INVESTIGATING THE ROLE OF RHO GTPases IN *Entamoeba histolytica*

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CERTIFICATE

This is to certify that the thesis entitled "**Investigating the role of Rho GTPases in Entamoeba histolytica"** was successfully carried out and submitted by**Ms. Yajarva Trivedi** in the partial fulfillment of requirements for the degree of Master of Science in Biochemistry & Biotechnology respectively, to the **Institute of Science, Nirma University**, Ahmedabad The contents of this report, in full or in parts, have not been submitted for the award of any other degree or certificate in this or any other university or institute.

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DECLARATION

I, hereby declare that the dissertation thesis entitled "**Investigating the role of Rho GTPases in *Entamoeba histolytica***" Submitted to **Institute of Science, Nirma University, Ahmedabad.** Under the guidance of **Dr. Kuldeep Verma & Dr. Sunando Datta** which is not being submitted as a partial fulfillment of the degree of Master of Science. We further declare that this manuscript written by us has not been previously submitted to this or any other University/Institute/College for any degree/diploma/certificate.

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1. Introduction

Entamoeba histolytica is a parasitic organism. *E.histolytica* causes amoebic dysentery and is identified as one of the top seven pathogens causing dysentery in the children under the age of 5 years (Stauffer W *et al.,* 2006). It accounts for 100,000 deaths annually. Apart for causing dysentery, it can also invade to liver causing liver abscess. This infection is mainly prevalent in developing countries; in India, 15% to 20% of population are infected with this pathogen. (Dhanalaksmi*et al.*,2016).

E.histolytica exists in two forms: the infective cyst form, which plays a major role in transmission and the motile trophozoite form. The cyst form is found mainly in the contaminated water which later disseminates to form the motile trophozoite form. Human-to-human and faecal–oral transmission are the major sources of human infection. The virulence of the parasite mainly results into tissue damage; starting from the invasion of the mucus of the intestine and later might invade to other tissues such as liver resulting into liver abscess (Swagata Ghosh *et al.,* 2018).

The virulence of this parasite is due to: adhesion to the cell, invasion after dissemination from the cyst form and killing of the cell mediated by two mechanisms, namely: Phagocytosis and Trogocytosis. Phagocytosis is the engulfment of the whole cell whereas, trogocytosis is the nibbling process of the cell, wherein the parasite takes the bites of the live cell (Ralston KS *et al.,* 2014).In the killing of the host cells, the actin dynamics and secretion of various proteases by the parasites (Tillack*et al.,* 2007) help to invade and degrade the extracellular matrix (ECM), ultimately resulting in tissue destruction (Neal, 1956, Gadasi*et al.,* 1983). During the infection, the parasite first encounters with the host's ECM and receives stimuli from the various components of ECM. This signalling could in turn lead to activation of actin remodelling in the parasite (Meza I., 2000). The actin cytoskeleton plays an important role in the motility of the parasite and hence involved in invasion. On adhesion to ubiquitinated component of ECM, called fibronectin, the parasite is known to induce the secretion of amoebic proteases and, these proteases plays a vital role in the degradation of ECM and motility of the parasite (Franco.E*et al*.,1997, Talamas-Rohana P *et al*., 2000). Role of fibronectin in the formation of actin rich protrusive structures in amoeba which visually resembles the mammalian invadosomes is known (Rios A et al., 2007). Thus, the role of fibronectin as a potent activator of actin dynamics is well known in the literature, the identity of the downstream effector molecules is barely explored.

Prominent actin rich structures of the eukaryotic cells includes: focal adhesion, lamelliopodia, filopodia and invadosomes, which plays a role in sensing the surrounding (Ridley AJ., 2011, McNiven MA., 2013). Invadosomes, found in the transformed cell and cells of monocytic origin, are formed as a result of an interplay between the structural proteins viz. vinculin, paxillin and actin regulatory and nucleating factors like Arp2/3 (actin related protein 2/3), N-WASP (Neuronal Wiskott Aldrich syndrome protein) and Rho GTPases (Linder S.,2009). These are the molecular players playing a role in actin dynamics, resulting into the formation of invadosomes. *Entamoeba histolytica* forms an actin rich protrusive structures named as 'Actin Dots' which resembles the invadosomes, are formed in a Rab21 dependent manner. These actin dots plays a role in invasion and lead to the severity of amoebic infection (Merlyn *et al*., 2019).RabGTPases is a member of ArfGTPases superfamily.

2. Review and literature

Entamoeba histolytica is considered to have an extraordinary motile behaviour that aids in developing the pathogenicity of the organism with the involvement of many diverse molecular players. Membrane trafficking aids in the pathogenicity of the organism as it is involved in the phenomena of phagocytosis, motility and invading the tissues to ultimately resulting into infection (Voigt et al., 1999). Also, it is considered as a linking parasitic organism between prokaryotes and eukaryotes (Brown *et al.*, 1997), which makes it an organism to study.

2.1 Small GTPases:

Superfamily small GTPases are classified into five families based on their primary sequence, namely: Ras, Rho, Ran, Arf and Rab (Bourne *et al.*, 1990, Takai *et al.*, 2001). Small GTPases are critical regulators of cytoskeletal and membrane dynamics underlying cell motility, cell polarity and cell growth.These acts as an effector molecule and functions in downstream signalling. Small GTPases acts as a molecular switches and exists in two forms, inactive in GDP bound state and active in GTP bound state. Binding of GTP and its hydrolysis triggers reciprocal conformational changes within a switch region within the catalytic domain (Stephen R., 1997). In the active state (bound to GTP), they acts on downstream effectors. The transition between active and inactive states may be limited by the intrinsic rate of GTP hydrolysis, or it may be accelerated by the binding of a GTPase-activating protein (GAPs). Guanine nucleotide dissociation inhibitors (GDIs) inhibit the release of GDP from certain G proteins, whereas guanine nucleotide exchange or release factors (GEFs) stimulate this process. These accessory factors that control G protein state may themselves be subject to regulation. Figure-1 depicts the regulation of small GTPases.

