Matrix stiffness dependent regulation of Golgi organization

A THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

OF

BS-MS DUAL DEGREE

BY

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Certificate

This is to certify that this dissertation entitled "**Matrix stiffness dependent regulation of Golgi organization"** towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by "**Vishakha Kasherwal at IISER PUNE"** under the supervision of "**Dr Nagaraj Balasubramanian**, Associate Professor", Biology Division, IISER PUNE during the academic year 2017-2018.

Dr Nagaraj Balasubramanian

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Declaration

I hereby declare that the matter embodied in the report entitled "**Matrix stiffness dependent regulation of Golgi organization**" are the results of the work carried out by me at the Department of Biology at Indian Institute of Science Education and Research, Pune under the supervision of Dr.Nagaraj Balasubramanian and the same has not been submitted elsewhere for any other degree

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Abstract:

Cell-matrix adhesion plays an important role to regulate anchorage-dependent signaling. Earlier studies in the lab have shown integrin-dependent cell-matrix adhesion regulates Arf1 activation to control Golgi organization. The Golgi complex plays an essential role in sorting and processing membrane proteins. In this study, I look at the role AMPK and ceramide synthase could have in mediating adhesion-dependent Golgi organization. Integrin-mediated adhesion and signaling have been seen to be distinctly regulated by matrix stiffness and crosslinking in 3D microenvironments. Our studies have shown cells grown in 3D collagen gels of varying concentration and stiffness to differential regulate endocytosis (unpublished data). In this study, we tested and identify the Golgi organization to be distinctly regulated by changing stiffness and crosslinking in 3D collagen gels. Using Caveolin1 lacking WTMEFs I have further tested the role of Caveolin1 has in regulating matrix dependent Golgi organization in 3D gels. The results from these studies show ceramide synthesis to play a role in regulating the adhesion-dependent Golgi organization and further reveal 3D collagen organization and stiffness to control Golgi organization in a caveolin-1 dependent manner.

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1.Introduction:

1.1 Extracellular matrix:

Extracellular matrices (ECM) are composed of plethora of secreted molecules like glycoproteins, collagen, glycosaminoglycan, proteoglycans which comprises the microenvironment of the cell and helps in preservation of the shape of tissues (Dalton et al., 1995). In vitro, most animal cells are anchorage dependent, where cells grow only after they attach to the substrate through ECM. One of the most abundant primary protein present in the entire body and ECM is collagen. Structure and chemical composition of microenvironment can regulate behavior of the cell by connecting intracellular and extracellular cues like growth factors (Ehrbar et al., 2011). As a consequence, it has been suggested cells have an ability to sense the microenvironment which occurs at the cell-ECM interface, especially through association of integrin assembly of ECM proteins such as collagen, fibronectin which allows cells to respond against mechanical cues. Most of the mechanical properties of tissue depending on the nanostructure of these collagen fibers and its interactions. The fibrous structure of the gel is stabilized by the by multiple posttranslational modifications which permits the formation of intermolecular crosslinking. Alteration in ECM stiffness may lead to the development of diseases, such as cancer and fibrosis. Since all the cells are embedded within a 3D microenvironment with cells all around it (Kim et al., 2011)(Wells, 2008)(Katz et al., 2000). Various methods are used to grow cells and mimic 3D microenvironment like cell-derived matrices, matrigel, and collagen gels which is one of the abundant protein of the ECM and 2D polyacrylamide gels of varying stiffness.

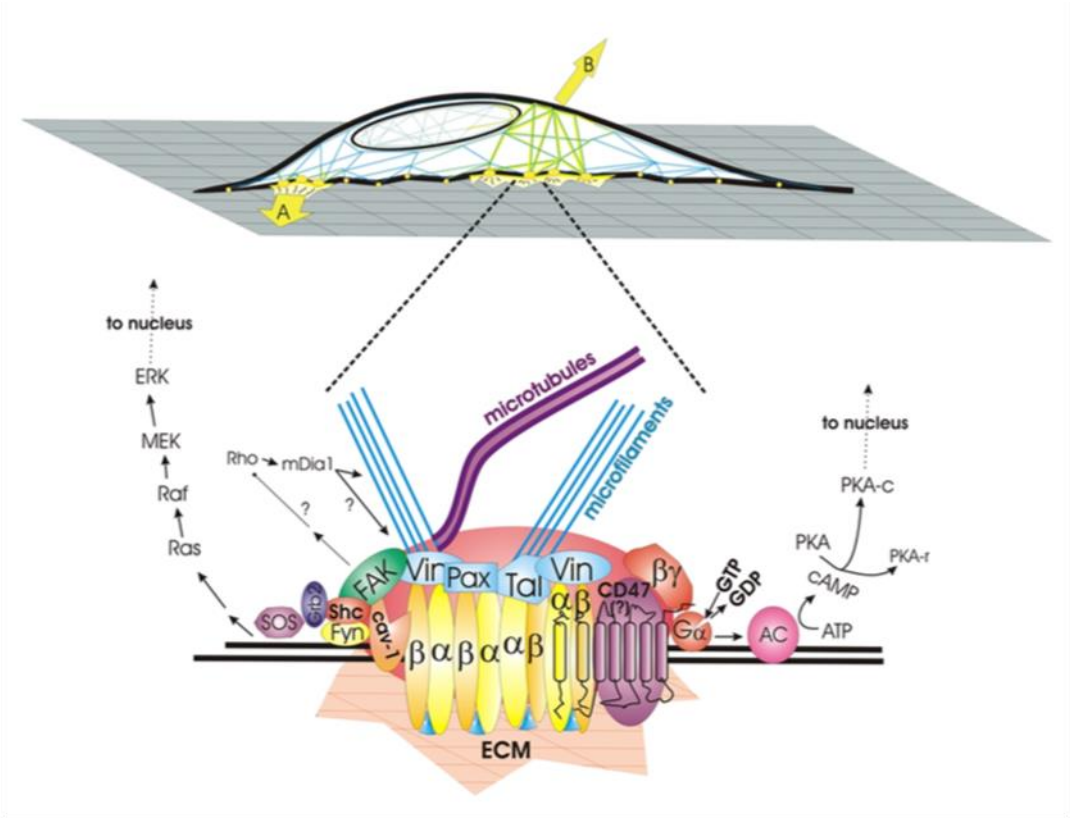


Figure 1. ECM and its regulation of forces. ECM applies force directly to the cell and allow the integrin clustering and FA complexes which in turn modulates the cytoskeletal filaments and regulate downstream signalling pathways which determines the behaviour of the cell like proliferation, migration, spreading , survival etc. (Alenghat and Ingber, 2002)

1.1.1.2D microenvironment:

Cells grown on 2D gel substrate includes adhesion of the cell with the ECM protein (Collagen) coated on top of the gel via integrin clustering that induces other focal adhesion molecules to assemble and help in sensing the chemical properties of the matrix and converting into biological signals which regulates the cell behaviour like cell proliferation, migration and spreading etc.(Wells, 2008) Cells on 2D gel substrate incudes attachment only from one side of the cell which allows the cell to sense the force only from one side of the cell i.e. integrin signalling is unidirectional and also it determines the apical-basal polarity of the cell. Migration on 2D surfaces involves the following highly coordinated steps: extension of leading edge, the formation of

adhesive contact with the surface, generation of traction force and subsequent retraction of the trailing edge. Effects of substrate stiffness in regulating behaviour of the cells has been conceded by many studies in literature. Most cells are adherent and require adhesion for survival called anchorage dependence where they adhere via integrin following FA complexes (Baker and Chen, 2012; Trappmann and Chen, 2013).The porous nature of polyacrylamide gels allows the supply of all the nutrients to the cell. Stiffer substrates with minimal pore size offer more anchorage points to the cell and help in integrin clustering and other FA complexes for further downstream processes like spreading, migration etc. compared to the soft substrate where pore size is big and doesn't allow the cell to make adhesion complexes. As a consequence of it, cell spreading is known to be known on stiffer compared to the soft substrate.(Byfield et al., 2009)

1.1.3 .3D microenvironment:

Cells in our body are embedded within tissues which not only the cells to withstand stress but also regulate their behavior like spreading, proliferation, migration. The most abundant protein present in our body is collagen. Various methods are used to grow cells in 3D like cell-derived matrices, matrigel and one of the most abundant components of the ECM, Collagen Gels (Ehrbar et al., 2011). ECM proteins have a capability to self-organize itself in a fibrous structure in which cells can be embedded within the gels thus mimic in vivo environment. Matrix stiffness of the gel is basically controlled by the crosslinking of the matrix which in turn impacts integrin signaling hence behavior of the cells like proliferation. Cells in 3D microenvironment are embedded where it senses the signaling from all the directions i.e. integrin is multidirectional which in turn regulate the cell behavior very distinctly from 2D microenvironments. Despite being a physiological system to study cell behavior, 3D has its own disadvantages and limitation of increasing stiffness and crosslinking compared to the 2D microenvironment. There are few points which give us the comparison between 2D and 3D microenvironments. (Wells, 2008),(Baker and Chen, 2012; Trappmann and Chen, 2013)

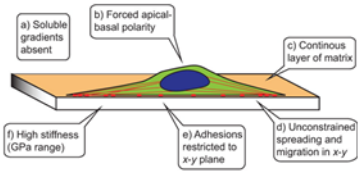
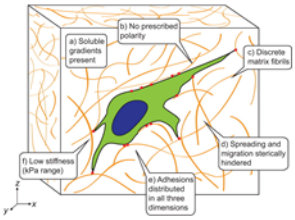
2D POLYACRYLAMIDE GEL	3D COLLAGEN GEL
	
• Cells spread on attachment • Cell adhesion is more robust • More focal adhesions visible • More polarized (Apico-Basal) • Cytoskeletal organization more robust • Possible to control stiffness without changing ECM concentration • Less physiological	• Cells make protrusions and don't spread • Cell adhesion seems less robust • Very few and small focal adhesions seen • Less polarized (Apico- Basal) • Cytoskeletal organization less robust • Stiffness is linked to ECM concentration and we cannot segregate both here • More physiological

Figure 2. Comparison between 2D and 3D gel: Here are the various differences listed out in the table which compares 2D and 3D environment and how does it regulate cell behavior and function.

1.2. Mechanotransduction:

The ability of the cell sensing the extracellular matrix and responding towards it. This process is known as "Mechanotransduction" which plays a very role in regulating the cell behavior like spreading, proliferation etc. (Provenzano et al., 2009)(Alenghat and Ingber, 2002). Cell senses the chemical properties of the substrate and respond against it accordingly and convert it into biological signals.(Discher, 2005) There are various known regulators of mechanotransduction includes adhesion receptors

includes integrins, FA (focal adhesion) complexes, caveolin-1(Solon et al., 2007)(Ehrbar et al., 2011).

1.2.1 Integrins:

Integrins consist of heterodimeric transmembrane receptors which is comprised of eighteen α(alpha) subunits and eight β(Beta) subunits. These can be covalently assembled into 24 possible combinations. Integrin clustering gets affected by the ECM composition like stiffness and hence regulates the cytoskeleton and control its behavior. (Hynes, 1992).

Integrins recognize Arg-Gly-Asp (RGD) motif and allow cell to attach and activates other downstream signaling. Later other ECM proteins like vinculin, talin, etc. are also know to recognize this motif during adhesion. One of the crucial receptors required for cell adhesion and migration are integrin where they attach to the cytoskeletal structure through Focal adhesion complex (FA) complex (Hynes, 1992)(Frisch and Ruoslahti, 1997)(Damsky and Ili, 2002). Talin initiates the assembly of focal adhesion complexes and later the synergistic effects of talin and integrin upon activation was further enhanced by binding to vinculin which triggers the further clustering and promotes adhesion. The advancement in adhesion complex upon vinculin recruitment leads to the formation of larger FA complexes and conformational change in integrin allows interactions with ECM ligands to a greater extent (Damsky and Ili, 2002). There are various factors which can control integrin signalling either from outside or inside of the cells that persuade changes in extracellular region of integrin to modulate its conformation in such a way which strengthens the ligand interactions. Integrin affinity towards extracellular ligands can be regulated from exterior of the cell and this process is termed as “Outside-in” signaling (Shyy and Chien, 2002)(Kim et al., 2011).The most known regulators are divalent cations, monoclonal antibodies and integrin ligand themselves whereas the process where integrin affinity is regulated from intracellular region of the cell itself, is known as “Inside-out” signalling and this type of signalling is reported in intracellular regulation of affinity in β1, β2 and β3 integrin families (Aplin et al., 1998)

Apart from the integrin-mediated assembly of cytoskeletal linkages, integrin association triggers a plethora of signaling pathways that allow modulation of several

aspects of the cell behavior including proliferation, survival or apoptosis, shape, polarity, migration and differentiation (Fielding et al., 2008)m(Dalton et al., 1995). Integrin-mediated signaling is really crucial to block apoptosis vis PI3K-kinase and Akt pathway and also stimulate cell cycle progression via ERK and cyclin D1 (Moreno-Layseca and Streuli, 2014)(Schwartz and Assoian, 2001).

