely independent processes, recent data have shown that they share many features and roles interconnected in the maintenance of cellular homeostasis (Cohen-Kaplan et al., 2016). Cross-talk among these systems is reflected in their potential to regulate similar cellular processes, for example apoptosis, stress responses, protein homeostasis and immune defenses. These systems also share cellular substrates for degradation, including ubiquitinated substrates and misfolded proteins. In addition, it has been reported that components or mediators of one degradative machinery are degraded by the other to exert mutual regulation between the two systems. Therefore, perturbation of one of the systems can affect the activity and flux of the other (Cohen-Kaplan et al., 2016; Korolchuk, Mansilla, Menzies, & Rubinsztein, 2009).

### 1.4.1 Ub-conjugation

The targeting of specific substrates by the UPS and autophagy requires the recognition of specific signals. One of these signals is a post-translational modification termed ubiquitination (also known as ubiquitylation), which involves the conjugation of small Ub-peptides (76 aa length, 8.5kDa). These small peptides can be covalently conjugated to the proteins by an isopeptide linkage to K-residues and in less extent to thiol groups of cysteine residues (Komander & Rape, 2012). Ub is conjugated to seven K residues, K6, K11, K27, K29, K33, K48, and K63, being among these the K48 and the K63 the most abundant and known types. Ub is linked as a single moiety to one (monoubiquitination) or to multiple residues (multimonoubiquitination). Additional Ub-molecules can be linked to these single moieties to

form di-, tri- tetra-Ub, or polymeric chains (polyubiquitination) (Sadowski, Suryadinata, Tan, Roesley, & Sarcevic, 2012). Ub-conjugation is adenosine-5'-triphosphate (ATP)-dependent and requires the coordinated action of three types of enzymes: the Ub-activating enzyme (E1), the Ub-conjugating enzyme (E2), and the Ub-ligase (E3) (Fig. 7) (Buetow & Huang, 2016; Pickart & Eddins, 2004). The E3 transfers Ub-peptides to target proteins and elongates poly-Ub chains (Buetow & Huang, 2016). The human genome encodes two E1 enzymes, tens of E2s enzymes and several hundred of E3 enzymes, where the E3 enzymes confer substrate specificity (Buetow & Huang, 2016).The Ub-peptides can be removed from the targeted proteins by deubiquitinating enzymes (DUBs) (Hanpude, Bhattacharya, Dey, & Maiti, 2015). Ubiquitination is a very dynamic and typically transient posttranslational modification that not only acts as a signal to target proteins for degradation, but also it represents a complex system that determines protein localization, trafficking, and activity (Buetow & Huang, 2016).

Due to the relevance of ubiquitination in determine the fate and activity of many proteins and cellular mechanisms, it is not surprising that many viruses have evolved mechanisms to modulate this posttranslational modification in order to promote their replication (Calistri, Munegato, Carli, Parolin, & Palù, 2014).

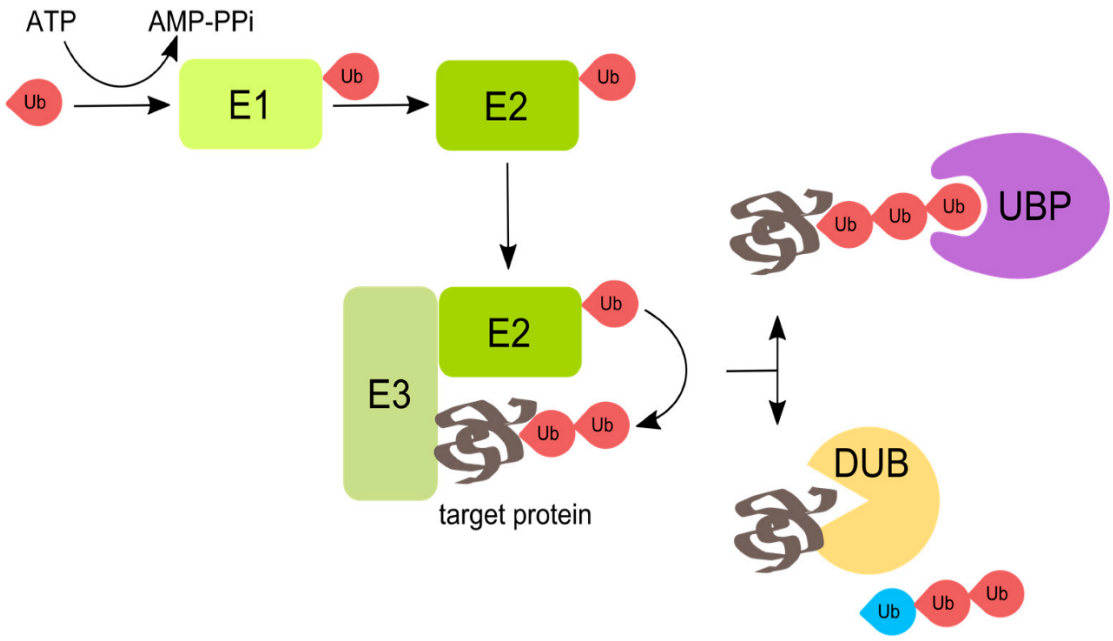


**Figure 7. Ubiquitin-conjugation.** Ub-conjugation requires the coordinated action of three types of enzymes, the Ub-activating enzyme (E1), the Ub-conjugating enzyme (E2) and the Ub-ligase (E3). The Ub is linked to the E1 in an ATP-dependent manner. The E1 transfers the Ub peptide to the E2 protein. Finally, the E3 bridges the Ub linked to the E2 and the target protein and catalyzes the formation of an isopeptide bond to conjugate the Ub to the target protein K residues. Ubiquitinated proteins can be targeted by ubiquitin binding proteins (UBPs) to mediate diverse cellular functions. The Ub peptides are removed or modified by deubiquitinases (DUBs) enzymes.

Image from Rudnicka and Yamauchi, *Viruses*, 2016 (Rudnicka & Yamauchi, 2016)

### 1.5.2 The Ubiquitin-Proteasome System

The UPS mediates the proteolytic degradation of the majority of the cellular proteins into small peptides (Yu & Matouschek, 2017). This system typically mediates the degradation of short-lived soluble proteins, but it cannot process stable or insoluble protein complexes, including oligomers or aggregates, or big structures like organelles and intracellular bacteria.

The UPS is a highly regulated degradation process that requires the coordinated action of many cellular factors that allow the recognition, processing and translocation of selective substrates into the proteasome. The 26S proteasome is the catalytic unit of the UPS, this unit is a cylindrical structure composed by two main complexes: the 20S which is the core center where the substrates are proteolytically processed, and the 19S complex that cap the 20S and mediates the recognition and entry of the targeted substrates (Gallastegui & Groll, 2010). The proteins designated for proteasome degradation typically contain two signals termed “degrons”. One mediates the affinity for the proteasome, typically a covalent Ub-conjugated signal, and the other is an unstructured region that acts as an initiation site for the substrate translocation into the proteasome (Ronai, 2016). The proteasome typically recognizes proteins linked with K48 Poly-Ub chains and in less extent proteins linked to K63-Ub short chains (Yu & Matouschek, 2017). Some studies have also reported that monoubiquitination is a sufficient signal to mediate UPS-degradation (Ronai, 2016). In addition, it has been shown that some substrates are target to the proteasome in an ATP and Ub-independent manner, where the presence of structural degrons inside the targeted protein are sufficient to mediate recognition by the proteasome (Erales & Coffino, 2014). Other proteins degraded by the UPS have been shown to be selectively targeted and delivered by adaptor proteins or chaperones into the proteasome (Shiber & Ravid, 2014). Targeted proteins must be deubiquitinated and unfolded prior to their translocation into the proteasome.

In the context of viral infection the correct functionality of the UPS has been proved to be required for the replication of relevant viruses such as retroviruses, adenoviruses, herpesviruses, rotaviruses, picornaviruses and paramyxoviruses (Calistri et al., 2014).

### 1.5.3 Autophagy-lysosomal degradation system

Autophagy is a cellular process that is upregulated in response to many cellular stresses, including nutrient starvation, hypoxia, disruption of proteostasis, oxidative stress, ER stress and pathogen infections (Kroemer, Marino, & Levine, 2010).

There are at least three types of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy. The description in this part is oriented to macroautophagy (referred from here on simply as autophagy). Autophagy can degrade bulk cytoplasmic content (nonselective autophagy) typically initiated upon nutrient deprivation, or remove specific components that can be harmful for the cell (selective autophagy) (Zaffagnini & Martens, 2016). The autophagy pathway is highly regulated and relies in the formation of double membrane structures called autophagosomes that sequester different substrates for its lysosomal degradation. The formation of the autophagosome requires different steps: nucleation, elongation and substrate sequestration. The nucleation is the process that initiates the formation of the isolation membrane (phagophore) and it is coordinated by two main protein complexes: the ULK1 and the lipid phosphatidylinositol 3-kinase catalytic subunit type III complex I (PI3KC3–C1) (Hurley & Young, 2017). The ULK1 complex is negatively regulated by mTORC1 which under homeostatic conditions is phosphorylated and bonded to this complex to inhibit is activity (Lamb, Yoshimori, & Tooze, 2013). The ULK1 and the PI3KC3-C1 complexes signal the formation of the phagophore and the recruitment of different autophagy factors, including the autophagy-related proteins (Atg) 5 and 12, to induce membrane elongation and autophagosome formation (Mizushima, Yoshimori, & Ohsumi, 2011). During this process the cytosolic LC3-I is cleaved by an ATG4(B) protease to expose its C-terminal glycine residue that is then, by sequential activation of the Atg7, Atg3 and Atg12 complex, conjugated to phosphatidylethanolamine to form the lipidated form of LC3 (LC3-II). The LC3-II is attached to the autophagosome membrane and acts as a docking site for autophagy receptors like p62 to mediate selective autophagy (Tanida, Ueno, & Kominami, 2008) (Mizushima et al., 2011). The cytoplasmic substrates and the autophagy receptors are sequestered inside the autophagosome, which subsequently fuses with early endosomes, late endosomes and lysosomes to form the autolysosome (Fig. 8). The lysosome delivers degradative proteases that convert the cytoplasmic

content sequestered in the autolysosome into amino acids and other macromolecules that are recycled by the cells (Lamb et al., 2013).

Autophagy has been associated with the recycling of protein aggregates, damaged or excessive organelles and intracellular pathogens.

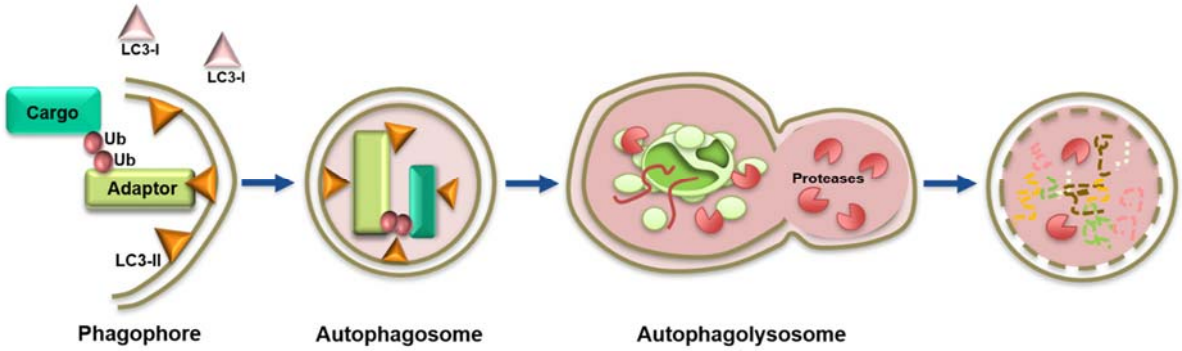


**Figure 8. Selective autophagy.** Selective substrates (cargo) conjugated with Ub-peptides are recognized by adaptor proteins. These adaptors and their cargos are recruited to the phagophore by interaction with the conjugated form LC3-II. The phagophore is enclosed forming the autophagosome in which the adaptors and their cargos are sequestered together with the LC3-II form. The autophagosome fuses with lysosomes which deliver degradative proteases that digest the autophagosome content in amino acids and molecules that are recycled by the cells.