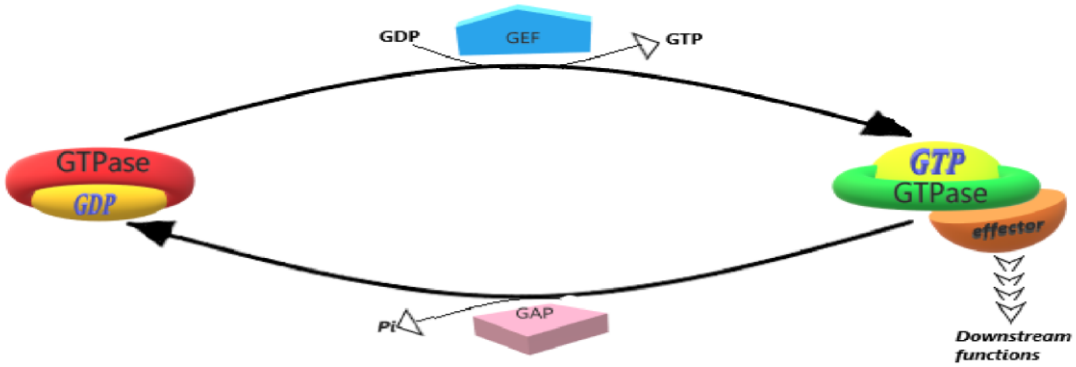


Figure-1 depicts the GTPase cycle. The conformation change due to the binding of GDP and GTP, is resulting into inactive and active forms respectively. GEFs are responsible for exchange of GDP to GTP and GAPs are responsible for accelerating the GTP hydrolysis. GTPase in its active conformation (when bound to GTP), can bind to the effector molecules, resulting into downstream functions.

Rho GTPases

Rho GTPases are members of the Ras superfamily of monomeric 20–30 kDa GTP-binding proteins. Rho

(A, B, C isoforms), Rac (1, 2, 3 isoforms), Cdc42 (Cdc42Hs, G25K isoforms), Rnd1/Rho6, Rnd2/Rho7, Rnd3/ RhoE, RhoD, RhoG, TC10 and TTF are the ten different Rho GTPases that have been identified in wide range of eukaryotes (Ridley *et al.,* 2000). The most extensively characterized members are Rho, Rac and Cdc42. Rho GTPases acts as a molecular switches, they have been implicated in many basic cellular processes that influence cell proliferation, differentiation, motility, adhesion, survival, or secretion**.** Rho GTPasesplays a major role in regulating the assembly and organisation of actin cytoskeleton (Hall *et al.,* 1998), basically, controlling the dynamics of F-actin cytoskeleton. About 30 guanosine nucleotide exchange factors (GEFs) have been identified that facilitate the exchange of GDP for GTP (Kjoller*et al.,* 1999). All Rho GEFs contain a Dbl-homology (DH) domain which encodes the catalytic activity (Cherfils *et al.,* 1999, Hart *et al.,* 1994) and an adjacent pleckstrin homology (PH) domain. The PH domain is thought to mediate membrane localization through lipid binding (Rameh *et al.,* 1994, Zheng*et al.,* 1996), but, in addition, structural and biochemical evidence suggests that it might also directly affect the activity of the DH domain.Approximately, 20 Rho GAPs (GTPase-activating proteins) have been identified, which increase the intrinsic rate of GTP hydrolysis. It is shown that there is a 20° rotation between GTPase and GAP, from ground state to transition state, allows an arginine residue in the GAP protein, the 'arginine finger', to enter the GTPase active site and participate in the stabilization of the transition stateand hence, increase the intrinsic rate of GTP hydrolysis (Anne L *et al.,* 2000).

Rho GTPases are able to interact with membranes via a posttranslational C-terminal geranyl-geranyl lipid modification. In the unactivated state, Rho and Rac can be isolated as a soluble complex associated with RhoGDI (guanosine nucleotide dissociation inhibitor). Rho GDI appears to sequester GDP-bound Rho GTPases in the cytoplasm and inhibit their spontaneous GDP to GTP exchange activity (Cox *et al., 1992*). Rho GTPases have been found to play a role in a variety of cellular processes that are dependent on the actin cytoskeleton, such as cytokinesis (Mabuchi *et al.,* 1993, Drechsel*et al.,* 1997) , phagocytosis (Cox *et al.,* 1997, Caron *et al.,* 1998), pinocytosis (Ridley *et al.,* 1992), cell migration (Nobes *et al.,* 1999, Allen *et al.,* 1998), morphogenesis (Settleman*et al.,* 1999) and axon guidance (Luo*et al.,*1997).

2.2 Parasitic Rho GTPases

E.histolytica genome encodes several Rho family GTPases and expresses approximately 20 Rho family GTPases. Rho family GTPases modulates through multiple effectors, including PAK's (p21 Activated Kinases): seven isoforms of PAK's have been identified out of which two are important in amoebic motility, invasion and host cell phagocytosis (Dustin E.*et al.,* 2014). EhRho1 (*E.histolytica* Rho1) is activated by conserved switch mechanism, but it diverges from mammalian Rho GTPases in lacking a signature Rho insert helix. EhRho1 is a homolog of molecules like mDia and EhFormin1, which plays a role in mediating serum-stimulated actin reorganisation (Erika S.*et al.,* 2011). Fibronectin, a component of ECM (Extracellular Matrix) regulates many aspects of cell behaviour involving Actin cytoskeleton and members of Rho family of small GTPases; it induced signalling in *E.histolytica* trophozoites and has implication in actin cytoskeleton, aiding in invasive behaviour during pathogenesis (Rios A*etal*., 2008).

2.3 Trigger vector:

Knock-down cell lines were made using Trigger vector. *Entamoeba histolytica* contains a large number of 27 nucleotide long antisense small RNAs with 5'-polyphosphate termini which can fuse to the region on Trigger vector and silence the gene fused to it (Laura *etal*., 2013). Thus, this approach was used to create RhoGTPases knock-down cell lines.

- Here, we propose to identify the role of Rho GTPases in the formation of 'Actin Dots' and thereby identifying the role in attack and migration.

