Elucidating the role of GTPase Rab43 in influenza A virus infection

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A dissertation submitted for the partial fulfilment of BS-MS dual degree in Science

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Indian Institute of Science Education and Research Mohali
May 2021

Certificate of Examination

This is to certify that the dissertation titled **'Elucidating the role of GTPase Rab43 in Influenza A virus infection'** submitted by Ms. Mamta, (Registration number MS16004) for the partial fulfilment of BS-MS dual degree programme of the institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report is accepted.

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Dated- May 9th, 2021

Declaration

The work presented in the dissertation has been carried out by me under the guidance of Dr. Indranil Banerjee at the Indian Institute of Science Education and Research Mohali.

This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effect is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Mamta

Dated- May 9th, 2021

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Indranil Banerjee

(Supervisor)

This thesis is dedicated to my papa,
Mr. Pawan Singh,
with love.

Actually, out of all the fathers in the world, I think I got the best one!

Acknowledgement

I would like to thank Dr. Indranil Banerjee for giving me the opportunity to work in his lab for the last years and also for the support and countless discussions about whatever. For me, you have been highly motivational as a supervisor and more than that, truly inspirational as a human being.

I would like to thank 'Nirmal and Kajal' for not only being my mentor but also for showering the best part of you two on me, you are the best guardian as well as friend anyone could get. I would like to particularly mention Gagan, Tejal, Prince and Akanksha for their friendship and support in the last years. You all are awesome people who have made CV lab a homely place to work in.

I would like express my appreciation and special thanks to the losers, 'Celina, Shubhangi, Kshitiz, Vishal and Priya'. You all are the most valuable asset I have earned in these five years and not to mention, of course, Celina your brand value is pretty high:). A special mention of Shubhangi for always being the moral support in my hard times. I am also grateful to Ravi for his persistent support and encouragement to pursue things in a better way.

I don't have words to utter the contribution of my parents to this journey. Thank you Papa and Mummy, I owe this to both of you. Not to forget, a big thanks to you, my brother, Pankaj.

I would like to extend my special thanks to the DBS faculty members for being supportive and helping all the time. I also appreciate IISER Mohali computer center and library for the resources and facilities. Finally, a big thanks to DST-INSPIRE for the financial support.

List of abbreviations:

Abbreviation	Description
IAV	Influenza A virus
vRNP	Viral ribonucleoprotein
ssRNA	Single stranded RNA
HA	Hemagglutinin
NA	Neuraminidase
NP	Nucleoprotein
NPC	Nuclear pore complex
LE	Late endosomes
EE	Early endosomes
EEA1	Early endosomal antigen 1
LAMP1	Lysosomal-associated membrane protein 1
NLS	Nuclear localization signal
ER	Endoplasmic reticulum
DN	Dominant negative
siRNA	Small interfering RNA
PCR	Polymerase chain reaction
Rab	Ras like from brain
KO	Knock-out
GFP	Green fluorescent protein
GDP	Guanosine 5’ triphosphate
GDI	GDP dissociation inhibitor
GTP	Guanosine 5’ diphosphate
GAP	GTPase activating protein
GEF	Guanine nucleotide exchange factor
GDF	GDI displacement factor

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Abstract

Influenza A virus (IAV) is a global threat as it causes millions of infections worldwide each year. Being a RNA virus, it is more susceptible to mutations which leads to the emergence of new antigenically variable strain of the virus. Also, the genome of the virus can undergo independent assortment leading to the emergence of new strain. Since most of the drugs available in the market target the virus itself, the mutations in the virus leads to the failure of the existing drugs and vaccines. A novel approach to deal with the problem of drug resistance and vaccine ineffectiveness is to target the host cell factors which are manipulated by the virus for its own propagation. IAV uses a variety of the host proteins for getting the access to the host cell machinery and multiplication in the cell.

A Genome wide RNAi screen was done in search for the druggable targets in the human genome which are required for IAV infection. Rab43, a small GTPase belonging to Ras superfamily was one of the potential hits in the screen and was showing a significant block in infection (>50%) upon depletion with the siRNA. This work was carried with an objective to elucidate the role of RAB43 in Influenza A cellular entry. Sequential steps of IAV infection were analyzed in a high throughput manner and it was observed that upon depletion of Rab43, IAV vRNP nuclear import is unexpectedly blocked. Following up the preliminary observations, work was done to gain mechanistic insights of the role of Rab43 in promoting vRNP nuclear import. The peculiar upregulation in the levels of early endosome, late endosome and lysosome was observed upon Rab43 depletion.

Also, the results suggested that the levels of Rab43 were increased with infection with time-dependent progression of infection and it shows a remarkable co-localization with one of the surface protein i.e. HA of the IAV in late stages of infection which indicated the involvement of Rab43 in late stages of infection as well. Together, this works gives preliminary insights of Rab43 as a proviral factor playing critical role in IAV infection.

Chapter 1: Introduction

1.1 Influenza A virus

1.2 The Rab proteins

Chapter1: Introduction

1.1 Influenza A virus (IAV)

Influenza A virus has a global impact on health and economy because of the potential to cause annual epidemic and seasonal pandemic (1). The virus can occasionally cross the species barrier and can infect numerous other species. A detailed understanding of the viral life cycle is not only required to design the anti-viral drugs but also to predict the impact of the circulating strain as well as the newly emerging viruses. Since viral entry is the earliest step required for successful propagation of virus, inhibition of first step would lead to efficient block in viral propagation. One approach is to target the proteins of the virus i.e. HA and alternative is to target the cellular factors required for entry of IAV. As the segmented RNA genome offers the possibility of mutations as well as independent re-assortment, it can lead to development of resistance against the drugs targeting the viral proteins. While in the latter, toxicity can be a detrimental factor, resistance is less likely to be observed. Also, many viruses using the similar route can be targeted by the same set of drugs leading to the possibility of development of broad-spectrum antibiotic which can be useful when a new strain with the possibility to cause outbreak emerges suddenly (2).

1.1.1 Structure of IAV

Influenza A virus is a member Orthomyxoviridae family (3). It is a ssRNA virus having the negative sense genome which is divided into eight segments. The virus derives its outer membrane from the host and the membrane consists of three transmembrane proteins i.e. two are the surface glycoproteins which are designated as HA(Hemagglutinin) and NA(Neuraminidase) and the other is the proton channel matrix protein (M2). The different strains of influenza A virus are divided on the type of HA antigen and NA antigen. There are 18 different type of known HA antigens (H1 to H18) and 11 different type of NA antigen (N1 to N11) (4). HA is the protein which mediates the binding of the virus to the host cell via siyalated receptor. NA is involved in release from the abundant non-productive attachment sites present in mucus and it also helps in the release of progeny virions from infected cells (5). M2 channel protein forms an essential part of the envelope which is activated by the low pH and helps in the influx of protons inside the viral core.

The nucleoprotein of the virus acts as a capsid for the negative sense RNA genome and protects it from the activity of nucleases. The viral RNA encapsulated by the NP together with the three polymerase subunits (PA, PB1 and PB2) forms the vRNP complex. M1 is the most abundant protein forms the core protein of the virus by forming bridge like matrix which connects the vRNP core and outer surface proteins of the virus (6).

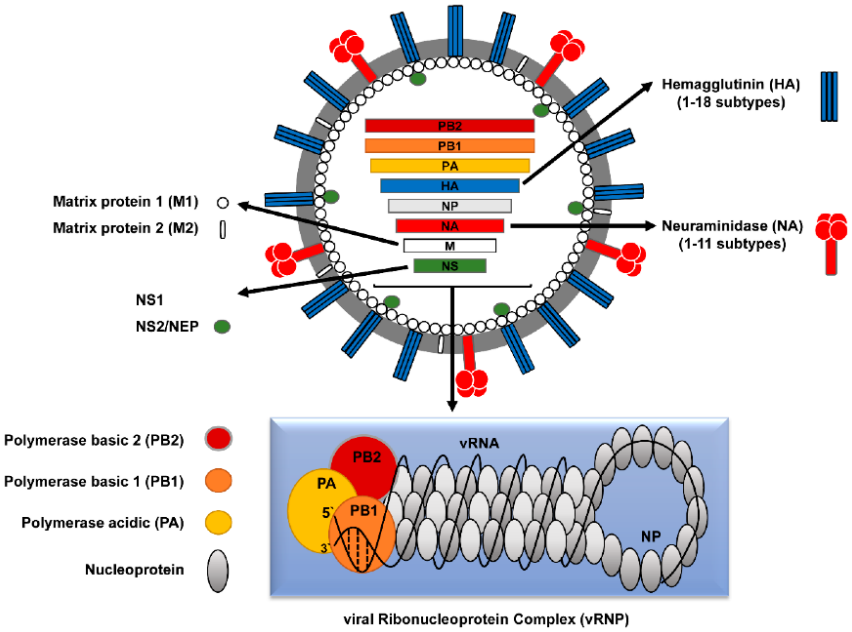

Figure a). Diagrammatic representation of structure of Influenza A virus along with all of its proteins. The viral-RNA is complex with NP (VRNP) has also been shown. (Source: Mostafa et. al, 2018, (94)).

1.1.2 Cellular entry of IAV

IAV follows a sequential set of steps in order to get entry into the host cell. The HA receptor binding site present on the HA protein of the IAV helps in the attachment of the virus to the siyalated receptors present on the surface of the host cell. Sialidase function of the NA helps to remove the local Sialic acid residues and remove the non-productive association of HA. HA mediated binding to the siyalated receptor of IAV triggers endocytosis of the virion. The endocytosis of the viruses can occur via clathrin mediated endocytosis or micropinocytosis (7,8). After entry into the cell, the virus is directed to the early endosomes. Due to pH change in the early endosomes, the M2 ion channel gets activated and there is a conformation change in the HA protein

of the virus. The HA of the virus is cleaved by host cell protease into two subunits HA1 and HA2. Due to the cleavage, the fusion peptide present on the N-terminus of the HA2 subunit of the virus is exposed. The fusion peptide of the virus is inserted into the endosomal membrane and the C-terminal transmembrane domain anchors HA2 into the viral membrane which brings the two membranes in close proximity forming a hair pin conformation. The hair pin bundle further come in close proximity leading to the formation of six-helix bundle leading to the formation of lipid stalk and fusion the two membranes (2). This fusion leads to the disruption of the viral core and the release of the viral genome into the cytoplasm i.e. uncoating of the virus. The NLS on the newly released vRNPs is recognized by the adapter protein importin- α and the vRNPs are carried to the nucleoplasm via NPC using the importin-α and importin-β family of proteins via nuclear import pathway. All the entry steps of the virus can be broadly divided into six steps which are described below:

1.1.2.1 Attachment of IAV to the plasma membrane

The preliminary step for IAV entry into the cell is the attachment of IAV to the host cell. IAV binds to the siyalated receptors present on the host cell which is recognized and bound by the outer membrane protein of the virus i.e. HA (9). HA is present in trimeric form on outer membrane and is a multifunctional protein mediating attachment and fusion of the virus. The receptor binding pocket of HA is present on the distal end of HA trimer at the head which is globular in shape. The specificity and conformation of HA determines the specificity for the receptor for IAV. Avian Influenza virus prefers siyalated receptors with α-2,3 linkage while humans IAV strains prefer α-2,6 linked siyalated receptors (10,11). It has been observed that binding of IAV to the siyalated receptor does not always result in internalization of the virus (12). Surprisingly, some of the desiyalated receptors also retain the ability of virus internalization (13) which gives us the hint of involvement of some other alternative route for IAV internalization. Some additional receptors like 6-sulfo sialyl Lewis X are also proposed to be the receptor for IAV attachment (14). However, it remains to elucidate that if these receptors are the sole receptors for attachment or some co-receptors are required along with them.

1.1.2.2 Internalization of IAV

After its binding to the host cell, IAV needs to be internalized inside the cells. Preliminary imaging studies revealed the internalization by receptor mediated endocytosis. IAV was shown to be internalized by clathrin coated vesicles as well as uncoated vesicles. It was also seen that virus can also infect the cells which are defective in clathrin and caveolin pathways (15). Imaging studies with single virus depicted the use of both clathrin dependent and independent pathway (16). One of the alternate pathways which was discovered is micropinocytosis. IAV enters the cells by clathrin mediated endocytosis in absence of serum which can be completely blocked by using dynamin inhibitor dynasore. However, if serum is present in the media during infection, the virus can use both dynamin dependent and independent routes (17). Further studies reveal cell type dependence of the internalization route (18). Also, the filamentous viral particles are more likely internalized by micropinocytosis (19). Several studies indicate the possible involvement of other receptors in addition to siyalated glycan for the efficient internalization of IAV. There is evidence of activation of RTK cascades which involves activation of PKC, MEK/ERK and PI3K/AKT by IAV infection. However, more studies are required to determine the contribution of these pathways to entry of IAV (20, 21, 23).

