Role of Grb2 in myogenesis and epithelial-to-mesenchymal transition

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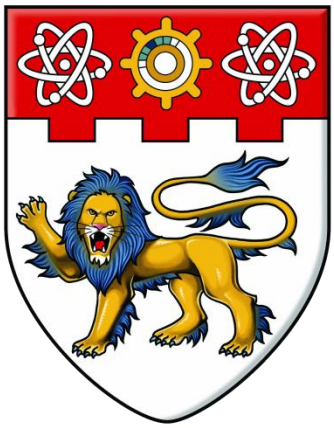
2016

Mitra, P. (2016). Role of Grb2 in myogenesis and epithelial-to-mesenchymal transition. Doctoral thesis, Nanyang Technological University, Singapore.

https://hdl.handle.net/10356/68857

https://doi.org/10.32657/10356/68857



NANYANG TECHNOLOGICAL UNIVERSITY

Role of Grb2 in Myogenesis and Epithelial-to-Mesenchymal transition

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School of Biological Sciences

April, 2016

Role of Grb2 in Myogenesis and Epithelial-to-Mesenchymal Transition

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A thesis submitted to Nanyang Technological University

In fulfillment of the requirement for the degree of

Doctor of Philosophy

April, 2016

ACKNOWLEDGEMENTS:

The four years of graduate studies has been a huge learning experience for me, both professionally and personally. I learnt something new and valuable from every person that I met and interacted with during my PhD. So, now it's time for me to thank them for making my stay here such a pleasant one. I first begin by thanking my supervisor Dr Thirumaran Thanabalu immensely for his constant guidance and support throughout these four years of PhD. I know there have been many occasions when I have digressed from my work, lost my focus and was not hard working enough, but he did not lose his patience and continuously encouraged me to work and think harder about my project. He was always available for suggestions and discussions. He has patiently corrected me and helped me improve my laboratory working and scientific writing skills. I will forever value his knowledge of cell and molecular biology that he has shared with me and hope to make him proud in my future endeavors.

I would also like to thank my Thesis Advisory Committee (TAC) members- Asso Prof Koh Cheng Gee and Tan Nguan Soon Andrew for their constant support and guidance throughout the four years of my PhD. The suggestions and advice that I received during my TAC meetings from them really helped me to give a positive direction to my projects for which i am immensely thankful to them.

Next, I would like to thank all the seniors in my lab such as Rajmohan, ChuRou, Raodah, Swagata, Neeraj and Bhawana with all of them patiently teaching me some or the other lab techniques that I know today. I know I could trouble them with my silly questions as many times as I wanted to and they would still smilingly answer them. But I must individually thank two of them, Neeraj and Bhawana, who have stood by me through thick and thin in these four years. Neeraj was at the end of his graduate studies when I joined this lab. But he never gave me the feeling that he was one of the senior most students in the lab around that time. He was friendly, warm and funny. He always lightened the mood in the lab with his jokes. At the same time, he was very knowledgeable and approachable with any difficulties I encountered, be it personal or professional. He will always be an inspiration and a good friend to me. I will always be grateful for his guidance and support. Bhawana was a year junior to him and she worked on the same cell line that I did. So my interaction with her started on a more professional front with me bugging her with all the technical questions. Later on, she became a good friend as well and gave me a lot of valuable suggestions on

work and life in general. Her positive attitude and encouragement has really helped me a lot especially in difficult situations. I have found true and lifelong friends in Neeraj and Bhawana.

Then, I would like to thank my batch mates, lab mates and friends for standing with me through both happy and rough times. The list is long but a special thank you goes to Amrita, Akshaya, Viswanadh and Divyanshu. Amrita has not only been a very friendly lab mate but also one of my good friends here. We joined the lab at the same time and have literally grown in these four years together. We have shared all our good and bad experiences together and supported each other thoroughly. I have also learnt a lot from her professionally. I wish her all the very best in all her future endeavors. Akshaya joined as an intern in our lab but soon became a good friend. Even though I was supposed to be her mentor, I was new and learning many techniques myself for the first time. But she was patient enough with me and we learned a lot of things together. Vishwanadh and Divyanshu are from our neighboring lab but we became friends and I have shared many professional advices and fun moments with them as well which I will always cherish.I would also like to thank my current lab mates Tan Hui Bing, Pazhanichamy, Chung Yat Joong and Apoorva Verma who have been quite friendly and helpful throughout my PhD.

Last but not the least, I would like to give a big thanks to my friends and family without whom I would not have had the strength and courage to complete my PhD. My friends, especially Romita, Vidhi, Kripa and Aditi, have truly supported me at every moment and encouraged me whenever I have needed support. My family has been instrumental in making me the person I am today and bearing with all the frustrations and anger that I hurled on them. They have been my constant source of encouragement, support, guidance and hope in my four years of PhD and I hope that I have been able to make them proud of me. These four years have given me a lifetime of experiences, memories and lessons which I will remember, cherish and follow for the rest of my life.

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ABBREVIATIONS:

A	Acidic
ADAMs	A Disintegrin and matelloprotease
AA	Amino acid
APS	Ammonium persulfate
ATP	Adenosine 5´-triphosphate
Arp2/3	Actin related protein complex
BCR-ABL	Break point cluster-Abelson
bHLH	Basic helix-loop-helix
BSA	Bovine serum albumin
cDNA	Complementary DNA
CRIB	Cdc42/Rac interactive binding motif
Cdc42	Cell division cycle 42
C	Cofilin homology domain
DAPI	4',6-diamidino-2-phenylindole
DEPC	Diethylpyrocarbonate
DM	Differentiation media
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethysulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol

ECM	Extracellular matrix
E.coli	*Escherichia coli*
EGF	Epidermal growth factor
EHD	Eps15 homology domain protein
EDTA	Ethylenediamine tetraacetic acid
EMT	Epithelial-to-Mesenchymal transition
Erk1/2	Extracellular signal-regulated kinases 1/2
FA	Focal adhesions
F-actin	Filamentous actin
FAK	Focal adhesion kinase
FC	Focal complex
FBS	Fetal bovine serum
FGF	Fibroblast Growth Factor
G-actin	Monomeric globular actin
GBD	GTPase binding domain
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
Grb2 (protein-mouse), GRB2 (protein-human), grb2(gene-mouse), *GRB2*(gene- human)	Growth factor receptor-bound protein 2
GTP	Guanosine triphosphate
HGF	Hepatocyte growth factor

HRP	Horse radish peroxidase
IGF-1	Insulin like Growth Factor-1
IRS-1	Insulin receptor substrate-1
JNK	c-Jun N-terminal kinase
KGF	Keratinocyte Growth Factor
LAP	Latency associated Proteins
LB	Luria – Bertani medium
MAPK	Mitogen activated protein kinases
MAPs	Microtubule associated proteins
MCS	Multiple cloning site
MCK	Muscle Creatine kinase
MEFs	Mouse emryonic fibroblasts
MECs	Mammary epithelial cells
MEF2	Myocyte enhancer factor 2
MKK	Mitogen activated protein kinase kinase
MPCs	Myogenic precursor cells
MMP	Matrix metalloproteinase
MRF	Myogenic regulatory factor
MyHC	Myosin Heavy Chain
MyLC	Myosin light Chain
mTOR	Mammalian Target of Rapamycin
MTT	3-(4,5-Dimethylthiazol-2-yl)-2-5,diphenyltetrazolium bromide)