Thus, we propose to address the following objective:

- Sub-cloning of Rho9GTPases, Rho5 GTPases and Rho13 GTPases from pEhEx vector where they are overexpressed into the trigger vector. The objective is to first study any changes occurring due to the absence of these GTPases.
- Preparing the Rho9 GTPase, Rho13 GTPase knock-down cell lines of *Entamoeba histolytica* and perform the actin dot assay and the RBC uptake assay. Rho5 GTPase knock down cell line was already available in the lab.
- Following are the Accession ID mentioned in the table-1 of the Rho gene taken into consideration. These are obtained from Amoebic Database:

Table-1:

Sr.No.	Gene	Accession ID
1.	Rho-5	EHI_012240
2.	Rho-9	EHI_180430
3.	Rho-13	EHI_135450

3. Experimentation Methodology

3.1 Sub-cloning of Rho from pEhEx vector to Trigger vector; creating Rho knockdown constructs:

3.11 Transformation in DH5α cells:

- The plasmid was present in the lab of which transformation was done to have multiple number of colonies of the plasmid containing bacteria. 3µl of plasmid (<100ng) on ice and 25µl of competent cells (DH5α) in an eppendorf was taken. (DH5α was pre-thawed on ice for 10 minutes).
- This was incubated for 15 minutes in ice.
- Heat shock at 42 ◌̊C for 40 seconds was given on a heat blocker.

- Kept on ice for 2 minutes after which 200 µl of LB broth (Luria Bertani broth) was added.
- It was incubated at 37 ◌̊C, 200 rpm for 1 hour on a shaker incubator.
- The transformed plasmid was plated on LB agar plates containing ampicillin and kept for 12-14 hours at 37 ◌̊C in an incubator after which the colonies appear.

3.12 Primary culture for transformed Rho:

 The isolated single colony from the LB plates were transferred to the test tubes containing 5 ml of LB media containing 5 µl of ampicillin for selection for primary culture. This was incubated for 13-14 hours on a shaker incubator at 37 ◌̊C.

3.13 Plasmid isolation (MINI-PREP.):

- Plasmid isolation was performed using Qiagen kit.
- The cells were harvested at 10,000 rpm for 2 minutes and the supernatant was discarded.
- 250 µl of resuspension buffer and 250 µl of lysis buffer was added and mixed by inverting for 4-6 times.
- Incubated for 4 minutes.
- 350 µl of cold neutralisation buffer was added and mixed by inverting for 4-6 times.
- The supernatant was taken in a spin column and spin at 15000g for 1 minute.
- The flow-through was discarded and 0.5 ml of PB buffer was added again spin at 15000g.
- PE buffer was added and spin at 15000g for 2 minutes.
- The column was dry-spin for 2 minutes and was moved to a fresh tube.
- 40 µl of AMQ (autoclaved milliQ water) was added to the column after waiting for 2 minutes and spin at 15000g for 2 minutes.

3.14 Agarose Gel Electrophoresis*:*

- For the gel electrophoresis a 0.5x stock solution of TBE buffer in 100ml of distilled H_2O was prepared. Sufficient amount of electrophoresis buffer had been prepared in order to fill the electrophoresis tank and cast gel.
- A 0.7% solution of agarose gel was prepared. For this 0.49 grams of agarose was added to 70ml of 0.5X TBE buffer.
- The solution was then allowed to heat in the oven till it boils. The solution was taken out of the oven and allowed to cool slightly.
- Upon cooling a tracking dye, ethidium bromide (EtBr) was added to the solution and it was poured in the casting tray and allowed to solidify.
- After this, the sample was mixed with 6x acrydine orange loading dye and the whole is added into one well using a micropipette and 1Kb DNA Ladder was added into another well for comparison.
- The electrophoresis apparatus were joined to the battery and the electrophoresis was carried on at 90V.
- The bands were then observed under the Gel Doc.

3.15 Restriction digestion:

Two restriction sites flanked by the Rho gene in the over-expression pEhEx vector were:

• *Sma1 and Xho1* recognizes the following sites as mentioned in the table-2:

Table-2:

Sr.no.	Restriction enzyme	Restriction site
1.	*Sma1*	CCC ⇓GGG GGG ⇑CCC
2	*Xho1*	C⇓TCGAG GAGCT⇑C

• For digestion, a 40 µl of reaction was set as under, by addition of the following components in sequence:

Table-3:

Sr.no.	Components	Volume (µl)
1.	AMQ	25.5
2.	Cut smart buffer (10x)	4
3.	Plasmid	10
4.	Sma/ Xho	0.4/ 0.4

• This reaction was kept overnight at 25 ◌̊C and after that was kept on water (37 ◌̊C) bath for 2 hours.

3.16 Agarose gel electrophoresis:

• To check if the digestion too place or not, the agarose gel was run again as previously.
• The gel was observed under U.V transilluminator. There were two bands observed; one of the trigger vector while the other of the gene. The band with gene Rho was cut with the help of a blade.

3.17 Gel elution:

• Gel elution was performed using Gmini gel extraction kit.
• The band containing the Rho gene was cut and weighed.
• 3 gel volumes of GGMB buffer was added (gel melting buffer) and incubated at 65C, 600 rpm for 10 ◌̊ minutes with occasional invert mixing.
• 10 µl of 3M sodium acetate was added (pH=5.2) and mixed thoroughly.
• 1 gel volume of Isopropanol was added and this solution was added to the Gmini spin column and centrifuged at 8500 g for 1 minute.
• 600 µl of membrane wash buffer was added and centrifuged at 8500g for 1 minute. Again centrifuged for additional 2 minutes.

• The gmini spin was placed in a fresh tube and 20 µl of nuclease free water was added to it, allowing it to stand for 2 minutes.
• The plasmid from the gel was obtained after centrifugation at 8500g for 2 minutes.
(Plasmid has to be stored at -20 ◌̊C).

3.18 Quantification of plasmid:

• Quantification was done using spectrophotometer, in terms of nanogram (ng).
• 260/280 nm ratio was noted.

3.19 Ligation:

• Ligation of the Rho gene in Trigger vector.
• The components were added as per the sequence followed in the table:-

Table-4:

Sr.No.	Components	Volume (µl)
1.	AMQ	5.5
2.	Ligase Buffer	1
3.	Rho gene	1.8
4.	Trigger vector	1.8
5.	T4 DNA ligase enzyme	0.5

• Ligation was completely performed on ice and was kept overnight (16-24 hours) on heat blocker set at 16 ◌̊C.

3.20 Transformation of ligated Rho gene in Trigger vector:

 Transformation of the ligated product was done in the same manner as above.

3.21 Setting up primary culture for ligated colonies obtained:

• To 5 ml of LB broth, 5 µl of ampicillin was added and one ligated colony was transferred to it.
• This inoculated culture was kept on shaker incubator (37 ◌̊C) overnight.