1.1.2.3 Endosomal trafficking of IAV

Upon internalization of the IAV, the virus exploits the endosomal pathway to gain further access to the host cell. Endosomal trafficking of the virus is actin as well as microtubule dependent (23, 24, 25). In the first step, the virus is transported to the cell periphery in actin dependent manner which is followed by the second step involving rapid dynein-directed movement. The third stage involves the movement of the virus along the microtubules to the perinuclear region. This transport pattern is similar to the endosomal maturation pathway in the cell. The cargos containing early endosomes are transported from the cell surface by actin pathway. EEs are transported by motor proteins kinesin and dynein along the microtubules (26). Early endosomes constantly exchange vesicles with trans-Golgi complex during the maturation process. Rab5 and other proteins such as EEA1 and PI(3)K are the regulators of this maturation and are used as markers for early endosomes (27, 28). The pH of early endosomes lies in the range between 6.8-5.9. Late endosomes are formed from early endosomes during their microtubule dependent transport. LE consists of integral

membrane protein LAMP1 and the pH range for late endosomes is in between 6.0-4.8 (29). The maturation of EE to LE is mediated by Rab switch i.e. from Rab5 in EE to Rab7 in late endosomes (30).

1.1.2.4 Acidification and fusion of Influenza A virus

During the acidification process of IAV the proton pump i.e. v-ATPase delivers protons into endosomal lumen (31, 32). The pump consists of the membrane associated V0 complex and a soluble cytosolic V1 complex which is required to hydrolyze the driving force for acidification i.e. ATP. Because of the low pH of the LE, there is a conformation change in the HA protein of the IAV, which exposes the fusion peptide and places it towards the endosomal membrane (33, 34, 35). After the final conformation change, the HA trimer is tilted at the fusion site leading to interaction of the outer leaflet of the viral membrane and endosomal membrane in hemi fusion state (36,37). Finally, both the membrane fuse with the formation of fusion pore (38,39).

1.1.2.5 Uncoating of IAV

During the IAV uncoating, the interaction of M1 protein with viral membrane as well as with vRNPs has to be removed in order to get completely uncoated. This requires the involvement of M2 protein. The M2 mediated change in pH is required for detachment of M1 from vRNPs resulting in the release of vRNPs in the cytoplasm (40). M1 is also shown to be separated from the vRNPs before they are imported to the nucleus (41). The uncoating of the IAV leads to the dispersal of M1 and release of vRNPs into the cytoplasm.

1.1.2.6 Nuclear import of vRNPs

Followed by uncoating, the vRNPs are to be transported to the nucleus. It has been shown that the RNPs without NP cannot be transported to the nucleus (42). Viral RNP are imported to the nucleus by karyopherin nuclear import pathway. All the protein component of RNP, the polymerase subunits, NP have the NLS. However, the NLS of NP is required for nuclear import of RNP. When the individual proteins of the polymerase have been imported, the NLS on the individual subunits

becomes important (43). After the import to the nucleus, the karyopherins bind to the RanGTP (44), which leads to release of cargo and the viral entry process ends at this step.

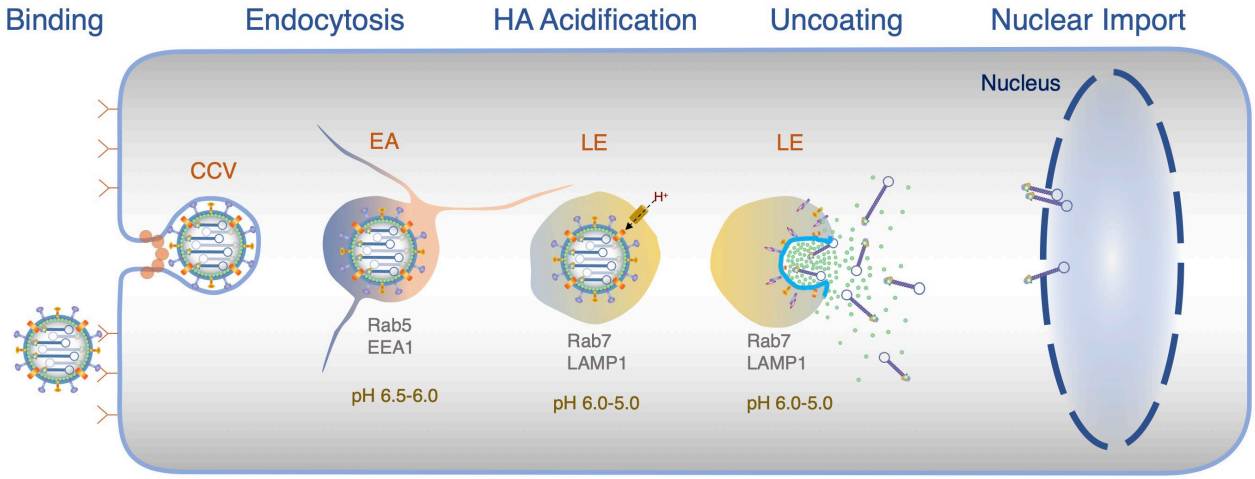

Fig b). Cellular entry steps of Influenza A virus in a sequential manner (Image courtesy: Dr. Banerjee).

1.1.3 Inhibitors for viral entry

There are various inhibitors of IAV which target the entry steps. Some of the drugs are classified as potential drugs for laboratory use (listed in Table 1).

Entry step	Drug
Internalization	Dynasore (45)
Internalization	Chlorpromazine (46)
Internalization	EIPA (47)
Endosomal Acidification	Bafilomycin A (48)
Endosomal Acidification	Ammonium Chloride (49)
Nuclear Import	Importazole (50)

1.1.4 Role of host cell factors in cellular entry of IAV

IAV depends on cellular factors to complete its infection cycle into the cell. To develop novel antiviral strategies, it is essential to understand the viral-host interaction in detail. Since entry is

the first essential step for viral replication, detailed insights into the host cell factors required for cellular entry can serve the purpose to develop novel entry inhibitors which can block infection efficiently (51). A list of host cell factors for which the role has been elucidated in IAV entry has been given in Table 2 given below:

Entry step	Host cell factor
Attachment	Sialic acid (52)
Attachment	Annexin V (53)
Attachment	C-type lectins (54)
Attachment	6-sulfo sialyl Lewis X (55)
Internalization	Dynamin (56)
Internalization	Epsin-1 (57)
Internalization	Clathrin (58)
Internalization	Actin (59)
	c-Met kinase (60)
	EGFR (60)
	PLC-γ1 (61)
Endosomal trafficking	Rab5 (62)
	Rab7 (62)
	PKC βII (63)
	Cullin3 (64)
	HDAC8 (65)
Endosomal Acidification	vATPase (66)
	CD81 (67)
IAV Uncoating	HDAC6 (68)
	ITCH (69)
Nuclear Import	Ran (70)
	p10 (70)
	Karyopherins (α1, α3 and α5) (71)
	CSE1L (72)
	TNPO1 (73)

2.1 The Rab proteins

2.1.1 Introduction

The small monomeric GTPases belong to the Ras subfamily which is mainly divided into five major groups: Ras, Rho, Ran. Arf and RAB. Rab43 belongs to the family of small GTPases called Rabs). The Rab family is composed of almost 60 members and is the largest family in the subgroup of Ras like monomeric GTPases. These proteins were initially discovered in the brain where they are present in abundant among and regulate various aspects of neuronal trafficking (74). They are mainly involved in recruiting the effector proteins to specific membrane and therefore, identify the identity of various organelles. Rabs are reported to be localized to specific sub-cellular compartments (75). Rabs function as the molecular switches i.e. in the active state they are bound to GTP while in inactive state, they are bound to GTP. Hydrolysis from GTP to GDP is stimulated by GAPs and the exchange of GDP to GTP is catalyzed by GEFs. The effectors i.e. the downstream interacting partners of the Rabs have high affinity to the Rabs when they are in the GTP bound state. Membrane trafficking of various proteins in the cell requires recruitment of various Rab GTPases to specific membranes.

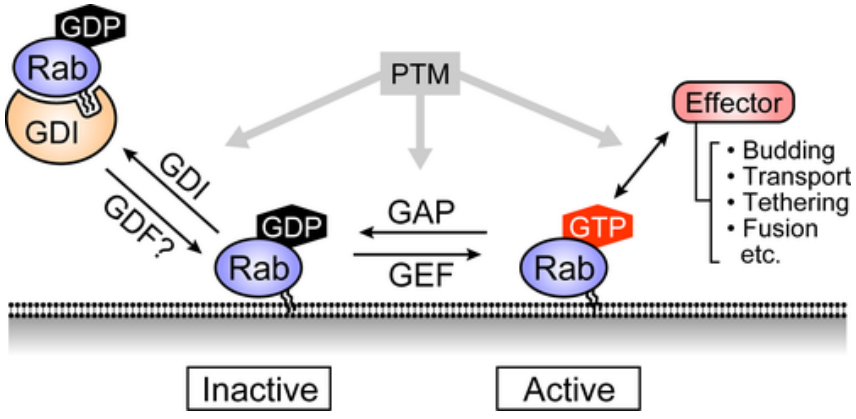

Figure c): The Rab GTPase cycle showing the molecular switching of Rab proteins by the action of GEFs and GAPs.

2.1.2 The Rab proteins in intracellular membrane trafficking

Specific set of Rab proteins are required for membrane compartmentalization and trafficking which is required for the functioning of the cell. Rab proteins are the GTPase which are recruited to the membrane of vesicles and have key role in regulating endosomal trafficking. Alteration in the Rab proteins and their effectors is associated with a variety of human diseases including neurodegeneration, cancer and infections. Rab proteins can regulate traffic in either direction by interacting with members of kinesin (plus-end directed motors) or dynein (minus-end directed motors) (76). Rab GTPases are used as the markers and identifiers of vesicles and organelles in the endocytic and secretory systems (77). The early endocytic pathway, the transport of clathrin coated vesicle from plasma membrane to early endosomes is mediated by Rab5 (77). Also, Rab5 is involved in the fusion of early endosomes (78). The maturation from EE to LE is also mediated by Rab switch i.e. Rab5 for early endosomes to Rab7 for late endosomes. Rab11 is shown to be associated with recycling endosomes and regulates the various aspects of vesicle trafficking (79). Many of the Rab proteins have also been studied for the transport pathways involved in neurodegeneration diseases. Fig. (d) represents the neuron and post-synaptic terminal and the trafficking pathways they regulate (74).

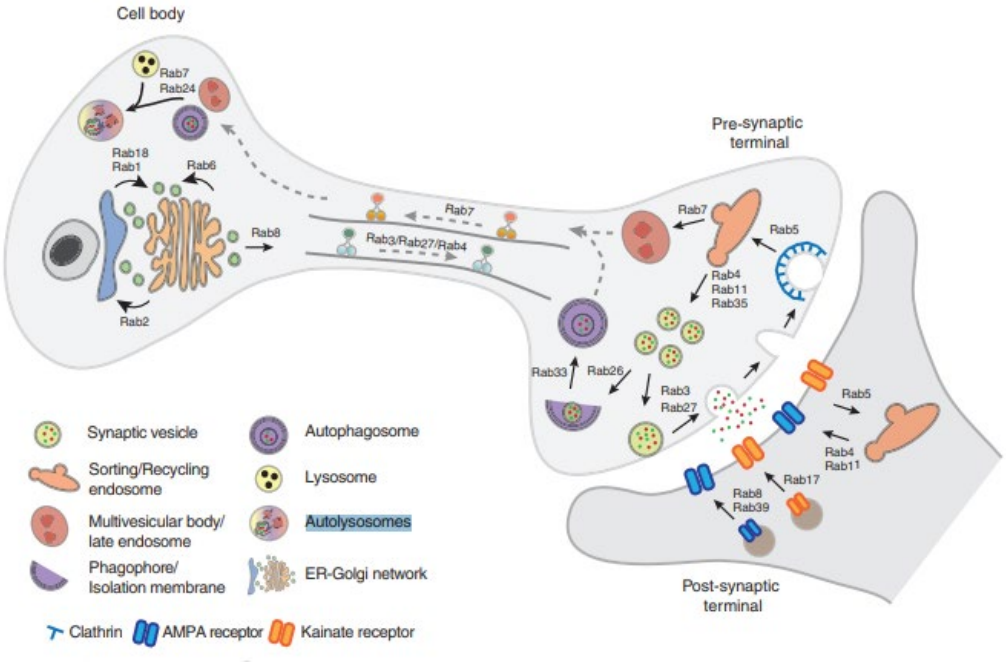

Figure d): Transport pathways regulated by Rab proteins in a neuron.