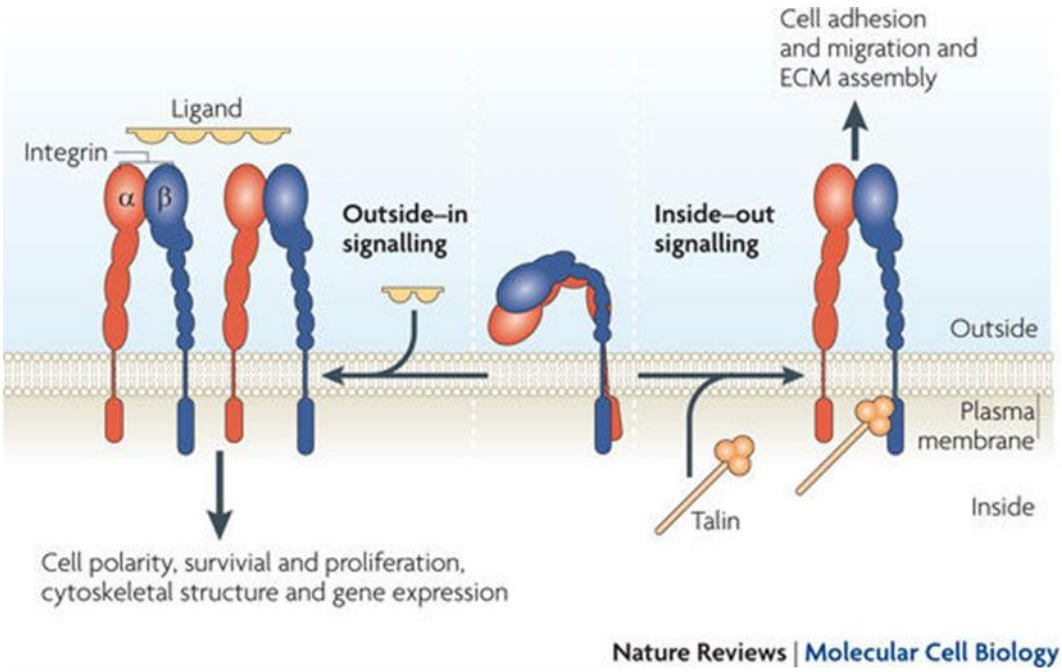


Figure 3. Integrin signalling upon activation. Integrin is one of the most common adhesion receptors of the ECM which connects intracellular environment of the cell to the extracellular compartment. This Integrin clustering can be regulated either form inside the cell which is known as "Inside-out signalling" whereas signalling can be regulated from outside as well which is known as "Outside-in signalling" which in turn activates downstream signalling pathways and coordinate cell behaviour. (Shattil et al., 2010)

1.2.2 Caveolin:

Caveolin-1 is also known to play a role in lipid transport, membrane trafficking, and cell signaling. Caveolin-1 is a crucial player in plasma membrane organization, transmission of signaling from outside to the interior of the cell and help in the remodeling of the extracellular environment (Shvets et al., 2014). Caveolin synthesis involves both ER (Endoplasmic reticulum) and Golgi apparatus. As a result, it is synthesized in a form of transmembrane protein in ER and then transported to the cell surface via Golgi apparatus. Most of the fraction localizes at the Golgi whereas in some cell types, it is mostly localized at the cell surface. Addition and removal of cholesterol accelerate and slow down the caveolin1 transportation from Golgi apparatus. Glycosylphosphatidiylinositol (GPI)-anchored proteins179 are involved in the exit of CAV1 from the Golgi apparatus whereas CAV1, although efficient GPI anchored-protein delivery does not require CAV1 to be present. There are various diseases known which are caused by mutations in caveolin1 which affects post-Golgi trafficking and results in the formation of reduced caveola formation at the cell surface.

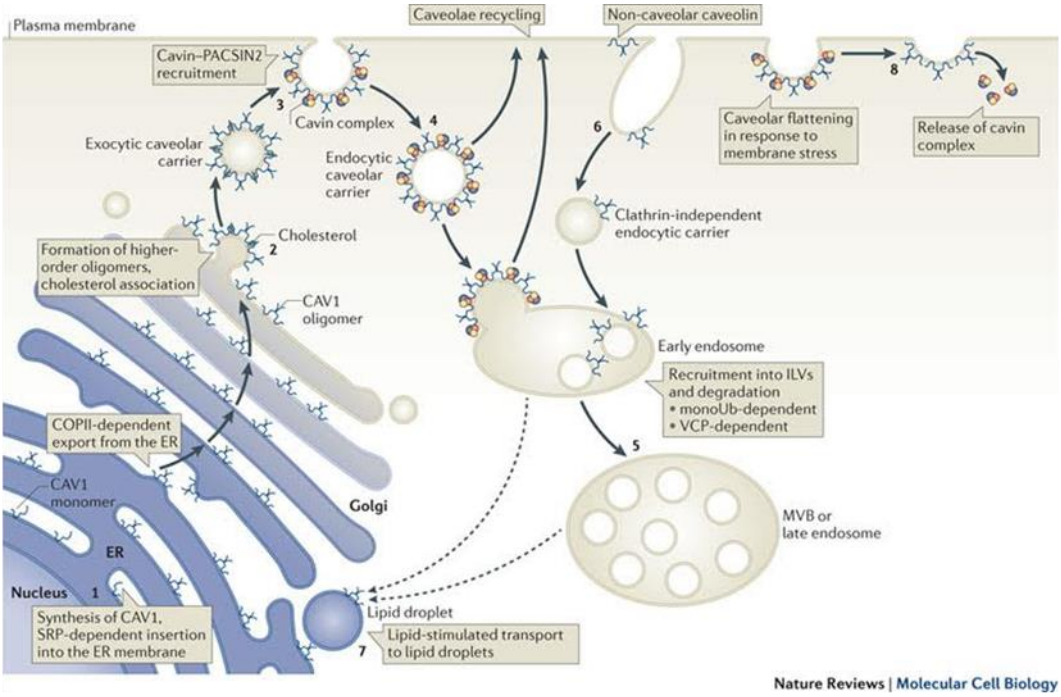


Figure 4. Role of Caveolin1 : Membrane trafficking of caveolins through exocytosis and endocytosis. Schematic showing the itinerary of caveolin 1 (CAV1) from synthesis in the rough endoplasmic reticulum (ER) and its trafficking through the Golgi complex to the cell surface.

1.3. Golgi:

Golgi is an intramembranous structure present in all eukaryotic cells. Golgi apparatus plays a key role in trafficking pathways, biosynthesis of lipid, modification, sorting and transportation of proteins. Golgi undergoes extensive fragmentation during the physiological process like mitosis, meiosis, cell division which is required for efficient portioning of Golgi into daughter cells that are associated with Golgi and pathological processes such as disease condition like cancer. (Shorter and Warren, 2002)(Colanzi et al., 2003)

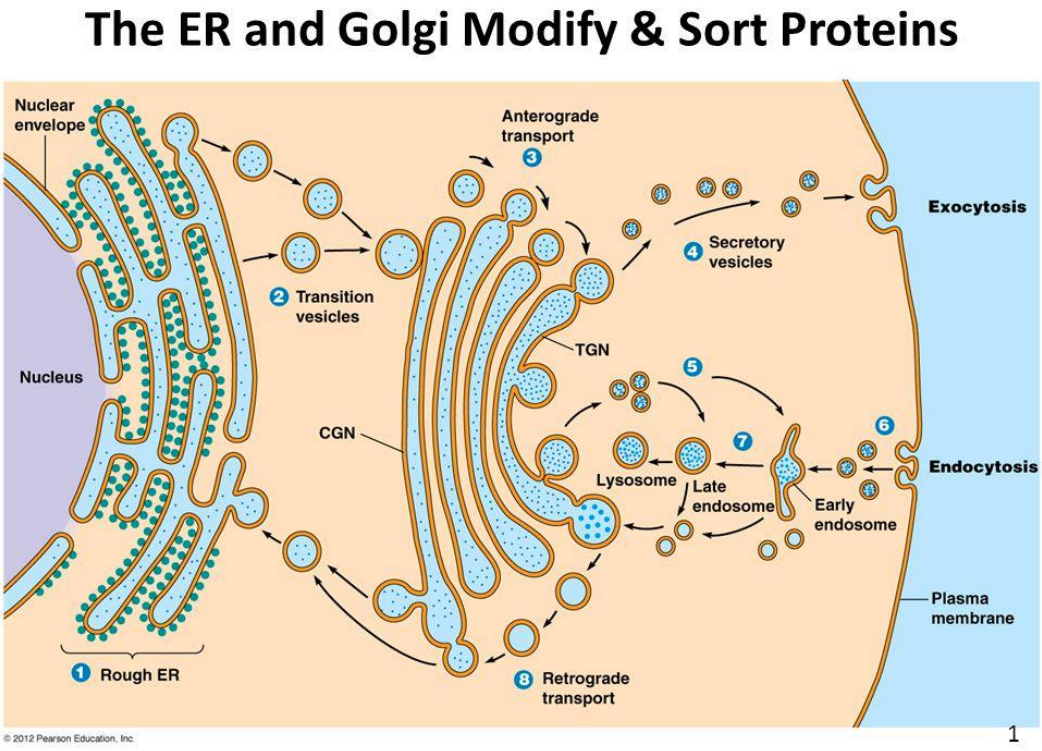


Figure 5. Role of the Golgi in cellular processes. The golgi is membranous structure which is composed of cis, medial and trans face. Golgi plays a crucial role in modifications of proteins, synthesis of lipid, secretion and form membrane bound vesicles of the material already synthesized in ER. The three main destinations of the processed proteins and lipids from the Golgi are lysosomes, plasma membrane and extra cellular secretion. Moreover, Golgi apparatus in mammalian cells fragments into vesicles at the start of the mitosis.

Golgi consists of cis, the medial and trans-compartment where proteins enter to the Golgi from the cis side and undergo modification in medial compartment and finally transported outside of the Golgi apparatus from the trans-Golgi network to ER. There are various regulators which are known to regulate the structure of Golgi incudes Small GTPases, Cytoskeleton, motor proteins, Golgins and GRASPS.(Andreeva et al., 1998)(Farhan and Hsu, 2016)

Role of small GTPase, Arf1 has already been shown in the lab in regulating Golgi fragmentation in an adhesion-dependent manner. GBF1, Guanine nucleotide

exchange factor is one of the regulators of Arf proteins and various studies suggested that GBF1 remains intact with Golgi and behaves as Arf-GEF that promotes the Golgi structure to remain intact. Phosphorylation of GBF1 leads to its activation through 5' AMP-activated protein kinase (AMPK) pathway and results in loss of GBF1 and Golgi complex that consequently lead to the Golgi fragmentation. Previous studies in the lab have shown that AMPK might also regulate Golgi organization. These regulators help in maintaining the Golgi structure and allow the completion of Golgi mediated cellular functions. (Altan-Bonnet et al., 2003)(Mihaylova and Shaw, 2011)

Hypothesis and Objectives of the thesis:

Hypothesis: Matrix stiffness plays a role in regulating the Golgi organization.

Objectives:

1. Confirm the adhesion-dependent regulation of Golgi organization.

2. Determine the role of AMPK in regulating adhesion-dependent Golgi organization.

3.Determine the role of ceramide in regulating adhesion-dependent Golgi organization.

4.Determine the regulation of Golgi organization in 3D collagen gels of varying stiffness.

5. Determine the role of Caveolin-1 has as a possible mechanotransducer in sensing 3D collagen matrix stiffness to regulate the organization of the Golgi.

6. Determine the effect changing stiffness in 2D gels has on Golgi organization.

2. Materials and methods:

2.1. MATERIALS:

2.1.1. Reagents:

DMEM (5%FBS and 0.2%FBS), Trypsin-EDTA and PBS, OPTIMEM was purchased from Invitrogen , PEI, Paraformaldehyde (PFA) was purchased from Sigma, Fluoramount, Glycerol, NAOH, Acrylamide, Bis-Acrylamide, Sulpho-SANPAH, DMSO was purchased from SIGMA, Comp C, FB1, Tetramethylethylenediamine (TEMED), Ammonium Persulfate (APS) from Abcam, Lamelli buffer, Collagen1 Rat-tail was purchased from Corning.

2.1.2. Antibodies:

Antibodies used for western includes Akt at a dilution of 1:1000, at a dilution of 1:2000 and actin at a dilution of 1:2000

Secondary antibodies conjugated with HRP were purchased from Jackson Immunoresearch and were used at a dilution n of 1:10000.

Antibodies used for immunofluorescence include Phalloidin Alexa fluor 594 and 488 at a dilution of 1:400.

2.1.3. Plasmids:

RFP tagged GalTase constructs were obtained from Dr.Jennifer Lippincott lab (Janelia Farms).

2.2 METHODS

2.2.1. Cell culture and transfections:

Mouse embryonic fibroblasts (MEFs) and Cav1 KO-MEFs were obtained from Dr. Richard Anderson (University of Texas Health Science Centre, Dallas TX) were cultured in complete Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) with 5% Fetal bovine serum (FBS) and penicillin and streptomycin (Invitrogen) at 37^{o}C in a 5% CO_2 incubator. MEFs were transfected using PEI. Transfections were done in a

6cm dish with 4µg DNA using PEI for 24hrs and then serum starved in DMEM with 0.2% FBS for 12hrs for suspension assay. Wild-type Mouse embryonic fibroblasts and Cav1 KO-MEFs were transfected using PEI and culture in DMEM with 5%FBS for 24hrs and then used for embedding in 3D collagen gel.

2.2.2. Transfection:

Mouse embryonic Fibroblasts- WTMEFs and Cav1 KO-MEF's were transfected with GalTase, Trans Golgi marker. 1X10^5 WTMEFs and Cav1 KO-MEFs were seeded in a 60mm petri dish, cultured in DMEM (5%FBS) for 12hrs. Fresh DMEM (5% FBS) was added and transfected with 4µg DNA (2µg/µl) using 12µl PEI in 450µl OPTIMEM (transfection medium) and incubated for 30min at RT. Post incubation, the mix was added to cells, incubated for 24hrs, then serum starved in DMEM (0.2%FBS) for 12hrs. Cells were further processed for suspension assay.

2.2.3. Suspension assay and re-adhesion of cells:

Mouse embryonic fibroblasts were grown in complete DMEM with 5% FBS to 70-80% confluency and then cultured in DMEM with 0.2%FBS for 12hrs, detached using Trypsin (Invitrogen) for 5min, neutralized with DMEM (0.2%FBS). Cells were held in suspension for 120min (120'SUS) with 2% methylcellulose. Post incubation cells were washed with DMEM (0.2%FBS) and spun down at 1,350 rpm at 4^{o}C for 10min, re-plated on fibronectin (10µg/ml) coated dishes for 5min and 4hrs, lysed using 1X Lamelli buffer and processed further for Western blotting. Suspension assay for WTMEFs transfected with GalTase was carried out in a similar fashion except the cells were re-plated on fibronectin (2µg/ml) coated coverslips for 5min (FN5') and 4hrs (SA).

2.2.4. Western blot protocol:

Cells were lysed in 1X Laemli buffer and cell equivalent volumes of lysates were resolved by SDS PAGE (12%, 1.5 mm gels), transferred to PVDF membrane and blocked with 5%non-fat dry milk in TBS+0.5% Tween-20 (TBS-T). Blots were probed with primary antibodies overnight at 4°C. Concentrations of primary antibodies were : Akt - 1:10,000, pAkt - 1:500, GAPDH - 1:5000, made in 5% BSA. Following the respective secondary antibody incubations (done at room temperature for 60

minutes), blots were developed using chemiluminescent substrates from Thermo-Fischer using the LAS 4000developing system (Fujifilm-GE). Densitometric analyses of blots were done using Image J software (NIH) to calculate the pAkt/total Akt ratio.