Nap1	Nucleosome assembly protein 1
N-WASP	Neural-WASP
NPFs	Nucleation promoting factors
NF κ β	Nuclear Factor κβ
NSCLC	Non small cell lung cancer
PAK	P21 activating kinases
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEI	Poly-ethylene imine
PI3K	Phosphatidylinositide 3-kinases
PIP2	Phosphatidylinositol (4,5) bisphosphate
PMSF	Phenylmethylsulfonyl fluoride
PRD	Proline rich domain
PS	Phosphatidylserine
qRT-PCR	Quantitative real time PCR
Rac1	Ras-related C3 botulinum toxin substrate1
RhoA	Ras homolog gene family, member A
RNA	Ribonucleic acid
RNase	Ribonuclease
SDS	Sodium dodecyl sulphate
SH2	Src homology 2
SH3	Src homology 3

SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
Shc	Src homology 2 domain-containing
shRNA	Small hairpin RNA
Sos	Son of Sevenless
SRF	Serum Response Fcator
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF-β	Transforming growth factor beta
TNF- α	Tumor Necrosis factor- α
WASP	Wiskott–Aldrich-Syndrome protein
WAVE	WASP-family verprolin homologous proteins
WIP	WASP-interacting protein
WIRE	WIP-Related
WH1	WASP homology domain
V	Verprolin homology domain
VEGF	Vascular Endothelial Growth Factor
ZEB	Zinc Finger E-box binding Homeobox 1

ABSTRACT

Grb2 is an adaptor protein whose SH2 domain has been shown to interact with phosphorylated tyrosine residues of activated receptors such as EGF and TGF-β and activate intracellular Ras/MAPK signaling pathway which controls the expression of genes required for biological processes such as proliferation and migration necessary for both myogenesis and EMT. The role of Grb2, though passive, is crucial in regulating the initiation of downstream signaling processes. It was hypothesized that Grb2 could play independent role in activating downstream interacting proteins such as N-WASP was tested in the two independent processes of myogenesis and EMT.

It was found that expression of Grb2 increased at the beginning of differentiation and was relatively constant during differentiation in C2C12 muscle myoblast cell line. Knockdown of Grb2 expression increased the myoblast fusion significantly compared to the control. Alternatively, over expression of exogenous Grb2 reduced the differentiation potential of Grb2 overexpressing cells significantly compared to the control indicating that Grb2 negatively regulates muscle differentiation. Grb2 domain structure consists of N and C-terminal SH3 domains flanking a central SH2 domain. Expression of point mutant $Grb2^{P206L}$, which is known to abolish the interaction of Grb2 with Neural-Wiskott Aldrich Syndrome Protein (N-WASP), did not inhibit muscle differentiation mediated by Grb2 indicating that differentiation inhibition by Grb2 may involve its interaction with N-WASP. Overexpression of exogenous N-WASP in Grb2 overexpressing cells was found to rescue the inhibition of differentiation mediated by Grb2 indicating that inhibition of differentiation by Grb2 occurs through N-WASP.

EMT, in contrast to myogenesis, involves the loss of cell-cell adhesion of epithelial cells, making them more motile and invasive and acquires mesenchymal phenotype to invade distant organs and tissues. TGF-β has been shown to induce EMT characterized by the loss of E-Cadherin expression and up regulation of mesenchymal markers such as Snail, vimentin etc. It was found that GRB2 expression increased significantly in the presence of TGF-β in lung adenocarcinoma cell line, A549. It was further observed that GRB2, even in the absence of TGF-β could cause drastic reduction in the expression of E-Cadherin, epithelial marker, compared to the control strengthening the hypothesis that Grb2 could play an independent role in controlling EMT. The migratory and invasive properties of A549 cells

were found to increase by overexpression of GRB2. The expression of one of the main mesenchymal transcription factor, Snail was found to increase in GRB2 over expressing cells, before TGF-β stimulation suggesting that the reduction of E-Cadherin expression observed on GRB2 over expression may be mediated by Snail in A549 cells.

The possibility of Grb2 playing a role in myogenesis and EMT, independent of growth factor activation, suggests that intracellular regulation of signaling pathways controlling myogenesis and EMT involves Grb2 in a much more prominent function than being just an adaptor protein. Both myogenesis and EMT are also pathophysiologically critical processes i.e. any abnormality in the process of muscle fusion can lead to muscle related disorders whereas EMT is an important hallmark of tumor progression. Hence, identifying an independent role of Grb2 in these processes will be crucial for designing targeted drug therapies.

Chapter 1: INTRODUCTION

Cell survival and almost every aspect of cell function in metazoans is dependent on interactions between the intracellular environment and external stimuli. These interactions involve a precisely orchestrated sequence of signaling events which generally begin with the activation of receptors present on the cell surface where the signal is generated [1]. Receptor activation then leads to a systematic relay and amplification of signals through protein-protein interactions and protein complex formations. This process is known as signal transduction. It finally culminates in the activation of various downstream target genes specific for different cell processes which include cell growth, differentiation, metastasis, immune response, apoptosis, development and proliferation and many more.

Receptor activation generally requires binding of external ligands in the form of growth factors, hormones, neurotransmitters, extracellular matrix (ECM) components etc. Upon receptor activation, the first groups of signal transducing molecules that respond and subsequently coordinate the downstream signaling cascade are called adaptor proteins. As the name suggests, these adaptors form an important link between activated receptors and the downstream signaling network. They mainly consist of Src homology domains 2 and 3 (SH2 and SH3) along with some other modular domains for e.g. kinase domains present in Src family kinases or DNA binding domain present in STAT family proteins [1]. The most evolutionarily conserved type of adaptor proteins are those without modular domains critical for their cellular and biological function [1]. These proteins generally have 1 SH2 domain and 2 or more SH3 domains. SH domains contain around 60-100 amino acids which represent non catalytic regions that are critical for a variety of protein-protein interactions [1]. Typically, SH2 domains interact with phosphorylated tyrosine residues of other proteins whereas SH3 domains interact with proteins which contain proline rich domains. Different combinations of SH2 and SH3 domains give these proteins remarkable specificity and control over a myriad range of intracellular signaling pathways that are responsible for different biological functions. One such adaptor is **Gr**owth Factor Receptor-**B**ound Protein **2 (Grb2)(mouse)**. Other examples of such proteins include Nck1/2, CrkL etc. Despite the simplicity of their structures, these proteins have a complex effect on the regulation of various downstream signaling proteins. The fact that they physically bridge the changing external environment to the adaptive internal environment is what makes their study

challenging. This work focuses on the adaptor functions of Grb2 during two biological processes- myogenesis and Epithelial-to-Mesenchymal Transition (EMT). The following section provides a general overview of Grb2 and its many roles.