Rab proteins are localized to distinct organelles and are involved in various transport pathway in the cell. Fig (e) summarizes the localization of different Rab proteins as well the transport pathways associated with them (79).

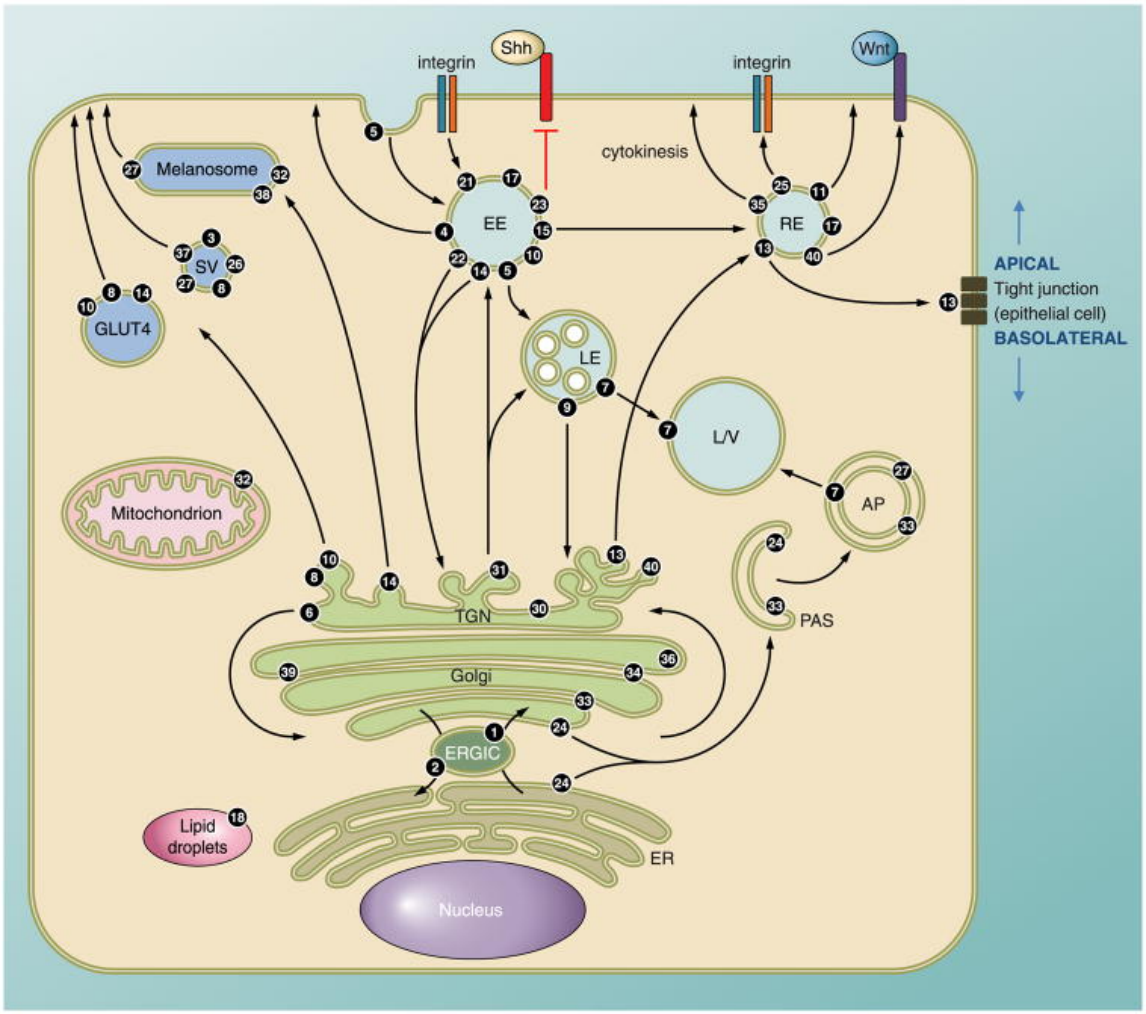

Fig e): Diagrammatic representation of different transport pathways associated with the Rab proteins and their cellular localization.

2.1.3 The Rab cascade:

The Rab GEF and GAP act in a cascade. After the insertion of RabA in the membrane, it is activated by the respective GEF and this activated Rab in turn activates the GEF for the downstream Rab (RabB). The GTP-bound RabB performs two functions: the activation of the GEF

for the downstream Rab (Rab C) and the inactivation of RabA. The coordinated actions of GAP and GEFs define the membrane boundaries of different compartments which is determined by the action of the Rab proteins associated by the compartment. Fig (f) depicts the functioning of the Rab cascade (76).

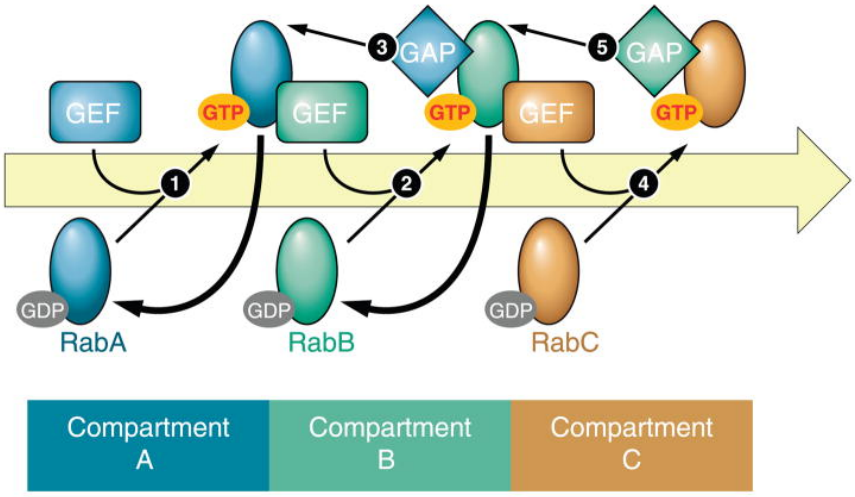

Fig (f): The cascade depicting the functioning of Rab proteins in cell.

2.1.4 Rab Proteins in viral infection:

Many of the Rab proteins have been widely studies for their role in viral infection. Rab11 is one such factor which is involved in vRNP transport and genome assembly of Influenza A virus (80). Dominant negative mutants of Rab5 and Rab7 were also shown to disrupt the entry of IAV. Rab7 plays a role in the maturation of HIV-1 envelope and egress (82).

Rab11-FIP2 is involved in the budding of respiratory syncytial virus (RSV) (83). Rab27a is shown to co-localize with HSV-1 glycoproteins in the TGN and the depletion of Rab27a reduces HSV-1 production (83). Depletion of Rab1a/b and Rab43 has also been shown to be involved in envelopment of HSV (84). human cytomegalovirus (HCMV) has also been shown to be enveloped into Rab3 containing vesicles (85). Fig (g) illustrates the Rab related pathways used by the viruses:

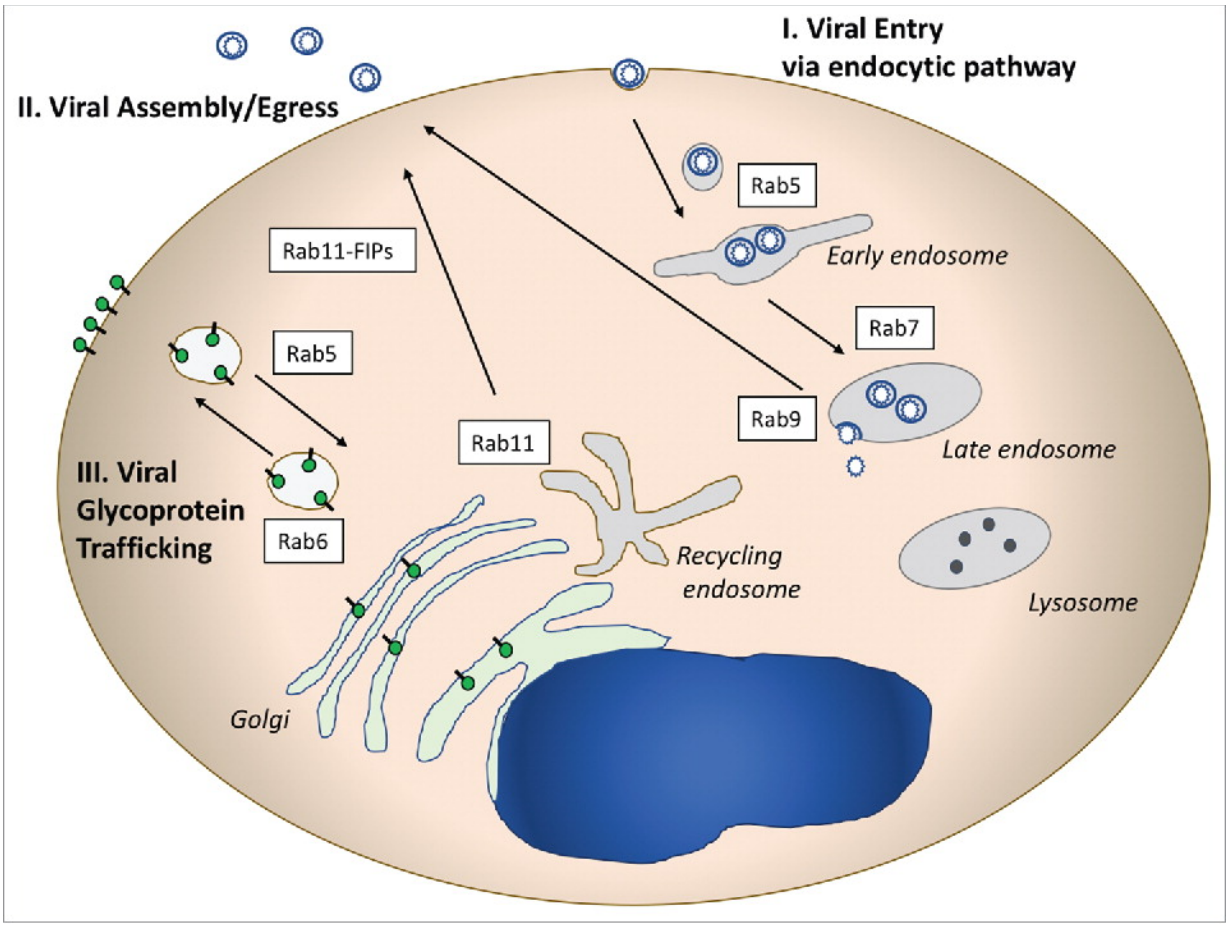

Fig g): Schematic representation of the known roles of Rab Proteins in infection cycle of various viruses.

2.1.5 Rab43 (Ras-related protein Rab43):

Rab43, a member of Ras family is a key member shown to be important for ER-Golgi trafficking (86). The GTPase activity of Rab43 is activated by USP6NL. Rab43 is mainly localized to Golgi complex and is also responsible for maintaining the integrity of Golgi complex. The expression of DN mutant of Rab43 disrupts the integrity of Golgi complex (86). It is shown to control the anterograde transport of GPCRs from ER to Golgi and helps in sorting of GPCRs (87). It is also involved in the transport of Shiga toxin from early and recycling endosomes to the trans-Golgi network (88). Rab43 also plays a role in the maturation of phagosomes by recruitment of Cathepsin D which are required for engulfment of pathogens such as *S. aureus and M.Tuberculosis* (89).

Role of Rab43 has been widely studied in cancer. Rab43 is shown to promote cell proliferation and metastasis in Gastric cancer (90). Also, it promotes the cell proliferation and Epithelial to

mesenchymal transition in hepatocellular carcinoma cells (91). Poor prognosis and epithelial-mesenchymal transition in gliomas is also associated with high expression of Rab43 (92).
In context of viral infection, Rab43 is also shown to be involved in the secondary envelopment of HSV. Upon depletion of Rab43, the virus is not able to get its secondary envelope which it derives from the Golgi complex (93).