## 1.6 Hypothesis of study and objectives

RSV represents a high risk for certain groups with immune deficits, including premature babies, infants and elderly with chronic cardiopulmonary diseases and immunocompromised individuals. At present, therapies to diminish RSV infection are restricted to costly prophylactic treatments with short-life monoclonal antibodies. Development of alternative therapies or vaccine remains challenging because of a lack of understanding of RSV physiopathology. Because this virus is known to interfere with the establishment of an efficient adaptive immune response, recent efforts have concentrated in targeting the early steps of infection, including entry, replication and transcription (Gomez, Guisle-Marsollier, Bohmwald, Bueno, & Kalergis, 2014). Ciliated AECs are the main target of RSV infection and also represent the first line of defense through the induction of diverse mechanisms including, but not restricted to the production of intracellular antiviral proteins that counteract virus replication. The importance of these intracellular proteins is also highlighted by the capacity of many viruses to manipulate or block their action to facilitate viral replication and pathogenesis.

In this study we focussed on the host scaffold protein SQSTM1/p62, which has been identified as an important intracellular factor capable of modulating the replication cycle of several viruses (e.g. JEV, DENV and influenza), where its activity stands out as proviral or antiviral depending on the virus. The p62 protein is a multifunctional signaling hub and autophagy receptor that serves as an adaptor to mediate diverse functions, including cell signaling regulation, regulation of proteotoxic stress and sequestration of specific ubiquitinated protein cargo (i.e. long-lived proteins, damaged organelles and intracellular bacteria) for degradation by autophagy (Katsuragi et al., 2015). During bacterial infections, the sequestration of ubiquitinated bacteria is further enhanced by the phosphorylation at Ser403 by the TBK1 kinase (Pilli et al., 2012).

As p62 is an important multi-adaptor protein involved in the regulation of several cellular processes in basal and stress-inducible conditions, with evidence highlighting its multiple roles at different levels of the replication cycle of several viruses, we hypothesized that **p62 could act as a regulator of RSV infection in AECs**.

To test our hypothesis, we have established two objectives:

**1)** Determine the impact of p62 in the regulation of RSV replication cycle.

**2)** Evaluate the role of the cargo function of p62 during RSV infection.

# 3. Materials and Methods

**Pharmacological inhibitors**

The dual IKKε and TBK1 inhibitor MRT67307 was purchased from Calbiochem®. For its reconstitution, the pharmacological inhibitor was dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 2mM and stored at -20ºC for a maximum of three months. The inhibitor MRT67307 was used to a final concentration of 2μM.

The proteasome inhibitor MG132 was reconstituted in DMSO at a concentration of 10mM and stored at -20ºC protected from light. MG132 was used to a final concentration of 5μM.

Where indicated, pharmacological inhibitors were added in serum free medium for 1h prior to infection and remained throughout the experiment.

**Cell culture and reagents**

All cells were cultured by incubation at 37°C in an atmosphere of 5% $CO_2$. Media and supplements were acquired from Gibco™. Cells were kept under antibiotic-free conditions.

A549 cell line was obtained from the American Type Culture Collection (ATCC) and cultured in F-12 nutrient mixture medium (Ham) supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS) from Gibco™ or Wisent Inc and 1% L-Glutamine (L-Glu).

Hep-2 and Vero cells (ATCC) were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal clone® III from Life sciences and 1% L-Glu.

The clone-selected A549-mRFP-LC3 cells stably express the LC3 protein conjugated to the mRFP tag (red fluorescence). A549-mRFP-LC3 cells and the parental A549 (control cells) were generously provided by Dr. Craig McCormick from Dalhousie University. Both cell lines were cultured in DMEM supplemented with 10% HI-FBS and 1% L-Glu. For the maintenance of stable A549-mRFP-LC3 cells, the medium was complemented with 1μg/mL of Puromycin. The antibiotic was removed before infection of the cells.

**Plasmids and transfection**

The IKKεK38A-pEGFPC1, TBK1K38A-pEGFPC1, TBK1-pEGFPC1, IKKε-pEGFPC1 plasmids were provided by Dr. R. Lin, McGill University. The TBK1-FLAG-pcDNA3.1Zeo, TBK1K38A-FLAG-pcDNA3.1Zeo, IKKε-flag-pcDNA3.1Zeo, IKKεK38A-FLAG-pcDNA3.1Zeo, were described in (Sharma et al., 2003). The p62-mCherry-pDEST plasmid was a gift from Dr. Terje Johansen (University of Tromso, Norway). The plasmid pDEST-Red (Addgene) was used as control for transfections with p62-mCherry-pDEST. The p62-pCMV-3FLAG-1C plasmid was shared by Dr. Diaz-Meco from Sanford Burnham Prebys Medical Discovery Institute (SBP).

Plasmid transfections in A549 cells were carried out using the transfection reagent TransIT®-LT1 from Mirus Bio following the manufacturer's protocol. The cells were transfected at 60-70% confluency using a ratio of 1:2 DNA/transfection reagent. All transfections were followed for 24h.

**RNAi oligonucleotides and transfection**

RNAi oligonucleotides (Dharmacon) transfection was performed using the Oligofectamine transfection reagent (Invitrogen). A non-targeting sequence (siCtrl) was used as control (Table 2). For the silencing of p62 the combination of two specific oligonucleotide sequences was used (Table 2). For the silencing of TBK1 three distinct sequences were used: TBK1(1) was used alone; TBK1(2) and (3) were used as mix (Table 2). For the silencing of IKKε, one specific sequence (Table 2) was used.

A549 cells were plated to reach 30-40% confluency at the time of transfection. The transfection was performed in opti-MEM (Gibco$^{TM}$). Silencing of p62 was followed for 24h before infection. The silencing of TBK1 and IKKε was pursued for 48h.

**Table 2.** RNAi oligonucleotide sequences

| RNAi sequence name | Target | Sense sequence (5' to 3') |
|---|---|---|
| siCtrl | No target | CAU AGC GUC CUU GAU CAC AUU |
| sip62(1) | SQSTM1/p62 | GAA CAG AUG GAG UCG GAU AUU |
| sip62(2) | SQSTM1/p62 | CCA CAG GGC UGA AGG AGG CUU |
| siTBK1(1) | TBK1 | GCG GCA GAG UUA GGU GAA A dT dT |
| siTBK1(2) | TBK1 | CCA CUG UUA UAC UGG GAU AUU |
| siTBK1(3) | TBK1 | CCU CAG UGA UCA CGA GAA GUU |
| siIKKε(1) | IKKε | AAG AAG CAU CCAGCA GAU UCA dT dT |

**RSV-A2 and RecRSV-GFP amplification and purification**

To generate the working stock of the RSV-A2 strain, the virus initially acquired from Advanced Biotechnologies (P1) was amplified in Hep-2 cells (maximum to P3). For this purpose, RSV-A2 at a multiplicity of infection (MOI) of 0.1 was used to infect twelve 175cm$^2$ flasks of Hep-2 cells at 50-60% confluency in DMEM 2% fetal clone® III and 1% L-Glu. The infection was terminated when syncytia and cytopathic effects were observed in 50% of the cells. For harvesting, the cells were scraped in the infection medium complemented with 2.5mL of 50mM HEPES, 0.1M $MgSO_4$. The cells and the medium were transferred into conical tubes and centrifuged at 3200g for 20min at 4°C. The supernatant (S1) containing the cell-free virions was transferred into prechilled sterile Nalgene bottles. To collect the cell-associated virions, the remaining cell pellets were resuspended in a low volume of the supernatant, frozen (liquid nitrogen) and thawed three times before centrifugation at 3200g for 20min at 4°C. The supernatant (S2) was added to the Nalgene bottles containing the S1 supernatant. To pellet the

virus, the supernatant was centrifuged overnight at 8,000g at 4°C. The virus pellet was resuspended in 2mL of cold 50mM HEPES, 5% sucrose prepared in OptiMEM using a Dounce homogenizer. The homogenized virus was purified on a 30% sucrose cushion by ultracentrifugation at 100,000g for 1h at 4°C. The purified virus was resuspended in ~6mL of 50mM HEPES, 5% sucrose prepared in OptiMEM, homogenized using a Dounce, aliquoted, flash frozen in liquid nitrogen and stored at -80°C.

The recombinant RSV virus expressing GFP protein (recRSV-GFP) was a generous gift from Dr. PL Collins (NIH, Bethesda). The recRSV-GFP was amplified following the same protocol as the RSV-A2 strain.

The virus titer of RSV-A2 and recRSV-GFP working stocks were determined as described in the viral titration section.

**RSV-A2 and recRSV-GFP infections**

For RSV-A2 and recRSV-GFP infection with kinetics no longer than 36h, an MOI of 3 was used. For kinetics of up to 72h, an MOI of 1 was used to reduce the cytotoxic effects that were observed with an MOI of 3. Infections of subconfluent cells were performed by addition of RSV-A2/recRSV-GFP in the culture medium supplemented with 2% HI-FBS and 1% L-Glu. The infection kinetics and cell type, A549, A549-LC3, Hep-2 or Vero, are specified in each figure.

**Viral titration**

Virus titer was quantified by plaque assay. Detection of plaques was performed either by crystal violet staining, anti-RSV immunodetection (immune plaque assay), or GFP fluorescence. Serial dilutions of the purified virions, for the titration of RSV stock, or of freshly harvested supernatant of infected cells were used. Before titration of supernatant, cell debris were removed by centrifugation for 3min at 2500g.

Virus dilutions were used to inoculate a monolayer of confluent Hep-2 cells (RSV-A2) or Vero cells (recRSV-GFP) cultured in 6 wells with 1 mL of DMEM supplemented with 2%

fetal clone and 1% L-Glu. The infection was conducted for 2h with agitation each 30min to allow viral attachment and entry. Following this period and to restrict the spread of the new viral progeny, the medium was replaced with 3 mL of 1% methylcellulose in DMEM containing 2% fetal clone and 1% L-Glu. Infection was followed for 5-6 days to allow the formation of plaques.

- For crystal violet staining, once the period of infection was completed, the methylcellulose was removed and the cells were fixed in 4% formaldehyde for 30 min at RT. After fixation, the plaques were stained with 0.2% of crystal violet for 5-10min and rinsed with water until the plaques were clear. The plaques were counted manually.

- For the immune plaque assay, the methylcellulose was removed and the cells fixed in 80% methanol for 30min at RT. The methanol was removed and the plates were left to dry. The cell monolayers were blocked in PBS, pH 7.2, 0.05% tween-20 (PBS-T), 2% nonfat dry milk without agitation for 15min. The immune detection of the plaques was performed using goat anti-RSV from Chemicon International (#AB1128, 1/4000) for 3h at RT. As a secondary antibody, horseradish peroxidase conjugated rabbit anti-goat from Jackson ImmunoResearch (#305-035-045, 1/10000) was used. The monolayers were incubated with the secondary antibody for 1h at RT. The plaques were visualized by addition of Western Lighting® Plus-ECL (PerkinElmer) using the ImageQuant (LAS 4000 mini) for acquisition. The counting was performed using the ImageQuant TL colony counting software.

- For titration of recRSV-GFP, cells were fixed in 4% formaldehyde for 30min at RT. To avoid photobleaching, from the harvesting all the steps were performed in the dark. The formaldehyde was removed and the cells washed with ddPBS and then with dd$H_2O$. The fixed monolayers were left to dry before analysis. GFP-positive plaques were visualized using a Typhoon apparatus (Molecular Dynamics) and counted with ImageQuant TL colony counting analysis software.

**Detection of the RSV proteins in the supernatant of infected cells by immunoblot**

To analyze the protein expression of the free RSV viral particles, A549 cells were infected with RSV-A2 using Bronchial Epithelial Cell Growth Medium (BGEM) from Lonza (#CC-4175) as medium for infection. This medium does not need serum supplementation and

therefore lacks albumin that interferes with the analysis of RSV proteins by immunoblot. The infection was pursued 2h with agitation each 30min to allow viral attachment and entry. Following this period, the cells were washed two times with BGEM to remove the initial inoculum. Infection was followed in BGEM for the time points indicated in the figure.

The fresh supernatant of four technical replicates (500µL per technical replicate) was cleared from cell debris by centrifugation at 10,000g for 2min. The cleared supernatant was transferred to a new microcentrifuge tube. To concentrate the viral proteins present in the supernatant, the pooled cleared supernatant (2mL total) were poured on a Microsep column from PALL (#MCP030C41), 30K molecular weight cut off, and centrifuged at 3,500g for 7min at 4°C. The 10X concentrated supernatant was transferred to a new tube. To expose all the viral proteins, 150µL of the concentrated supernatant was complemented with protease inhibitors, 10µg/mL leupeptin and 10µg/mL aprotinin. The same volume of the treated supernatant (30µL) for each condition was subjected to SDS-PAGE electrophoresis followed by immunoblot analysis as described below. The membrane was subjected to amido black staining to control for equal total protein levels in all samples.