1.1 Growth Factor receptor-bound protein 2(Grb2):

The adaptor Grb2 is a 25KDa molecular weight protein which consists of one SH2 domain flanked by two SH3 domains (Fig 1). The first Grb2 homolog was discovered in *C.elegans* and was called Sem-5. This protein was found to be important for downstream signaling events in the Let- 60 pathway (Let-60 is a *C.elegans* homolog of Ras) [2].The Let-60 signaling in *C.elegans* is required for vulval development and sex myoblast migration [3]. Drk, the Grb2 homolog in Drosophila was also shown to have an important role in several receptor tyrosine kinase activated pathways by binding specifically to a guanine nucleotide exchange factor called Sos (**S**on **O**f **S**evenless). Loss of function mutants in both *C.elegans* and *Drosophila* were found to die at the larval stages [3].

Shortly thereafter, many studies of mammalian Grb2 showed that the SH2 domain can bind to specific tyrosine phosphorylated residues of several growth factor activated receptor tyrosine kinases which include Epidermal Growth Factor Receptor (EGFR), Met(HGF receptor) among others. The SH3 domains have been found to interact with proline rich domain containing proteins one of which is Sos. The C terminus of Sos is rich in proline motifs which bind to Grb2 with high affinity [3]. In fact, Grb2 is presumed to be constitutively associated with Sos in the cytoplasm of the cell. Upon activation and subsequent auto phosphorylation of receptor tyrosine kinases, the Grb2 SH2 domain interacts with the tyrosine phosphorylated residues bringing Sos close to its target protein, Ras, near the plasma membrane allowing it to catalyze the interchange of GDP to GTP and activate Ras. GTP hydrolysis by Ras then initiates activation of kinase Raf and stimulates downstream Ras-mitogenactivated protein kinase (MAPK) pathway [4]. Thus Grb2/Sos complex has been found to participate in various signaling mechanisms in different cell types [3]. The structure of Grb2 has been completely solved which makes it the best characterized member of its family of adaptor proteins [5].

1.2 Structure of Grb2:

Grb2 crystallizes as a dimer with the SH2 domain of one Grb2 molecule in contact with the C- SH3 domain of the other Grb2 protein and vice versa leaving the two N-

SH3 domains available for binding to other proteins. At high concentrations it also forms a dimer in solution whereas at low concentrations it exists as a monomer [3]. The SH2 domain links the two SH3 domains by long flexible arms which helps all the three domains to function and interact independently [6]. Cussac D. et al, 1994 observed that the interaction of phosphorylated tyrosine residues with the SH2 domain does not cause any effect on the interaction of other proline rich residues with the SH3 domains and vice versa [7]. However, the two SH3 domains may make some contacts with each other. Sos has been observed to contain several proline rich residues which can interact with both the N and C-SH3 Grb2 domains. It has also been reported that in vivo, both the domains are necessary to stably bind to Sos. Individually their binding is weak (4-35 µM), however their combined binding probably increases the overall affinity. A specific proline rich sequence PVPPPVPPRR has been observed to have the highest binding affinity to both SH3 domains [6] .

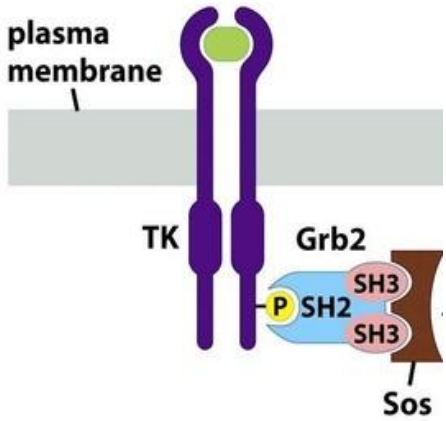


Fig 1.1: Localization of Grb2 at the plasma membrane along with Sos protein: The domain structure of Grb2 consists of one SH2 domain (amino acid residues:59-160) flanked by N-terminal SH3 domain (amino acid residues: 1-58) and C-terminal SH3 domain (aminoacid residues:161-217). The SH3 domains associate with Guanine nucleotide exchange factor, Sos. (*The Biology Of Cancer, 2nd Edition, Chapter 6*).

1.2.1 Grb2 SH2 domains:

The Grb2 SH2 domain contains a central antiparallel β sheet surrounded by two α helices [3]. The phosphotyrosine binding pocket is similar in most of the SH2 domain containing proteins which include Nck, Shc, Src family (Grb2 R67, R86, S88, S90, H107, K109). The binding site also maps a interacting surface for 3 or 4 residues after it which gives specificity to each SH2 [3]. It recognizes short phosphotyrosine motifs such as YxNx on the C terminus of activated receptor tyrosine kinases. This consensus sequence can be found in two proteins, Epidermal Growth Factor Receptor and Src homology 2 domain

containing transforming protein 1 (SHC) , both of which are known to strongly bind Grb2 [8]. Similar sites can be found on other receptors such as Hepatocyte Growth Factor receptor (HGFR), Insulin Receptor, Insulin like Growth Factor (IGF-1) receptors etc. The tyrosine motif on the SHC protein seems to be one of the most preferred interacting sites for Grb2 SH2 domain. Tyr1068 on EGFR also has a strong affinity for Grb2 SH2 domain. Point mutation on the Arg 86 located in the SH2 domain has been found to disrupt interaction of Grb2 SH2 domain with phosphotyrosine residues [9].

1.2.2 Grb2 SH3 domains:

The N- and C-SH3 Grb2 domains each form a β barrel [3] which can interact with the proline rich sequences at the C- terminus of Sos. A short proline rich sequence VPVPPPVPPRRRP in Sos is enough to bind to Grb2 [10]. The arginine residues in the above sequence seems to be a pre-requisite for binding to Grb2 SH3 domains; since substitution of arginine with lysine was observed to greatly reduce the affinity of Grb2 for these proline rich sequences. Moreover, addition of another arginine after the first one in the sequence has been shown to increase the affinity of the proline rich sequences to Grb2 since two times higher tryptophan signal (fluorescence) has been observed on doing so. Tryptophan fluorescence is a sensitive and intrinsic measurement of the fluorescence of the proteins containing aromatic amino acid residues such as tryptophan. Such measurements give an idea regarding the conformational state of a protein. The NMR structure has shown that the 2nd arginine by itself does not bind to Grb2 but changes the conformation of the peptide such that the first arginine is placed in the right position [3]. Mutant studies have shown that most of the fluorescence changes in the tryptophan signal takes place with the binding of the ligand to the C-SH3 domain compared to the N-SH3 domain [7]. One tryptophan is strictly present and conserved in both the SH3 domains (W36 in N SH3 and W193 in C SH3). Most probably, this tryptophan has a critical role in ligand binding to the domains. This tryptophan is followed by a second hydrophobic residue which is again a tryptophan for the C SH3 domain whereas a tyrosine for N SH3 domain [7]. This second tryptophan makes hydrophobic contacts with several residues within the ligand binding site. The SH3 Grb2 domains have been found to specifically recognize the proline rich motifs which suggest the presence of specific residues which are conserved in the domains. It has been predicted that specificity comes from the presence of acidic loops at each end of the binding clefts in the SH3 domains [7].