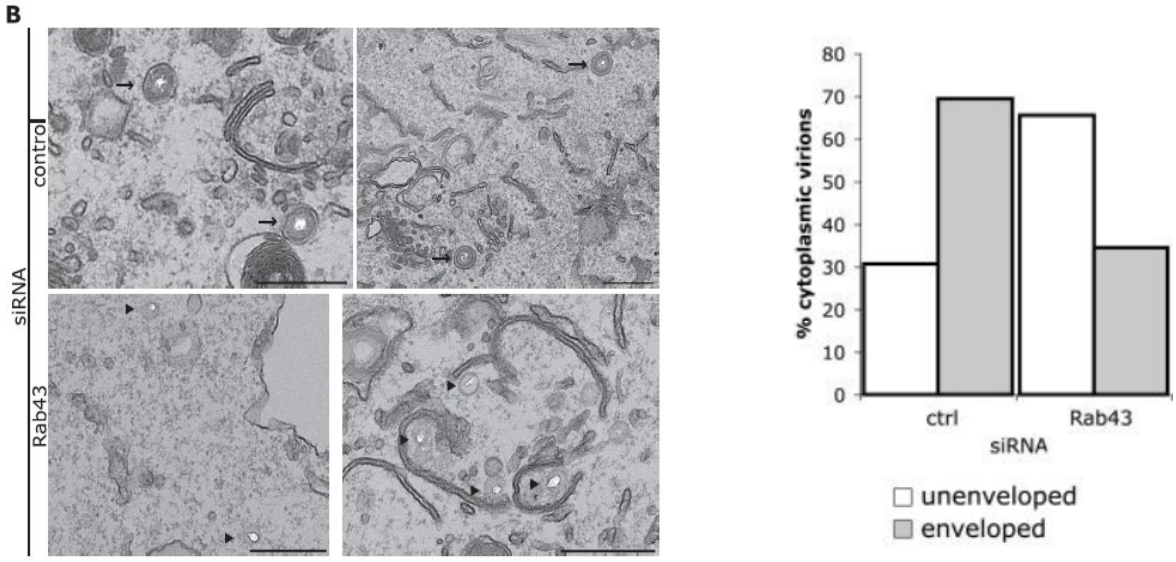

Figure h): Figure depicting involvement of Rab43 in secondary envelopment of HSV. a) TEM images showing unenveloped viruses in Rab43 depleted cells compared to the negative control. b) Quantification of the virus particles which are enveloped and unenveloped in control and Rab43 depleted cells.

Chapter 2: Materials and methods

2.1 Materials
2.2 Bacterial methods
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Chapter 2: Material and methods

1.2 Materials

1.2.1 Reagents

Reagents were purchased from Sigma-Aldrich, Himedia, VWR, Qiagen and Thermo scientific unless stated in the text.

2.1.2 Solutions

Solution	**Composition**
1xPBS	0.137M NaCl, 0.0027M KCl, 0.01M Na_2HPO_4 , 0.0018M KH_2PO_4.
1XTBST	50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% TWEEN20.
50X TAE	242 g Tris base, 57.1ml Glacial Acetic acid, 0.5 EDTA (pH 8.0). Mix the solutions in 100 ml of ddH2O and adjust the volume to 1000 ml.
10X SDS Running Buffer	30 g Tris base, 144g glycine and 10 g of SDS in 1000 ml of H_2O (pH-8).
1X Transfer Buffer	28.8 g Glycine, 6.04 g Tris base, 200 ml methanol, 1.6 L ddH_2O.
Permeabilization solution	1%BSA, 5%FBS in PBS, 0.1% Saponin in PBS.
Blocking solution	1%BSA, 5%FBS in PBS.

2.1.3 PCR Primers

Target gene	Forward, Reverse primer (including restriction sites)
RAB43	GCATAAAGCTTTGATGGCAGGGCCGGGCCCA, GCATAGGATCCTCAGCACCCGCAGCCCCAGC
RAB43 RT	TGCCTACGACATCACCAAGA, GACGTCTCAATGGCACACAG
RAB11A	CAACAAGAAGCATCCAGGTTGA, GCACCTACAGCTCCACGATAAT
RAB11B RT	CAAGCACCTGACCTATGAGAAC, GCTTCCTCTACGTTAGTGGAATC
RAB43gRNA1	CACCGGGTGCGGAACCGCTCCTGGC
GAPDH RT	GAGTCAACGGATTTGGTCGT, GACAAGCTTCCCGTTCTCAG
Rab43N131I	CAGCTGCTGATCGGGAACAAGTCAGACCTCAGCA, GCTGAGGTCTGACTTGTTCCCGATCAGCAGCTG
Rab43Q77L	CTGGGACACGGCCGGCCTGGAGCGGTTCCGCACCATCACCCAGA, CTGGGTGATGGTGCGGAACCGCTCCAGGCCGGCCGTGTCCCAG
Rab43T32N	GGCGACGCAAGCGTGGGCAAGAACTGCGTGGTGCAGCGCTTCAAGA, CTTGAAGCGCTGCACCACGCAGTTCTTGCCCACGCTTGCGTCGCC

2.1.4 siRNA Oligonucleotides

siRNA nucleotides for mRNA mediated degradation of specific genes were purchased from Qiagen. The sequence of the siRNA used in the study are listed in the table below:

ASN (All stars negative)	Scrambled siRNA (Qiagen sequence)
ASD (All stars death)	Qiagen sequence
RAB43#2	CCGAGCGTGGGTCCCAGTCTA
RAB43#4	GCAGTACGATTTCCTGTTCAA
ATP6V1B2	CACGGTTAATGAAGTCTGCTA
ATP6AP2	GGGAACGAGTTTAGTATATTA

2.1.5 Antibodies

The antibodies used during this work are listed in the table below. The species indicated are: M-Mouse, R-Rabbit.

2.1.5.1 Primary Antibodies

Name	**Source**
RAB43	SANTA CRUZ (SC 81923)
HA1 MOUSE Mab	Source: Dr. Indranil Banerjee
HA1 RB PAb (PINDA)	Source: Dr. Indranil Banerjee
A1	Source: Dr. Indranil Banerjee
M1 (HB64)	Source: Dr. Indranil Banerjee
NP (HB65)	Source: Dr. Indranil Banerjee
Golgin	Invitrogen
EEA1	CST
LAMP1	SIGMA
GAPDH	CST

DAPI	INVITROGEN
PHALLOIDIN 568	INVITROGEN

2.1.5.2 Secondary Antibodies

A488 MOUSE	INVITROGEN
A568 MOUSE	INVITROGEN
A647 MOUSE	INVITROGEN
A488 RABBIT	INVITROGEN
A568 RABBIT	INVITROGEN
A647 RABBIT	INVITROGEN
MOUSE HRP	CST
RABBIT HRP	CST

2.2 Bacterial Methods

2.2.1 Growth and maintenance of E. coli

Bacteria were grown at 37°C in LB medium containing appropriate antibiotics: Ampicillin (100 µg/ml) and kanamycin (50 µg/ml). Short term storage of the plates was done at 4 °C along with antibiotics. For all the cloning, Stabl3 strain of E. coli was used for transformation.

2.2.2 Preparation and transformation of chemical competent bacteria

Bacteria was grown overnight in 10ml LB medium (Primary culture). 1% of the primary culture was added to 500 ml of LB (secondary culture) and incubated till 0.4-0.5 O.D. is reached. Culture was pelleted down at 3000 rpm for 20 min, 4°C. Pellet was resuspended very gently in 25 ml of 100mM MgCl2 followed by 1hr incubation on ice. The mixture was centrifuged at 3000 rpm for

20min and pellet was very gently resuspended in 20ml of CaCl2. The mixture was again allowed to pellet down at 3000 rpm, 20 min at 4°C. Pellet was then resuspended in 20ml of 15% glycerol and aliquots of 100ul were made. Competent bacteria were stored at -80°C. For transformation, desired volume of DNA was added to 100ul of competent bacteria and incubated on ice for 30 min. Mixture was incubated at 42°C for 1 minute followed by 5 minutes of incubation on ice. LB was added to the mix and was incubated at 37 °C for 1 hour. The grown bacteria were pellet down at 13000 rpm for 5 min and supernatant was discarded. Pellet was resuspended in the leftover LB and was plated on appropriate antibiotic resistance plate.

2.2.3 Plasmid DNA isolation from bacteria

Plasmid DNA from the bacteria was prepared using mini-preparation kit purchased from Qiagen. Miniprep were done from 10 ml of bacterial culture grown overnight at 37˚C.

2.3 Cloning

2.3.1 PCR and cloning of PCR products

Oligonucleotides used for PCR were obtained from Europhins and IDT. Oligonucleotides were pre- dissolved to 100 µM. For all PCR reactions, PCR cycler (Applied Bio systems) was used. Reactions were carried out in total 50 µl volume and contained Q5 Polymerase (0.02 U/µl), 1x GC enhancer, 1x Q5 reaction buffer, 0.5 uM Forward and Reverse primer, Template (variable), dNTPs (200mM) and NFW (up to 50 µl).

The reaction cycle used as per the following table:

Steps	Time	Temperature
Initial denaturation	30 sec	98˚C
35 cycles	10 sec	98˚C
	45 sec	variable
	30 sec	72˚C

Final extension	2 minutes	72˚C
Hold	Infinite	4˚C

Depending on the length of amplicon, elongation time and temperature were altered. PCR products were analyzed using Agarose gel electrophoresis. Gel extraction was performed using Gel extraction kit purchased from Qiagen according to the instruction manual.

2.3.2 Site directed mutagenesis

For cloning the active and dominant negative mutants of RAB43, point mutation was carried out using SOE-PCR. Primer pair containing the particular mutation were used to generate two fragments which were finally annealed using end primers. The cycling parameters used for the amplification were same as 2.3.1.

2.3.3 Restriction digestion and agarose gel electrophoresis of DNA

Restriction digestion was carried out using total volume of 20 µl using 0.5 µl of each enzyme and appropriate buffer to digest 1 µg of DNA. Digestion was carried out at 37°C for 2.5 hours. 5 µl of 4x dye was added to the whole mixture and it was loaded on 0.8-2% agarose gel (depending on the size of DNA fragment). For gel pouring, appropriate amount of agarose was added to 1xtae by heating the mixture. EtBr was added in the concentration of for visualizing the gel. The gel was run at 90v for 30 minutes. DNA was visualized on UV trans illuminator.

Restriction digestion for the vector was carried using 2.5 µg of DNA. Vector DNA was given quick-CIP treatment for 10 minutes at 37°C and followed by inactivation of CIP for 2 minutes at 80°C.

Bands of appropriate size were cut after agarose gel electrophoresis using scalpel blade and DNA was purified using Qiagen gel extraction kit as per the instruction manual.

2.3.4 Cloning of the digested fragments of DNA

Total reaction of 20ul was set to carry the ligation. 1ul of T4 DNA ligase (NEB) was used in 1X T4 ligase buffer (NEB). 100 ng of vector was used along with 3:1 molar ratio of insert to vector DNA using NEB ligation calculator. Ligation was carried overnight at 16°C. 20 µl of the ligated product was transformed in the competent bacteria.

2.4 RNA isolation and cDNA synthesis

2.4.1 RNA isolation

1. 80% confluent A549 cells were taken and media was aspirated out.
2. Cells were trypsinised and pelleted down.
3. The pellet was resuspended very gently in 1ml trizol and incubated on ice for 7min (5-10min).
4. Chloroform was added and vigorously shaken vigorously.
5. Tube was incubated on ice for 10 minutes and then centrifuged at 12000g for 15min at 4°C.
6. Topmost aqueous layer was picked and added in other fresh autoclaved MCT.
7. To this 500 µl isopropanol was added and content were mixed very gently and then kept on ice.
8. Tube was then incubated at -80°C overnight and next day thawed on ice.
9. As soon as it was thawed, tube was centrifuged at 12000 g for 20minutes at 4°C.
10. Supernatant was discarded by inverting the tube on tissue paper.
11. Pellet was resuspended very gently by pipetting 2-3 times in chilled 75% ethanol prepared in DEPC treated water.
12. Tube was centrifuged at 7600 g at 4°C for 15 minutes.
13. Supernatant was discarded and tube was kept in an inverted position on the tissue paper for 5min.
14. Pellet was allowed to get dry for 15min inside 37°C incubator.
15. Pellet was resuspended in 20 µl DEPC treated water by pipetting very gently 4-5 times.
16. RNA was then kept at 65°C for 10 min and then placed immediately on ice.
17. RNA was stored at -80 °C.

2.4.2 cDNA synthesis

cDNA was prepared using kit purchased from takara for which a reaction of 1 µg of RNA, 50 uM of oligo dT primer and 10mM of dNTP mixture was diluted to make the total reaction of 20 µl. The mixture was heated at 65 °C for 10 minutes and then cooled immediately on ice. This mixture was supplemented with 1x Prime Script Buffer, 40U/µl RNase inhibitor and 200 U/µl of Prime Script RTase. The total mixture was mixed gently and reaction mixture was incubated at the following conditions:

25 °C/ 5min	46 °C/ 20min	95 °C/ 1min	4 °C/ infinite

cDNA samples were stored at -20˚C.

2.5 qRT-PCR (quantitative reverse transcription-PCR)

qRT-PCR was performed as per manufacture's instruction mentioned in sybrgreen kit (Bio-Rad). Equipment: Bio-Rad qPCR CFX96.