2.2.5. Confocal Imaging:

GalTase transfected MEFs were fixed using 3.5%PFA, washed twice with 1X PBS, mounted with fluoramount for required timepoints (120'SUS, FN5' and SA). For confocal imaging, the sample was imaged using 63x oil immersion with 2% laser power at 1AU (Pinhole) with gain around 700-900 approximately. Z-stacks were taken using confocal microscope at 0.2µm intervals whereas we go from top to bottom at a scan speed of 8 per µm at zoom size 6.

2.2.6. Analysis using Huygen's Software:

2.2.6.1. Deconvolution:

Z-stacks of GalTase transfected WTMEFs taken at confocal was processed for deconvolution with following settings:

Iterations-30, Threshold-0.0001, Signal to Noise ratio (SNR)-20 and Background estimation radius – 1.

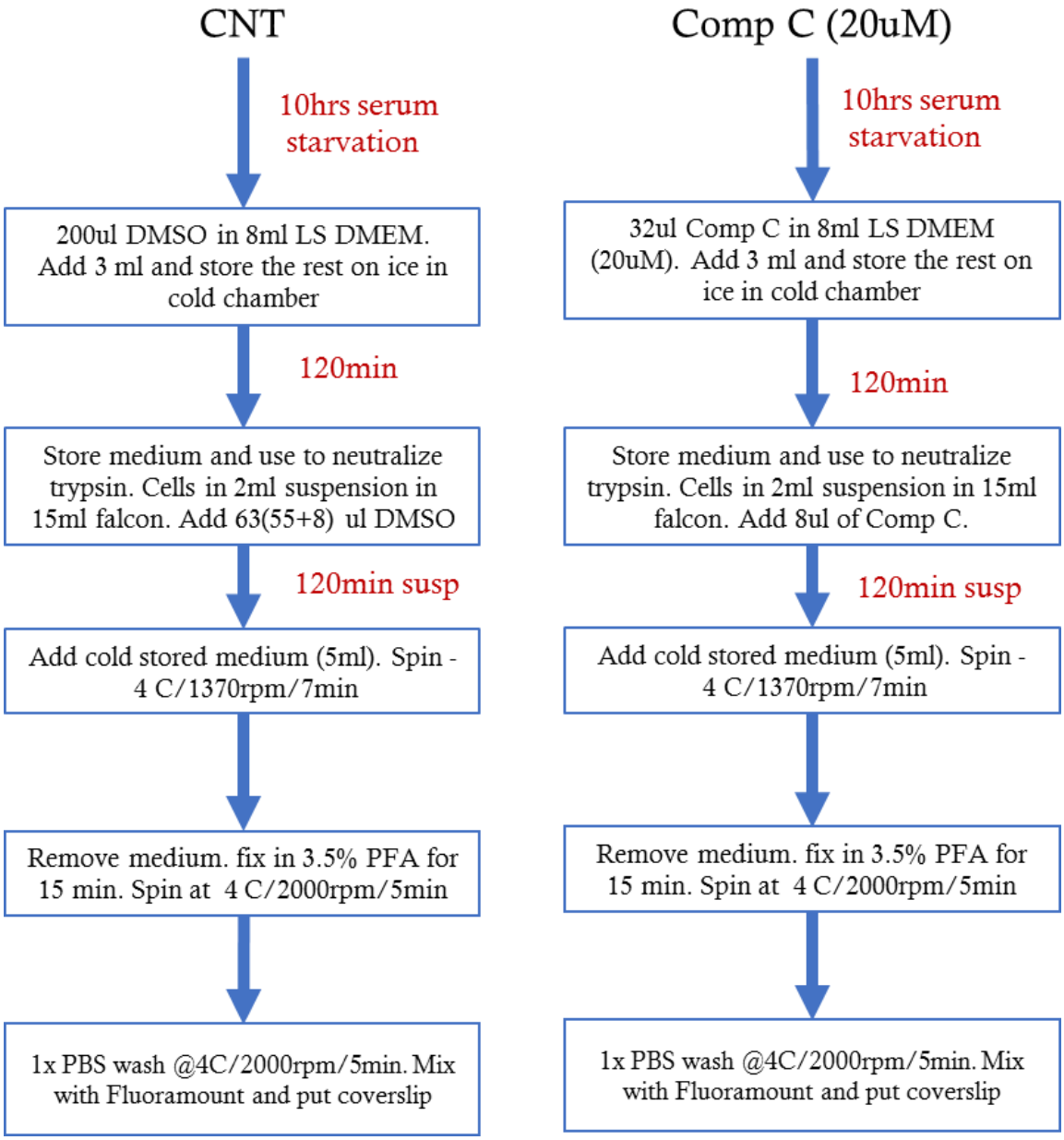
2.2.6.2. Counting Golgi object:

Deconvoluted images of confocal z-stacks were processes in Huygen's software for quantifying Point spread function (PSF) with the threshold set to 50%, seed untick and Garbage value set to 1. For quantifying Golgi objects threshold was set to 15%, seed untick and garbage value was set to the calculated PSF value.

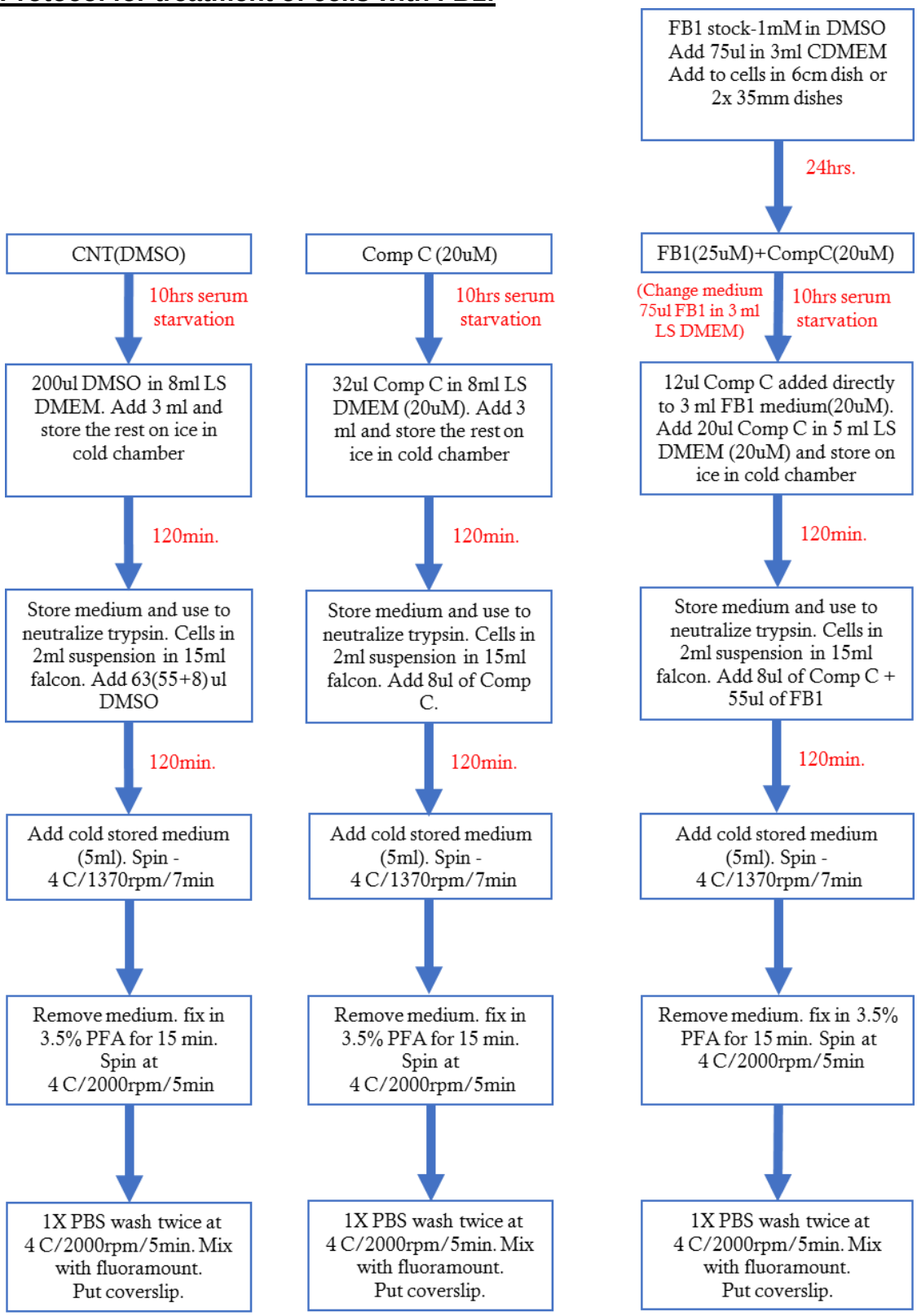
2.2.6.3. MIP and Surface rendered Images:

Deconvoluted z-stacks were processed further for preparing Maximum intensity progression (MIP) and Surface rendered images with the threshold set to 15%, zoom set to 1.51, tilt set at 0, seed untick, Garbage value was set at calculated PSF and frame size was set at 1024 X 768.

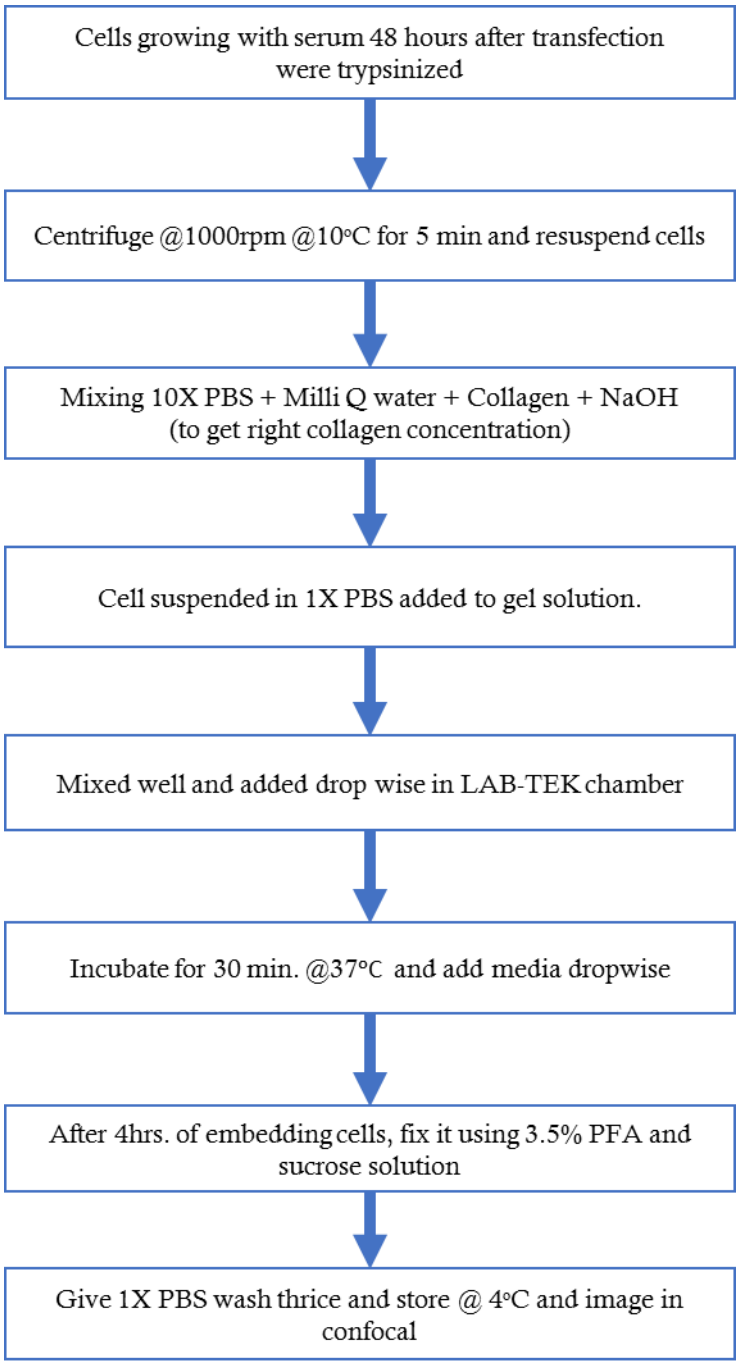
2.2.7. Protocol for treatment of cells with Compound C:



2.2.8. Protocol for treatment of cells with FB1:

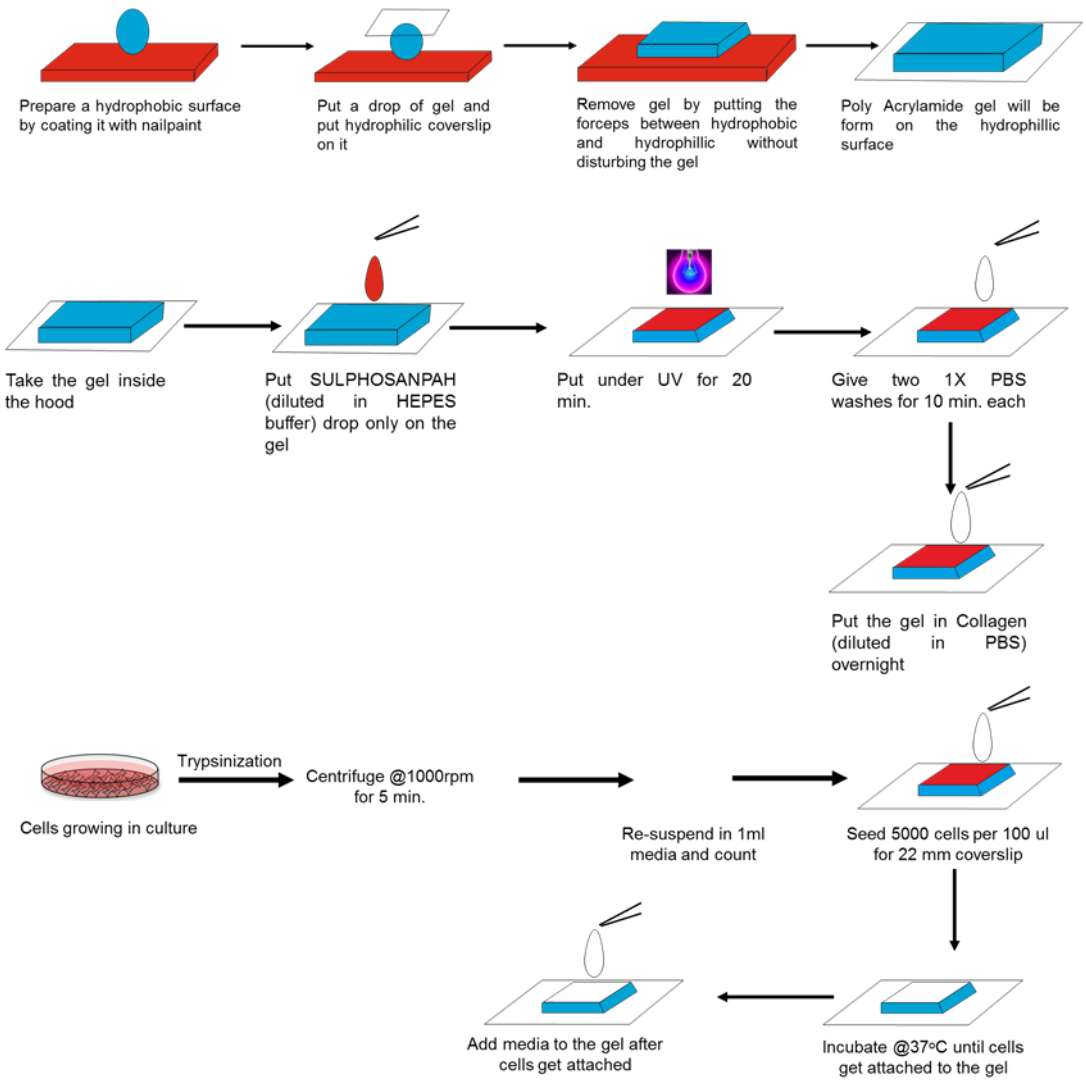


2.2.9. Protocol for preparing 3D collagen gel of varying stiffness:



2.2.10. Protocol for preparing 2D Polyacrylamide gel of varying stiffness:

40% Acrylamide and 2% BIS-Acrylamide solution were prepared in MQ water. Mixing 40%Acrylamide, 2%Bis-Acrylamide, 10%APS and TEMED to get proper stiffness. Coverslips were made hydrophobic using Toluene: Silane (1:9) ratio.