$Grb2^{P49L}$ and $Grb2^{G203R}$ are two commonly studied mutants in the N-SH3 and C-SH3 Grb2 domains respectively. In *C.elegans*, $Grb2^{P49L}$mutants do not survive whereas the $Grb2^{G203R}$ mutant has a milder phenotype [7]. In mammals, the $Grb2^{P49L}$ mutation has been shown to greatly impact the interaction of Sos1 with Grb2 when compared to the $Grb2^{G203R}$ mutation. This could mean either that the N-SH3 binds to Sos more than C-SH3 or that the Proline to Leucine substitution changes the structure of Grb2 much more drastically than the Glycine to Arginine substitution [7]. To eliminate the second possibility a second mutant $Grb2^{P206L}$ at the C-terminal SH3 was used. It was then confirmed that N-SH3 contributes more than C-SH3 to the binding to Sos [7].

1.3 Interacting partners of Grb2 at the plasma membrane:

The SH2 Grb2 domain has been observed to interact directly with the phosphotyrosine motifs of several proteins and growth factor receptors. One such receptor is the activated EGFR. Treatment of cells with EGF leads to the translocation of Grb2 and Sos to the plasma membrane and subsequent activation of Ras by Sos [11]. Other tyrosine phosphorylated proteins shown to interact with Grb2 include Insulin Receptor Substrate (IRS-1), the adaptor protein Shc, the chimeric oncogenic product BCR-ABL and the middle T antigen of the polyoma virus [11]. The SH2 domain of Shc protein has been found to be tyrosine phosphorylated on activation by various stimuli including EGF. The SH2 Grb2 domain has been found to interact with tyrosine phosphorylated Shc [12]. Shc has been shown to cause transformation of fibroblasts when over expressed and to cause neurite growth in PC12 cells [11]. Most of the interactions of Grb2 SH2 domains with tyrosine phosphorylated residues of upstream signaling molecules have been shown to lead to Ras activation via the formation of the Grb2/Sos complex. However, interaction of Grb2 SH2 and C-SH3 domain with a receptor like protein tyrosine phosphatase called, PTPα (protein tyrosine phosphatase-α), has been shown to be independent of Grb2/Sos complex formation and probably Ras activation([13],[14, 15],[16, 17]).

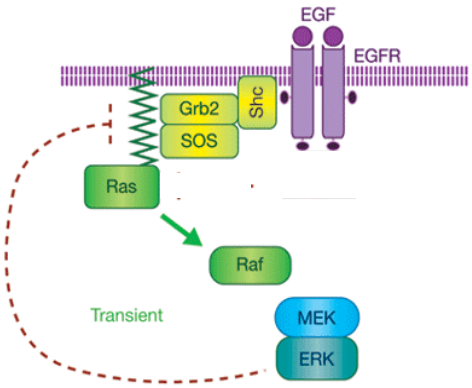


Fig 1.2 Interaction of Grb2 with EGFR: Grb2 can interact with EGFR either independently or through Shc and lead to activation of Ras which has been thought to be transient *[18]*

The Grb2 SH3 domains interacts with the C- terminal Sos domain with high affinity and form a stable complex in mammalian cells [3]. The Grb2/Sos complex has been found to be constitutive in most cell types; in few others such as Rat fibroblasts, it has been shown to be induced by EGF [11]. Although Grb2 exists mainly in complex with Sos, it has also been observed that the proportion of Grb2 is higher than Sos, at least in fibroblasts [3]. This means that a pool of free Grb2 exists in the cell which does not bind to Sos but may bind to other signaling proteins activating other pathways. In fact, it has been found that over expression of Grb2 can reduce Ras signaling [11]. However under certain circumstances such as stimulation by EGF or over expression of EGFR can increase Ras signaling in Grb2 over expressing cells [3]. This suggests that the release of unbound Grb2 is tightly regulated in the cell. This may be possible via the relocation of Grb2 to a distinct sub compartment within the cell. Also, the complex of Grb2 with Sos may be in a more proximal position, allowing it to be activated by the receptors at the cell surface and to subsequently stimulate Ras signaling close to the membrane. In contrast, the population of Grb2 bound to other proteins within the cytosol may not have enough access to these receptors at the membrane [3].

Grb2 was found to be localized to the membrane ruffles, structures formed during epithelial cell migration, by immunofluorescence assay. Subsequenlty, it was shown that mutations within the SH3 domain of the protein could affect this localization [19]. The localization of Grb2 to these ruffles suggests that it may be capable of interacting with proteins participating in the regulation of actin cytoskeleton formation. One such protein is Neural Wiskott Aldrich Syndrome Protein (N-WASP).

N-WASP is a protein belonging to the WASP/SCAR family of proteins and is expressed ubiquitously. These multi-domain WASP family proteins have been found to interact with Arp2/3 complex, a protein complex that is responsible for branched actin polymerisation [20]. Specifically, this interaction has been found to be responsible for the formation of structures rich in actin such as filopodia, lamellipodia, invadopodia etc that play critical roles in cell migration and adhesion [20]. N-WASP contains a N- terminal WASP homology 1(WH1) domain which is followed by a GTPase binding domain (GBD), proline rich domain and a VCA region (V- verprolin, C-cofilin, A-acidic) at the C-terminus interacting with Arp2/3 complex [20]. It has been observed that full length N-WASP activates Arp2/3 complex to a lesser extent when compared to the ability of just the VCA domain of the protein to do so. This suggests that N-WASP exists in an auto inhibited conformation in the cell [20]. In the presence of GTPases such as Cdc42 which binds to the GBD of N-WASP, this inhibition is released and N-WASP can activate the Arp2/3 complex to a greater extent [21] thus causing a burst of branched actin polymerisation. She,H.Y et al, 1997 showed that Grb2 causes the translocation of WASP from the cytoplasm to the plasma membrane after stimulation by EGF and that the Grb2/WASP complex probably plays an important part in connecting the RTKs to the actin cytoskeleton [22]. It has also been observed that the SH3 domains of Grb2 binds to the proline rich domain of N-WASP and activate it [20]. By using point mutants of Grb2 such as $Grb2^{P49L}$, $Grb2^{P206L}$, $Grb2^{G203R}$ along with in vitro actin polymerisation assays , it was observed that the binding of N-WASP with the C-SH3 Grb2 domain is responsible for the activation of Arp2/3 complex [20]. Actin polymerisation was found to be higher in the presence of both Grb2 and Cdc42 than with either protein separately. This suggests that both these effector proteins may bind N-WASP simultaneously in order to cause an enhanced activation of N-WASP and consequent actin polymerisation [20].