**Tandem Ub-Binding Entities (TUBEs) assay:**

Whole cell extracts (WCE) were prepared by sonication in NP-40 lysis buffer with 50mM Hepes pH7.4, 150mM NaCl, 5mM EDTA, 10% (p/v) Glycerol, and 1% Nonidet P-40 complemented with the protease inhibitors 50µM PR-619 and 5mM 1,10-phenanthroline (o-PA) from LifeSensors and phosphatase inhibitors 30mM NaF, 1mM Na3VO4, 10mM PNPP, and 25mM glycerophosphate. All the following steps were performed in siliconized tubes (Fisher, #02-681-320) blocked with 5% Bovine serum albumin (BSA)-Fatty acid free (SIGMA-ALDRICH). Prior to the pull-down of polyubiquitinated proteins, WCE were precleared to avoid non-specific binding using the Control-agarose beads (LifeSensors, #UM400) following the manufacturer's protocol. Precleared WCE were quantified using the reagent Bio-Rad protein assay from Bio-Rad. For the pull-down of ubiquitinated proteins, 1mg of precleared WCE was incubated with 10µL of Agarose-TUBE1 beads (UM401, Lifesensors) for 3h at 4ºC in continued rotation. Elution of the polyubiquitinated proteins was performed using SDS reducing sample

buffer boiled for 5min and centrifuged at 13,000g for 5min. The elution was analyzed by SDS-PAGE and immunoblot in parallel with 5% of the input WCE.

**Immunoblot analysis:**

WCE were prepared on ice in RIPA lysis buffer 50mM Tris pH 7.5, 150mM NaCl, 2mM EDTA, 0.1% SDS, 1% Triton X-100, and 1% NaDoc complemented with the protease inhibitors, 10µg/mL leupeptin, 10µg/mL aprotinin and 10µg/mL pepstatin, and the phosphatase inhibitors, 30mM NaF, 1mM Na3VO4, 10mM PNPP, and 25mM glycerolphosphate. Quantification of the WCE was performed using the DC protein assay (Bio-Rad). A quantify of 10-50µg (specified in each figure) were subjected to SDS-PAGE electrophoresis followed by immunoblot analysis as in the reference (Robitaille, Mariani, Fortin, & Grandvaux, 2016), using the following primary antibodies: anti-TBK1 from Imgenex (#IMG-270A, 1/6000), anti-IKKε from Cell Signaling (#D20G4, 1/2000), anti-p62 from BD biosciences (#610832, 1/3000), anti-RSV from Chemicon International (#AB1128, 1/3000), anti-Ub from LifeSensors (VU101, 1/1000) that targets poly, mono and free Ub-proteins, Anti-GFP from Roche (#11814460001, 1/1000) and anti-actin clone AC-15 (#A5441, 1/7500) and anti-Flag M2 (#F1804) antibodies from Sigma-Aldrich. For the phosphospecific antibodies, the following were used: Anti-p62 phospho-Ser403 from Millipore (#MABC186-I, 1/2000), Anti-TBK1 phospho-Ser172 (D52C2, 1/3000) and Anti-IKKε phospho-Ser172 (#D1B7, 1/750) from Cell Signaling. As secondary antibodies, horseradish peroxidase coupled antibodies were used: anti-mouse from KPL (#074-1806) and anti-Rabbit (#111-035-144), anti-goat (#305-035-045), anti-rat (#112-035-143) and anti-mouse (#115-035-062) from Jackson ImmunoResearch. The dilution of the total antibodies was carried out in PBS-T complemented with 5% nonfat dry milk, while the dilutions of the phosphospecific antibodies was performed in PBS-T containing 5% BSA. The proteins targeted were visualized by addition of Western Lighting® Plus-ECL (PerkinElmer) and visualized using ImageQuant (LAS 4000 mini).

**RNA extraction and real-time PCR**

Extraction of total RNA was performed using the RNAqueous-96 Total RNA Isolation Kit from Ambion (#AM1920) in RNase free conditions following the manufacturer's instructions.

The RNA was quantified and 1µg of total RNA was subjected to reverse transcription using the QuantiTect Reverse Transcription kit (Qiagen) according to the manufacturer's protocol.

Quantitative PCR (qPCR) was performed using the FastStart SYBR Green system from Roche. The expression of RSV-N mRNA and the reporter gene S9 were analyzed using the set of primers described below (Table 3). The qPCR reactions were performed with the Rotor-Gene 3000 Real Time Thermal Cycler (Corbett Research). The standard curves for the absolute quantification were obtained by serial dilutions of the plasmids S9-pCR2.1-TOPO and RSVA2N-PCR4-TOPO, which were generated by subcloning. The normalization of the number of copies of the RSV-N mRNA was performed with the S9 mRNA absolute copy numbers.

**Table 3.** Primer sequences used in qRT-PCR analysis.

| Gene | Accession number | Primer sequences 5' to 3'<br>Sense (S) and antisense (AS) |
|---|---|---|
| RSV-A2 N | U39661.1 | **S:** AGATCAACTTCTGTCATCCAGCAA<br>**AS:** TTCTGCACATCATAATTAGGAGTATCAAT |
| S9 | NM_001013.3 | **S:** CGTCTCGACCAAGAGCTGA<br>**AS:** GGTCCTTCTCATCAAGCGTC |

**Immunofluorescence and confocal microscopy:**

A549 cells were grown on glass coverslips from Fisher Scientific (#12CIR-1.5) and infected with RSV-A2. After infection, the cells were fixed in 3.7% formaldehyde diluted in F-12 nutrient mixture medium (Ham) supplemented with 10% HI-FBS and 1% L-Glu for 15min at 37°C. Fixed cells were permeabilized with 0.5% Triton X-100 diluted in PBS containing 0.5mM $CaCl_2$ and 1mM $MgCl_2$, for 10min. The blocking of the cells was performed with 3% BSA for 1h at RT. The washes were performed in PBS containing 0.5mM $CaCl_2$ and 1mM $MgCl_2$. For the detection of the proteins of interest, immunostaining was performed with the following primary antibodies: mouse anti-p62 from BD biosciences (#610832, 0.83µg/mL), guinea pig anti-p62 C-terminal from abcam (#ab194129, 1/300), goat anti-RSV from Chemicon International (#AB1128, 1/20000). The primary antibodies were incubated for 3h at RT. Cells were then incubated with a fluorochrome-labelled secondary antibodies: donkey anti-mouse Alexa 594 from Jackson Immuno Research (#715-585-150, 1/1000), rabbit anti-goat Alexa 488 from Invitrogen (#A11078, 1/1000), rabbit Anti-guinea pig Texas red from termofisher (#PA1-28595, 1/1000). The nucleus of the cells was stained with TO-PRO3 from Invitrogen (#T3605, 1/300) diluted in Prolong Antifade mounting medium (Invitrogen, #P7481) while performing the mounting of the coverslips. The image acquisition was performed with an Inverted Confocal Microscope TCS SP5 from Leica Microsystems. Image analysis was performed using Fiji software.

The A549-mRFP-LC3 cells were grown on glass coverslips (as described above) and infected with recRSV-GFP at an MOI of 3. After infection, the cells were fixed in 3% paraformaldehyde for 10min at RT, protected from light. Nuclear staining was performed using DAPI (1/6000). Image acquisition was performed using an Axio Imager.M2 microscope from Zeiss. Images from three different fields were acquired to obtain a minimal of 100 cells per condition. The total and GFP-positive cells were counted using the cell counting tool from Fiji to determine the percentage of RSV-GFP positive cells.

# 4. Results

## 4.1 Impact of p62 in RSV infectivity.

P62 has been reported to act at different stages of the replication cycle of some viruses, however this is not always reflected in *de novo* production of viral particles or their infectivity (Orvedahl et al., 2010; Shi et al., 2013). Taking this into account, we evaluated if p62 impacts the production of RSV infectious particles. For this, we used the alveolar epithelial cell line A549, which is a cellular model routinely employed for mechanistic studies of RSV infection. A549 cells were transfected with small interfering RNAs (siRNAs) specifically targeting p62 (sip62) or with a non-targeting sequence (siCtrl), and the silencing efficiency was evaluated by immunoblot (Fig.9A). A549 cells transfected with control- or p62-specific siRNA were infected with the prototype strain RSV-A2 at an MOI of 1 for 72h and the supernatant titrated by plaque assay in Hep-2 cells. We observed that silencing of p62 significantly increases the production of infectious particles (Fig. 9B). As RSV has been reported to naturally generate defective particles that lack full-length genomes but are strong inducers of an innate antiviral response mediated by type I and III IFNs production (Sun et al., 2015), we wanted to investigate if the decrease in the infectivity of RSV by p62 is associated with the production of defective particles. For this purpose, we used the recRSV-GFP virus (Hallak, Collins, Knudson, & Peeples, 2000) in which at the end of the genome has been inserted a reporter gene encoding a green fluorescent protein (GFP). Taking into account that the viral gene expression of RSV occurs in an obligatorily sequential manner (Collins et al., 2013), the GFP protein will be expressed only when full-length genomes are transcribed. Thus, A549 cells transfected with control- or p62-specific siRNA were infected with the recRSV-GFP virus at an MOI of 1 for 72h and the supernatant was titrated by plaque assay in IFN-deficient Vero cells to discriminate a response mediated by IFNs. The viral titer was evaluated based on the formation of GFP-plaques. In here, we also observed a significant increase in the production of full-length genome viral particles, when p62 was silenced, independently of IFNs production (Fig. 9C). Then, we evaluated if the reduction in the infectious titer by p62 was associated with a decreased production of free viral particles. To test this, we analyzed the levels of the viral proteins present in the cell supernatants that correlate with an increased production of free virions. For this, A549 cells transfected with control- or p62-specific siRNA were infected with RSV-A2 at an MOI of 1 and the cell supernatants subjected to immunoblot analysis for the RSV proteins. Preliminary data suggest

that p62 does not decrease the overall quantity of RSV proteins, which would have correlated with a lower production of free virions. Instead we observed a differential protein composition between the free virions produced in control and p62-silenced A549 cells (Fig. 9D). Together these data indicate that p62 reduces the production of infectious RSV particles, while preliminary data suggest that this effect might be associated with an impact of p62 on the protein composition of the mature free virions.