2.2.11. Culturing cells on 2D Polyacrylamide gels of Varying stiffness:

WTMEFs were cultured cells in DMEM (5% FBS) up to 60% confluency, detached with Trypsin-EDTA (Invitrogen) for 5min, neutralized in DMEM (5%FBS), spun down at 1000 rpm at 10°C for 5 min. Cells were re-suspended in DMEM(5%FBS) and plated

on Collagen coated gels of varying stiffness (0.5kPa, 2kPa, 5kPa, 10kPa, 20kPa and CNT (Coated with collagen). Cells were attached within 45min then cultured in DMEM (5% FBS) for 6hrs and 24hrs for cell spreading analysis and Phalloidin staining respectively.

2.2.11.1. Cell spreading Analysis:

WTMEFs were cultured on gels of varying stiffness (0.5kPa, 2kPa, 5kPa, 10kPa, 20kPa and CNT (Coated with collagen) in DMEM (5%FBS), performed live imaging on EVOS microscope for 6hrs. Post-imaging cells, area analysis was done using Image J software.

2.2.11.2. Actin staining:

WTMEFs were cultured on gels of varying stiffness (0.5kPa, 2kPa, 5kPa, 10kPa, 20kPa and CNT (Coated with collagen) in DMEM (5%FBS), incubated for 24hrs. at 37^{o}C in 5% CO_2 incubator. Post incubation cells were permeabilized using 1% Triton-X for 2min, fixed using 3.5%PFA for 5min, blocked in 2%BSA for 20min and stained for Actin (Alexa flour 488 diluted in 2%BSA (1:400)) overnight at 4^{o}C. Post incubation washed twice using 1X PBS for 5min each. Gels were mounted using fluoramount, imaged on EVOS microscope at 40X resolution.

3. Result:

3.1. Role of adhesion in regulating Golgi architecture. Possible role of AMPK?

3.1.1 To confirm the role of Adhesion in regulating Golgi organization:

To determine if adhesion plays any role in regulating Golgi organization, studies in the lab have corroborated that serum-starved WTMEFs upon loss of adhesion that is a loss of Integrin-mediated signaling, triggered loss of Akt activation in suspended cells whereas it recovers Akt activation upon re-adhesion on the fibronectin-coated coverslip. I have also confirmed the role of adhesion in regulating Akt activation where we have used serum starved WTMEFs in order to negotiate the role of growth factors and to look the role of adhesion fully. Cells were detached using Trypsin for 5min and held in suspension for 2hrs (120'SUS), re-plated them on fibronectin (10µg/ml) coated dishes for 5min (FN5') and 10min (FN 10'). Cells were lysed using 1X laemilli's for required time points. We confirmed the Akt activation by western blotting by probing for Akt, pAkt, and GAPDH and we have observed that upon loss of adhesion Akt activation is very low and again recovers its activation upon re-adhesion which corroborates that adhesion does play a role in integrin-mediated signaling (Figure 6). Simultaneously, Adhesion was observed to play a role in regulating Golgi architecture upon loss of adhesion and re-adhesion. We have used serum starved WTMEFs transfected with GalTase tagged with RFP in order to confirm the role of integrin-mediated adhesion in regulating Golgi architecture and negotiating the possibility of growth factors. Cells were detached using Trypsin-EDTA and held in suspension for 2hrs (120'SUS) using 2% Methylcellulose, re-plated on Fibronectin (2µg/ml) for 5min (FN5') and 4hrs (SA). Cells were washed with DMEM (0.2%FBS), fixed using 3.5% PFA and mounted using fluoramount and processed further for confocal imaging. We imaged the Golgi architecture using confocal microscopy where we have counted cells with disorganized and organized phenotype for required time points and further processed for deconvolution for quantifying Golgi objects and represented as Maximum intensity projection (MIP) and Surface rendered (Figure 7A). We have observed that most of the cells have completely disorganized phenotype in suspended cells that is Golgi undergoes complete disorganization upon of loss of adhesion and

regain its intact phenotype upon re-adhesion on fibronectin-coated coverslip within five minutes or less where most of the cells have organized phenotype (Figure 7B) which was further confirmed by counting the Golgi objects for the same and we have observed that cells held in suspension for 2hrs (120'SUS) have more no. of objects as compared to the re-plated WTMEFs (Figure 7C). Statistical analysis was done using Mann-Whitney test which shows a significant reduction in Golgi disorganization upon re-adhesion as compared to the loss of adhesion. This data has corroborated that adhesion plays a role in integrin-mediated downstream signaling and Golgi organization.

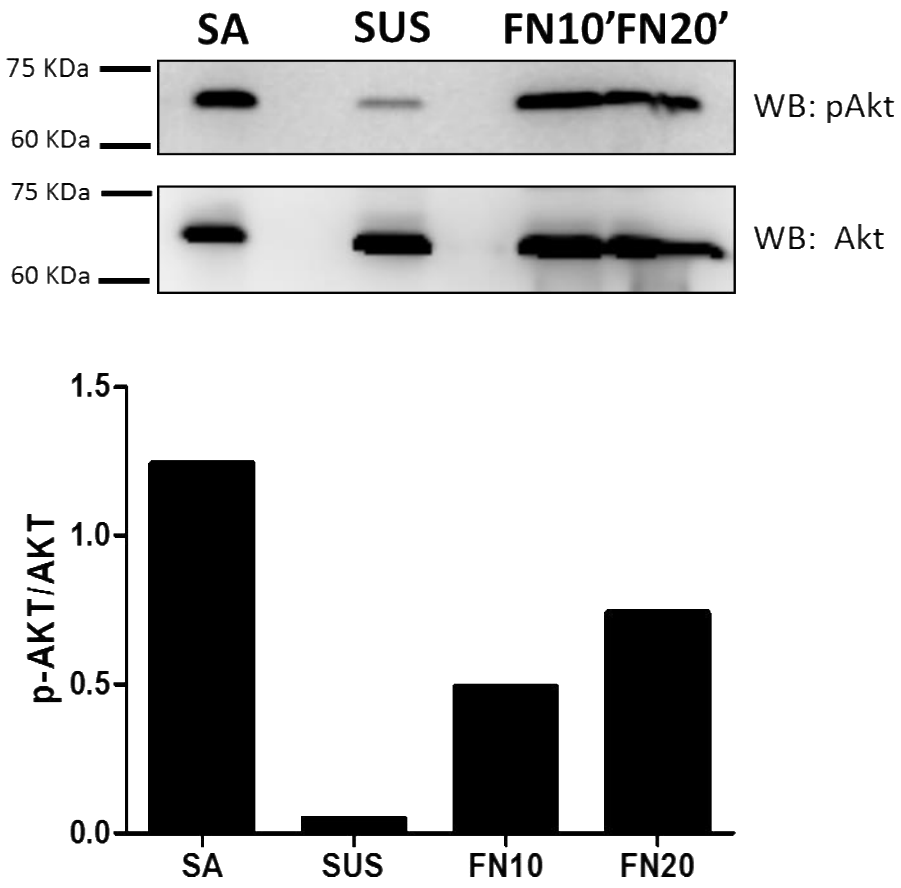


Figure 6. Activation of Akt on loss of adhesion and re-ahesion in WTMEFs. WTMEFs cells were put in suspension for 120 minutes, replated on Fibronectin coated dishes (10ug/ml) for 10 min (FN 10'), 20 min (FN 20') and 4 hrs (Stable Adherent) respectively. Activation of Akt was determined by detecting its phosphorylation at Serine 473 (WB:pAkt) and normalizing the same to total Akt levels (WB:Akt). Graph represents thr ratio of pAkt/Akt and shows loss of adhesion to expectedly caused a dramatic decrease in Akt activation that recovers on replating to fibronectin for 15min. Data reported here is from one experiment.

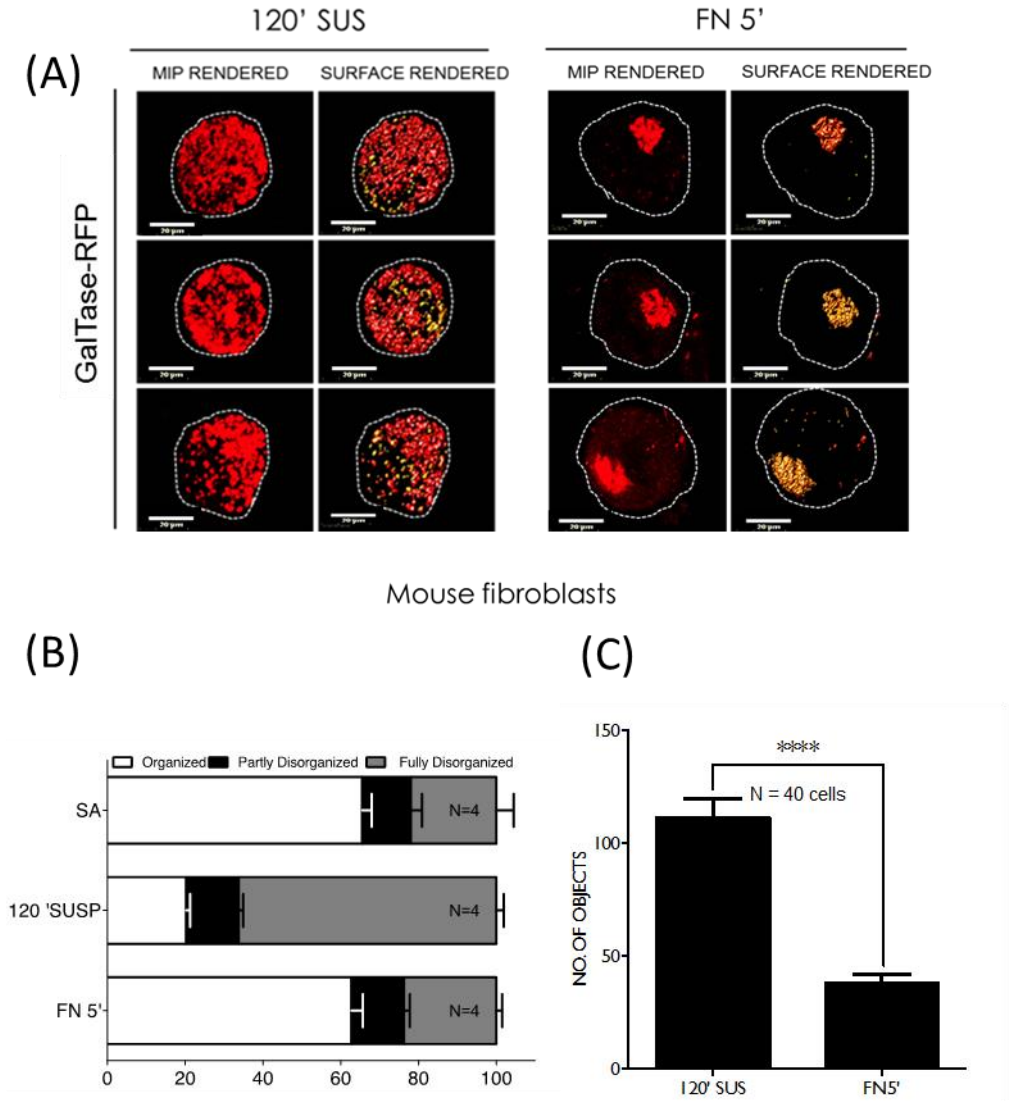


Figure 7. Golgi organization upon loss of adhesion and re-adhesion: (A)WTMEFs were transfected with GALTASE-RFP and after 48hrs kept in suspension(SUS) for 2hrs. After 2hrs, Cells were fixed using 3.5%PFA and then plated upon fibronectin coated coverslips for 5min (FN5') and mounted for imaging for Golgi phenotype. Confocal Z stacks were rendered using Huygens Professional Image Analysis software and respresented as maximum intensity projections (MIP) and surface rendered images. (B) The distribution profile of cells with a organized or disorganized phenotype confirmed the same. (C) Cell images were further deconvoluted and number of golgi objects determined using the Huygens Professional Image Analysis software and $p<0.001$, significance calculated by unpaired two tailed T-test(ann-Whitney test). This confirmed the presence of the partially dispersed golgi phenotype in suspended cells compared to re-plated cells.