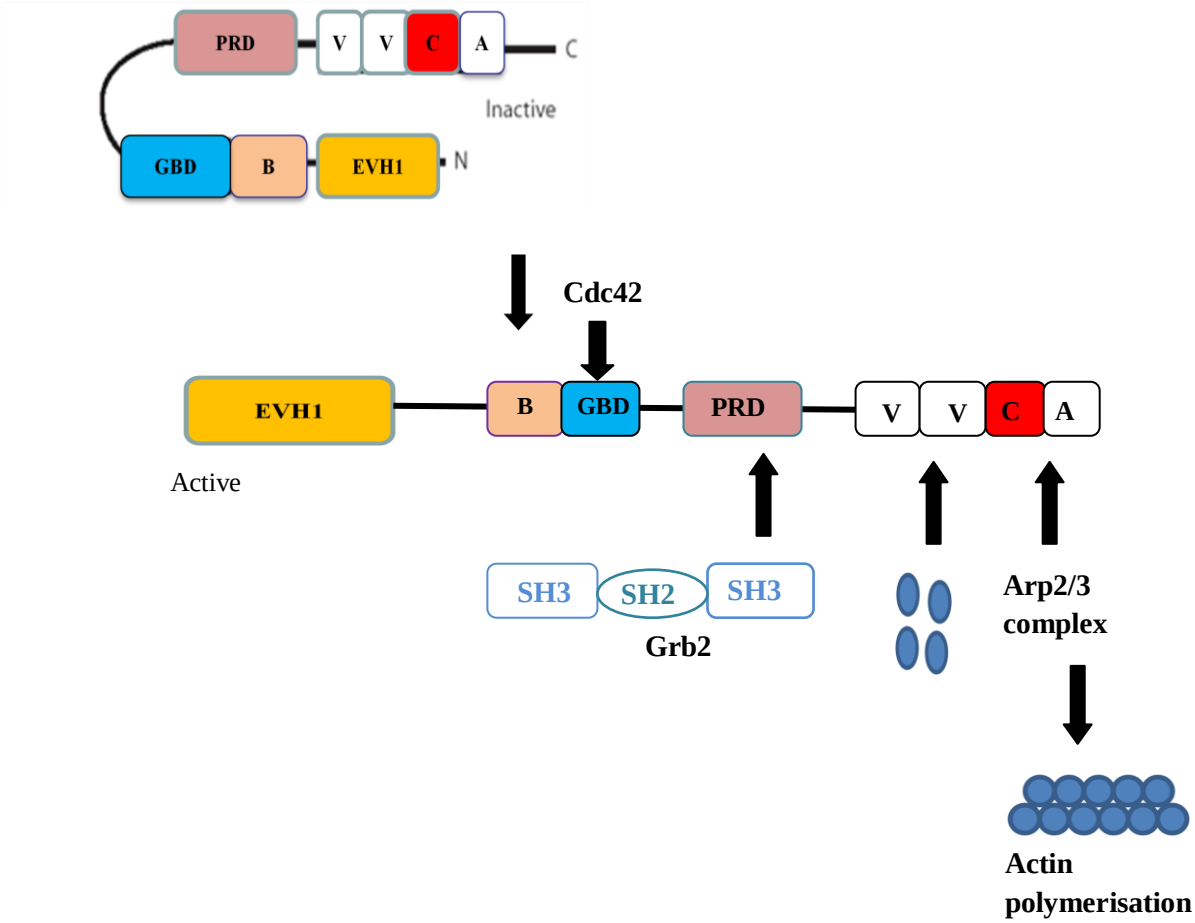


Fig 1.3 Interaction of Grb2 with N-WASP: C- SH3 Grb2 domain has been shown to interact with the proline rich domain of N-WASP and act as an effector to stimulate actin polymerisation through the formation of N-WASP-Arp2/3 complex *[20]*.

1.4 FUNCTIONS OF Grb2:

Grb2 has been studied extensively as a pivotal signal transducer, capable of linking the RTKs to Ras signaling**.** After the role of Sem-5 (*C.elegans* homolog of Grb2) in vulval formation was established, mammalian Grb2 was identified during a screen for phosphorylated receptor targets [23]. The search for the missing link between phosphorylated receptors and Grb2 led to the discovery of Sos, a GEF [23]. A direct interaction between Grb2 and Sos was shown to be responsible for the translocation of Sos from the cytoplasm close to the plasma membrane and thus proximal to its target Ras; which can then initiate the downstream signaling pathway. Thus, Grb2 is a vital adaptor molecule that can, by binding to Sos, physically connect growth factor activated receptors to Ras signaling [23]. Ras signaling is an important pathway that controls normal cell proliferation [24]. Ras modulates different aspects of cell proliferation and survival by binding to several downstream effector proteins. The first among these is Raf, a Serine/Threonine kinase[24]. Raf further activates a

series of kinases which finally leads to the phosphorylation of ERK1/2 (extracellular signal-regulated-kinases 1 and 2) [24]. ERK1/2 have several cytosolic and nuclear targets. Nuclear targets include many transcription factors. One example is ELK-1, which is the member of the ETS transcription factor family and can regulate c-Fos expression and c-Jun. Activation of these transcription factors finally stimulates cell cycle regulators such as cyclins which then enable cell cycle progression[24]. There are many other proteins and pathways controlled directly or indirectly by Ras such as AKT (cell survival/apoptosis), Rac (actin cytoskeleton), Phospholipase Cε (calcium signaling) etc [24]. Hence, any aberrations or mutations in the Ras proteins can lead to uncontrolled cell proliferation and promote malignant transformations [24].

Grb2 was initially studied for its role in cell proliferation. Due to its importance as an adaptor protein that can interact with different downstream molecules and thereby activate various signaling mechanisms, recent studies have stressed on its function in cancer metastasis and cell motility. To study these aspects of Grb2 function, different cell lines and mouse models were used. Studies have shown that Grb2 knockout in mice is embryonically lethal [25]. Hypomorphic mutants of Grb2 in mice also show major defects in cardiovascular development and processes such as cell migration and vasculogenesis [26]. Additionally, GRB2 expression is elevated in many types of cancers including breast cancer, where GRB2 has been shown to enhance MAPK signaling [27]. In MCF-7 cells, GRB2 has been shown to be involved in Keratinocyte Growth Factor (KGF) induced motility. This underscores the importance of Grb2 function in cancer cell invasion and metastasis [28]. Even in cell lines where the immediate upstream activator of Grb2, EGFR, is not over expressed such as bladder cancer cells, Grb2 has been observed to be highly expressed and together with Sos 1 has been found to be mainly responsible for tumor metastasis[29].