3.22 Screening of ligated Rho and trigger vector:

A. Plasmid isolation (conventional):

• Overnight culture was harvested at 10000g for 2 minutes and supernatant was discarded.
• 250 µl of resuspension buffer was added and was made homogeneous by pipette mixing.
• 250 µl of lysis buffer was added andmixed gently 3-4 times. This was incubated for 4 minutes at room temperature.
• 350 µl of neutralization buffer was added and mixed vigorously. This was incubated for 4-5 minutes on ice and centrifuged at 19000g at 4 ◌̊C for 10 minutes.

• To 700 µl of clean supernatant, 500 µl of ice-cold isopropanol was added and kept on ice for one minute.
• This was centrifuged at 19000g for 10 minutes at 4 ◌̊C and the supernatant was discarded.
• To the pellet, 800 µl of 70% ethanol and the pellet was dislodged.
• This was spin again at 19000g for 10 minutes and the alcohol was removed and dried.
• 30 µl of AMQ was added and mixed.
• **B. Agarose gel electrophoresis:**
• The gel was run to see the quality of the isolated plasmid.
• 2 µl of plasmid was loaded with 1 µl of loading orange dye.
• **C. Digestion :**
• Digestion was performed to see if the ligation happened or not.
• Digestion was performed at room temperature and a 20 µl of reaction was set. The following components were added in sequence:-

Table-5:

Sr.no.	Components	Volume (µl)
1.	AMQ	14.6
2.	10X cut smart buffer	2
3	Plasmid	3
4..	SmaI/Xho	0.2/0.2

• Digestion was kept at room temperature for 2 hours and then incubated at 37 ◌̊C in water bath.

D. Agarose gel electrophoresis:

• Digestion was performed to see if the ligation happened or not.
• 10 µl of plasmid was loaded with 2 µl of loading orange dye.
• Appearance of two bands implicates the ligation has happened after digestion with the restriction enzymes that were used for ligation.

3.23 Sequencing:

 The plasmid positive for screening were sent for sequencing.

3.2. Maintaining trophozoites:

3.21 Culturing:

• Media components:
• For 1 unit of Incomplete media following components has to added as mentioned in the table-6**:-**

Table-6:

Sr.no.	Components	Amount
1.	Biosate	3g
2.	Glucose	2g

3.	NaCl	200mg
4.	KH_2PO_4	60mg
5.	K_2HPO_4	100mg
6.	L –Cysteine	100mg
7.	L-Ascorbic acid	20mg
8.	Ferric Ammonium Citrate	2.28mg
9.	Distilled water	88ml

*****1 unit media= 88ml of Incomplete media.

• To prepare Complete Media following components has to be added:

Table-7:

Sr.no.	Components	Volume (ml)
1.	Incomplete media	88
2.	Heat inactivated Adult Bovine Serum*	15
3.	Vitamin Mix	2
4.	Penicillin-streptavidin (Pen-Strep)**	1

The other components have to be added into the autoclaved incomplete media in the laminar hood only.

* ABS provides growth factors.

**Pen-strep to prevent bacterial contamination in trophozoite culture.

Procedure:

- For sub-culturing, complete media was filter sterilized with a steric cup.
- For sub-culturing, dead cells in the form of white pellet at the bottom were removed with a pipette, by removing approximately 6.5 ml of media (One culture tube contains 13ml of media along with cells).
- The culture tube was kept on ice for 5-6 minutes, during which it was shaken very well such that all the adhered cells on the wall of the culture tubes gets detached.
- After this, with the help of the remaining media, the walls are flushed to remove the adhered cells and 0.6-1 ml depending on the requirement was kept as inoculum.
- Filtered sterilized complete media to make a total of 13ml and 54 µl (6µg) of G-418 was added to the tube as a selection antibiotic, for a selective growth of trophozoites.

3.3 Creating knock-down cell lines:

3.31 Transfection:

• Buffers for Transfection (Incomplete Cytomix Buffer):

Sr.no.	Incomplete Cytomix Buffer components	Concentration-10x (M)	Weight in grams
1.	K_2HPO4/KH_2PO4 (pH=7.6)	0.1	0.522/0.408

2.	KCl	1.2	2.6
3.	$CaCl_2$	0.15	0.00661
4.	HEPES (pH=7.4)	0.25	1.787
5.	EGTA	0.02	0.228
6.	$MgCl_2$	0.05	0.1428

Table-8:

- All these components were weighed and each was dissolved in 30 ml Autoclaved MilliQ Water (AMQ) and stored at 4 ◌̊C.
- During transfection, 1 ml of all the six components were taken and added to 4 ml of AMQ. This constitutes 10 ml of Incomplete Cytomix Buffer. They are filter sterilised.

- Other requirements for transfection:

Table-9:

Sr.no.	Components	Stock concentration(M)	Working concentration (mM)	Volume required (µl)
1.	Glutathione (GSH)	0.1	10	100
2.	ATP	0.5	4	8

- 100 µl and 8 µl of GSH and ATP respectively were added to 892 µl of filter sterilized incomplete cytomix buffer. This constitutes to Complete Cytomix Buffer and was kept on ice.

Transfection Procedure:

- Three wild-type trophozoites tubes with 90 to 100% confluent cells were taken, dead cells were removed (pellet) and kept on ice for 5-6 minutes.
- The culture tubes were flushed from walls by pipette such that most of the cells adhered to the wall gets detached. These cells were transferred to a new falcon and centrifuged for 600 rpm for 6 minutes at
  4 ◌̊C.
- The supernatant was removed and 5ml of filter sterilized antibiotic-free media to give wash and the cells were resuspended and again centrifuged for 600 rpm for 6 minutes at 4 ◌̊C.
- The supernatant was removed and 5ml of filter sterilized incomplete cytomix buffer was added. 10 µl of this resuspended cells were taken and counted on haemocytometer. For transfection, $3\text{-}4*10^6$ cells must be there.
- And the cells were resuspended and again centrifuged for 600 rpm for 6 minutes at 4 ◌̊C and discard the supernatant.
- The cells were then resuspended in 400 µl of filter sterilized complete cytomix buffer.
- 100 µl of isolated plasmid and 400 µl of cells resuspended in complete cytomix buffer was taken in an electroporation tube and kept on ice for 2 minutes.