3.1.2 Role of AMPK in regulating Golgi organization in suspended cells:

To determine the role of AMPK, a regulator of Golgi organization in suspended cells. Studies from the literature have been suggested that Comp C is a potential inhibitor of AMPK at a specific concentration which doesn't interfere with any other pathway. We have used serum starved WTMEFs transfected with RFP tagged GalTase in DMEM (0.2%FBS) for 10hrs and then cells were treated with DMSO (CNT) and treated (20µM Comp C) for 2hrs. Post incubation cells were held in suspension for 2hrs (120'SUS) and processed further for confocal imaging. For confocal imaging, cells were washed using cold DMEM containing DMSO and Comp C (0.2% FBS), fixed using 3.5%PFA for 15min, washed with 1X PBS twice for 5min each, mounted using fluoramount. Z-stacks were taken and processed further for deconvolution and quantifying Golgi objects and represented as Maximum intensity projection (MIP) and Surface rendered images for visualization using Huygen's software (Figure 8A). We have counted the cells having a disorganized and organized phenotype which reveals that most of the cells have partial disorganized phenotype upon Comp C treatment as compared to CNT where cells are treated with DMSO in suspended cells (Figure 8B). This was further confirmed by counting no of objects using Huygen's software where we observed that cells undergo partial reversal of disorganized phenotype upon Comp C treatment in suspended cells as compared to the suspended cells treated with DMSO which is highlighted in the graph (Figure 8C). This data has suggested that AMPK might play some role in the regulation of Golgi architecture and its organization.

Later in the lab, studies have been done to check AMPK levels in suspended cells using western blot and AMPK levels were observed to be very low in suspended cells which suggests partial reversal of fully disorganized Golgi architecture is regulated via some other pathway which is independent of AMPK (Figure 9). This data led encouraged us to look back into the literature for determining the role of Comp C is playing in regulating Golgi architecture. Studies from the literature have been suggested that Comp C treatment to the cells lead to the increase in short chain ceramide (C16 and C18) via ceramide synthase activity which plays a crucial part in Golgi assembly. Mechanisms are not very clearly known in the literature about regulating Golgi architecture but ceramide does affect Golgi assembly or disassembly which supported us to determine the role of ceramide in Golgi organization (Figure 10).

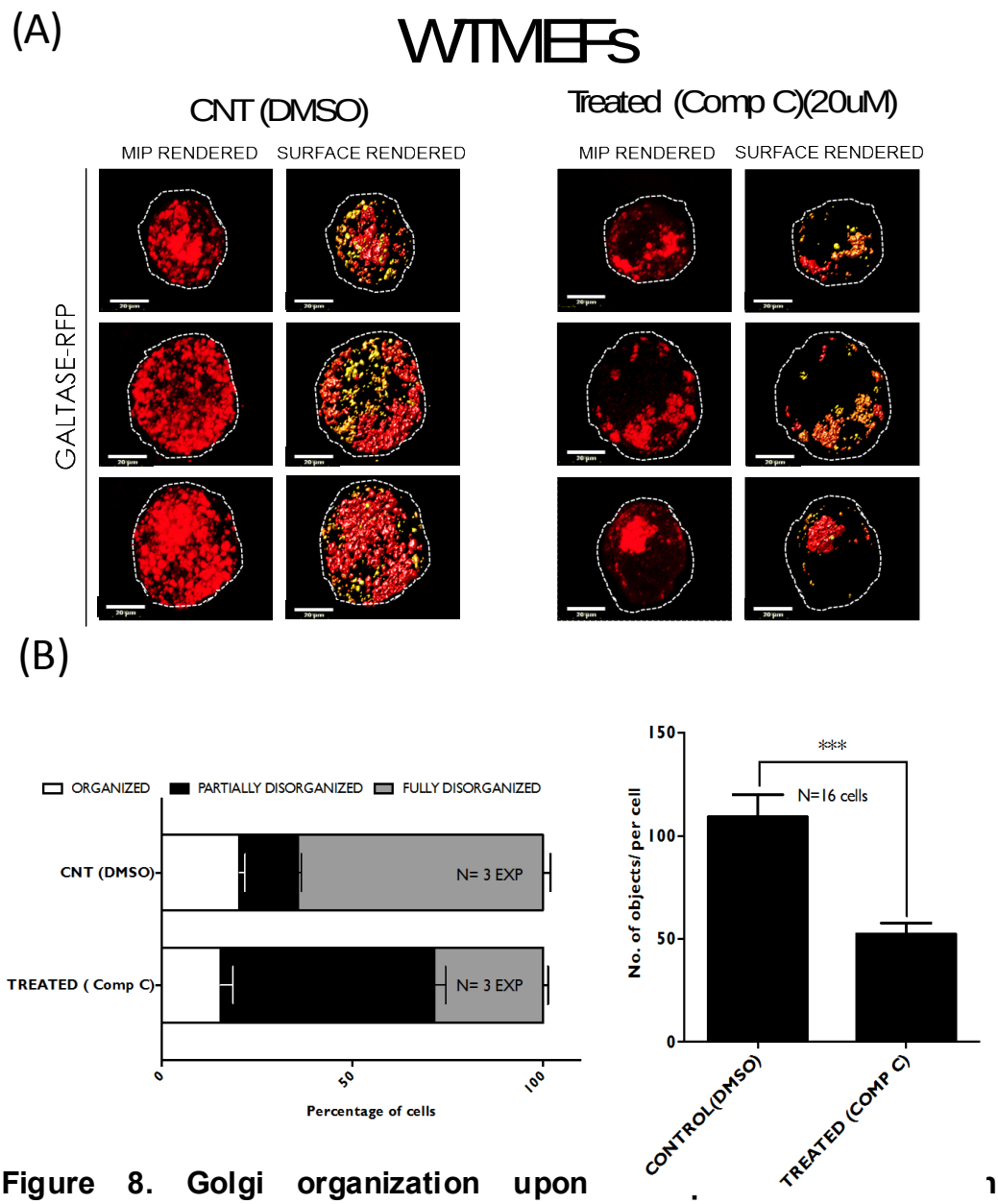
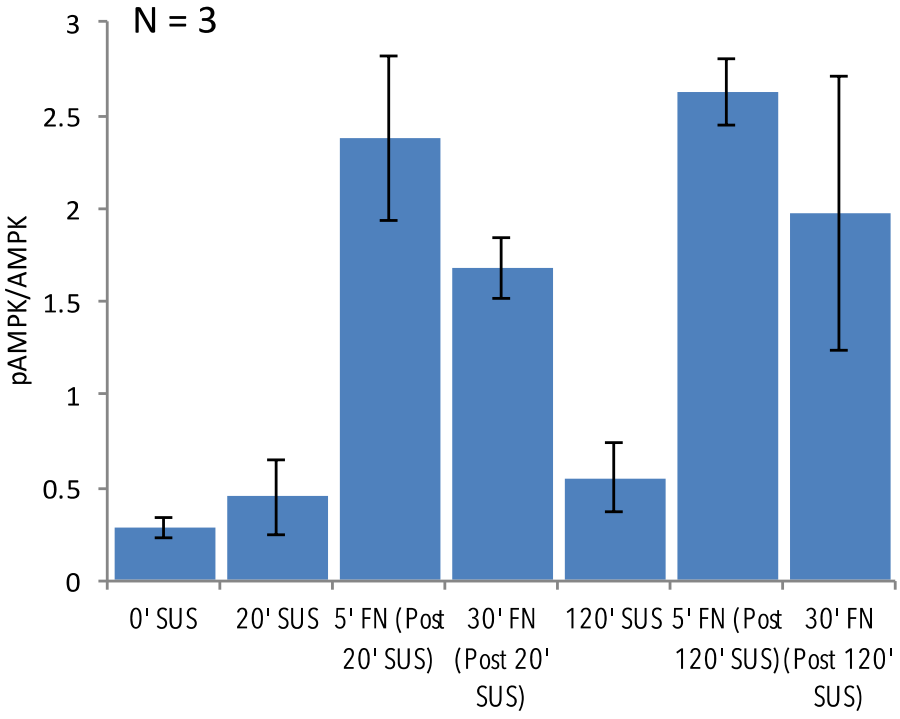


Figure 8. Golgi organization upon . ı

WTMEFs:(A)WTMEFs were transfected with GALTASE-RFP and treated them with DMSO (CNT) and Comp C (Treated-AMPK inhibitor) for 2hrs and then kept in suspension for 2hrs. After 2hrs, Cells were fixed using 3.5%PFA and then mounted for imaging for Golgi phenotype. Confocal Z stacks were rendered using Huygens Professional Image Analysis software and represented as maximum intensity projections (MIP) and surface rendered images.(B) The distribution profile of cells with a organized or disorganized phenotype confirmed the same. (C) Cell images were further deconvoluted and number of golgi objects determined using the Huygens Professional Image Analysis software and $p<0.0001$, significance calculated by unpaired two tailed T-test(ann-Whitney test). . This confirmed the presence of the partially dispersed golgi phenotype in Comp C treated cells compared to DMSO(CNT) treated WTMEFs



(Vibha's data)

Figure 9. AMPK activation upon loss of adhesion and re-adhesion : WTMEFs cells were put in suspension (SUS) for 0, 20, 30 and 120 minutes, replated on Fibronectin coated dishes (10ug/ml) for 5 and 30 min (5' FN and 30' FN). Activation of AMPK was determined by detecting its phosphorylation. Graph represents the ratio of pAMPK/AMPK and shows AMPK activation is very low upon loss of adhesion as compare to re-adhesion. (Vibha's data)

3.1.3. Role of Ceramide in regulating Golgi organization in suspended cells:

Literature has revealed some studies where people have shown that treatment of Comp C, a potential inhibitor of AMPK, lead to the increase in short chain ceramide (C16 and C17) via ceramide synthase 5 (LASS5). Studies revealed that treatment of Compound C leads to the accumulation in ceramide levels (C16 and C17) compared to DMSO and Scr (CNT) which is further confirmed by knockdown LASS5 which showed a significant decrease in ceramide levels compared to Untr-CC (Untransfected-Compound C). This data suggests that ceramide might play a role in regulating Golgi architecture in suspended cells (Figure 10) (Jin et al., 2009).

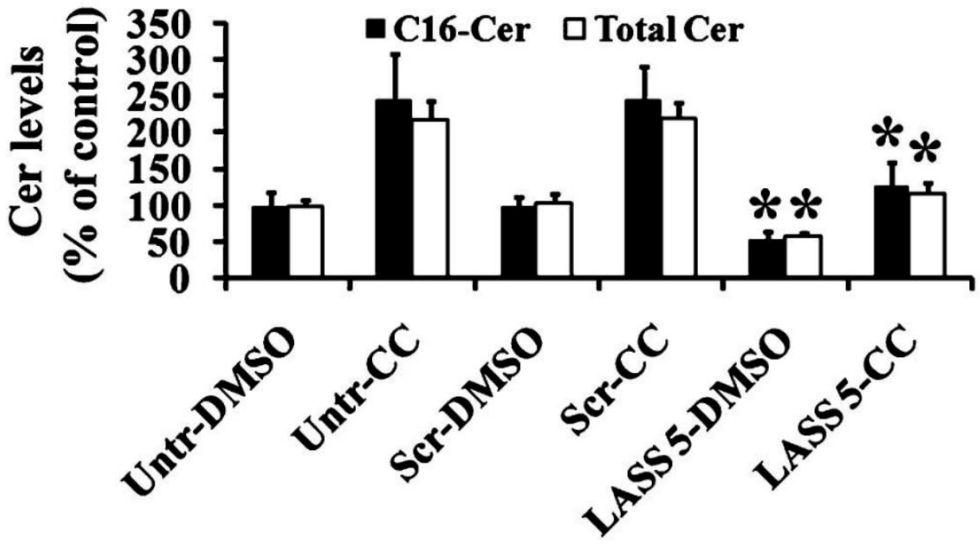


Figure 10. Regulation of Ceramide levels through Comp C: Comp C (CC) has been used as a potential inhibitor of AMPK in different cellular systems. Studies have been suggested that upon treatment of cells with Comp C, it induces ceramide (17C16 and 18C16) accumulation. Ceramide is a signalling molecule which plays a vital role in many cellular functioning like growth, differentiation and apoptosis. It is synthesized in ER and transport to Golgi apparatus via ATP dependent pathway. Studies have been shown that LASS/CerS 5 is involved in Compound C-mediated ceramide accumulation. The levels of C 16-ceramide and total ceramide in cells were measured by tandem MS and expressed as a percentage of the untransfected (Untr) and untreated cell

In order to determine the role of ceramide in regulating the Golgi organization is independent of AMPK role, Fuminobiosin1 (FB1) was used as a potential inhibitor of ceramide in suspended cells (Figure11). We have used serum starved WTMEFs transfected with RFP tagged GalTase, trans Golgi marker, cultured in DMEM (5%FBS) with FB1 (25µm) for 24hrs. Post incubation cells were serum starved in DMEM (0.2%FBS) with FB1 (25µM) for 10hrs. Post incubation cells were treated with DMSO (Control), Treated with Comp C and FB1 in presence of Compound C for 2hrs and then held in suspension for 2hrs (120'SUS) using 2% Methylcellulose in presence of DMSO, Compound C and FB1 in presence of Compound C and further processed for confocal imaging. Cells were washed with DMEM (0.2%FBS) incubated with DMSO, Comp C, and FB1 in presence of Compound C, fixed using 3.5%PFA for 15min, washed twice using 1X PBS for 5min each and then mounted using fluoramount. Confocal z-stacks were taken which was processes further for deconvolution and quantifying Golgi objects. Deconvoluted images were further processed for representing as Maximum intensity projection (MIP) and Surface rendered images using Huygen's software (Figure 12A). We have observed that Cells undergoes complete disorganization of Golgi upon FB1 treatment in presence of Comp C and cells treated with DMSO as compared to the cell treated with Comp C in suspended cells similar was corroborated by counting the cells having disorganized or organized phenotype upon DMSO, Comp C and FB1 treatment in Comp C which reveals that most of the cells have disorganized phenotype in DMSO treated (CNT), FB1 in presence of Comp C as compared to the cells treatment upon Comp C only where most of the cells have partial disorganized Golgi phenotype prominently (Figure 12B). This was further confirmed by quantifying Golgi objects using Hugyen's software which also confirms the same. Statistical analysis (Mann-Whitney test) have also shown a significant increase in increase in Golgi objects upon FB1 treatment in presence of Comp C (Figure 12C).