Cell adhesion and migration are important functional characteristics that are affected during tumor metastasis and invasion. When cancer cells begin to metastasize, they lose cell-to-cell and cell- ECM contact and become highly motile. Cell-ECM interaction is mediated by the integrin family of cell membrane receptors [30]. Integrin is a part of a larger protein complex known as the focal adhesion complexes. Dynamic interactions between the different proteins of this complex form the basis of continuous assembly and disassembly of focal adhesions during cell migration. Since integrins lack inherent catalytic activity, they depend on the kinase activity of another focal adhesion protein- Focal Adhesion Kinase (FAK) for their function [30]. The association of FAK with integrins is a crucial event in cell

migration [31] and FAK activity depends on its phosphorylation [30]. Upon activation, FAK has been shown to bind other proteins. One such protein is Grb2, whose SH2 domain has been shown to interact with high affinity around Tyr925 in FAK, which is also an important phosphorylation site [32]. Several studies have shown an important role of FAK in cancer metastasis which partly stresses on the novel role of Grb2 in tumor metastasis as well.

The next step of cancer metastasis involves tissue degradation and subsequent invasion of blood vessels (extravasation), allowing the metastatic cells to reach distant sites in the body. This process is carried out by Matrix Metalloproteinases (MMP), diverse proteins family capable of degrading several components of ECM [33]. ADAMs (A Disintegrin and Metalloprotease), a family of MMPs, have been implicated in several tumors [34]. ADAM12 has been found to interact directly with the Grb2 SH3 domains through its proline rich cytoplasmic domain [34]. Both these proteins have also been observed on the membrane ruffles, structures formed during cell migration in epithelial cells [34].

Grb2 has been observed to bind to several proteins involved in the regulation of the actin cytoskeleton. It thereby links upstream signaling proteins to the actin cytoskeleton and regulates cell motility. Grb2, as mentioned earlier, binds directly to WASP family members such as WASP and N-WASP, through its SH3 domain and thus translocates them from the cytoplasm to the membrane. Here, they are further activated by GTPases such as Cdc42 and can then cause downstream actin polymerisation via interactions with the Arp2/3 complex ([35], [36]). PAKs (p21 activating kinases) are serine/threonine kinases which are known to regulate the actin cytoskeleton through the formation of actin rich motile structures such as filopodia and lamellipodia. Hence, PAKs have been implicated widely in cancers caused by responses to external stimuli. PAKs have been found to bind directly to Grb2 through their proline rich sequence and this complex is required for EGF induced lamellipodia formation ([37],[38]). Podosome is another type of motile actin rich structure found to contain Grb2 specifically, along with cortactin, Arp2/3, N-WASP and VASP. These structures have been found to be present in many cell types [39]. Other than the actin cytoskeleton, Grb2 also affects microtubule formation. For example, Grb2 has been found to interact with dynactin in vivo, through its N-SH3 domain in osteoclasts. Dynactin is a multiple subunit containing activator of the microtubule protein dynein which regulates cell polarization during cell migration [40]. Another microtubule associated protein, MAP2, present abundantly within neurons, has also been shown to bind to Grb2 [41].

In summary, Grb2 has been studied extensively as a receptor proximal adaptor protein. Various growth factor receptors have been shown to, either directly or indirectly, bind to Grb2. EGF is one of the most extensively studied growth factor known to activate Grb2 and thus trigger downstream Ras/MAPK pathways involved in cell proliferation [42]. Binding of EGF to EGF receptor causes its activation and autophosphorylation at several tyrosine residues which form the docking site for several adaptor proteins. Tyr1068 has been shown to be the direct binding site for Grb2 [42]. This has been shown to cause the complex formation of Grb2, Sos and EGFR and activate downstream Ras signaling pathway [42]. Growth factors, other than stimulating "growth" modulate a plethora of functions such as cell survival, cell migration and tumor invasion to name a few. Vascular Endothelial Growth Factor (VEGF) and Fibroblast Growth Factor 2 (FGF2), which play important roles in forming new blood vessels in both normal and pathological conditions such as tumor angiogenesis have been shown to interact with Grb2 ([43],[44]).Transforming Growth Factor-β (TGF-β) is a part of a large family of around 40 secreted cytokines known to regulate a wide variety of cell functions such as cell differentiation, proliferation, migration, apoptosis, stem cell maintenance etc.[45]. It has been shown to perform tumor suppressing as well as tumor promoting functions in breast cancer cells [46]. TGF-β interacts with and activates TGF-β receptor II and I (TBRII and I) by auto and transphosphorylations respectively which then activate downstream signaling either by Smad dependent or independent mechanisms[45]. GRB2 binding to Tyr284 in TBRII has been found to be necessary for TGF-β induced mammary tumor metastasis in NMuMG breast cancer cells [46]. HGF is another growth factor which has been shown to induce various biological responses such as proliferation, migration, invasion etc [47]. It is known to bind to a receptor tyrosine kinase, c-Met [47]. This binding induces a phenomenon known as scattering, wherein cell clusters lose polarity, dissociate through the disruption of cell- cell contacts and subsequently migrate [48]. Binding of HGF to c-Met induces phosphorylation at the several tyrosine residues at the cytoplasmic domain of c-Met, one of which is Tyr1356. This phosphorylated tyrosine residue has been shown to be the binding site for the Grb2 SH2 domain ([48],[47]). It has been further shown that binding of Grb2 to c-Met is essential for HGF induced inhibition of muscle differentiation [49].

1.5 Muscle Differentiation (Myogenesis):

Myogenesis is the process of generation of muscle tissues from muscle precursor cells in the body. It occurs both during embryonic and post natal development (muscle regeneration). There are three types of muscle tissues- Smooth, cardiac and skeletal [50]. Smooth muscles are involuntary muscles lining the walls of organs (intestines, stomach, urethra etc) and vessels (blood and lymphatic) responsible for controlling shape and diameter changes. Cardiac muscles are also involuntary muscles found only in the heart; controlling its contraction and the dynamics of blood circulation. Skeletal or striated muscles are voluntary muscles that control the body posture, movement and body temperature. Striations are due to the alignment of sarcomeres, which represent the functional unit of muscle. They consist of multinucleated muscle fibres. Skeletal muscles are further divided in two types: slow twitch and fast twitch. These skeletal muscles make up 35-40% of total human body weight.

In adults, skeletal muscles are attached by tendons to bones. Each muscle is made up of numerous muscle fascicles which are surrounded by a layer of connective tissues called the perimycium [51]. Each fasciculus consists of bundles of multinucleated myofibres which are surrounded by an endomycium. In between the basement membrane and plasma lemma, special type of mononucleated cells, called satellite cells, exists in a quiescent state. Myofibres contain myofibrils which are surrounded by a tubular network, the sarcoplasmic reticulum. Each myofibril consists of successive repeats of sarcomeres [51]. Each sarcomere contains alternating rows of thick filaments of myosin and thin filaments of F-actin which lend the muscle their contractile properties [51] (Fig 1.4).