- This was given an electric pulse in an electroporation machine, prior to which two times the pulse was given with filter sterilized complete cytomix buffer to get the pulse constant in an electroporation machine.
- After the electric pulse, incubated on ice for 2 minutes and the cells were added to a culture tube containing 12.5 ml media containing heat inactivated ABS(Adult bovine serum) and vitamin mix, deprived of any antibiotics and was kept in incubator.
- The cells are observed after 48hrs and accordingly media is changed every alternate day. After two to three days G418 in concentration 2ug/ml is added. Gradually it is increased to 6ug/ml.
- The cells are splitted once the confluency of the cells in G418 is 100 percent

3.4 Assays with knock-down cell lines:

3.41 Actin Dot Assay:

- 50 µl of fibronectin (100 µg/ml) in three wells was added each and 50 µl PBS (1x; control) was added in control wells. The slide was allowed to dry in laminar hood for 1 hour.
- The wells were briefly washed with PBS and allowed to dry under LAF.
- Meanwhile, the cells were harvested by keeping on ice and centrifugation at 1200xg for 5 minutes followed by the removal of dead cells.
- The slide was kept in a humid chamber and incubated at 37 ◌̊C.
- The harvested cells added onto the wells and incubated for 1 hour.
- The cells were fixed with 4% of 50 µl PFA for 20 minutes.
- PFA was aspirated and cells were given wash with PBS thrice, 5 minutes each.
- 15 µl of 0.05% TritonX-100 was added to wells for permeabilisation and incubated for 10 minutes.
- Again wash with PBS was given thrice.
- 50 µl of blocking solution (5% FBS in PBS) was added to each well and incubated for 1hour at room temperature.
- Phalloidin(1:60) and DAPI ((1:1000) were diluted in blocking solution and was incubated for 1 hour in dark.
- The cells were washed twice with blocking solution and once with AMQ.
- The wells were mounted with 5 µl mowiol and air dried overnight under dark.

*Autoclaved and filter sterilized PBS was used.

3.42 RBC Uptake Assay:

RBC Isolation:

- Human blood ~5 was taken and transferred to 15 ml of falcon containing EDTA-Na (1.5mg EDTA-Na per ml blood) and mixed for ~5 minutes to prevent blood from coagulating.
- It was then centrifuged at 500xg for 10 minutes at 4 ◌̊C.
- Plasma and erythrocytes were removed by aspiration and discarded in bleach solution. Wash buffer components:

Table-10:

Sr.no.	Components	Concentration	Weight (grams) for 50 ml
1.	Tris base	21mM	0.127
2.	KCl	4.7mM	0.017
3.	$CaCl_2$	2.0mM	0.0147
4.	NaCl	140.5mM	0.41
5.	$MgCl_2$	1.2mM	0.0057
6.	Glucose	5.5mM	0.049
7.	Bovine serum albumin (BSA)	0.5%	0.25

(pH was maintained 7.4 by HCl).

- RBCs were washed 3 times with wash buffer and centrifuged at 500xg for 5 minutes at 4 ◌̊C (the pellet was mixed thoroughly prior to centrifugation).
- RBCs were then washed with 1% BSA in PBS and the supernatant was discarded.

RBC labelling:

- 50 µl of RBCs from the pellet was taken in an eppendorf and 50 µl of cell tracker orange dye (50 µl/ml) was added and mixed.
- 400 µl of 1% BSA in PBS was added and mixed.
- The tube was kept at 37 ◌̊C for 1 hour and centrifuged at 500xg for 5 minutes at room temperature. Discard supernatant.
- Three washes with 1% BSA in PBS were given and a final was with incomplete media.
- The supernatant was discarded and wrapped with aluminium foil.
- After labelling, RBCs were resuspended in 1ml of serum free media and 20 µl of it was taken for counting using a haemocytometer.
- The ratio of the RBC:amoebic cells should be 50:1.
- They are resuspended in such a way that there are cells in 50:1 in 10ul and 50ul of RBCs and amoeba each.
- The RBCs and amoebic cells are added onto the wells and kept at 37 for 10 mins.
- The cells were fixed with 4% of 50 µl PFA for 20 minutes.
- PFA was aspirated and cells were given wash with PBS thrice, 5 minutes each.
- 15 µl of 0.05% TritonX-100 was added to wells for permeabilisation and incubated for 10 minutes.
- Again wash with PBS was given thrice.
- 50 µl of blocking solution (5% FBS in PBS) was added to each well and incubated for 1hour at room temperature.
- The cells were washed twice with blocking solution and once with AMQ.
- The wells were mounted with 5 µl mowiol and air dried overnight under dark.

4. Results and Inferences

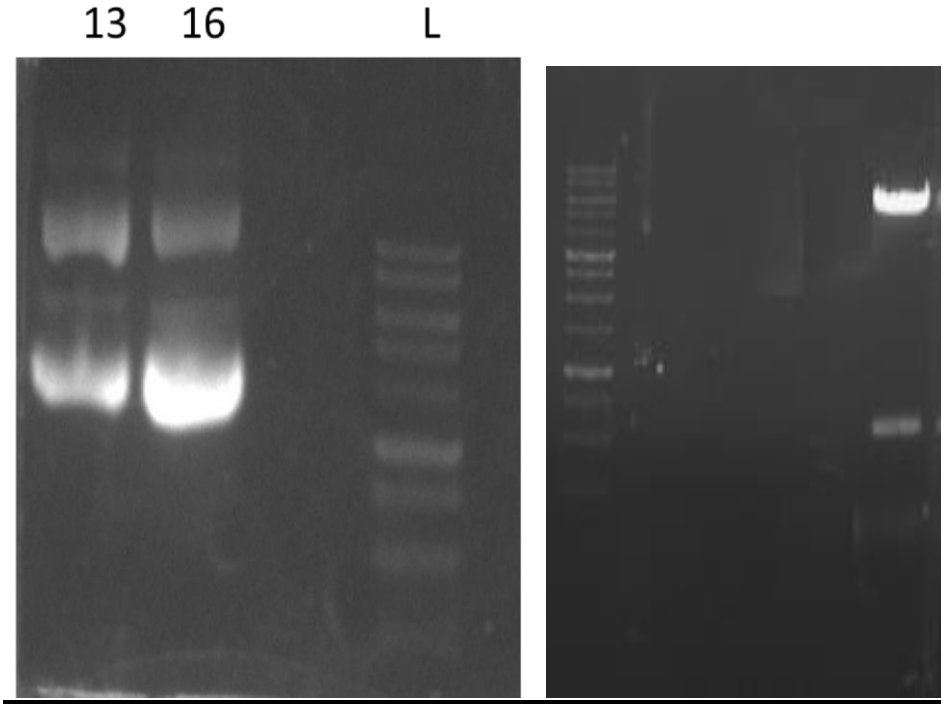


Figure-2 : Plasmid isolation of two Rho GTPases Rho 13, Rho 16. I worked with Rho 13. The next gel is showing the positive colony screening of Rho13. The lower band in the second figure show the gene of interest.