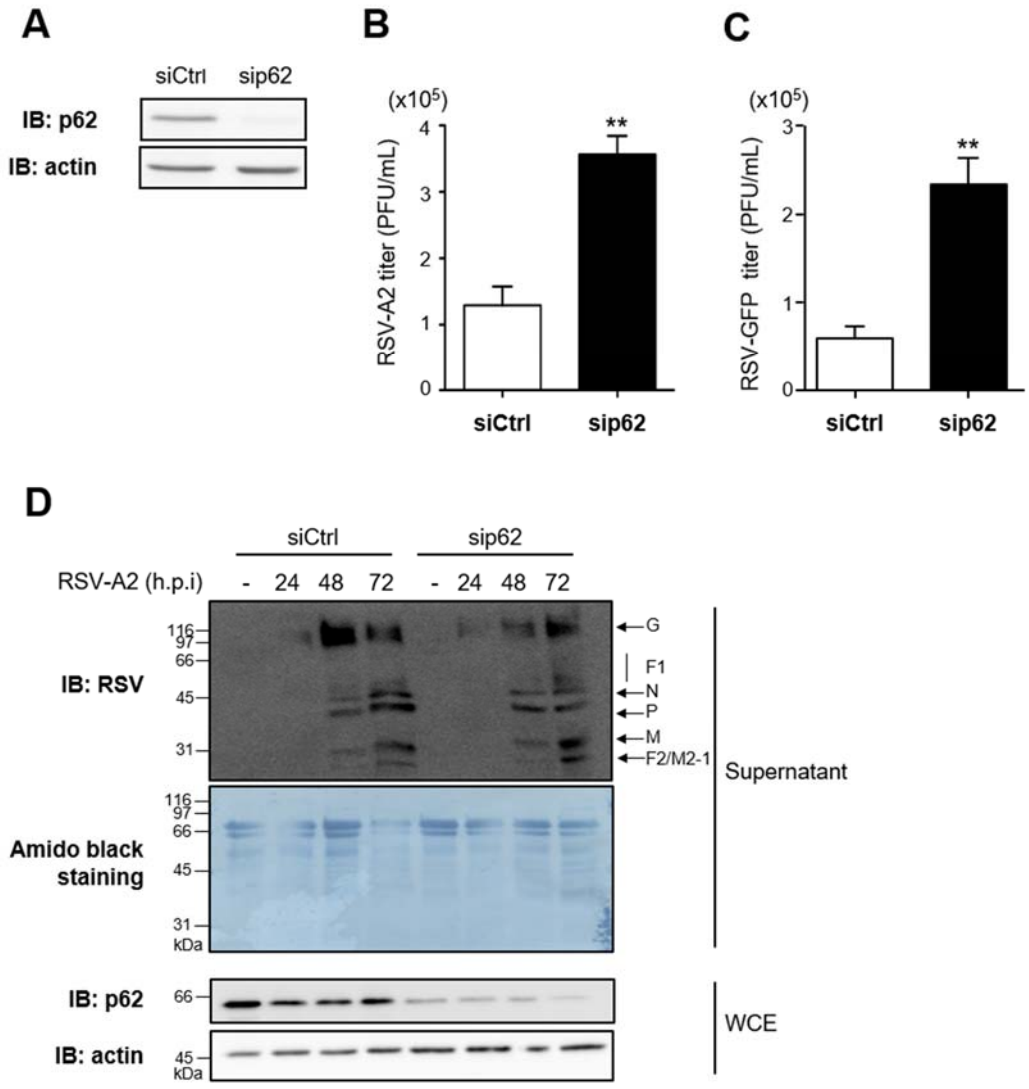


**Figure 9. Impact of p62 in the production of RSV infectious particles**. (A-D) A549 cells were transfected with control- or p62-specific siRNA for 24h. (A) WCE were analyzed by immunoblot for the indicated antibodies. (B-C) The cells were infected with RSV-A2 (B) or recRSV-GFP (C) at an MOI of 1 for 72h and the supernatants (SN) were titrated by plaque assay in Hep-2 cells (B) or Vero cells (C). The PFU titers are presented as mean +/- SEM; n=3; unpaired t-test, p values < 0.01=**. Results are representative of 3 independent experiments. (D) The cells were infected with RSV-A2 at an MOI of 1 for the indicated time points and the supernatants were concentrated using 30kDa Microsep columns. Concentrated supernatants were subjected to immunoblot analysis for the RSV proteins. Amido black staining is showed as control of equal protein levels between samples. WCE were subjected to immunoblot analysis with the indicated antibodies to monitor p62 silencing. Preliminary results. *Figure performed in collaboration with Xiaochun Guan*.

## 4.2 The intracellular impact of p62 on RSV replication cycle.

As we observed in the previous section, p62 might have the potential to drive a differential protein composition on the RSV-free virions and taking into account that p62, via its cargo function, has the potential to interact and sequester viral proteins (Orvedahl et al., 2010), we sought to evaluate its impact on the levels of RSV proteins. First, we analyzed the intracellular effect of p62 on the expression of the RSV proteins. Thus, A549 cells were transfected with control- or p62-specific siRNA and infected with RSV-A2 at an MOI of 1 for the indicated time points. WCE were subjected to immunoblot analysis to evaluate the expression of the cell-associated viral proteins. Here, we observed that p62-silenced A549 cells have reduced levels of the cell-associated G, $F_1$, and N viral proteins after 48-72h post-infection (Fig. 10A). We sought to confirm this result through ectopic expression of p62. For this purpose, A549 cells were transfected with an empty or a p62-mCherry encoding plasmid and infected with RSV-A2 at an MOI of 1 for the indicated time points. WCE were subjected to immunoblot for the analysis of the cell-associated RSV proteins. Preliminary results show that ectopic expression of p62 increases the expression of the cell-associated RSV G, $F_1$ and N proteins (Fig. 10B). This is in accordance with the results observed when p62 is silenced. Because we observed that p62 reduces the expression of the cell-associated RSV G, $F_1$ and N proteins, we sought to evaluate the impact of p62 on RSV mRNA levels. Thus, A549 cells were transfected with control- or p62-specific siRNA and infected with RSV-A2 at an MOI of 3 for the indicated time points. Total RNA was extracted. Then, we monitored by real-time PCR the expression of RSV-N mRNA. The results indicate that p62 does not regulate the levels of viral mRNA (Fig. 10C). Together, these results show that p62 has the capacity to increase the expression of the cell-associated $F_1$ and G glycoproteins, and in less extent the expression of the N protein.

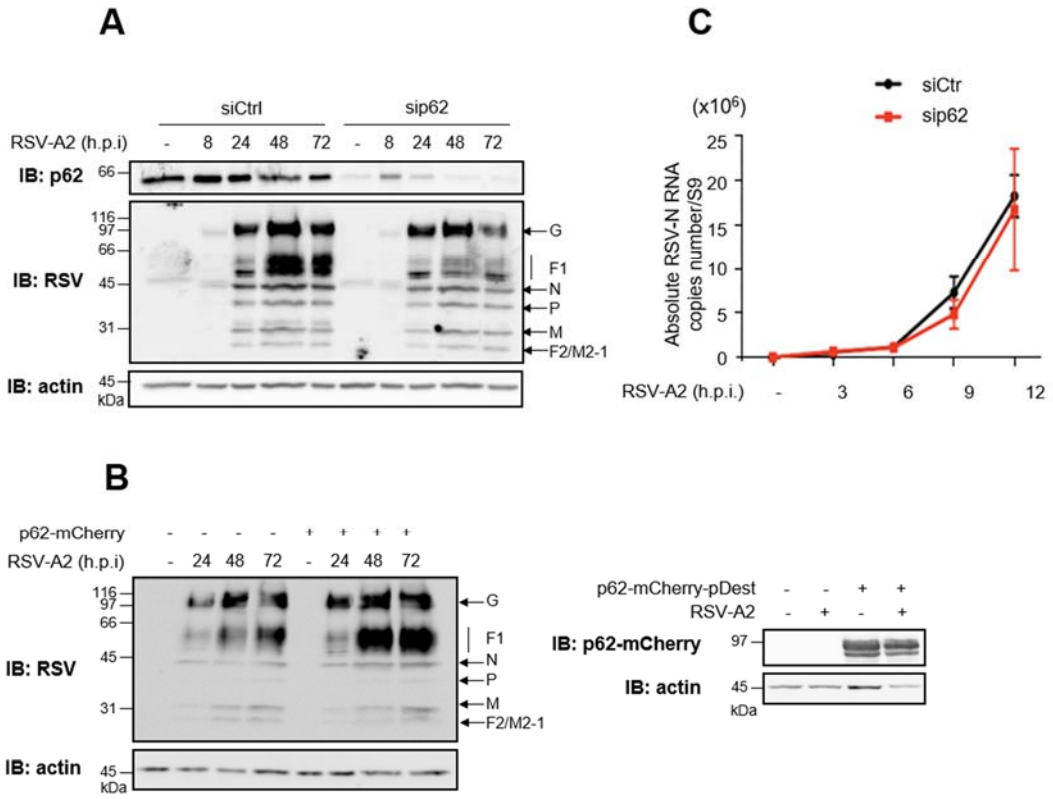


**Figure 10. P62 modulates the levels of cell-associated RSV G, $F_1$, and N proteins.** (A) A549 cells were transfected with control- or p62-specific siRNA for 24h and infected with RSV-A2 at an MOI of 1 for the indicated time points. WCE were subjected to immunoblot analysis for the indicated antibodies. Results are representative of 4 independent experiments. (B) A549 cells were transfected with an empty or a p62-mCherry encoding plasmid for 24h and infected as in (A). WCE were subjected to immunoblot analysis for the indicated antibodies. Effect of the ectopic expression of p62 during RSV infection (left panel), transfection control (right panel). Preliminary results. (C) A549 were transfected as in (A) and infected with RSV-A2 at an MOI of 3. Total RNA was extracted and RT-qPCR performed for RSV-N and the reporter gene S9, the copies number of RSV-N RNA were normalized with the S9 mRNA and presented as the mean and SDs of three technical replicates (error bars) using an unpaired T-test $p<0.05$=ns. *Figure performed in collaboration with Xiaochun Guan*.

## 4.3 P62 is regulated during RSV infection at the level of expression and phosphorylation, and degraded by the proteasome.

In the previous section, we showed that p62 increases the cell-associated $F_1$, G, and N protein levels. Previous reports have shown that p62 directly interacts with cellular and viral proteins (Isogai et al., 2011; Orvedahl et al., 2010), and this interaction can be mediated by the p62-UBA domain after phosphorylation on Ser403. To gain insight into the mechanisms by which p62 could be mediating the increased expression of the viral proteins, we evaluated the profile of expression and phosphorylation of p62 during RSV infection. For this, A549 cells were infected with RSV-A2 at an MOI of 3 for time points up to 32h. WCE were subjected to immunoblot analysis to examine the levels of p62 and its phosphorylation on Ser403. We observed that during RSV infection p62 is initially induced but from 12h and for the rest of the infection, the amount of p62 progressively decreases (Fig. 11A). We also observed that phosphorylation of p62 on Ser403 is induced during RSV infection reaching its maximum expression at 16h of infection (Fig. 11A). Very interestingly the peak of phosphorylation correlates with the time points at which total p62 levels start to decrease, suggesting that the phosphorylation could be linked to the formation of p62 aggregate-like structures that are further targeted via autophagy for degradation. As a note, the regulation of p62 was observed only in RSV-infected conditions and not in mock cells (data not shown) following the same kinetic. As we observed a decrease in the expression of total p62 during infection and this protein acts as substrate for degradation by autophagy and the UPS, we wanted to investigate if this decrease could reflect p62 degradation by autophagy or the UPS. To date, we evaluated the expression of p62 during RSV infection after inhibition of the proteasome. A549 cells were treated for 1h with the vehicle (DMSO) or the proteasome inhibitor MG132 and then infected with RSV-A2 at an MOI of 3 for time points up to 32h. WCE were subjected to immunoblot analysis to evaluate the levels of total- and Ser403 phosphorylated-p62. We observed that when the proteasome is inhibited, the decrease of the total- and phosphorylated-p62 is delayed, but not blocked, as accumulation of the levels of this protein were not observed (Fig. 11B). Together, these results suggest that the cargo function of p62-UBA domain, which is regulated by phosphorylation on Ser403, might be implicated in the restriction of RSV infection. In addition, we observed that in some extent the proteasome is implicated in the degradation of p62 during

RSV infection; however, as we did not observe accumulation in the levels of p62, another mechanism might be implicated.

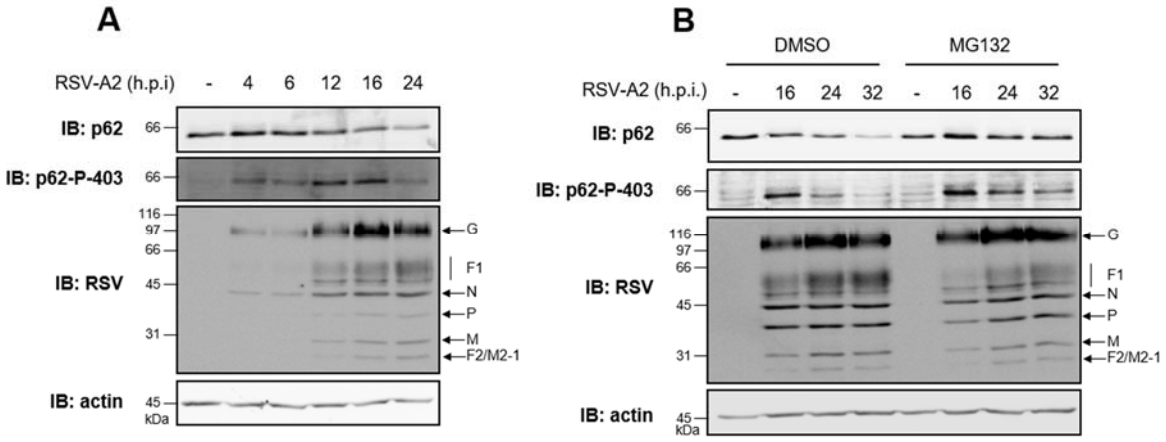


**Figure 11. Regulation of p62 during RSV infection.** (A) A549 cells were infected with RSV-A2 at an MOI of 3 for the indicated time points, and the WCE subjected to immunoblot analysis for the indicated antibodies. For the detection of phosphorylated p62 on Ser403 a phosphospecific antibody (anti-p62-P403) was used. Results are representative of 4 independent experiments. (B) A549 cells were treated with DMSO or the proteasome inhibitor MG132 (5μM) and infected as in (A) for the indicating time points. WCE were subjected to immunoblot analysis for the indicated antibodies. Results are representative of three independent experiments.