2.6 Mammalian cell culture

2.6.1 Cell culture

All the cell lines used (A549-ATCC, A549-N, Hela, HEK293T and Beas-2b) were cultured in DMEM (Invitrogen) supplemented with 10% FBS, 1% Pen strap and 1% non-essential amino acid. Cells were allowed to grow as monolayer and were trypsinised and passaged biweekly at 70 percent confluency.

2.6.2 Transient transfection of mammalian cell lines

1. 1.0ul siRNA (10 µM) was added in 150 µl NFW for all siRNA.
2. Lipofectamine RNAiMax was added to plain DMEM (1:150 dilution).
3. Equal amount of diluted siRNA and diluted RNAi max was mixed.
4. 30 µl of the mix was added to each well.
5. Plate was incubated at RT for 1 hour.
6. Cells were counted and stock of 4.28 X 104 cells/ml was made.
7. After the incubation was completed, 70ul (3000 cells/70 µl) of cell suspension was added to each well.
8. Plate was incubated at 37˚C in CO2 incubator for 72 hours.
9. All stars death siRNA (ASD) was used as the control of transfection efficiency. If the cells transfected with ASD died 72 hrs. post transfection, transfection was considered efficient enough.

2.7 Mammalian culture methods

2.7.1 Immunofluorescence

1. For Immunofluorescence, cells were fixed with 4% formaldehyde and incubated for 20 min at RT.
2. Formaldehyde was discarded and cells washed with 1X PBS twice.
3. Blocking solution was added and incubated at RT for 30 minutes.
4. 50µl permeabilization solution was added and incubated for 30min at RT.
5. 50µl of primary antibody HB65 (NP, 1:10 diluted in PS) was added to each well and incubated for 2 hours at RT.
6. 3 times washing was done with PBS for 10min each.
7. 50 µl of secondary antibody anti mouse IgG AF488 (1:1000 In PS) along with DAPI (1:10000 in PS) was added for 1 hour at RT.
8. 3 times washing was done with PBS for 10min each.

2.8 Cellular and Biochemical assays

2.8.1 EGF and transferrin uptake assay

1. Cells were starved in serum free DMEM for 24 hrs. before proceeding with the respective assays.
2. Cells were pulsed with 50 ng/ml EGF-A488 and 30 µg/ml Trasnferrin-A488 for 30 minutes on ice.
3. Serum free DMEM was used to thoroughly wash each well twice.
4. Complete media was added to each well and the cells were incubated at 37˚C (10 min for transferrin uptake and 30 min for EGF uptake).
5. Cells were re-washed with PBS twice.
6. Cells were fixed with 4%PFA followed by nuclear staining with DAPI.

2.9 Viruses

2.9.1 IAV strain

Influenza A X31 strain (an H3N2 reassorted strain derived from the A/Puerto Rico/8/34 (PR8) and A/Hong Kong/1/68 strains) was purchased from Virapur (CA, USA) in purified form.

2.9.2 IAV infection assay

1. One day before infection assay, X31 virus was kept for thawing at 4˚C.
2. Wells were washed thrice with 1X PBS (at RT).
3. Virus dilution was made by adding 1ul X31 IAV in 100µl infection media (50mM HEPES, 0.2% BSA in serum free DMEM) and then this 5µl of this was added to 5ml of infection media and vortexed.
4. 100 µl of diluted virus was added in all the wells.
5. Plates were incubated in CO2 incubator at 37˚C for 10 hours.

6. Cells were then fixed with 4% PFA and were stained for NP.

2.9.3 IAV entry assays

Infection media (DMEM+50mM HEPES+0.2% BSA) was prepared. 1mM cyclohexamide was added to the infection media. For each assay, already standardized volumes of virus X31 was used (Ref: Banerjee et. al., Plos one, 2013)

IAV stock volume per well (ul)	Time period of detection
0.5	30 min
0.25	60 min
0.5	2.5 hr.
0.5	4 hr.

1. X31 virus was thawed overnight and diluted before assays in infection media.
2. Wells were washed with chilled infection media.
3. Nuclear import infection media (50µl) was added to respective wells followed by uncoating, acidification and cells were fixed after 4 hrs., 2.5hrs and 1 hr. respectively to fix all assays at same time.
4. 25 µg/ml Chlorpromazine pretreatment was done in respective wells of endocytosis for 10 minutes.
5. Endocytosis infection media was added to respective wells and fixed after 30 minutes.

2.9.4 Immunostaining for the entry assays:

2.9.4 a Endocytosis:

1. 4% PFA was aspirated and cells were washed with 1x PBS.
2. Blocking solution was added and cells were incubated at RT for 1hr.

3. 1:1000 PINDA in blocking solution was added to respective wells to block the membrane bound viruses and incubated overnight at 4˚C
4. Next day unbound Rabbit polyclonal antibody (PINDA) was washed off by PBS washing 3*10mins.
5. Anti-Rabbit AF568 1:1000 in BS was added and incubated for 1hr at RT.
6. Unbound Ab was washed with 3*10 min with 1xPBS.
7. Cells were fixed again with 4% PFA 20 min at RT.

2.9.4 b Staining for all the assays:

1. 50 µl permeabilization solution added and incubated for 30 minutes.
2. Anti-mouse NP (1:10), anti-mouse M1 (1:250), anti-mouse A1 (1:1000) and anti-mouse HA1 (H3SKE) (1:100) was added to respective wells.
3. Cells were washed with 1x PBS for 3 times, 10 minutes each.
4. 50uL anti-mouse AF488 (1:1000 in PS) and DAPI (1:10000 in PS) added to all wells and incubated for 1 hour at RT.
5. Cells were washed with 100uL 1x PBS 3 times 10 minutes each.

2.10 Rab43 KO Beas-2b cell line

1. Independent guide RNA sequences targeting different regions of the gene were designed using various algorithm (CRISPOR, Thermo guide tool etc.) and were cloned in Lenti-CRISPR V2 vector having puromycin resistance.
2. The gRNA sequences were transfected in HEK-293 T cell line using PEI for lenti viruses generation using packaging plasmid (PCMVR 8.74) and envelope plasmid (PMD2.G).
3. The lenti-viral soup was transduced in Beas-2b cell line (50% confluent) in 1:1 ratio with complete DMEM.
4. KO cells were selected in puromycin (2µg/ml) and were scanned using indirect-immunofluorescence using Golgi as a marker for Rab43 KO cells.

2.11 Protein methods

2.11.1 Lysate preparation

For preparing the lysate, 100 mM dish full of A549 cells was taken and media was aspirated out and cells were washed with 1xPBS. Cell lysis buffer (protease inhibitor added) was added and cells were scrapped. The mixture was transferred to MCT and was vortexed after every 10 minutes for 5-6 times. The MCT was incubated on ice for 40 minutes and was centrifuged for 20 minutes at 12,000 rpm. The supernatant was transferred to fresh MCT and the lysate was stored at -80 °C.

2.11.2 SDS-PAGE

Small SDS-acrylamide gels were prepared consisting of resolving gel (lower) and stacking gel (upper). 10% Resolving gel and 4% stacking gel was prepared as follows:

	Stacking gel (3ml)	Resolving gel (6ml)
ddH20	1.8 ml	2.4 ml
30% acrylamide	0.4 ml	2.0 ml
Tris	0.75 ml (pH-6.8)	1.5 ml (pH-8.8)
10% SDS	30 µl	60 µl
10% APS	50 µl	60 µl
TEMED	5 µl	6 µl

APS and TEMED were added just before pouring. Resolving gel was poured overlaid with water to allow the polymerization. After full polymerization, the overlaid water was removed and stacking gel was poured with the comb inserted on the top.

After the polymerization of the gel, lysates were boiled at 95 °C for 10 minutes and was mixed with 4x Bromophenol Blue. The samples were loaded in Bio-Rad mini PROTEAN gel apparatus and gel was run in SDS-running buffer at 15V for 45 minutes.

2.11.3 Western-Blotting

Protein from the gel were transferred to nitro-cellulose membrane by semi-dry botting in transfer apparatus (1xSDS-PAGE running buffer plus 10%methanol) at 15V for 45 minutes. Blots were than blocked in skimmed milk or 5% BSA for 1 hour at room temperature. Primary antibody was diluted in 1xTBST and was incubated overnight at 4°C followed by three washes with 1xTBST. Secondary antibody was diluted in 1xTBST and membrane was incubated in that for 1 hour followed by three washes with 1xTBST. Bound antibodies were detected using 1:1 mixture of HRP and H_20_2 (ECL substrate from Bio-Rad) followed by development of blot using X-ray machine.

Chapter 3: Results

3.1 Regulation of Influenza A virus entry by Rab43

3.2 Regulation of late stages of IAV infection by Rab43

Chapter 3: Results

3.1 Regulation of Influenza A virus entry by RAB43

3.1.1 Aim of this work

IAV uses a variety of host cell factors for its propagation and replication in the host cell. Genome wide RNAi screen (Banerjee et.al., 2013, in collaboration with 3V-Biosciences) revealed RAB43 as one such factor which was observed to be a proviral factor supporting IAV infection. RABs coordinate many processes in membrane trafficking (76) and many of them are reported to be involved in viral infection as well i.e., Rab11 dependent vesicles in carrying IAV vRNPs to plasma membrane via recycling endosome pathway (79), Rab5 and Rab7 positive early and late endosomes are involved in the endocytosis of IAV (77).

To find the step at which RAB43 is playing a role in IAV infection, Entry assays were done. The distribution of early and late endosomes was also checked upon depletion of RAB43.

3.1.2 IAV infection is reduced to a significant level in RAB43 depleted cells

To elucidate the role of RAB43 in IAV infection, Infection assay with X31 IAV was done upon depletion of RAB43 by transient transfection for 72 hrs. ASN (All-stars negative) siRNA was used as the scrambled control. siRNA targeting the ATP6V1B2 subunit of vacuolar ATPase was used as positive control. Also, ASN control was supplemented with NH_4CL prior to infection as another positive control. Two siRNA sequences targeting RAB43 were used i.e., RAB43#2 and RAB43#4.

The results showed that upon depletion of RAB43, IAV infection was reduced by ~70% (Fig. 1) in cells relative to ASN which suggested that RAB43 is acting as a proviral factor for IAV infection.

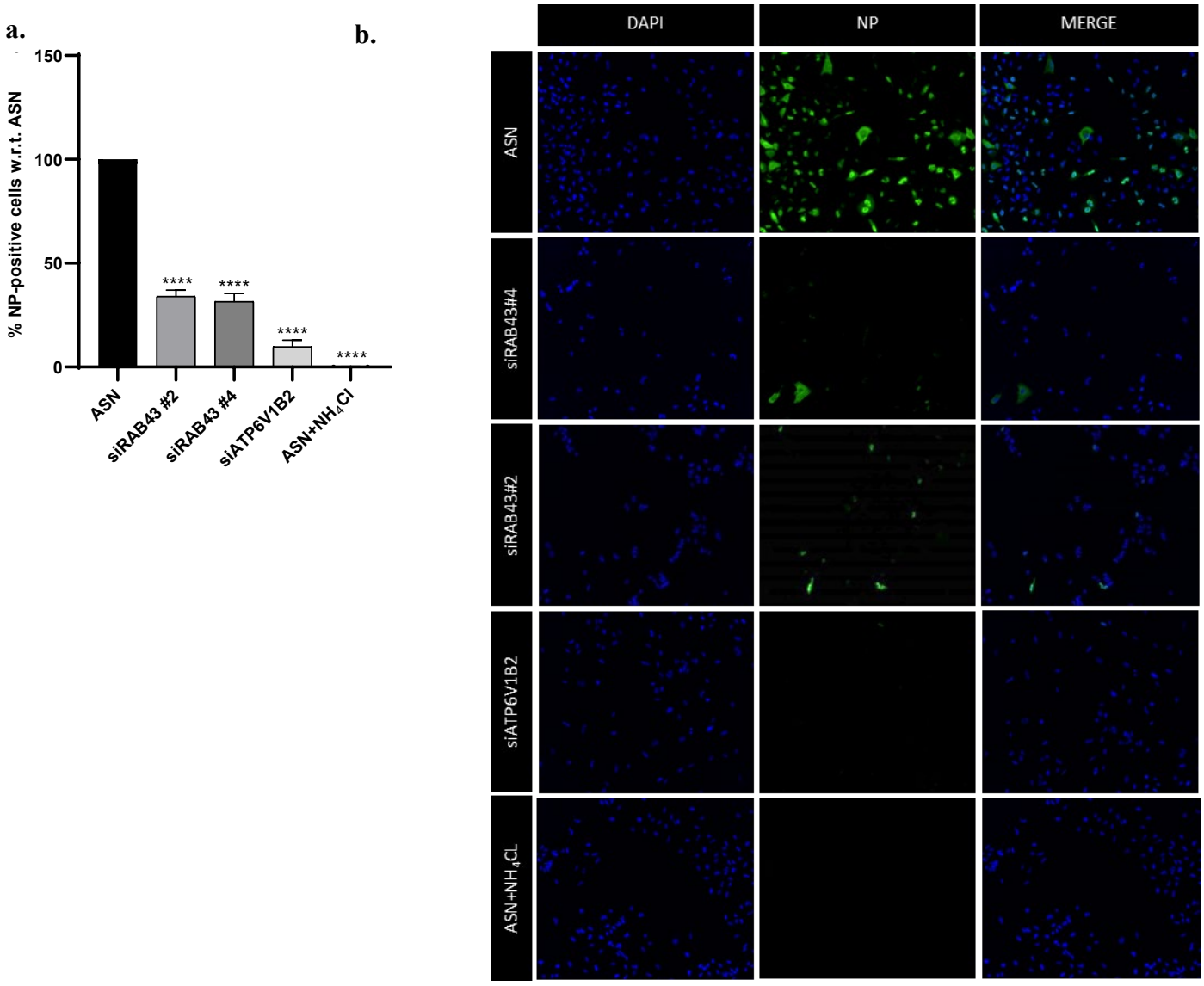


Fig 1. IAV infection reduced by a significant level in Rab43 depleted cells. a) Cells were infected with IAV for 10 hrs. and fixed, permeabilized and stained for NP b) Quantification plot for NP positive cells upon depletion with various siRNAs.