- Aiming to sub-clone, first thing I did was to isolate the plasmid with pEhEx vector with Rho13 gene and Rho16 gene insert, the left agarose gel picture shows isolation of plasmid was efficient. After isolating plasmid, restriction digestion was performed in order to get the gene of interest (Rho13 and Rho16 gene) from the overexpression vector (pEhEx); using the two restriction enzymes: *Sma1* and *Xho1*. The restriction site these two enzyme recognizes are as mentioned in table-2. After restriction digestion, once gene of interest is obtained, it was ligated in Trigger vector (sub-cloning was performed). To confirm the ligation of gene happened in trigger vector happened or not, the ligated plasmid was transformed in the DH5α cells and screening was performed of the obtained colonies. The colony positive for screening was obtained for Rho13 was obtained, where the restriction digestion was performed again with *Sma1* and *Xho1* restriction enzymes. The right side agarose gel picture indicates the lower band on the gel, Rho13 gene of 764 base-pairs and upper band on the gel, trigger vector of 1275 base-pairs when compared with ladder.

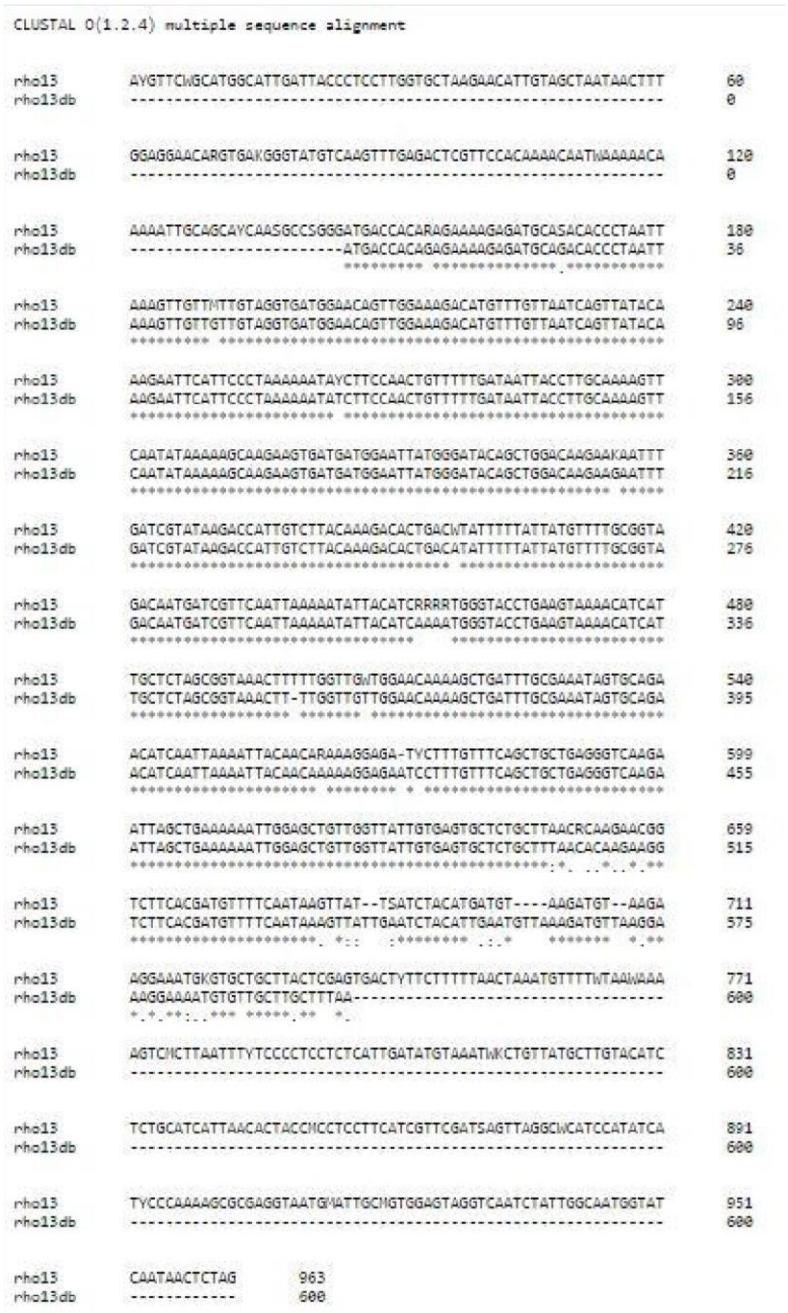


Figure-3: Sequencing of the Rho 13 in trigger vector construct. The rho13db depicts the original sequence taken from amoeba database AMOEBADB while rho13 is the construct of which the sequence was checked.

- The colony positive for screening was sent for sequencing. For which, plasmid was isolated from the same colony positive for screening was sent for sequencing to confirm the sub-cloning of gene of interest (Rho13 gene) from pEhEx vector to Trigger vector. Figure-3 indicates the sequencing results where the rho13database depicts the original sequence taken from amoeba database AMOEBADB while rho13 is the construct of which the sequence was checked. The alignment results were positive since most of the nucleotide matched indicated by the asterisk mark in the figure. Thus, sub-cloning of Rho13 gene in the Trigger vector was successfully performed.