## 4.4 P62 phosphorylation on Ser403 during RSV infection is mediated by TBK1.

It has been reported that different kinases phosphorylate p62 on serine 403: TBK1 in HEK293 cells, CK2 in Neuro2a cells, and ULK1 in HEK293 cells (Matsumoto et al., 2011; Pilli et al., 2012; Ro et al., 2014). Among them, only TBK1 has been reported to phosphorylate p62 in the context of self-defence against intracellular pathogens (Pilli et al., 2012). In addition, TBK1 is central to the signaling cascades leading to type-I IFN production in response to pathogen recognition (Fitzgerald et al., 2003; Sharma et al., 2003). TBK1 and its closely related family member IKKε, are two kinases that have significant functional redundancy but also additional functions and distinct activation profiles that shape the innate immune signaling differently (Pham & tenOever, 2010). Taking the previous into account, we focused on evaluating if the protein kinase TBK1 is responsible for phosphorylating p62 on Ser403 during RSV infection in airway epithelial cells. We also studied the potential involvement of IKKε. First, we co-transfected a p62-mCherry encoding plasmid with the plasmids pEGFPC1-TBK1 or pEGFPC1-IKKε in A549 cells. To verify if their kinase activity was required we also expressed the respective kinase defective mutants TBK1K38A and IKKε K38A. We observed that TBK1, and to a lesser extent IKKε, phosphorylate p62 on Ser403 in A549 cells. This effect was not observed in cells co-transfected with the kinase defective mutants (Fig. 12A) indicating that their kinase activity is required to mediate p62 phosphorylation on Ser403. Next, to evaluate if TBK1 or IKKε were implicated in the phosphorylation of p62 on Ser403 during RSV infection, we used the dual TBK1 and IKKε inhibitor MRT67307 (Clark et al., 2011). A549 cells were treated with DMSO or MRT67307 for 1h before infection with RSV-A2 at an MOI of 3. The cells were infected for time points up to 32h and the WCE analyzed by immunoblot. A549 cells treated with MRT67307 show a suppression of p62 phosphorylation on Ser403 suggesting that the activity of these kinases is required for the cargo activity of p62 during RSV infection (Fig. 12B). MRT67307 is a dual inhibitor of TBK1 and IKKε and therefore does not allow to discriminate the activity of each kinase. To evaluate which kinase was mediating the phosphorylation of p62 during RSV infection, we evaluated the profile of activation of both kinases by looking at their phosphorylation on serine 172 (Ser172), a post-transcriptional modification that reflects their activation (Kishore et al., 2002). A549 cells were infected with

RSV-A2 at an MOI of 3 for up to 32h and the WCE were subjected to immunoblot using the phosphospecific antibodies anti-TBK1-P172, anti-IKKε-P172. During RSV infection, TBK1 phosphorylation is detected at 6h post infection and peaks at 16h following a kinetic of activation similar to the phosphorylation on Ser403 of p62, while IKKε phosphorylation was observed only at 24h post-infection, a time point at which p62 levels and phosphorylation have decreased (Fig. 12C). These results suggest that TBK1 is mainly responsible for the phosphorylation of p62 at Ser403 during RSV infection. Finally, we sought to confirm these results by silencing of both kinases using siRNA. Unexpectedly, A549 cells transfected with two different siRNA specifically targeting TBK1 and one targeting IKKε, without another stimulation, display decreased expression of p62 detected by immunoblot analysis (Fig. 12D), hampering the analysis of p62 phosphorylation during RSV infection. Altogether, these results indicate that during RSV infection TBK1 phosphorylates p62 on Ser403, and that TBK1 and IKKε could be required to mediate p62 expression/stability, independently of their kinase activity.

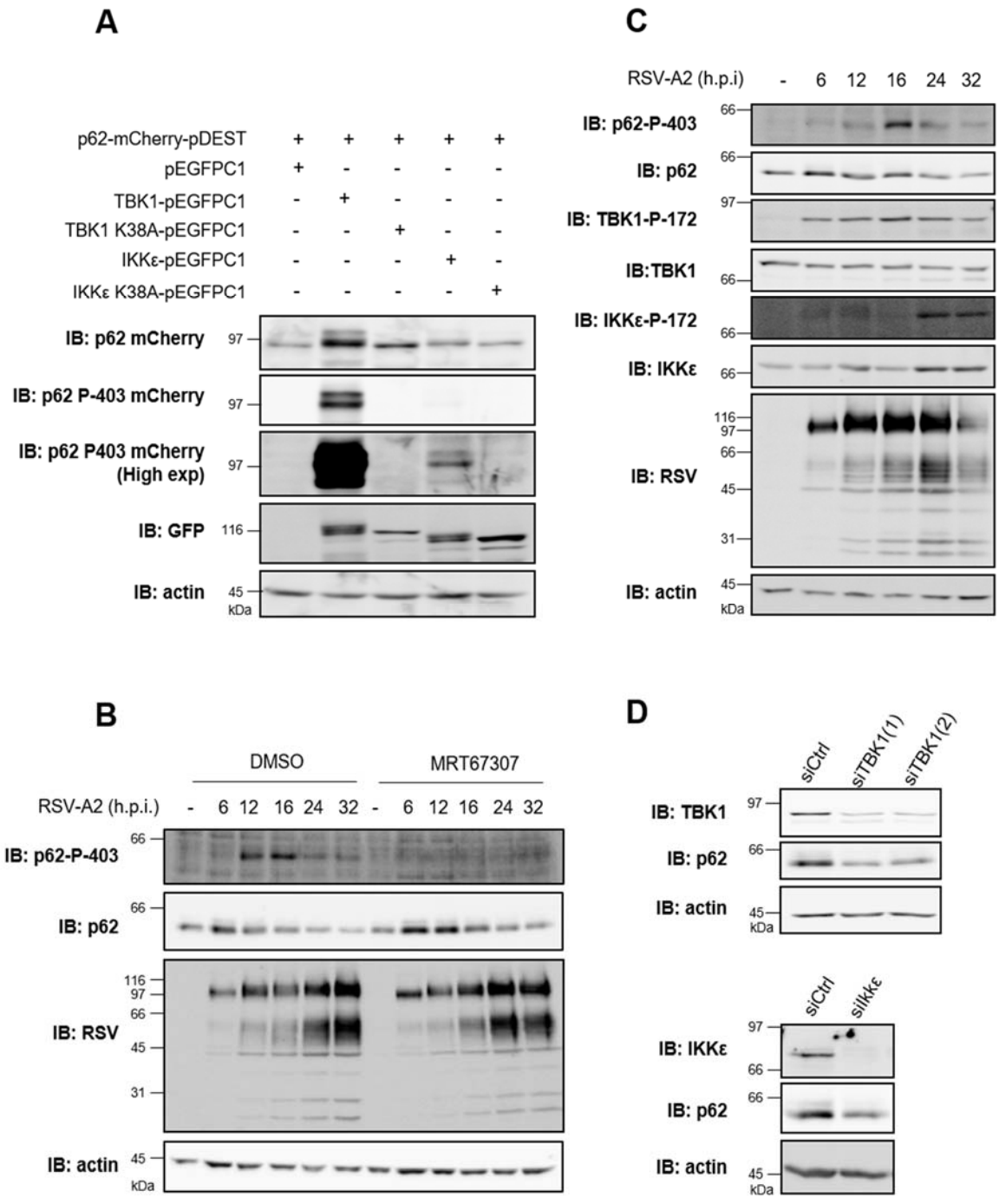


**Figure 12. P62 is phosphorylated on Ser403 by TBK1 during RSV infection.** (See legend in the next page).

**Legend Figure 12.** (A) A549 cells were co-transfected with p62-mCherry-pDEST and pEGFPC1-TBK1, IKKε or their defective kinase mutants K38A for 24h. (B) A549 cells were treated with DMSO or MRT67307 (2μM) for 1h before infection with RSV-A2 at an MOI of 3. The cells were infected for the indicated time points. (C) A549 cells were infected with RSV-A2 at an MOI of 3 for the indicated time points. (D) A549 cells were transfected for 48h with a non-targeting (siCtrl) and two different siRNA targeting TBK1 (siTBK1) (upper panel), and one targeting IKKε (siIKKε) (lower panel).From A-D, WCE were subjected to immunoblot analysis for the indicated antibodies. For the detection of TBK1 and IKKε phosphorylation on Ser172, the phosphospecific antibodies anti-TBK1 serine 172 (P172) and anti-IKKε-P172 were used. The results are representative of three independent experiments.

## 4.5 Co-localization between p62 and RSV proteins.

As mentioned before, p62 is a multi-domain protein that interacts with different binding partners in the regulation of diverse cellular signaling pathways in basal and stress inducible conditions (Katsuragi et al., 2015). In addition, p62 has a cargo function by which it targets various substrates, including cellular and viral proteins, for degradation by selective autophagy and in certain cases by the UPS (Babu, Geetha, & Wooten, 2005; Lim et al., 2015; Orvedahl et al., 2010). Taking into account that during RSV infection p62 is phosphorylated on Ser403, which regulates the cargo activity of its UBA domain, and that the expression of the cell-associated G, $F_1$, and N viral proteins are increased in presence of p62, we wanted to get insights about the localization of p62 and the RSV proteins that could suggest their possible interaction. For this, we cultured A549 cells on coverslips and we infected them with RSV-A2 at an MOI of 3 for 12h. Then, we performed indirect immunofluorescence using anti-RSV and anti-p62 antibodies, while the nuclei were counterstained with To-pro3. The localization of the RSV proteins and p62 was analyzed by confocal analysis. Preliminary results show that at 12h post-infection there is partial co-localization between the total RSV proteins and p62 (Fig.13). This indicates that p62 is close to the RSV proteins at 12h post-infection, one of the time points at which p62 displays the maximum levels of phosphorylation on Ser403. These results suggest a possible interaction between p62 and the RSV proteins mediated by the cargo function of p62.

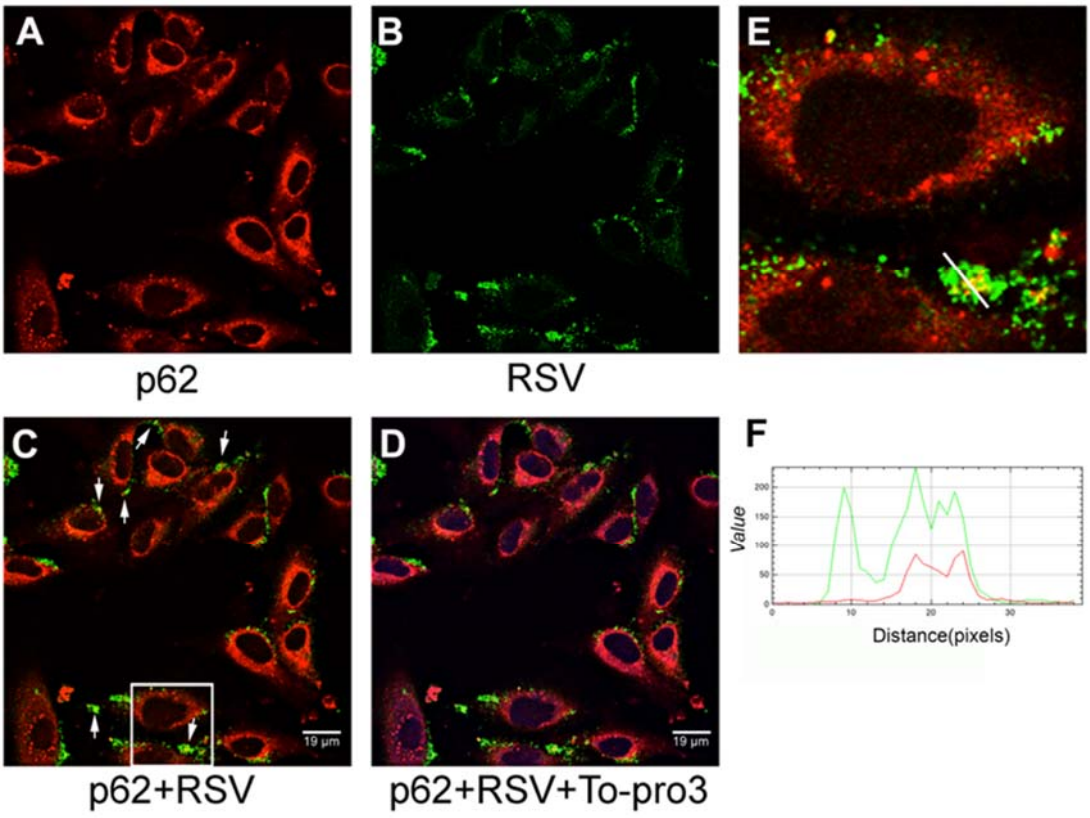


**Figure 13. Co-localization between p62 and RSV proteins.** A549 cells were grown on coverslips and infected with RSV-A2 at an MOI of 3 for 12h. The cells were fixed and stained with (A) anti-p62 (Alexa 594, red). (B) and anti-RSV (Alexa488, green). The nuclei were counterstained using To-pro3 (blue). Slides were scanned sequentially for each fluorochrome by confocal microscopy (63X). One confocal optical section is shown. (C) Merge of p62 and RSV and of (D) RSV, p62 and To-pro3. In C, arrows highlight areas of co-localization. (E) Enlargement of the white square section indicated in C. (F) Line scan (from the white line in E) was used to assess co-localization using Fiji software. Preliminary data. *Figure performed in collaboration with Elise Caron*.