3.1.2.a RAB43 mRNA levels in siRAB43 transfected cells is reduced to a significant level

To confirm the specificity if siRNA that whether it is depleting RAB43 specifically, mRNA levels of RAB43 were checked 72 hrs. post transfection by qRT- PCR. The results suggested that siRAB43#4 was showing a better knockdown efficiency among the two sequences used. So, this sequence was used for further experiments and was referred as siRAB43.

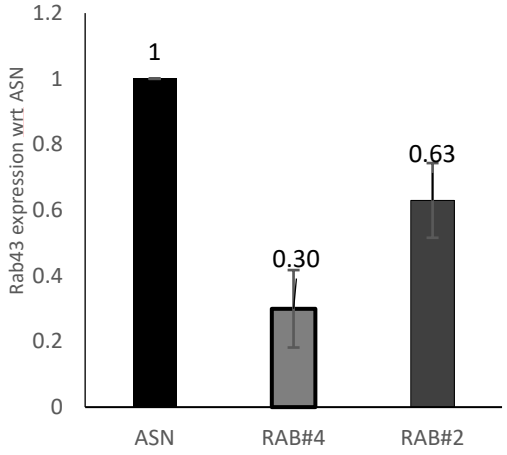


Fig 2: Rab43 mRNA levels were in All Stars Negative and two independent sequences targeting different regions of the gene. The levels were quantified as fold change, normalizing the ASN level to 1.

3.1.2.b RAB43 depletion using siRAB43 as well as the dominant negative mutant disrupts the integrity of Golgi apparatus

RAB43 is reported to be playing a role in the maintenance of structural integrity Golgi apparatus. The expression of dominant negative mutant of RAB43 (T32N) – the mutation which disrupts the functionality of the protein, is shown to disintegrate the Golgi as well. The same phenotype was replicated using the dominant negative and active mutant of Rab43.

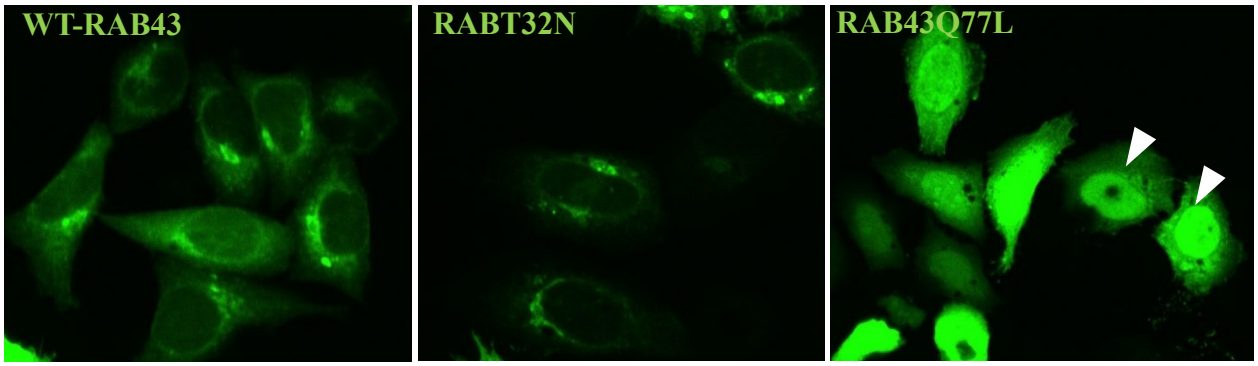


Fig 3: Rab43 mutants a) WT-RAB43 b) Rab43T32N c) Rab43Q77L

To confirm the same phenotype after the gene depletion mediated by siRNA, structure of Golgi was seen 72 hrs. post transfection and staining with Golgin. The results suggested that siRNA mediated depletion of RAB43 disrupts the structural integrity of Golgi (fig 4). Golgi fragmentation was used as marker for RAB43 knockdown validation in further experiments.

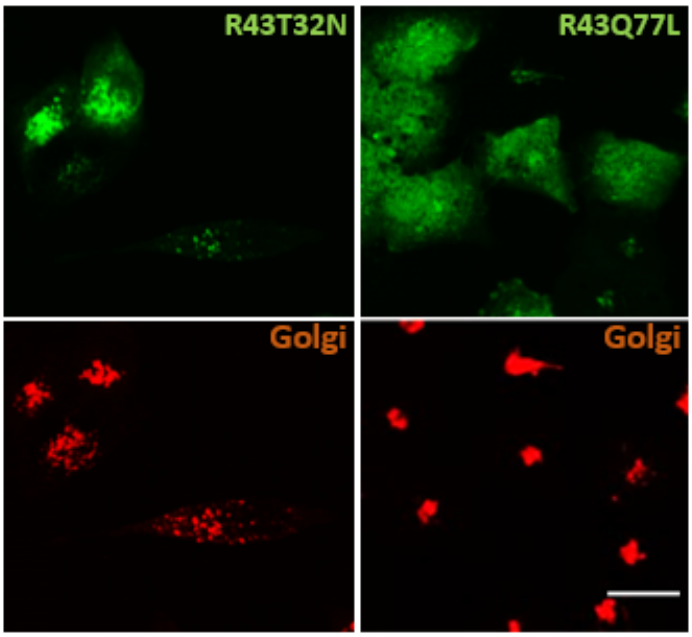


Fig 4: Golgi staining in the active and Dominant negative mutants of Rab43.

3.1.3 IAV infection is reduced substantaily in Rab43 KO Beas-2b cell line

Rab43 KO Beas-2b cell line was created as discussed in 2.10. Golgi disruption was used as an indirect criterion for scanning the KO population. Two clones showing disruption in integrity of Golgi complex were selected for infection assay. The results suggested a decrese in IAV infection relative to WT cells.

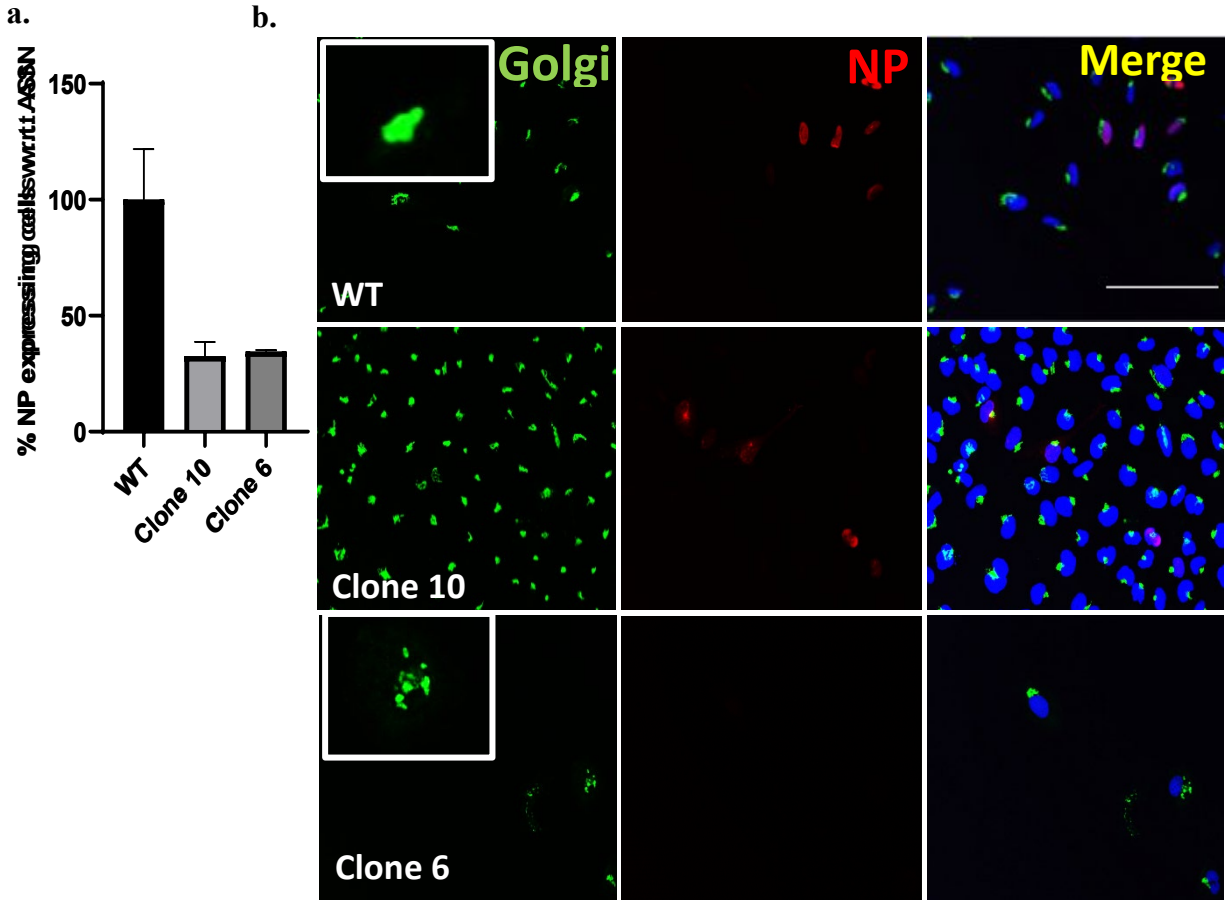


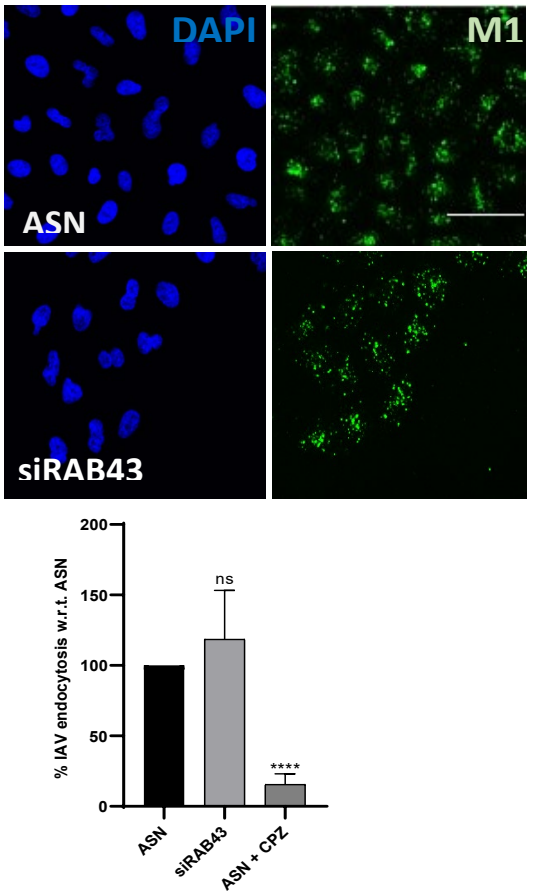
Fig 5: NP staining in Rab43 KO Beas-2b cell line 10 hrs. post infection a) Quantification of NP positive WT cells vs R43 KO Beaas-2b cell line. b) NP and Golgin staining in the Rab43 KO Beas-2b clones.