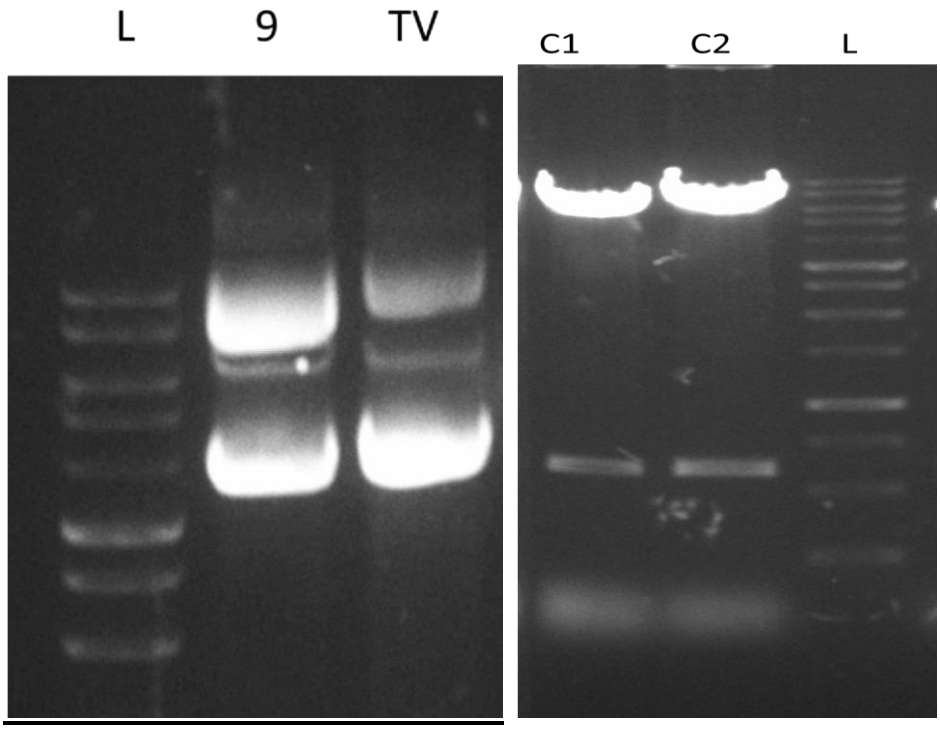


Figure-4: Plasmid isolation of Rho 9 and Trigger vector. The second gel shows the colony screening where C1 and C2 both are positive for digestion. The lower bands show the gene of interest. It was then send for sequencing.

- Aiming to sub-clone the other gene, Rho9 the same way I did for Rho13 gene. First thing I did was to isolate the plasmid with pEhEx vector with Rho9 gene insert, the left agarose gel picture shows isolation of plasmid was efficient. After isolating plasmid, restriction digestion was performed in order to get the gene of interest (Rho9 gene) from the overexpression vector (pEhEx); using the two restriction enzymes: *Sma1* and *Xho1*. The restriction site these two enzyme recognizes are as mentioned in table-2. After restriction digestion, once gene of interest is obtained, it was ligated in Trigger vector (sub-cloning was performed). To confirm the ligation of gene happened in trigger vector happened or not, the ligated plasmid was transformed in the DH5α cells and screening was performed of the obtained colonies. Two colonies positive for screening was obtained for Rho9, where the restriction digestion was performed again with *Sma1* and *Xho1* restriction enzymes. The right side agarose gel picture indicates the lower band on the gel, Rho9 gene of 702 base-pairs and upper band on the gel, trigger vector of 1275 base-pairs when compared with ladder.

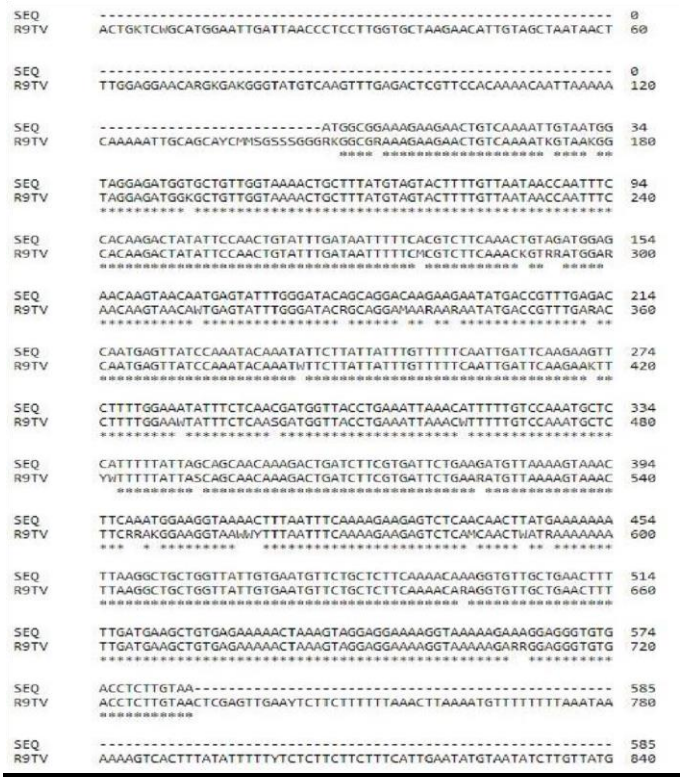
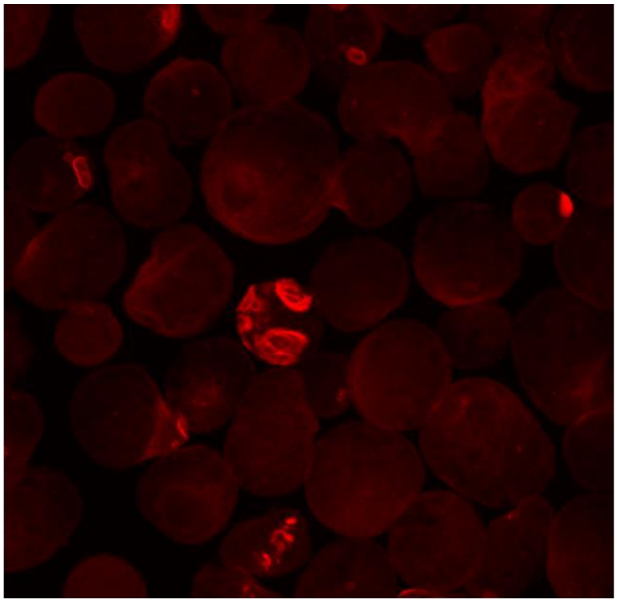
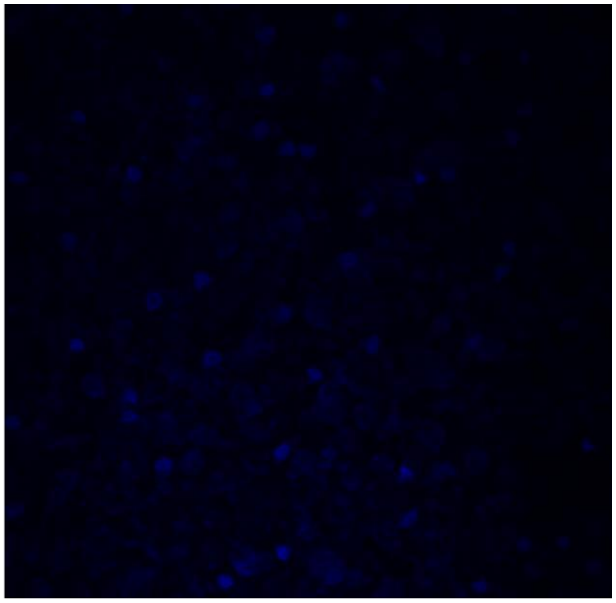