## 4.6 P62 and RSV glycoproteins G and $F_1$ are pull-down with polyubiquitinated proteins.

During RSV infection, we observed that p62 co-localizes with uncharacterized viral proteins and that it is phosphorylated on Ser403, which increases its affinity for ubiquitinated substrates (Matsumoto et al., 2011). We wondered if ubiquitination could be mediating an interaction between p62 and the viral proteins G, $F_1$, and N, thereby reflecting the accumulation of these proteins in the cells. To test this, our first approach was to determine if the RSV proteins and p62 were pulled-down with polyubiquitinated proteins during infection. To do this, we infected A549 with RSV-A2 at an MOI of 3 for the indicated time points. The WCE were incubated with TUBE-1 (tandem Ub-binding entities), which are tandem Ub binding associated domains (UBAs) that bind to all 7 poly-Ub linkage type proteins with high affinity in their native state (Hjerpe et al., 2009). The elution from the TUBE-1 beads per condition was analyzed by immunoblot. Our data demonstrate that the isolated fraction with the polyubiquitinated proteins was enriched with p62 phosphorylated on Ser403, in concordance with the Ub-cargo function of p62-UBA domain. Lastly, we detected the viral glycoproteins G and $F_1$ in the elution (Fig. 14). These results suggest that the viral proteins $F_1$ and G might be ubiquitinated during infection and recruited by the p62-UBA domain. It is important to mention that this assay also pulled down proteins interacting with polyubiquitinated proteins and therefore exists the possibility that an intermediate factor links the effect of p62 on the accumulation of the viral proteins.

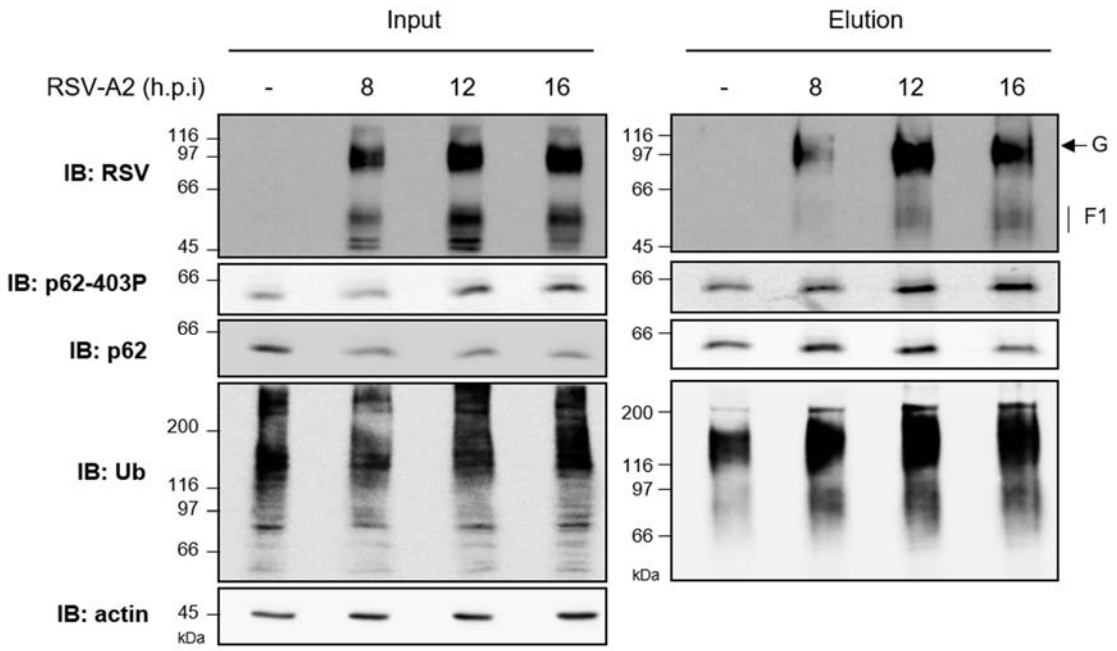


**Figure 14. Pull-down of RSV G and $F_1$ with polyubiquitinated proteins.** (A) A549 cells were infected with RSV-A2 to an MOI of 3 for the indicated time points. 1mg of WCE per condition was incubated with 10µL of TUBE1 agarose beads for 3h at 4°C. The polyubiquitinated proteins present in the WCE were analyzed by immunoblot in parallel with 5% of the INPUT. Anti-RSV was used for the detection of viral proteins, and anti-p62 and anti-p62-P403 for p62 expression and activation, respectively. Anti-Ub was used as a control of pull-down efficiency and anti-actin as input loading control. Results are representative of two independent experiments. *Figure performed in collaboration with Quentin Osseman*.

## 4.7 The p62 downstream LC3 protein reduces RSV replication.

P62 is a receptor that recruits ubiquitinated cargos to deliver them into autophagosomes for their degradation via autophagy. After cargo recruitment, p62 via its LIR region interacts with the autophagosome conjugated form LC3-II to deliver selective cargos to the autophagosome (Pankiv et al., 2007). During autophagy, LC3 is an essential protein required for the formation, maturation, and transport of the autophagosome (Lee & Lee, 2016). Autophagy impacts the replication cycle of several viruses, many of which have evolved mechanisms to block this pathway or even manipulate it to promote different stages of their replication cycle, ranging from entry to egress (Chakrabarti, Ghosh, Banerjee, Gaughan, & Silverman, 2012; Liu et al., 2016; Sharma et al., 2014). The direct role that autophagy plays in the replication cycle of RSV in AEC is unknown, and so far a strong induction of LC3-II (standard to monitor autophagy) by RSV has not been detected. Taking this into account, our first approach was to evaluate the effect of LC3 in the replication cycle of RSV using A549-mRFP-LC3 cells, which are LC3 stable transfected cells with a RFP tag (red) to monitor LC3. The parental (wt) A549 cells and the A549-mRFP-LC3 were infected with recRSV-GFP at an MOI of 3 for up to 36h. The replication of the virus and the expression of the LC3 were monitored by detection of GFP (green) and RFP (red) respectively, using immunofluorescence microscopy. The A549-mRFP-LC3 cells display reduced levels of recRSV-GFP replication compared with the wt-A549 cells throughout the infection (Fig. 15A). Interestingly, we also observed that A549-mRFP-LC3 cells with lower levels of LC3 expression are more susceptible to RecRSV-GFP replication (Fig. 15B) suggesting that the virus must overcome the expression and probably the activity of LC3 to replicate effectively. Finally, we evaluated the percentage of recRSV-GFP positive cells to gain insights into the extent of reduction of the viral replication in the A549-mRFP-LC3 cells compared with the wt-A549. Here we observe that the stable expression of LC3 reduces notably the percentage of recRSV-GFP positive cells even at late times of infection (Fig. 15C). These data suggest that LC3 has an antiviral effect over RSV by reducing its viral replication, however eventually the virus has the potential to overcome its antiviral effect, probably by blocking *de novo* synthesis or expression of LC3.

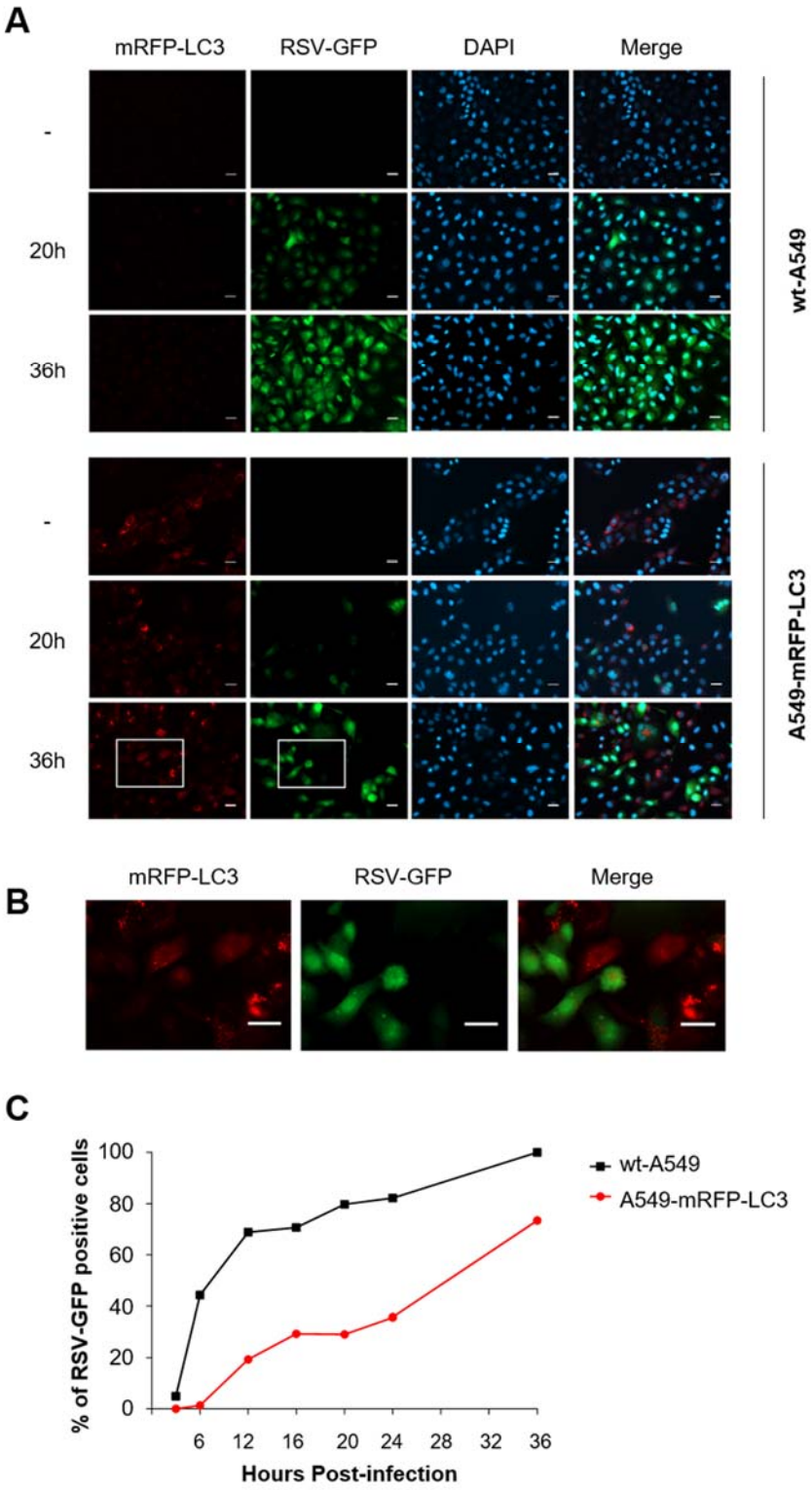


**Figure 15. Stable expression of LC3 in A549 cells restricts recRSV-GFP replication.** (See legend in the next page).

**Legend Figure 15.** Wt-A549 and A549-mRFP-LC3 cells were grown on glass coverslips and infected with recRSV-GFP at an MOI of 3. The coverslips were harvested for up to 36h and fixed. The nuclei were counterstained using DAPI (blue). (A) The recRSV-GFP replication (green) and the LC3-mRFP (red) expression were monitored by fluorescence microscopy. 20x images were acquired from all time points, here is depicted only 20h and 36h. (B) Enlargement of the white rectangle sections indicated in A, and the merge of both channels. In A and B, scale bars represent 25μm. (C) Quantification of recRSV-GFP positive cells. For the analysis, 100 cells per condition were analyzed using Fiji software, and the percentage of GFP-positive cells was calculated for all the time points. A-C, are results representative of 2 independent experiments.