3.1.4 Nuclear Import of vRNPs is perturbed upon RAB43 depletion in A549 cells

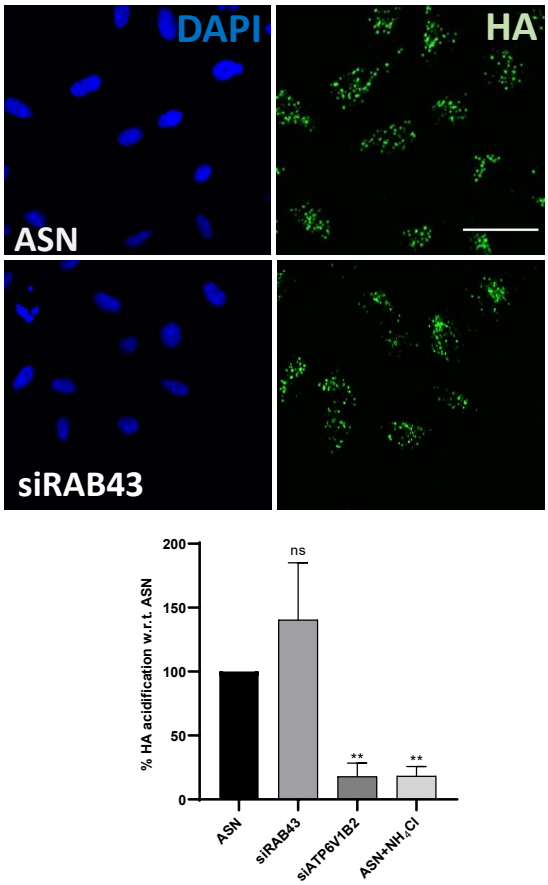
To check the steps at which Rab43 is acting as proviral factor for IAV entry, entry assays for the sequential steps of entry were performed: Endocytosis, Acidification, Uncoating and Nuclear import (described in material and methods). The cells were reverse transfected with the siRNA and all the assays were performed 72 hrs. post transfection. All the positive and negative controls were used as mentioned in 3.2.

The results suggested that the early steps Endocytosis, Acidification and Uncoating are unperturbed upon Rab43 depletion. The nuclear import of vRNPs is blocked up to a significant level in Rab43 depleted A549 cells. Also, a peculiar phenotype suggesting the movement of vRNPs to the plasma membrane was noted. The results suggested that Rab43 is a proviral factor which is helping in carrying the vRNPs to the nucleus.

a.



b.



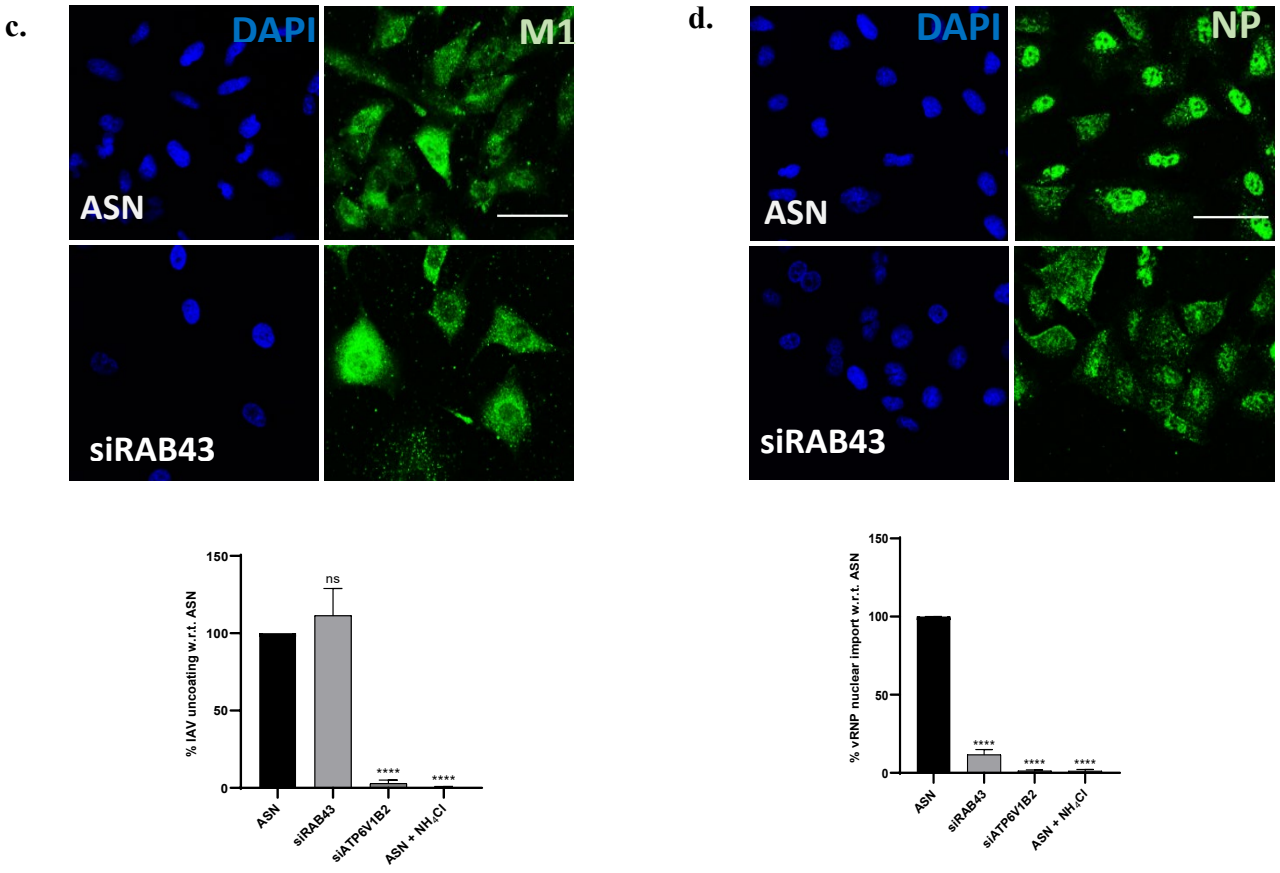
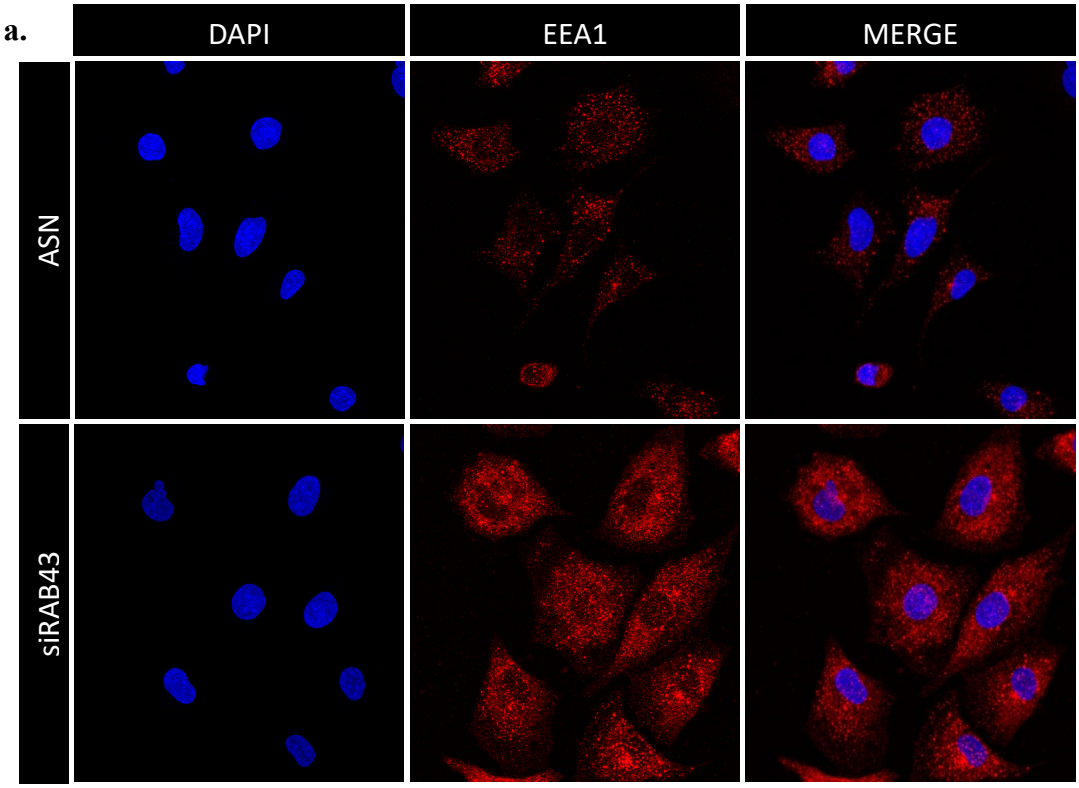


Fig 6: IAV entry assays a) IAV endocytosis was allowed for 30 min. Cells were than fixed, permeabilized and stained for HA. HA intensity was quantified as a measure for IAV endocytosis and IAV endocytosis was normalized w.r.t negative control. b) HA acidification was allowed for 1 hr. post infection. Cells were then fixed, permeabilized and stained for acidified HA (HA1). HA1 intensity was quantified as a measure for HA acidification and it was normalized to negative control. c) Viruses were allowed to uncoat for 2.5 hrs post infection and cells were fixed, permeabilized and stained for M1. Manual counting of uncoated cells was done and data was normalized with respect to negative control. d) IAV nuclear import was allowed for 4 hrs post infection and cells were fixed, permeabilized and stained for NP. Cells were manually counted as NP positive or negative and data was normalized in reference to negative control.

3.1.5 LAMP1 and EEA1 are upregulated and dispersed upon RAB43 depletion in A549 cells

Rab proteins are important players in endocytic trafficking. Rab43 is also widely studied for the secretory pathway, but less is known about its role in endocytic pathway. To elucidate the role of Rab43 in the endocytic trafficking pathway, the distribution and levels of early endosomes, late endosomes and lysosomes was checked upon Rab43 depletion using Indirect immunofluorescence and staining with the markers for all.

The results suggested that upon depletion of Rab43 in A549 cells, both EEA1 and LAMP1 are highly upregulated and the distribution is also changed. EEA1 and LAMP1 are more dispersed in the cell relative to the negative control.



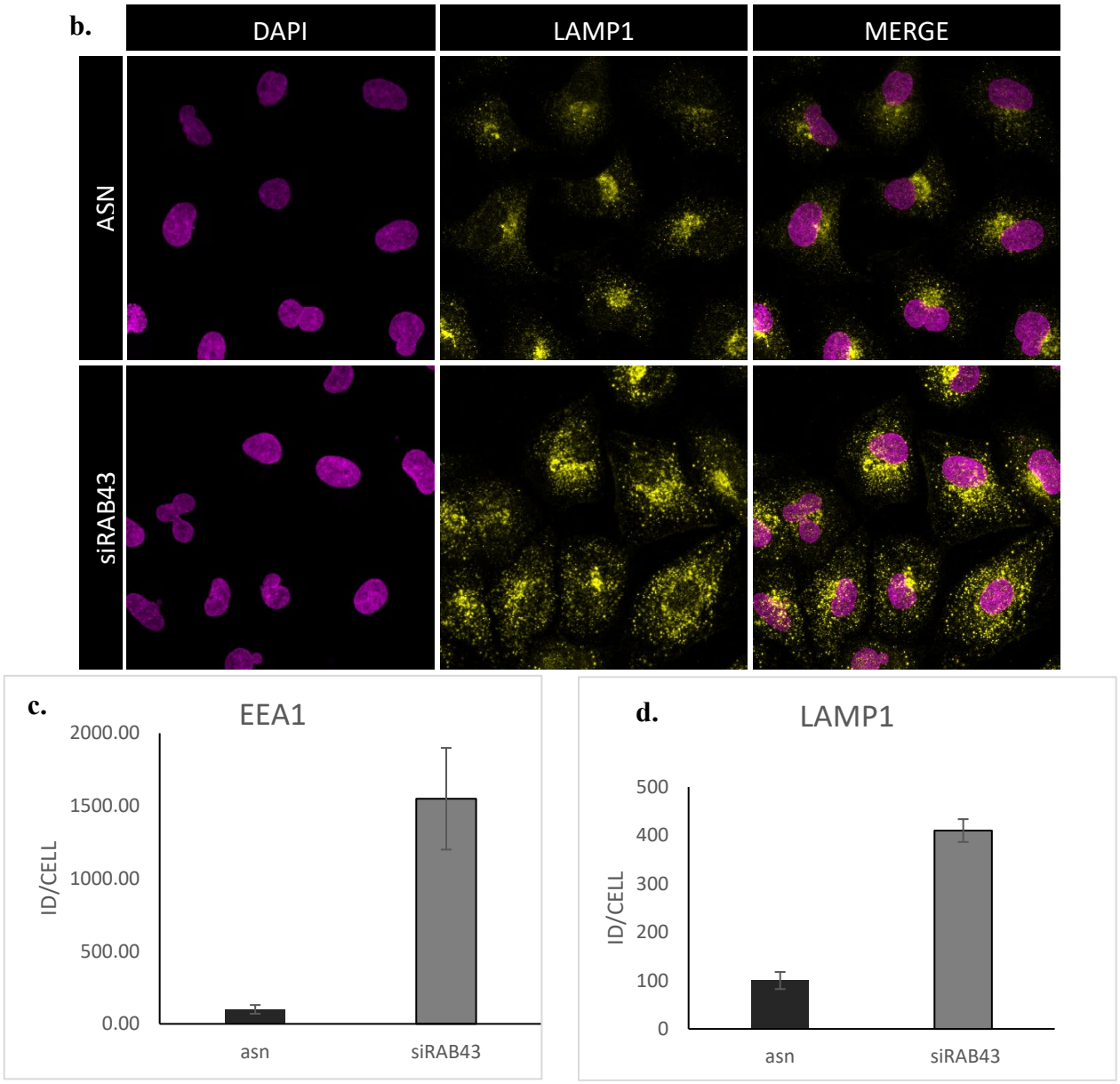


Fig 7: Confocal images for the cells which were transfected with respective siRNAs and were fixed, permeabilized and stained for a) EEA1 B) LAMP1 c) Quantification of EEA1 intensity normalized with respect to negative control d) Quantification of LAMP1 intensity normalized with respect to negative control.