Figure-5: Sequencing of the Rho 9 in trigger vector construct. The SEQ depicts the original sequence taken from amoeba database AMOEBADB while R9TV is the construct of which the sequence was checked.

- One colony positive for screening was sent out of the two, for sequencing. For which, plasmid was isolated from the same colony positive for screening was sent for sequencing to confirm the sub-cloning of gene of interest (Rho9 gene) from pEhEx vector to Trigger vector. Figure-5 indicates the sequencing results where the Rho9database depicts the original sequence taken from amoeba database AMOEBADB while Rho9 is the construct of which the sequence was checked. The alignment results were positive since most of the nucleotide matched indicated by the asterisk mark in the figure. Thus, sub-cloning of Rho9 gene in the Trigger vector was successfully performed.

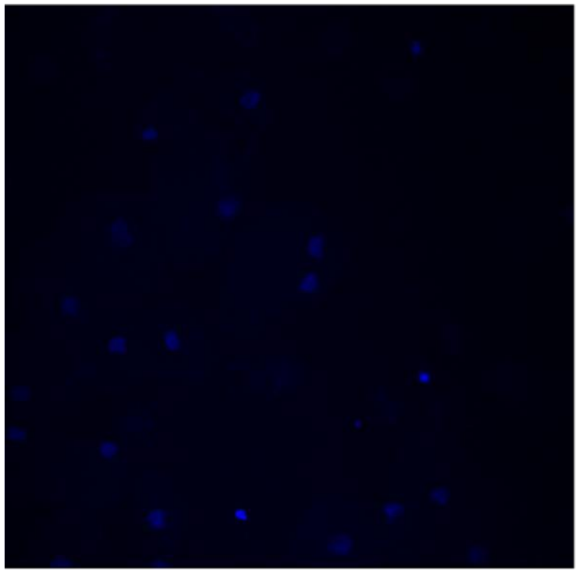


Phalloidin Staining

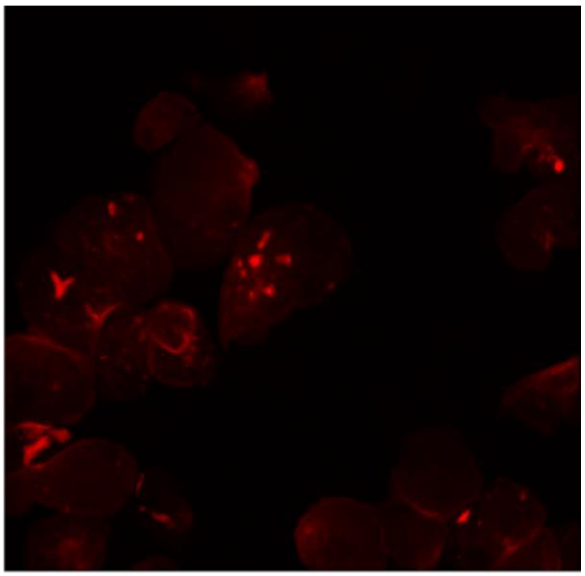


DAPI Staining

Figure-6: The control empty vector cells on fibronectin showing no actin dot formation.



DAPI Staining



Phalloidin Staining

Figure-7: The R5KD cells on fibronectin showing no actin dot formation but the formation of some different kind of stuctures. But since it was not repeated it is not sure that this is not an error but a real phenomenon.

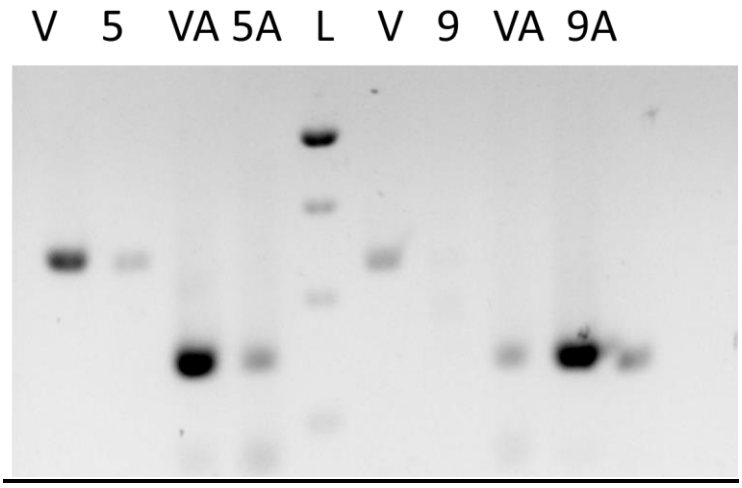


Figure-8: Rho 5 KD was available earlier and was shown to have a 30% knockdown efficiency. I tried checking it again by doing an RT-PCR of both Rho 5 and 9. Although the result was unsatisfactory. It has to be repeated again. Here, in this figure, V is empty vector, 5 is Rho 5KD, VA is actin control for vector, 5A is actin control for Rho 5KD, L is Ladder, 9 is Rho 9KD, 9A actin control for Rho 9KD

❖ **The inferences from all the experiments:**

- The knockdown cell lines of Rho5, 9 and 13 were made.
- The actin dot assay has to be repeated again. The RT-PCR is to be done again.

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