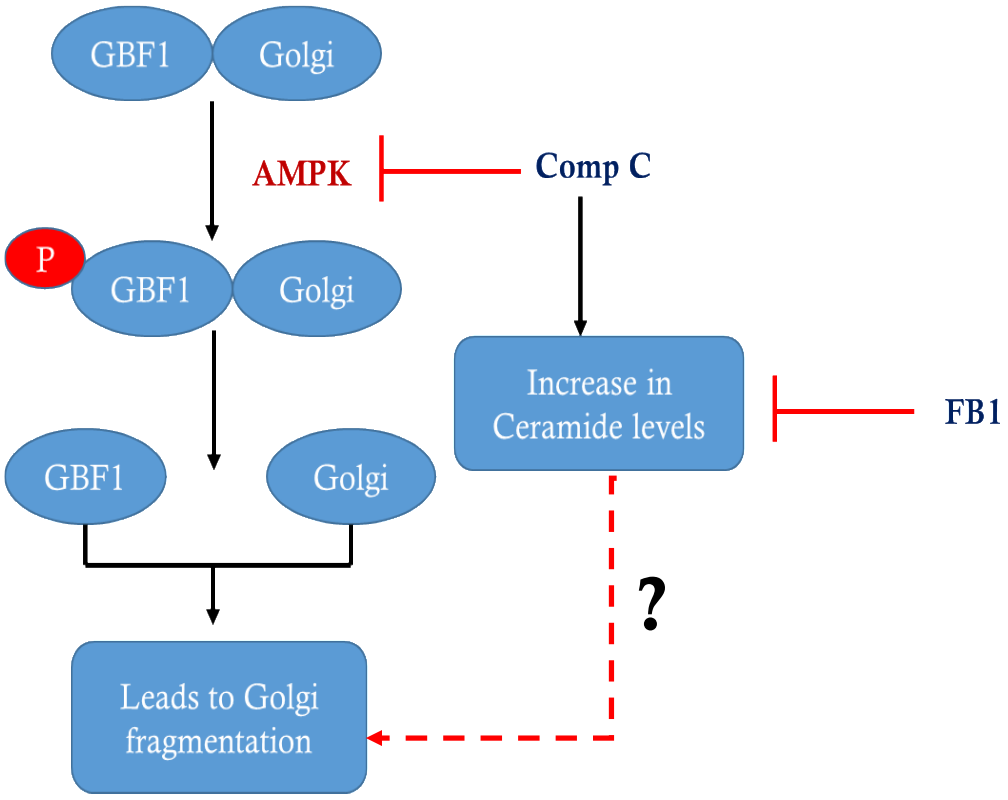


Figure 11. Does FB1 regulates golgi architecture in presence of Comp C mediating through ceramide levels : WTMEFs were treated with Comp C that results in increase in ceramide levels. To determine the role of FB1 whether FB1 rescues the golgi architecture in presence of Comp C mediating through ceramide levels in suspended cells.

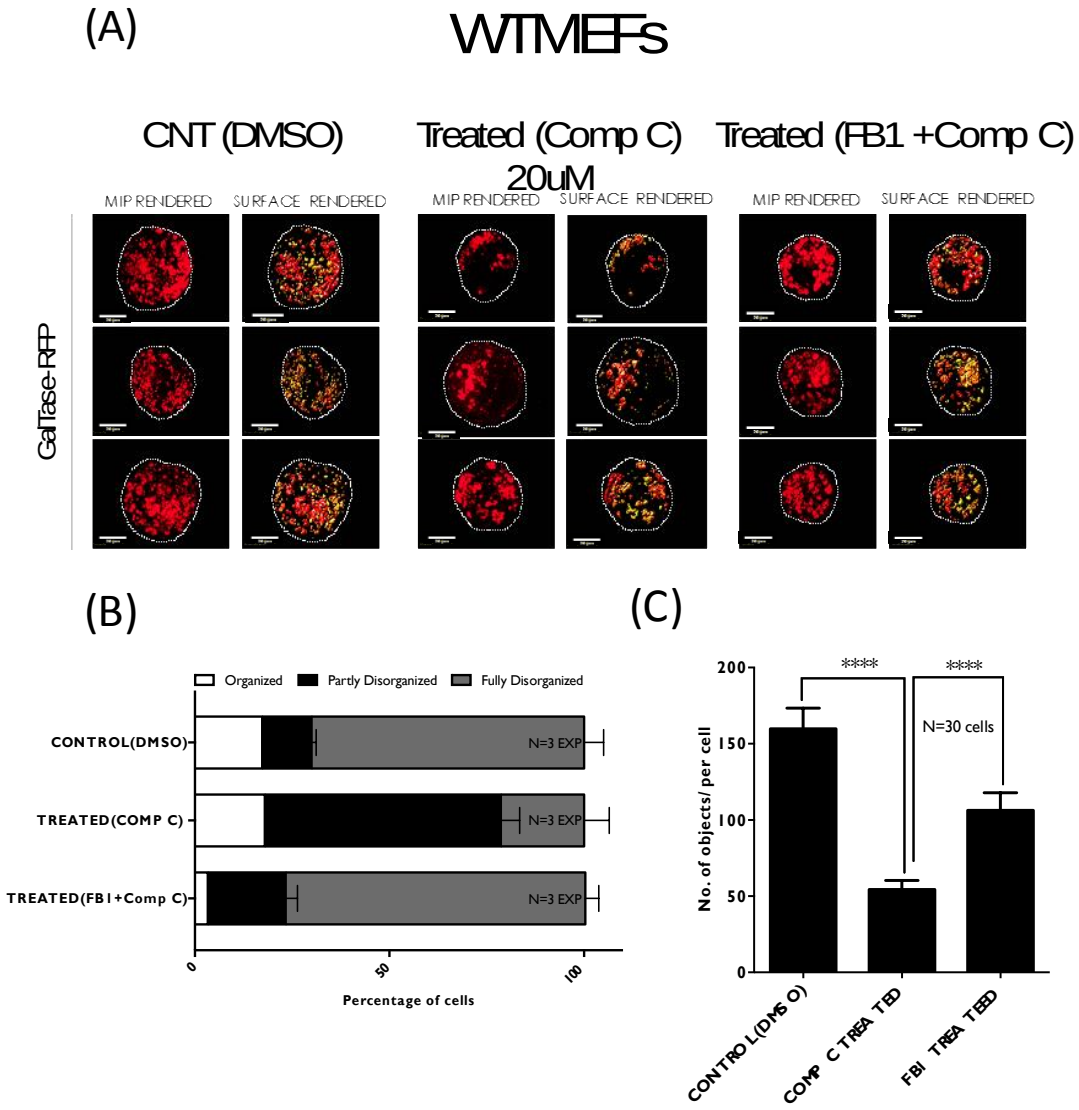


Figure 12. Golgi organization upon FB1 treatment in WTMEF's: (A) WTMEFs was transfected with GALTASE-RFP and treated them with DMSO (CNT) and Comp C (AMPK inhibitor) and FB1 and Comp C (Ceramide inhibitor) for 2hrs and then kept in suspension for 2hrs. After 2hrs, Cells were fixed using 3.5%PFA and imaged using confocal microscopy. Confocal Z stacks were rendered using Huygens Professional Image Analysis software and respresented as maximum intensity projections (MIP) and surface rendered images. (B) The distribution profile of cells with a organized or disorganized phenotype confirmed the same. (C) Cell images were further deconvoluted and number of Golgi objects determined using the Huygens Professional Image Analysis software and $p<0.0001$, significance calculated by unpaired two tailed T-test(ann-Whitney test). This confirmed the rescuing of disorganized golgi phenotype in FB1+ Comp C treated WTMEFs compared to Comp C treated.

3.2. Role of matrix stiffness in 3D and 2D in regulating Golgi architecture

3.2.1 Role of matrix stiffness in regulating Golgi organization in 3D collagen gels of varying stiffness:

Studies in the lab revealed that matrix stiffness can regulate differential endocytosis in WTMEFs embedded in 3D collagen gels of increasing stiffness. They have looked endocytosis in WTMEFs embedded in 3D collagen gels using a GM1-CTxB marker (Figure 12A). This was further corroborated by counting the WTMEFs with endocytosis and with no endocytosis which is shown in graph (Figure 12B). This data has suggested that certain endocytic pathways are getting affected by matrix stiffness which gives us enough evidence and encouragement to look the role of Golgi architecture in 3D collagen gels of varying stiffness.

In order to determine the role of Golgi architecture, we have used WTMEFs transfected with RFP tagged GalTase used to visualize trans Golgi were cultured in DMEM (5%FBS) for 24hrs. Post incubation cells were detached using Trypsin-EDTA for 5min, neutralized in DMEM (5%FBS), spun down at 1000rpm for 5min at 10^{o}C. Meanwhile, sterile 10XPBS, Milli Q water, and collagen solution were prepared and stored at 4^{o}C or on ice. Cells were re-suspended in 1X PBS, 40,000 cells were mixed in collagen gels solution, 1M NaOH was added to maintain the pH of the collagen gel, added to LAB-TEK chamber and incubated at 37^{o}C in 5%CO_2 incubators for 30min. Post incubation media was added and incubated for 4hrs and further processed for confocal imaging. For confocal imaging, Cells were fixed using 4%PFA and 5%sucrose for 20min, washed using 1X PBS twice for 10min each. Z-stacks were taken and further processed for deconvolution and quantifying Golgi objects using Huygen's software. Deconvoluted cells were processed for representing Maximum intensity projection (MIP) and Surface rendered images using Huygen's software (Figure 13A). Cells were counted with organized or disorganized phenotype where we observed that most of the cells in 1.5mg/ml have intact Golgi phenotype as compared to 1mg/ml in prominent phenotype was found to be fully disorganized (Figure 13B). This was further confirmed by quantifying no of Golgi objects where we found WTMEFs embedded in 1mg/ml have more Golgi objects as compared to 1.5mg/ml where Golgi objects were found to be very less (Figure 13C). Statistical analysis has

also shown a significant difference in Golgi objects in WTMEFs embedded in 3D collagen gels of varying stiffness.

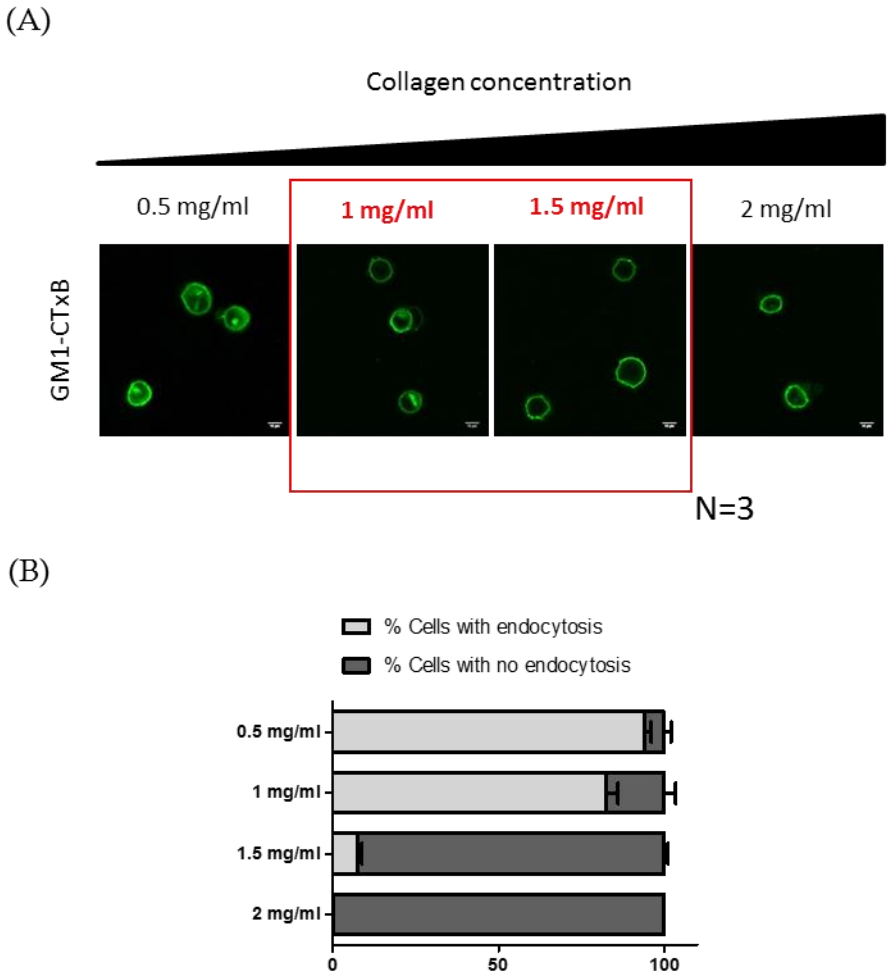


Figure 13 .Endocytosis of various markers in WT MEFs embedded in collagen gels of varying concentrations. (A) Endocytosis of GM1-CTxB (B) Profile for endocytosis in WT MEFs represented the same. Images are representative from 3 independent experiments. (from Thesis of Trupti Thite).