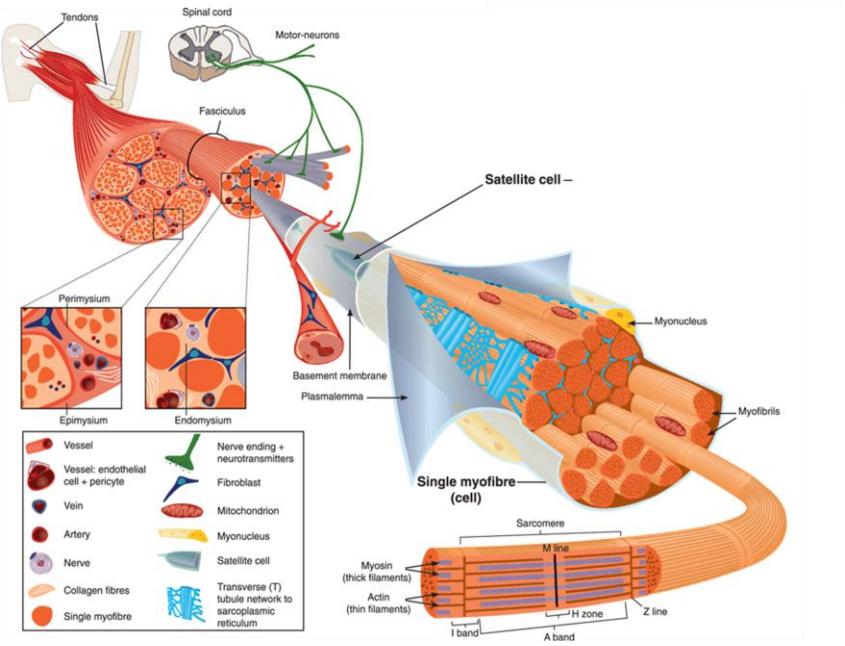


Fig 1.4 Skeletal muscle and its associated structures: Sarcomere is the basic unit of a skeletal muscle whose successive arrangement forms the myofibrils which then forms the myofibre. Satellite cells are located in between the basement membrane and plasma lemma of a myofibre *[51]*.

All the embryonic tissues that can give rise to skeletal muscles are mesodermal in origin, with most of them arising from the paraxial trunk mesoderm that consists of somites and head mesoderm [52]. Somites are segmented structures which arise from unsegmented paraxial mesoderms [53]. Somites then give rise to the dermomyotome which goes on to form the dermal precursor and myogenic precursor cells [54]. Expression of muscle specific markers commits the myogenic precursor cells to myogenesis. Muscle progenitor cells (MPCs) are multipotent and acquire myogenic identity through the expression of myogenic regulatory factors (MRFs). The progenitor cells then give rise to myoblasts. Myoblasts then differentiate in to multinucleated myofibres via the expression of markers such as Myogenin and MyoD [54]. These myofibres then form the myotome which is the first skeletal muscle mass to be formed in the somite. Subsequently, both the embryonic and fetal myoblasts contribute to muscle tissue formation by differentiating in to primary and secondary muscle fibres respectively [54]. Kassar-Duchossoy *et al.*, 2005 discovered a novel and distinct type of cell population called the satellite cells which only express Pax3 and Pax7 but not the MRFs. These non-committed, specific marker expressing cells were thought to form in order to maintain the stem cell pool. These cells could later form muscle precursor cells [54].

1.6 Myogenesis at the cellular and molecular level:

Myogenic differentiation is required for generation, maintenance and regeneration of myofibres. Cellular and molecular mechanisms and regulation at each step of myogenesis is quite complex and not well understood. Understanding each step during myoblast fusion will help to design therapies that can prevent various muscle degenerative diseases such as muscular dystrophy as well as muscle injuries and ageing.

Myogenesis is a regulated and coordinated series of events: beginning with proliferation of myoblasts followed by withdrawal from the cell cycle and expression of muscle specific marker genes, myocyte alignment and lastly fusion of mono-nucleated myocytes to form multi-nucleated myotubes ([55], [56], [57]). Different types of muscle specific genes are expressed at every step of differentiation. Not all myocytes differentiate into myotubes. Some of them revert back to a quiescent pool of cells called satellite cells which can again give rise to myotubes when needed. Satellite cells are a crucial cell type necessary during normal skeletal regeneration of muscles in adults such as during muscle injury, muscle trauma, ageing.

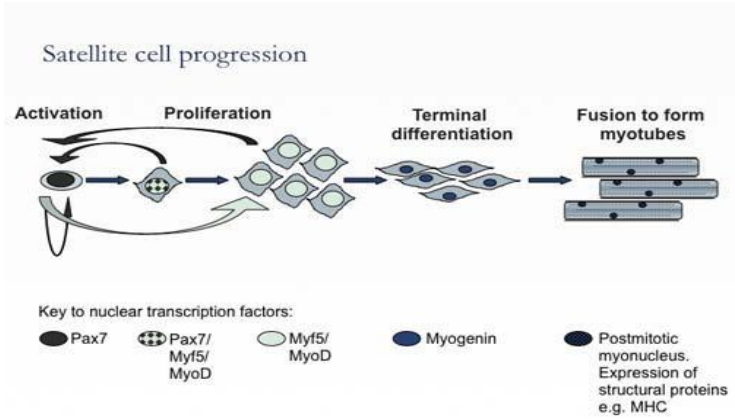


Fig 1.5 Steps of myogenic differentiation: Satellite cells undergo a well orchestrated differentiation mechanism with the expression of early and late differentiation markers to induce growth or repair muscles *(www.pub.phar.cam.ac.uk)*

1.6.1 Satellite Cells:

Satellite cells are a pool of quiescent, undifferentiated, mononuclear cells present in mammals [56]. They are so named because of their distinct location within the basal lamina around the myofibres; between the cell membrane and basement membrane. Other features of satellite cells include a higher ratio of nucleus to cytoplasm and a smaller

size of nucleus with more heterochromatin compared to the myofibre nuclei emphasizing the fact that these cells are dormant mitotically and less active transcriptionally than myonuclei [56]. Satellite cells are a distinct from fetal and embryonic myoblasts. However, their exact origin remains contentious with emerging data supporting a dual origin model. They are believed to have a somitic origin from the mesoderm as well as an endothelial origin from the embryonic dorsal aorta [56]. Satellite cells are found to be present in all skeletal muscle types. However, they show certain differences in distribution. For example, the motor neuron junction has a higher density of satellite cells compared to other regions. This may be specifically to regulate the satellite cell pool by concentrating their distribution at specific locations in the body [56].