# 5. Discussion

P62 is a scaffolding protein that regulates several cellular events, including proteostasis, redox control, apoptosis, and selective autophagy. P62 activity is required to maintain cellular homeostasis and counteract stress conditions. In past years many studies have provided evidence supporting that p62 is an important determinant in host defense against intracellular pathogens, including bacteria and viruses (Metz et al., 2015; Pilli et al., 2012), and in modulating the production of different cytokines to control excessive inflammation (Kim & Ozato, 2009). Recently, some studies have started to uncover the roles of p62 in the replication cycle of different viruses, including JEV and DENV where p62 has a proviral and an antiviral function respectively (Metz et al., 2015; Tasaki et al., 2016). However, to our knowledge, the potential of p62 to regulate the replication cycle of RSV had not been evaluated.

## P62 affects RSV infectivity and replication.

Based on the work presented in this memoire, we propose that p62 acts as an antiviral factor in the replication cycle of RSV. We show that silencing of p62 increases RSV infectivity and replication based on titration assays of RSV-A2 and recRSV-GFP. These results are important because p62 does not always have a direct contribution in the replication of the virus and because many viruses have evolved mechanisms to block or modulate p62 activity (Judith et al., 2013; Orvedahl et al., 2010). As an example, DENV promotes the early degradation of p62 to inhibit its antiviral function (Metz et al., 2015), while JEV uses p62 to promote its replication by an unknown mechanism (Tasaki et al., 2016). We show that when p62 is silenced RSV infectivity is enhanced suggesting that p62, without need to be overexpressed, has the potential to control RSV infectivity. Here we observed a two-fold increase in the viral titer when p62 is silenced. This is not very high, but comparable with other published reports where not more than 3 fold changes are observed after the silencing of different cellular proteins or after treatment with different inhibitors, including those involved in RSV transport, assembly and release (Brock et al., 2003; Hassan et al., 2014; Tayyari et al., 2011; Utley et al., 2008). Alternatively, it remains the possibility that the increase in infectivity is an indirect result of the intracellular effect of the regulation of p62 over RSV. Further analysis are required to evaluate if an opposite effect is observed when p62 is ectopically expressed in the cells. In addition, validation of the results with another RNAi sequence specifically targeting p62 will be supplementary to discard off targeting effects. It is being considered performing studies with

different clones of A549-p62 CRISPR/Cas9 knockout cells as an alternative method to the use of siRNA.

To evaluate the tentative mechanisms behind the increase in RSV infectivity following p62 silencing, A549 cells were infected with a recRSV-GFP virus and titration was performed in IFN-deficient Vero cells. Here we discard the involvement of an IFN-dependent mechanism or a process associated with an increased production of RSV-defective particles that lack full-length genomes. Preliminary data suggest that the increase of RSV infectivity after knockdown of p62 is not associated with a higher production of free viruses, as we did not observe a general increase in the total amount of viral proteins present in the supernatant that would correlate with a bigger production of free viruses. Interestingly in these preliminary results, we observe differential levels of viral proteins between free virions generated in A549 cells silenced of p62 and control cells. In other viruses like herpes simplex virus type I (HSV-1), protein heterogeneity within individual viral particles (specifically of the enveloped proteins) has been shown to modulate viral infectivity and fitness (El Bilali, Duron, Gingras, & Lippé, 2017). To test if the viral protein composition in the new viral progeny is altered by p62, we could purify the virions by ultracentrifugation using a sucrose cushion and examine the virion morphology by electron microscopy. The samples can be analyzed in parallel by tandem mass spectrometry to identify the structural proteins and their relative abundances (Chung et al., 2006). Additional studies need to include the impact of p62 on the protein composition of the cell associated viruses, which are the predominant morphology of RSV particles (Mitra et al., 2012; Shaikh & Crowe, 2013). As RSV assembles in lipid rafts, a tentative approach would be to analyze the lipid-raft cell membranes from infected cells by two dimensional nanoflow liquid chromatography mass spectrometry that would permit evaluation of the protein content of these structures (Brown et al., 2005).

## P62 increases the cell-associated RSV G, $F_1$ and N proteins.

Analysis of the cell-associated RSV proteins shows that silencing of p62 reduces the levels of the RSV G and $F_1$ glycoproteins and to a lesser extent of the N protein, without altering the levels of the other proteins that are incorporated into mature virions. In accordance with these results, ectopic expression of p62 induces increased levels of the cell-associated G, $F_1$ and

N proteins. We confirm that the modulation in the levels of the viral proteins by p62 is not at the transcriptional level as p62 does not alter the levels of viral mRNA using as reporter the mRNA of the N protein by qPCR. However, taking into account that p62 modulates the level of the viral protein at late times of infection (after 48 h.p.i) evaluating the levels of viral mRNA in longer kinetics of infection might be required as well as confirming these results performing biological triplicates.

P62 could be modulating the viral proteins at different stages, including their synthesis, folding, trafficking or degradation. It is important to note that the major proteins affected by p62 are the F and the G that follow the secretory pathway to become functional before their assembly into the new viral particles. Further studies are required to evaluate if p62 acts on this pathway to control protein trafficking during RSV infection and how it influences changes in the levels of the N protein. Recently, p62 has been shown to act as an adaptor for vesicular transport in the ER via association with the ubiquitin ligase Ring finger protein 26 (RNF26). Here p62 is recruited by the RFP26 to sequester specific vesicle adaptors via its UBA domain and therefore restrain the vesicles fast transport to other cellular compartments, including the Golgi apparatus, and the extracellular space (Jongsma et al., 2016). The fact that p62 mainly increases the cell-associated G and F protein could be associated with the retention or slow transit of these viral proteins to the cell membrane and their poor incorporation into the mature viral particles. Differential levels of the G and F glycoproteins within individual RSV free virions has been previously observed (Liljeroos et al., 2013), and it is generally recognized that the quantity and quality of these two proteins are important determinants of RSV infectivity. Mainly because they control the initial phase of the infection, attachment and entry (McLellan et al., 2013). Meanwhile, the N protein has the function of encapsidating the viral genome to protect it from degradation and recognition by cellular sensors that trigger an antiviral response to fight the infection (Fearns et al., 1997; Groskreutz et al., 2010). The next steps would be to evaluate if p62 is required to control ER vesicular transport during RSV infection, which might result in a poor transit and incorporation of the RSV glycoproteins into the cell surface and if this is due to the action of p62 and RNF26. Live imaging experiments to track the transit of the viral proteins might be useful.

Our results demonstrate that the cargo function of p62 is activated during RSV infection. Indeed, we found that RSV induces p62 phosphorylation on Ser403, a posttranslational modification that is known to promote the recruitment of polyubiquitinated cargos via the p62-UBA domain to form aggregate like-structures that are degradated via autophagy (Matsumoto et al., 2011). In accordance with this, we show that RSV infection increases phosphorylated p62 pulled down with polyubiquitinated proteins. Interestingly, in this assay we also show that the viral glycoproteins, G and F, are found in this fraction, suggesting that these proteins interacts with Ub-proteins or are ubiquitinated. We have previously observed the formation of p62 aggregate-like structures during RSV infection (data not shown here). Also, preliminary data show partial co-localization between unidentified/total RSV proteins and p62.It is possible that during infection p62 promotes the sequestration of the viral glycoproteins in a Ub-dependent manner. However, our results only indicate partial co-localization and this technique only shows that the proteins are in close proximity, but not interacting. Further studies are required to evaluate this, which viral proteins and if it is Ub-dependent. A tentative strategy to evaluate the interaction between p62 and the viral proteins would be to perform p62 immunoprecipitation (IP) and evaluating by immunoblot the co-immunoprecipitation of the viral proteins. Additionally, mass spectrometry analyses could be carried out on the immunoprecipitated material to determine the peptides that mediate this interaction. Also, it could be determined by ubiquitin enrichment mass spectrometry if the viral proteins are ubiquitinated and in which residues. Unbiased approaches may include IP-p62 and subjecting the immunoprecipitated material to TUBEs and mass spectrometry to determine p62-ubiquitin interactome.

Until now, it has not been reported if the RSV proteins can be ubiquitinated during the replication cycle of the virus, but in viruses like influenza this posttranslational modification plays important roles. For instance, ubiquitination has been associated with promoting the degradation of viral proteins and boosts cell resistance to the infection (Rudnicka & Yamauchi, 2016). However, it is also required for the virus to promote different stages of their replication cycle, for example its entry, and to evade the cell autonomous response (Banerjee et al., 2014; Rudnicka & Yamauchi, 2016).

In viruses, like SIN and CHIKV, p62 has been shown to directly recruit viral capsids for degradation by autophagy (Judith et al., 2013; Orvedahl et al., 2010). However, in our results

we observe that p62 increases the levels of RSV proteins (G, $F_1$ and N). This could be explained if the viral proteins are sequestered but not degraded, however in this hypothesis p62 should also be accumulated while our results show that the p62 expression decreases throughout the infection suggesting that its turnover is not affected. There is a possibility that p62 mediates an indirect effect over the viral proteins which might be dependent or independent of its cargo function. Alternatively, as mentioned previously p62 via its cargo function acts as an adaptor for ER-endosomal transport via interaction with the Ub-ligase RNF26, independently of a degradative pathway (Jongsma et al., 2016).

P62 directs its cargo for degradation by autophagy and by the UPS (Myeku & Figueiredo-Pereira, 2011). To date, it is still unknown if the RSV has the potential to modulate either autophagy or the UPS in AECs, and if so how this impacts the replication cycle of the virus. Unexpectedly, treatment with the proteasome inhibitor MG132 during RSV infection slightly delays the degradation of p62 and prolongs its phosphorylation on Ser403, although it is reported that p62 is mainly degraded by autophagy and not by the UPS (Myeku & Figueiredo-Pereira, 2011). However, this result must be taken with precaution because previous reports have shown that treatment with proteasome inhibitors can affect p62 expression and induce dramatic changes in its interactome (Milan et al., 2015). Additional controls are required to determine if the response of p62 after inhibition of the proteasome is due to the infection and not by the treatment itself. Additional experiments have been performed recently in our laboratory, showing that Bafilomycin strongly inhibits degradation of phosphorylated p62 (data not shown). The present results suggest that both systems contribute in the degradation of p62 during RSV infection, but the phosphorylated form of p62 is mainly directed for degradation via autophagy.

During DENV infection p62 is degraded by the UPS as a mechanism of viral evasion to block its antiviral activity (Metz et al., 2015). For RSV, several studies have reported the potential of the NS1 and NS2 proteins to promote the degradation of many cellular signaling proteins by the proteasome (Sun & Lopez, 2017). Whether degradation of p62 via autophagy and/or the UPS supports or restricts viral replication is still unknown.

In other viruses, like SIN and CHIKV, accumulation of viral capsids inside the cell induce cell-death and tissue damage (Judith et al., 2013; Orvedahl et al., 2010). As we observed

that p62 increases the levels of the viral proteins during RSV infection it would be required to evaluate if this is associated with the induction of cell-death. To test this we should perform cell-viability assays when p62 is expressed or silenced independently of RSV infection.

We demonstrate that TBK1, which plays a central role in the activation of IRF3 and the production of IFN (Fitzgerald et al., 2003; Sharma et al., 2003), is an important kinase in charge of phosphorylating p62 on Ser403 during RSV infection. This suggests that TBK1 has an alternative role during RSV infection that could be independent of IFN production. Unexpectedly, we observed that TBK1 not only is required for the phosphorylation of p62 but also impact the expression/stability of p62 independently of its kinase activity. This is contrary to what is reported in murine primary bone marrow macrophages, where inhibition/silencing of TBK1 effects autophagic maturation, which induces accumulation of p62 (Pilli et al., 2012). This may be a consequence of the fine-tune regulation in different cell types, however further studies are required to explain these contrasting results and how TBK1 affects the expression of p62 in AECs. As IKKε shares a lot of substrates and activities with TBK1, we evaluated its role in phosphorylating p62 during RSV infection. Our data demonstrate that IKKε has the potential to phosphorylate p62, but during RSV infection its activation is triggered when the levels of p62 have decreased in the cells.