This data suggest that matrix stiffness might play a role in regulating the cellular behavior of the cells which is confirmed by differential endocytosis in WTMEFs embedded in 3D collagen gel. Previous studies in the lab have shown that there

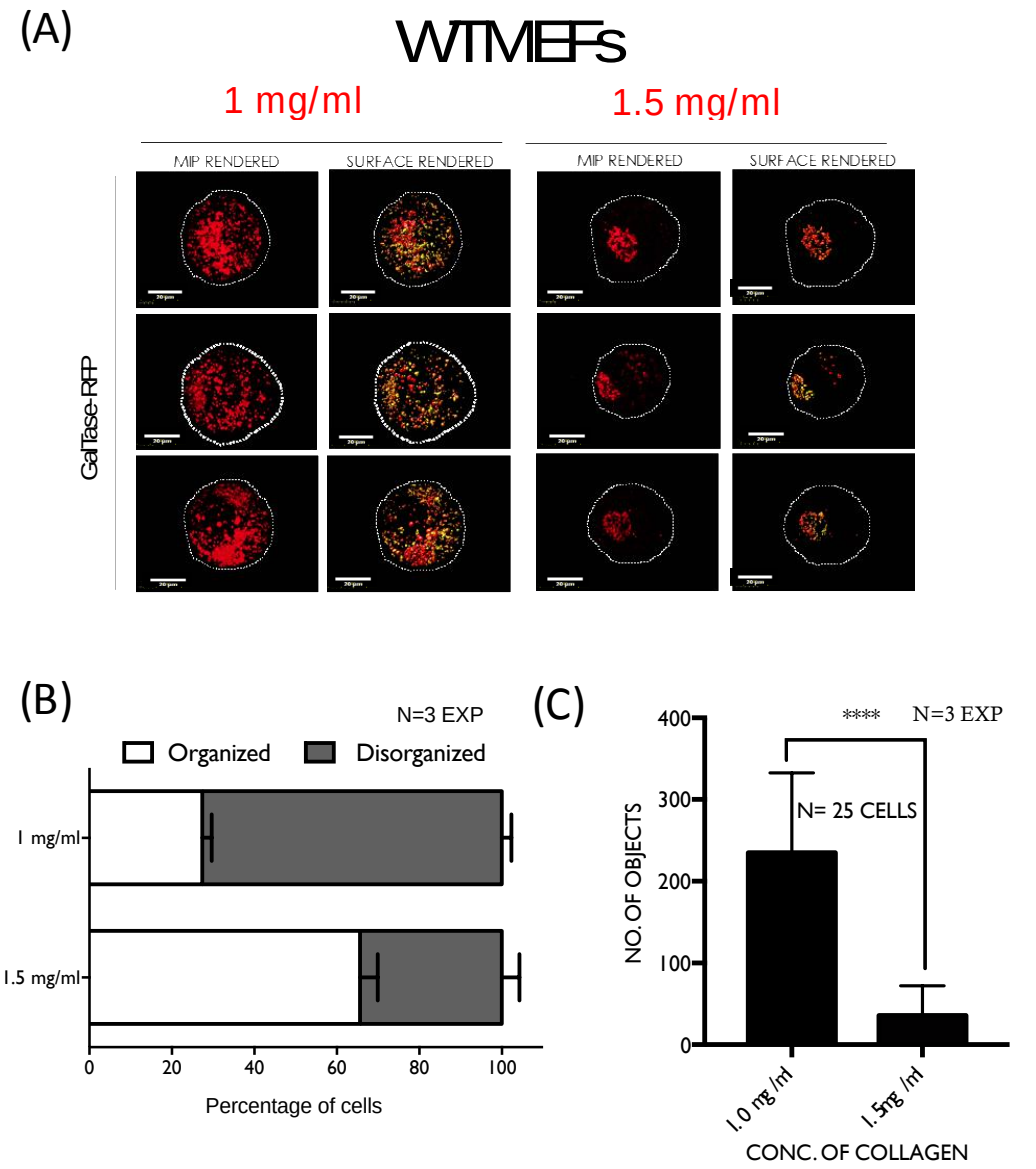


Figure 14. Golgi organization in 3D collagen gel in WTMEFs: (A) WTMEFs were transfected with GALTASE-RFP and after 48 hrs. of transfection, cells were embedded in collagen gel having a concentration of 1mg/ml or 1.5mg/ml. Cells were fixed after 4hrs. of embedding using 3.5% PFA and imaged using confocal microscopy. Confocal Z stacks were rendered using Huygens Professional Image Analysis software and respresented as maximum intensity projections (MIP) and surface rendered images. WTMEFs embeded in 1mg/ml collagen show a prominent disorganized phenotype while these same cells in 1.5mg/ml collagen gel have a prominently organized phenotype of Golgi. (B)The distribution profile of cells with a organized or disorganized phenotype confirmed the same. (C) Cell images were further deconvoluted and number of Golgi objects determined using the Huygens Professional Image Analysis software and $p<0.0001$, significance calculated by unpaired two tailed T-test(ann-Whitney test).This confirmed the presence of the dispersed Golgi phenotype in 1.0mg/ml collagen as compare to 1.5mg/ml collagen.

caveolin1 plays a role in endocytosis where they embedded Cav1 KO-MEFs in 3D collagen gels of increasing stiffness and observed endocytosis using CTxB marker. We observed that there is endocytosis in cells lacking caveolin1 upon embedding in the 3D collagen of increasing stiffness which suggested that caveolin1 might play a role in sensing the 3D microenvironment which encouraged us to look at the role of caveolin1 in regulating Golgi organization (Figure 15) (Data from Trupti's thesis)

In order to determine the role of caveolin1 in regulating Golgi organization, we have used transfected Cav1 KO-MEFs with GalTase tagged with RFP, culture them in DMEM (5%FBS) for 24hrs. Post incubation cells were detached using Trypsin-EDTA, neutralized using DMEM (5%FBS), spun down at 1000rpm at10^{o}C for 5min. Meanwhile, sterile 10XPBS, Milli Q water, and collagen gel was mixed and store at 4^{o}C. Cells were re-suspended in 1X PBS, 40,000 cells were added to the collagen gel mixture, added to the LAB-TEK chamber, incubated at 37^{o}C in 5% CO_2 incubator for 30min. Post incubation DMEM (5% FBS) was added to the gels and further processed for Confocal imaging. For confocal imaging, cells were fixed using 4%PFA and 5%sucrose for 20min, washed using 1X PBS for 10min each. Z-stacks were taken and further processed for deconvolution and counting Golgi objects using Huygen's software. Deconvoluted cells were further processed for representing as Maximum intensity projection (MIP) and Surface rendered images using Huygen's software (Figure 16A). We have observed that upon embedding Cav1KO-MEFs in 3D collagen gels of different stiffness (1 mg/ml and 1.5 mg/ml) has fully disorganized phenotype prominently which was confirmed by counting the no of cells having a disorganized or organized phenotype which shows the same (Figure 16B). This was further corroborated by counting Golgi objects using Huygen's software where we have observed that Cav1 KO-MEFs embedded in 3D collagen in 1mg/ml and 1.5mg/ml, both seem to have more Golgi objects which were shown in the graph and there was no significant difference among both the stiffness (1mg/ml and 1.5mg/ml) (Figure 16C). This data suggests that Caveolin1 might play a role in regulating Golgi organization. Furthermore, experiments are required to confirm the role of Caveolin1 in regulating Golgi organization.

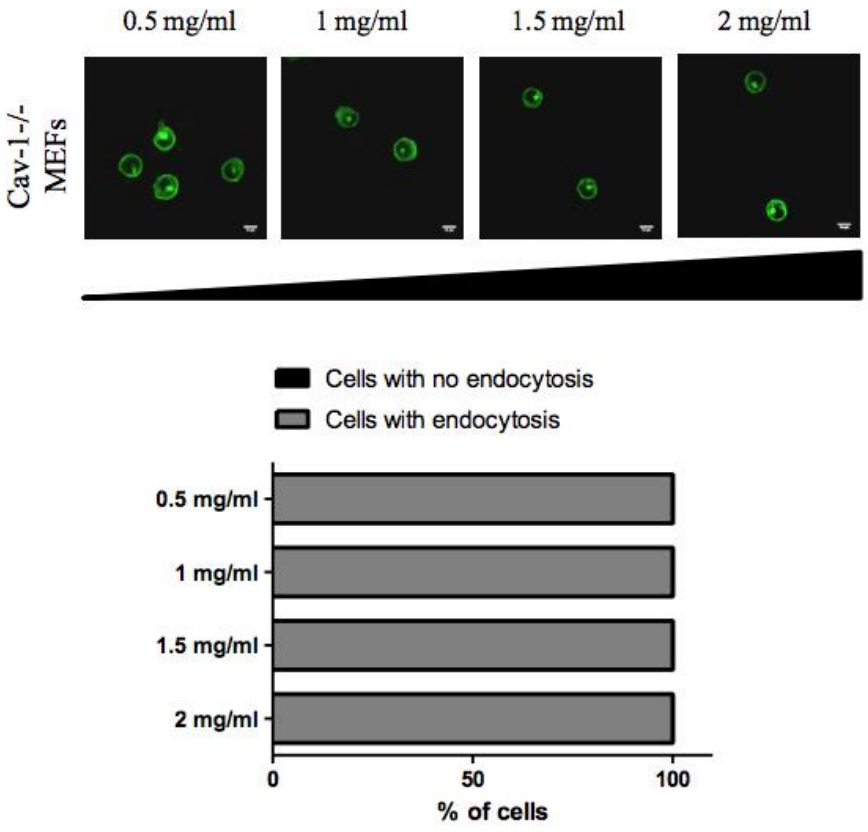


Figure 15. Endocytosis of CTxB in Cav1-KO MEFs at different collagen concentrations: Cells embedded at increasing collagen concentrations (0.5mg/ml to 2mg/ml) and labeled with CTxB were imaged and representative images shown here from 2 independent experiments. 100 cells were analyzed in each experiment. Graph below shows % distribution of cells with and without endocytosis and represents data from one experiment. Both experiments gave similar results.

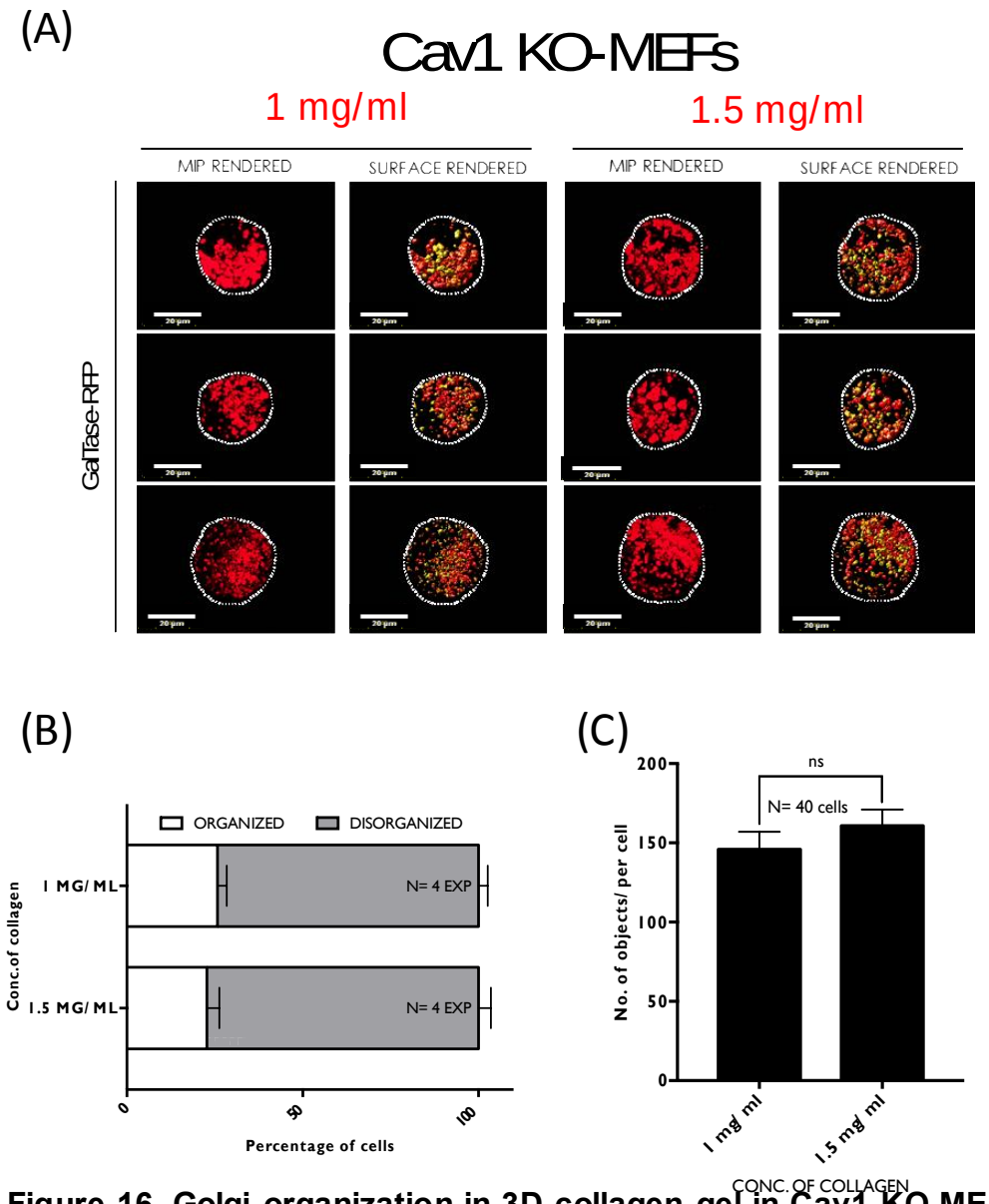


Figure 16. Golgi organization in 3D collagen gel in Cav1-KO MEFs : (A) Cav1-KO MEF's were transfected with GALTASE-RFP and after 48 hrs. of transfection, cells were embeded in collagen gel having a concentration of 1mg/ml or 1.5mg/ml. Cells were fixed after 4hrs. of embedding using 3.5% PFA and imaged using confocal microscopy. Confocal Z stacks were rendered using Huygens Professional Image Analysis software and respresented as maximum intensity projections (MIP) and surface rendered images. WTMEFs embeded in 1mg/ml and 1.5mg/ml collagen show a prominent disorganized phenotype of Golgi. (B) The distribution profile of cells with a organized or disorganized phenotype confirmed the same. (C) Cell images were further deconvoluted and number of golgi objects determined using the Huygens Professional Image Analysis software and there is no significant significance difference which is calculated by unpaired two tailed T-test(ann-Whitney test). This confirmed the presence of the dispersed golgi phenotype in 1.0mg/ml and 1.5mg/ml collagen.

3.2.2 Role of matrix stiffness in regulating Golgi organization on 2D polyacrylamide gels of varying stiffness:

While 3D collagen gels are indeed more physiological and suggest that changing stiffness does affect the Golgi organization. A significant amount of work in earlier studies is done in 2D gels of varying stiffness. We hence wanted to test if changing gel stiffness in 2D microenvironments could indeed also affect the Golgi organization. In order to determine the role of matrix stiffness on cells, we have accomplished a technique to prepare 2D polyacrylamide gels of varying stiffness coated with collagen in IIT, Bombay.

In order to look the role of matrix stiffness, we have prepared gels of varying stiffness using appropriate proportions of Acrylamide, Bis-Acrylamide, 10% APS and TEMED and allowed them to polymerize for 15-20min which makes sure the proper cross-linking of gels and gives us the appropriate stiffness. Post-polymerization, 2D PA gels of varying stiffness [0.5kPa, 2kPa, 5kPa, 10kPa, 20kPa and CNT(coverslip)]was coated with collagen (25µg/ml) using SULPHO-SANPAH for 12hrs at 4^{o}C. Post incubation, gels were washed with 1X PBS, incubated with PENSTRAP to avoid contamination, cells were seeded, incubated at 37^{o}C in 5%CO_2 incubator for 45min. Post incubation, DMEM (5%FBS) was added to the cells seeded on gels and further processed for live imaging on EVOS microscope. In order to look the spreading behavior of cells, we have used WTMEFs, seeded on gels of varying stiffness, imaged live using EVOS microscope for 6hrs. We have observed that cell spreading increases with the increase in stiffness of polyacrylamide gels (Figure 17B) which was further confirmed by performing area analysis using Image J software which also confirms the same (Figure 17B). This data have encouraged us to look at the cytoskeleton structure of the cell on 2D PA gels of varying stiffness. In order to look the organization of cytoskeleton of cells on 2D PA gels of varying stiffness, we have done Actin staining for which we have used WTMEFs, seeded on gels of varying stiffness (2kPa and 20kPa), cultured them in DMEM (5%FBS) for 24hrs, incubated at 37^{o}C in 5%CO_2 incubator and allowed them to attach. Post incubation, media was added to the cells seeded on gels of varying stiffness, incubated at 37^{o}C in 5%CO_2 incubator for 24hrs, and further processed for actin staining. In order to perform actin staining, cells seeded on 2D PA gels of different stiffness (2kPa and 20kPa) was permeabilized using 1%Triton-X for 2min, fixed using 3%PFA for 5min, blocked using 2%BSA for 20min

and stained with Alexa flour 488 (1:400 diluted in BSA) overnight at 4^{o}C. Post incubation, cells seeded on gels washed using 1X PBS for 10min each, mounted using Fluoramount and imaged using EVOS microscope which is shown in the figure below (Figure 18).