3.1.6 Transferrin uptake is highly upregulated in Rab43 depleted A549 cells while EGF remains the same

The predominant accumulation of the vRNPs to the plasma membrane suggested a defect in the recycling pathway. Since, the signaling pathway of EGFR and Tfr differs in the fact that EGFR is degraded after internalization in the cell while majority of Tfr is directed back to the plasma membrane. To look for the general endocytosis defect or recycling defect, Transferrin uptake and EGF uptake assays were performed as mentioned in 72 hours post transfection.

The results suggested that the EGF uptake remains same in the Rab43 depleted cells compared to the negative control, while the transferrin uptake is highly upregulated in Rab43 depleted cells compared to the negative control. The results indicated a defect in the recycling pathway upon depletion of Rab43 in either of the two ways: Recycling pathway is upregulated leading to the more amount of Transferrin in the cell or the recycling pathway is downregulated leading to more accumulation of transferrin in the cell.

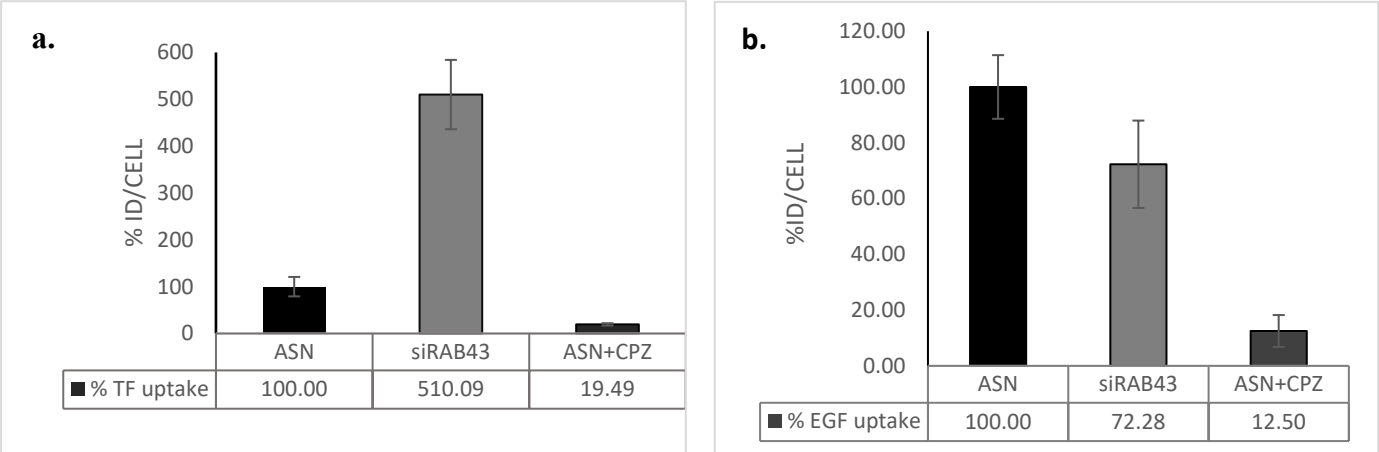


Fig 8: a) Transferrin uptake measured by intensity of Transferrin-A488 and normalized to negative control. b) EGF uptake measured by the intensity of EGF-488 and normalized to the negative control.

3.2 Role of Rab43 in late stages of IAV infection

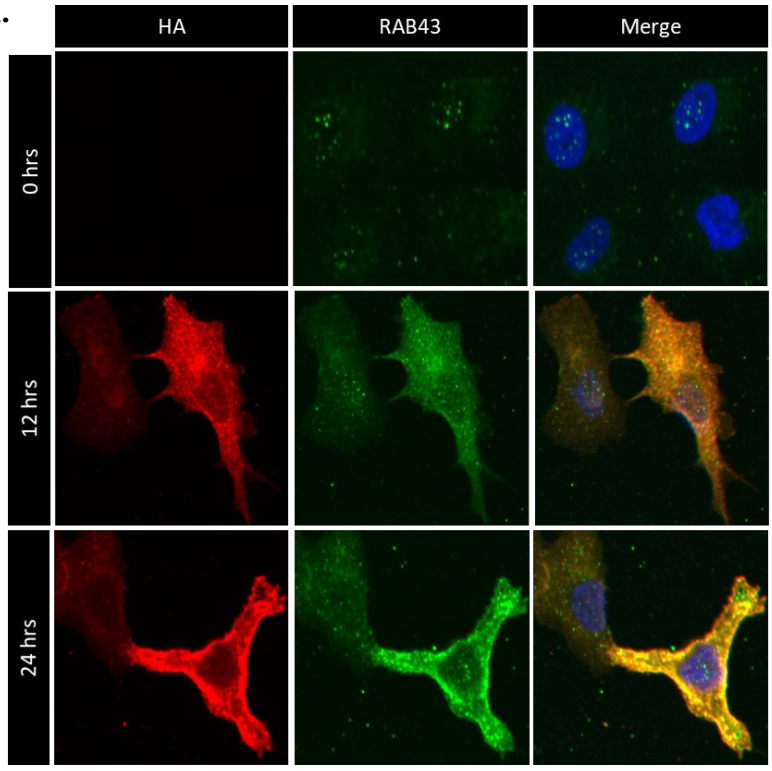
3.2.1 Aim of this work

Rab43 helps in the secondary envelopment of Herpes Simplex virus. HSV derives its secondary envelope from Golgi complex. It was seen that upon siRNA mediated depletion of Rab43, the viruses remain un-enveloped as seen by TEM. HA of the IAV, being a glycosylated protein is also processed via the ER-Golgi pathway and the Rab43 is also known to be localized to Golgi complex. This aim of this work was to check for the function of Rab43 with respect to HA processing.

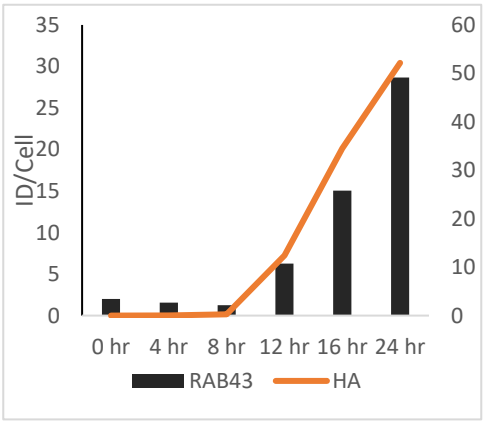
3.2.2 Rab43 level increase with progression in infection and co-localizes with HA

Time course infection assay was performed with high M.O.I of virus for the given time points: uninfected, 4hrs, 8 hrs., 12 hrs., 16 hrs. and 24 hrs. Cells were fixed and stained with HA and Rab43. The results suggested that there is an increase in the level of RAB43 as the infection progress and it co-localizes with HA (correlation coefficient calculated in Fig 9c).

a.



b.



c.

h.p.i	Pearson's R value
0 hr	0.13
4 hr	0.26
8 hr	0.47
12 hr	0.85
16 hr	0.87
24 hr	0.87

Fig 9*:* a) Immuno-staining of HA and Rab43 in a time course infection. Cells were infected with virus for the given time points and fixed, permeabilized and stained for Rab43 and HA. b) Levels of Rab43 and HA over the course of infection as measured by Intensity quantification. c) Correlation of HA and Rab43 with progression in infection as calculated using Pearson's R value.

3.2.3 Western Blotting to check for the levels of RAB43 with progression in Infection

Time course infection assay (uninfected, 4 hrs., 8 hrs., 16 hrs., 20 hrs. and 24 hrs.) was done and levels of RAB43 were checked using western blotting. Different concentration of antibodies was used ranging from 1:50-1:2000. Also, different types of blocking solutions were also used: SM-TBST, 1% BSA and 5% BSA.

The bands for Rab43 were not very clear and the blot turned black every time. It turned out from the literature search that since Rab43 is a phosphorylated protein, phosphorus signal may interfere and can turn the blot black. To solve the issue, the phosphatase inhibitor should be added freshly while preparing the lysate to avoid the turning of blot to black.

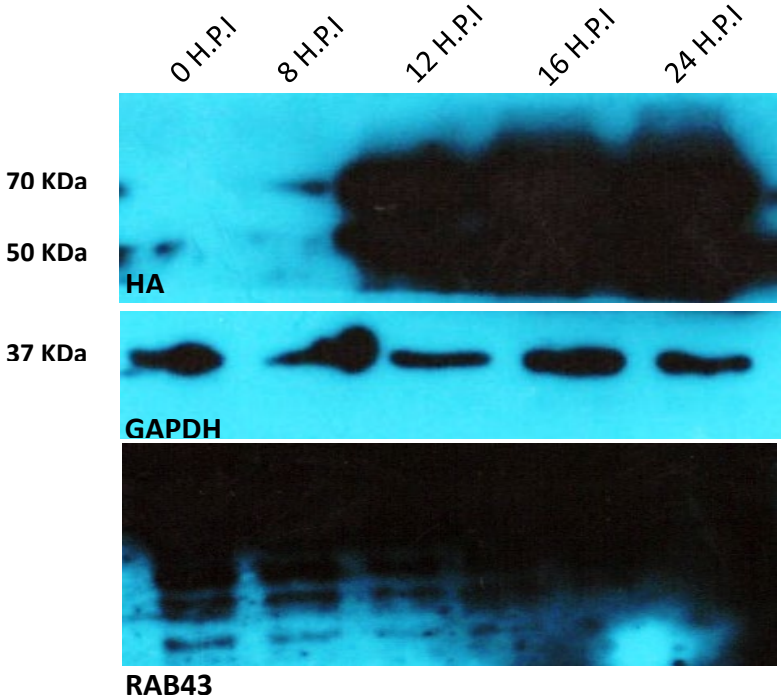


Fig 10: Immunoblot with Rab43 and HA over time course infection.

4 Future outlook of the work

This work provides preliminary insights of Rab43 as a proviral factor which is observed to be carrying the vRNPs into the nucleus. The vRNPs instead of going to the nucleus are directed to the plasma membrane. The preliminary results suggest an upregulation of Rab11 pathway which may be carrying the incoming vRNPs to the plasma membrane. Also, the active mutant of Rab43 (R43Q77L) also seems to be localized to the nucleus which gives the indication that working of Rab43 is similar to Ran GTPase. The level of EEA1 and LAMP1 upregulated upon Rab43 depletion along with upregulation of transferrin uptake.

Future aspects of the work remain to create Rab43 KO A549 cell line and to validate the entry assay in that cell lines with a clearer phenotype. Also, Rab43 expressing stable cell line needs to be created to check for the experiments dependent on expression of Rab43. The knockdown efficiency of siRab43#4 is required to be validated using Western blotting. The levels of EEA1 and LAMP1 upon Rab43 depletion should be checked using Western blotting.

The levels of Rab43 in a time course infection should be checked by western blotting. As Rab43 is co-localizing with HA, Co-IP needs to be done to check for the interaction of the two. Detailed insights to gain mechanistic understanding of Rab43 as a proviral factor is required to understand the role of Rab43 in promoting viral infection.

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