We focused in the cargo function of p62, however, we cannot discard that p62 by its different domains mediates cooperative or alternative cell-autonomous mechanisms to decrease RSV infectivity in AECs.

## Stable expression of LC3 reduces recRSV-GFP replication.

Some pathogens induce early stages of autophagy but block the late ones, *i.e.* fusion of the autophagosome with the lysosome (Deretic, 2011). In the context of RSV infection, a recent report has shown that LC3 deficiency in mice contributes to RSV induced lung pathology (Reed, Morris, Owczarczyk, & Lukacs, 2015). In this study it was demonstrated that $LC3^{-/-}$ murine primary AECs are incapable to upregulate autophagosome formation and therefore have increased inflammasome activation and elevated secretion of the cytokines IL-1 and IL-6 that promote $CD4^{+}$ T cell immunopathology (Reed et al., 2015). However, in the study of Reed *et al.*, it is not addressed whether RSV has the capacity to block or induce autophagy and how this

cellular system directly affects the replication of RSV. In this study, we evaluate the potential of stable LC3 expression to modulate RSV replication using A549-LC3-mRFP cells infected with a recRSV-GFP virus. Our data show that stable expression of LC3, which drives higher autophagy flux, negatively impacts RSV replication. A549-LC3-mRFP cells compared with A549-wt cells display a decrease in the percentage of GFP positive cells suggesting that the effect of stable ectopic LC3 happens before transcription of the virus. Interestingly, we observe that the cells with higher expression of LC3 through the infection are the ones with lower GFP expression and higher resistance against the virus, suggesting that the virus must reduce LC3-mRFP stable expression to establish an optimal replication. Current studies are undertaken to investigate how and in which stage of the infection LC3 decreases the replication of RSV in AECs and if RSV is able to modulate autophagy. Analysis of LC3 conversion (from LC3-I to LC3-II) by immunoblot performed in our laboratory have failed to demonstrate a significant induction of autophagy during RSV infection (data not shown). A recent report in $CD8^+$ T cells has shown that RSV induces mTOR activation by phosphorylating this protein at Ser2448 to impair memory $CD8^+$ T cells differentiation (de Souza et al., 2016). Phosphorylation of mTOR impairs ULK1 activation, one of the initiator complexes for autophagosome formation (Hurley & Young, 2017). The phosphorylation of mTOR is induced by the RSV F protein and it is independent of an active viral replication as UV-inactivated RSV also induces mTOR phosphorylation. Other viruses, like Epstein–Barr and human immunodeficiency virus, have been shown to modulate autophagy through interaction with the mTOR pathway (Chen et al., 2010; Nardacci et al., 2017). Hence, it would be interesting to evaluate if RSV uses this mechanism in AECs to control autophagosome formation and if the levels of the RSV F protein alter the potential of the virus to do it. To test this, live imaging could be performed to monitor autophagosome formation in A549-LC3-mRFP during infection with RSV-wt and RSV-ΔF, which has been previously engineered (Batonick & Wertz, 2011). mTOR phosphorylation by immunoblot could be evaluated during RSV infection as a mechanism of autophagy block. To monitor autophagy flux, A549 cells could be transfected with LC3-mCherry-GFP and infected with RSV. The LC3-mCherry-GFP construct allows to evaluate autophagosomes acidification, because the GFP is sensible to low pH and it is lost when the autophagosomes fuse with the lysosomes while the mCherry not.

## Working model.

Summarizing the results presented in this work we propose the following working model (Fig. 16). During RSV infection RIG-I recognizes the viral RNA and induces downstream activation of MAVS and TBK1 to induce IRF3 phosphorylation and translocation to the nucleus for the production of IFN. Additionally, TBK1 phosphorylates p62 at Ser403, which promotes the affinity of p62 for polyubiquitinated cargos. P62 might sequester directly or indirectly the F, G and N viral proteins in a Ub-dependent manner retaining these proteins inside of the cells, but without promoting their degradation. This can be mediated by the formation of aggregate-like structures or by delaying the transport of the viral proteins to the cellular membrane therefore affecting their transit or incorporation into the mature free virions. Virions with reduced levels of the viral proteins may present reduced fitness to infect new cells. During RSV infection p62 is degraded by autophagy and the UPS but the implications of this degradation are unknown. Finally, we observe that stable expression of LC3 reduces the replication of RSV before transcription. As the antiviral effect of LC3 is observed early during the infection, while p62 has an effect over the viral proteins and *de novo* production of infection progeny it could be possible that this two proteins act at different stages of the infection.

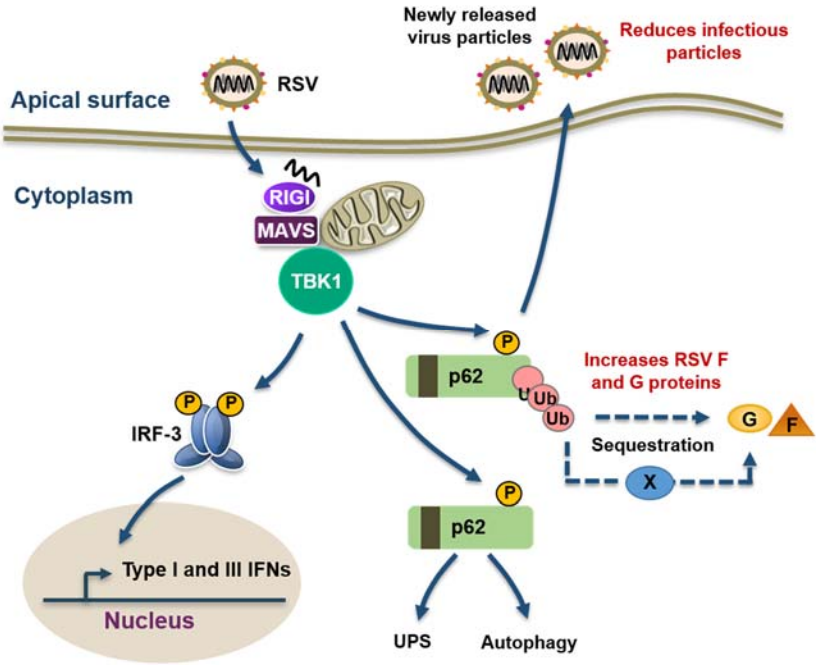


**Figure 16. Working model.**

## Limitations and further directions.

The studies performed in this work were carried out in the alveolar epithelial cells A549, which are routinely employed to model the cellular events occurring during acute infections and to study the different stages of the replication cycle of RSV and other respiratory viruses (Hassan et al., 2014; Zeng & Carlin, 2013). This is mainly because these cells resemble more closely, compared to other cell models, the response occurring in primary cells and because carrying out mechanistic studies require a great amount of cells and techniques that are not compatible with primary cells. However, as these cells are derived from lung adenocarcinoma, it is known that some pathways and cellular processes are modified and may not completely reflect the events occurring *in vivo* (Borchers et al., 2013). Therefore some findings obtained here should be further validated in human bronchial epithelial cells.

P62 is an important cellular protein which expression is highly regulated inside the cells, supporting this silencing of p62 in A549 decreases cell proliferation while overexpression of p62 is associated with increased apoptosis (Huang et al., 2013; Nihira, Miki, Ono, Suzuki, & Sasano, 2014). Taking this into account supplementary studies need to be performed to evaluate parallel cell effects of silencing and ectopic expression of p62 in our experimental conditions.

Further approaches for this project are planned to be performed in A549-p62 knockout cells using the CRISPR/Cas9 editing system in which p62 domain deletion mutants can be ectopically added-back without the background signal of the endogenous protein and to avoid saturation of the system.

# 6. Conclusion

The present work provides evidence that demonstrates that p62/SQSTM1 is a novel antiviral factor during the replication of RSV in AECs. Here we demonstrated that p62 reduces the production of RSV infectious titers while increasing the cell-associated G, $F_1$ and N viral proteins. Preliminary data demonstrates that p62 modulates the protein composition of the mature free RSV virions which may compromise the fitness of the new viral progeny to infect new cells. We propose that the cargo function of p62 promoted by phosphorylation on Ser403 could be important in mediating the effect on the viral proteins and reduce RSV infectivity. This is supported by isolation of p62 phosphorylated on Ser403 and the G and $F_1$ proteins with polyubiquitinated proteins. Additionally, we shown co-localization between p62 and total RSV proteins. Finally, we evaluated the role of stable transfection of LC3 in the replication of RSV and we observed an important decrease in the replication of the virus. Current studies are undertaken to evaluate if p62 is required to mediate the decrease in RSV replication by LC3. The results shown in this work are the initial evidence aimed to identify the molecular mechanisms involved in the regulation of RSV replication by p62. Further studies are required to determine if this cellular protein can represent a novel therapeutic target to fight RSV infection.

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# 8. Other contributions

# Peer-reviewed papers

**1. Cervantes-Ortiz SL**, Zamorano Cuervo N, Grandvaux N. Respiratory Syncytial Virus and Cellular Stress Responses: Impact on Replication and Physiopathology. Viruses, 2016 May 12;8(5). Author Contributions: S.L.C.O. (33%), N.Z.C. (33%) and N.G. (33%) wrote the manuscript. Funding: CIHR MOP-298478 and MOP-325322.

# Published abstracts

**1.** M. Mariani, **S. Cervantes**, E. Mukawera, M. Kalamujic, N. Grandvaux. (2015). Identification de la voie de signalisation mise en jeu dans la réponse antivirale tardive induite par l'action synergique de IFNβ/TNFα dans les cellules épithéliales pulmonaires. Can. Respir. J., 22 (3) e20.

# Oral and poster presentations

**- S. Cervantes**, E. Caron, Q. Osseman, A. Robitalle, X. Guan, C. McCormick, N. Grandvaux. Regulation and impact of the adaptor protein SQSTM1/p62 in the replication cycle of Respiratory Syncytial Virus in Airway Epithelial Cells. CRCHUM 19e Congrès des étudiants. May 4th, 2017. **(Poster)**.

**- S. Cervantes**, E. Caron, C. McCormick, N. Grandvaux, Regulation of Respiratory Syncytial Virus infection by SQSTM1/p62. CRCHUM 18e Congrès des étudiants. May 4th, 2016. **(Poster)**.

- M. Mariani, **S. Cervantes**, E. Mukawera, M. Kalamujic, N. Grandvaux. A novel STAT2/ IRF9-dependent pathway mediates the synergistic action of IFNb+TNFa to regulate delayed antiviral and immunoregulatory genes. Symposium Keystone, Cytokine JAK-STAT signaling in immunity and disease. Steamboat Springs, USA, January 2016. **(Oral presentation)**.

- **S. Cervantes**, N. Grandvaux. SQSTM1/p62: a potential regulator of Respiratory Syncytial Virus infection in Airway Epithelial Cells, Congrès québécois en Santé respiratoire, Québec, Canada, November 12th to 13th, 2016. **(Oral presentation)**.

- M. Mariani, **S. Cervantes**, E. Mukawera, M. Kalamujic, N. Grandvaux. Identification de la voie de signalisation impliquée dans le développement d'une réponse antivirale latente induite par l'action synergique du TNFa et de l'IFNb dans les cellules épithéliales pulmonaires, XXe Colloque Annuel d'Immuno-Inflammation, Eastman, Québec, Canada. June 19th, 2015. **(Oral presentation)**.

- **S. Cervantes**, J. Guan, K. Fink, N. Grandvaux. SQSTM1/p62 as potential regulator of RSV in Airway Epithelial Cells. Journal Club d'immuno-virologie, CRCHUM, Montréal, Canada. May 6th, 2015. **(Oral presentation)**.

- M. Mariani, **S. Cervantes**, E. Mukawera, M. Kalamujic, N. Grandvaux. Identification of the signaling pathway involved in the development of late antiviral response induced by the synergistic action of IFNβ/TNFα in lung epithelial cells. 18ème congrès annuel des étudiants du CRCHUM, Canada, Québec, Montréal. December 5th, 2014. **(Poster)**.

- M. Mariani, **S. Cervantes**, E. Mukawera, M. Kalamujic, N. Grandvaux. Identification de la voie de signalisation mise en jeu dans la réponse antivirale tardive induite par l'action synergique d'IFNβ/TNFα dans les cellules épithéliales pulmonaires. Congrès québécois en santé respiratoire. November, 2014 **(Poster)**