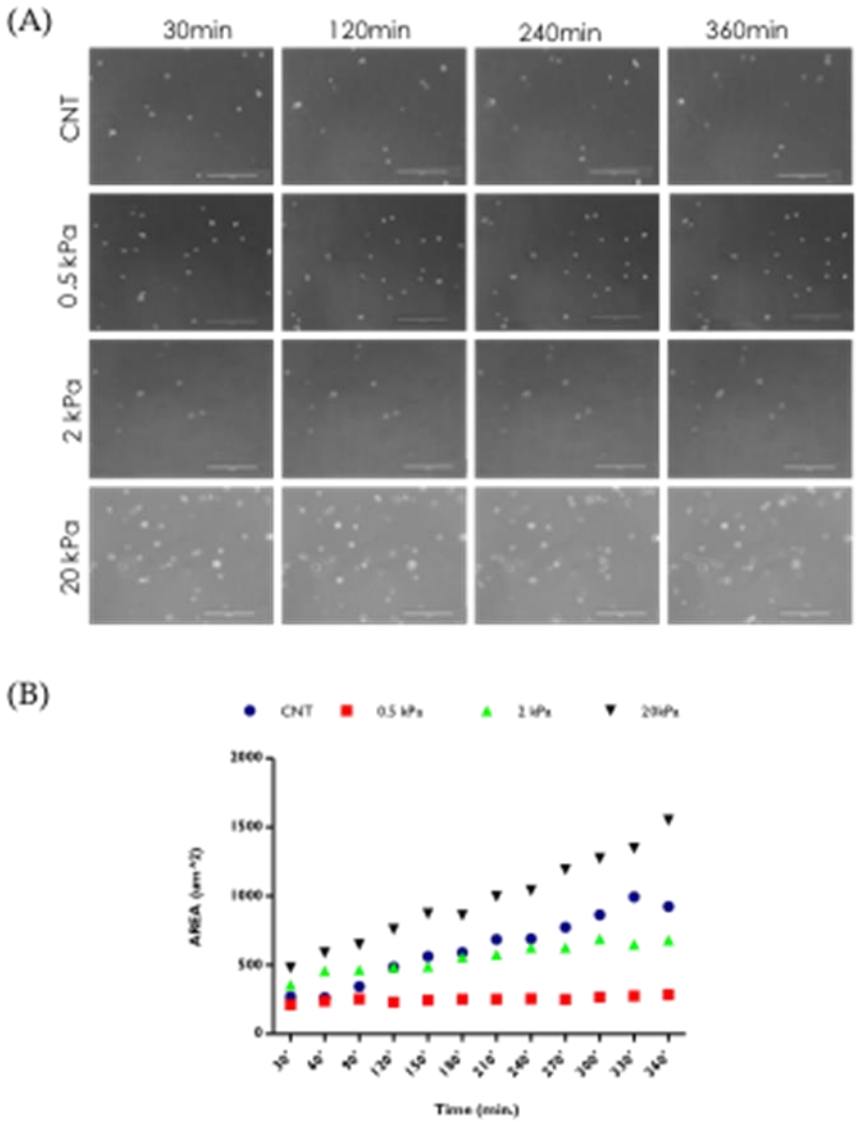


Figure 17. Dynamics of WTMEFs on PA(PolyacrylAmide gels) of varying stXiffness: (A) Polyacrylamide gels were made using 40% Acrylamide and 2% Bis- Acrylamide solution of 0.5kPa, 2kPa, 20kPa and CNT(coated with collagen). Gels were coated with collagen (25ug/ml) overnight using NHS-EDC treatment to the gels for 20 min. WTMEFs were cultures on gels and taken a time lapse for 6hrs. It was observed that WTMEF's spreading increases with the increment in the stiffness of the gel. (B) Area of the cells were calculated using Image J software which and plotted using Prism software with area (um^2) on Y-axis and time (min.) on X-axis which confirms the same.

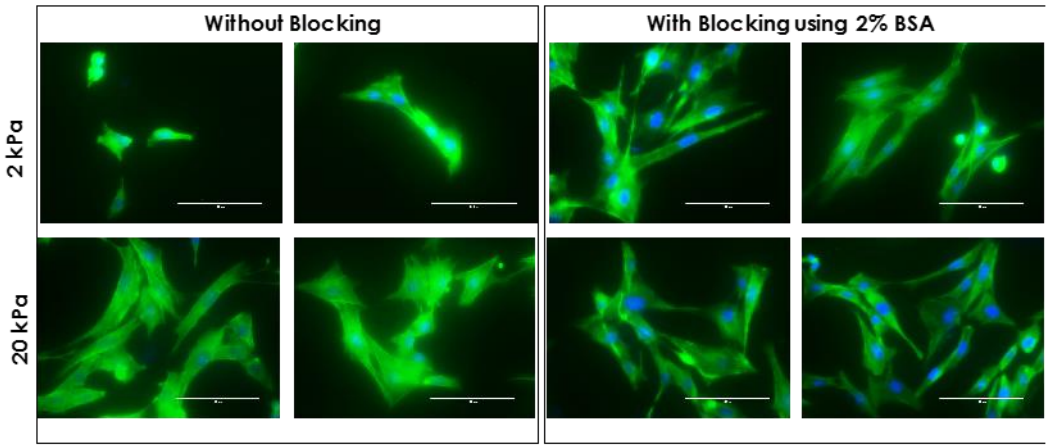


Figure 18. Phalloidin staning on gels of varying stiffness: Gels of varying stiffness were prepared and WTMEFs were cultured on them. After 24. hrs, cells were fixed using 3.5% PFA. Cells on one set of gels were blocked using 2% BSA and then stained for Actin whereas cells on another set of gels were directly stained for the Actin. Images have been captured using EVOS microscope at 40X magnification after mounting the gels using fluoramount media.

4. Discussion:

4.1. Adhesion-dependent regulation of the Golgi organization

Integrin-mediated adhesion plays a very critical role in regulating cellular signalling which in turn controls the behaviour of the cells like mitosis, meiosis, cell survival etc. This adhesion-dependent signalling is also deregulated in many pathological events like neurodegeneration, cancer. Studies have shown adhesion regulated membrane trafficking to drive anchorage-dependent signaling (Alonso and Millán, 2001). The Golgi is an important mediator membrane and vesicular trafficking. It is also seen to undergo extensive fragmentation during various physiological condition including meiosis, mitosis and pathological events like cancer. These known studies had encouraged the lab to look at if integrin-mediated adhesion plays any significant role in regulating Golgi organization. In order to determine the role of integrin in regulating downstream signaling processes. We have performed suspension assay where cells were serum starved, to negate the crosstalk adhesion has with growth factors, held in suspension in DMEM (0.2%FBS). This we find significantly reduces integrin-dependent signaling and the same was seen for Akt activation. Akt activation that drops on the loss of adhesion was restored on re-adhesion (Figure 6). Under these conditions we looked at Golgi organization in cells, using GalTAse RFP (trans Golgi marker) expressing cells. We observed that most of the cells have fully disorganized Golgi phenotype on the loss of adhesion that rapidly re-organizes and recovers its intact phenotype upon re-adhesion within five minutes or less (Figure 7). This data confirms earlier studies by Vibha et. al. from the lab that adhesion does play a role in regulating Golgi organization.

This led us to look into the literature for determining the role of the possible regulator which helps in regulating Golgi organization. Studies have been done in the lab which suggested that adhesion uses the Arf GEF BIG1/2 and GBF1 to regulate Arf1 activation to drive Golgi organization (Vibha et.al. - https://doi.org/10.1101/261842).

4.2. Role of AMPK and ceramide in Golgi organization:

AMPK is Adenosine mono-phosphate activated protein kinase which is a key player in regulating Golgi organization with the help of GBF1, Guanine nucleotide exchange factor, which is an Arf-GEF. AMPK phosphorylates GBF1 and allows the GBF1 to

dissociate from the membrane which results in Golgi disassembly. This led us to use Comp C, a known AMPK inhibitor, and determine how it affected the adhesion-dependent Golgi organization. We observed that Golgi reverses its disorganized phenotype to partially disorganized phenotype in suspended cells treated which suggests that AMPK does play a role in regulating Golgi organization. We wanted to confirm this regulation by checking activation of AMPK in suspended cells but interestingly we found that AMPK activating stayed very low in suspended cells. This made us wonder how Comp C affects the Golgi organization and if it could be doing this independent of AMPK pathway. Looking at literature we found Comp C at concentrations used (20µM) is also known to inhibit AMPK. Thus is seen to cause an accumulation of the ceramide (C16 and C18) affects Golgi disassembly or assembly (Jin et al., 2009). In order to determine the role of ceramide could have in compound C mediated regulation of Golgi organization, we have used a known ceramide synthase inhibitor FB1 in presence of Comp C in suspended cells. We have observed that FB1 treatment in presence of Comp C does cause a reversal of Golgi phenotype causing it to regain its fully disorganized phenotype. This data suggests that ceramide might play a role in regulating the Golgi organization. Further confirmatory experiments we want to do using R (+) – Methanandamide (AM 356), synthetically created a stable chiral analog of anandamide (Ramer et al., 2003) to cause an increase in ceramide levels independent of compound C treatment to ask if this affect the Golgi organization.

Ceramide plays important role in coordinating cellular responses to extracellular stimuli and stress. C_{16}-ceramide that can be generated by ceramide synthase 6 (CerS6) (Saddoughi and Ogretmen, 2013), have been implicated in cancer cell proliferation whereas C_{16}-ceramide is known to play a role in Golgi organization (Figure 12). Determining the role of ceramide in regulating Golgi organization might reveal its role in cancer which can be further prevented by therapeutic treatment targeting C_{16}-ceramide.

4.3. Changing gel stiffness (3D vs2D) effect on Golgi organization:

Studies done till now have focused to determine the role of adhesion in regulating Golgi organization, looking at the same on the loss of adhesion. Adhesion-dependent

signaling is seen to regulate cell morphology and function in the physiological 3D microenvironment. There are studies which have shown that adhesion complexes vary in behavior in different models to study cellular behavior. Adhesion complex assembly might get affected by the stiffness of the matrix on which cells are cultured. Various models have been developed to study the cellular behavior like 3D collagen gels, 2D Poly-acrylamide gels which has an ability to self-organize into the fibrous structure and mimic in vivo microenvironment. Studies have also been done in the lab where we have observed that increasing matrix stiffness in 3D collagen does regulate differential endocytosis among WTMEFs and further seen to be regulated by Cav1. This leads us to ask how changing matrix stiffness in 3D regulates the Golgi organization and the role this has in cellular function.

In order to determine the role of matrix stiffness in regulating Golgi organization, we have used WTMEFs expressing Galtase RFP, embedded in 3D collagen gels of varying stiffness and looked at their Golgi organization. We observed that the Golgi does seem to be extensively disorganized in 1mg/ml compared to 1.5mg/ml, suggesting that changing stiffness does regulate Golgi organization. This change in behavior between 1.0 vs 1.5 mg/ml collagen also mimics the difference seen in how WTMEFs regulate endocytosis differentially. That change we knew is dependent on the presence of caveolin, which could both act as the endocytic regulator or a mechanotransducer to mediate its effect. We hence asked if the presence of caveolin-1 does affect change in Golgi organization in 1.0 vs 1.5 mg/ml collagen as reported above. We find the Golgi be fully disorganized in both 1mg/ml and 1.5mg/ml 3D collagen gels. This suggests that the change in phenotype observed in 1.5mg/ml collagen for Golgi organization is indeed affected by the presence of caveolin-1. This in that sense mimics the change observed in GM1 endocytosis in these Cav-1 KO MEFs that now behave at 1.5mg/ml collagen-like they do at 1.0mg/ml. With caveolin-1 not known affect Golgi organization pathways and seen to also show the adhesion-dependent regulation of the Golgi (Vibha unpublished data). This observation in 3D does suggest a possible role for caveolin-1 as a mechanotransducer in allowing cells to sense changing stiffness in 3D microenvironments.

While 3D collagen gels are indeed more physiological and suggest that changing stiffness does affect the Golgi organization. A significant amount of work in earlier studies is done in 2D gels of varying stiffness. We hence wanted to test if changing

gel stiffness in 2D microenvironments could indeed also affect the Golgi organization. We have tried looking at cells on 2D Polyacrylamide gels of varying stiffness, coated with collagen. WTMEFs spreading behavior on 2D gels with varying stiff does mimic what has been reported with a distinct increase in spreading observed on increasing stiffness. Further experiments to determine the Golgi organization on 2D PA gels of varying stiffness are in progress.

The main aim of this project was two-fold, one to explore the regulation of adhesion-dependent Golgi organization and second to ask if changing stiffness and crosslinking in 3D microenvironments could affect this regulatory pathway. My work has been able to highlight the possible role ceramide dependent pathways may have in mediating adhesion-dependent Golgi organization. In the second half of my study, I have been able to show changing stiffness and crosslinking in 3D microenvironments to indeed be able to affect the Golgi organization. These studies extended to caveolin-1 null cells do suggest that caveolin-1 could act as sensor/transducer of mechanical forces in cells to affect their behavior in 3D microenvironments and control their Golgi organization. This could indeed have implications for how we understand what role the Golgi has in 3D. The role that caveolin-1 a known tumor suppressor (lost in cancers) could have in making these cancer cells respond similarly in 3D environments is also something we are exploring. The regulation and role of this pathway in 2D Polyacrylamide gels of varying stiffness which are coated with collagen could further reveal how this adhesion-Golgi pathway works in cells in response to changing mechanical cues.

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