The proximity of satellite cells to myofibres has made it easier to hypothesize their role in skeletal muscle growth and regeneration. Various experiments using radioactive labelling, electron microscopy and thymdine tracing have provided definite evidence that although satellite cells are mitotically dormant, they can quickly re-enter the cell cycle after events such as muscle injury [58]. Various studies have shown that the proliferation and differentiation of satellite cells to form mature myofibres requires the presence of many factors such as hormones, nutrition, extent of injury and so on. Satellite cells have been shown to have the ability to both proliferate and differentiate into muscle precursor cells as well as self renew. This was first proven by a single myofibre transplantation assay where it was observed that transplantation of a few satellite cells on a single myofibre could sustain many rounds of muscle regeneration [58]. With respect to gene expression, satellite cells are quite heterogenous differing in their gene expression signature profiles and propensity of myogenic differentiation. However, a universal marker for satellite cells has been the gene, Pax7, found to be expressed in both dormant as well as activated satellite cells *in vivo* [56]. Pax 7 is a part of paired E box containing transcription factor family and has been shown to be important for the development of skeletal muscles in adults [58]. Pax3, a paralog of Pax7, has also been observed to be present in satellite cells although in small amount. Pax3 has been shown to play a much more critical role in somitic myogenesis [58]. Although $Pax7^{-/-}$ mice have not been observed to have major defects in muscle development, Pax7 has been shown to be crucial for the formation of satellite cells. Hence in the absence of Pax7, mice with increased muscle wasting and failure to regenerate muscle after acute injury have been observed [59]. During embryogenesis, both Pax3/$Pax7^{+}$ cells exist and form post natal satellite cells where the expression of Pax3 is specifically down regulated at birth making

Pax7 a universal marker for satellite cells. This pool of Pax7 expressing, proliferating cells are continuously expressed and rapidly down-regulated, when required, during muscle differentiation [59]. Therefore, skeletal muscles always maintain a small population of Pax7/MyoD^{+} cells. In response to stimuli for muscle regeneration, Pax7 expression is maintained whereas MyoD expression is up-regulated giving rise to two distinct populations of satellite cells. Pax7^{+}/MyoD^{+} cells give rise to muscle precursor cells leading to eventual terminal differentiation and muscle regeneration. Pax7^{+}/MyoD^{-} cells form a smaller subset of satellite cell population which have been termed as reserve cells and maintain the satellite cell pool [54].

1.6.2 Myogenic precursor cells- Myoblasts, myocytes:

Expression of MyoD in satellite cells commits them to terminal differentiation. MyoD is considered to be a myogenic master gene as its ectopic expression in even non muscle cells can trigger the myogenic program [60]. Many studies have suggested that Pax7 can regulate the expression of MyoD indirectly, in order to control the rate of differentiation induction [59]. However, once myogenin, another myogenic regulatory factor, is expressed, Pax7 can no longer repress differentiation. In fact, in the presence of Myogenin, Pax7 levels are significantly reduced in myoblasts [59].

The molecular and cellular changes that occur in adult skeletal muscles during muscle regeneration are quite similar to those that occur during embryonic muscle development. Signals from a damaged environment such as muscle injury causes the satellite cells, which are mitotically dormant to activate and start proliferation [58]. Proliferating satellite cells form myogenic precursor cells (MPCs) or adult myoblast. Myoblast activation is characterized by the rapid increase in expression of two myogenic regulatory factors-Myf5 and MyoD [61]. MRFs belong to the basic- helix- loop- helix (bHLH) family of transcription factors. They regulate transcription of many target genes by binding to a DNA sequence, E Box (CANNTG) [62]. The expression levels of MyoD and Myf5 is temporally regulated in myoblasts suggesting that they may have different roles during myogenesis [58]. Studies in Myod$^{-/-}$ and Myf5$^{-/-}$ myoblasts suggest that MyoD is more essential for differentiation whereas Myf5 is crucial for proliferation [58]. Hence, it has been hypothesized that different expression profiles of MyoD and Myf5 can determine the myogenic fate of activated satellite cells. In cells where expression of MyoD is higher, muscle differentiation is reached early

whereas in cells where Myf5 expression predominates, enhanced proliferation and delayed differentiation has been observed ([63], [64]). However, several studies have shown that MyoD expression does not necessarily commit myoblasts to differentiation and that its expression is influenced by various other factors such as MEF2 and Serum Response Factor (SRF) among others [58]. Majority of the myoblasts fuse with one another or with existing damaged myotubes to form new myofibres. Late differentiation is initiated by the induction of Myogenin and Mrf6 [65]. Most of the genes expressed during the induction of terminal differentiation are the muscle specific genes encoding skeletal actin, myosin (Myosin Heavy Chain(MHC)) and troponins; all of which are required for the formation of skeletal muscles [66].

1.7 **Sub-cellular events regulating myogenesis:**

During the process of myogenesis, various changes have been observed even at the cellular level. These include changes in cell shape, cell-ECM contacts, cell-cell interactions, cell adhesion events and actin cytoskeleton dynamics. Two types of myogenesis have been characterized in the mammalian embryo as well as in skeletal muscles. Primary myogenesis, which is the fusion of differentiated myoblasts with one another involves primary myotubes, which are thin and contain few nuclei [67]. Secondary myogenesis, which is the addition of myofibres to existing ones involves secondary myotubes, which have a larger number of nuclei and begin expressing contractile proteins required for muscle growth [57]. It has been observed that different proteins are required for both these processes. For example, membrane proteins such as β1 integrins, VLA-4,VCAM and caveolin-3 have been shown to be important for primary myogenesis whereas IL-4 has been shown to be critical for secondary myogenesis([68], [69], [70], [71]). The sub cellular events regulating myogenesis are discussed in detail below.

1.7.1 Cell-ECM adhesion:

Integrin mediated cell adhesion to the proteins of the ECM has been found to regulate both myoblast proliferation and differentiation [72]. Upon entering the myogenic program, the myoblasts secrete fibronectin, an ECM protein which can activate and interact with integrins, especially the α5β1 integrin. This interaction is required for cell alignment and fusion of myoblasts to form myotubes. Hence blocking this interaction can inhibit

myogenesis by reducing the expression levels of promyogenic proteins such as MyoD and Cdc42 [73]. Cell- ECM adhesion has been shown to activate Focal Adhesion Kinase (FAK) which can further bind to several proteins such as Grb2, Shc, Paxillin, p130Cas and PI3-kinase thereby activating several downstream signaling pathways which regulate myoblast proliferation, differentiation, actin cytoskeleton rearrangement and migration among others [73]. Cell migration and adhesion are other crucial aspects of myoblast differentiation, since the cells need to migrate and attach to each other and to the substrate in order to fuse and form myotubes. Focal adhesions are dynamic multiprotein complexes which are activated after ECM- integrin interaction and are temporally expressed at the cell periphery to regulate the process of cell adhesion. Vinculin is a cytosolic focal adhesion protein which is recruited early in the development of focal adhesions and forms a direct connection between the actin cytoskeleton and integrins [74]. Vinculin has been shown to bind to the cytoplasmic tail of β integrins and stabilize focal adhesion complexes by integrin clustering [75]. Hence, vinculin has been commonly used as a marker for studying focal adhesions in different cell types. Different proteins of the ECM have also been directly implicated in the regulation of muscle differentiation. The Basal lamina is a part of the ECM that surrounds the muscle fibres where the satellite cells reside. Laminin is an important constituent of the basal lamina. It has been observed that laminin increases myoblast proliferation and change of morphology during differentiation and that its knockdown reduces myogenic differentiation [76]. Collagen is another important constituent of the ECM and has been shown to be required for muscle differentiation in mouse C2C12 myoblasts and rat L6 myoblasts [76].

1.7.2 Elongation and Migration:

At the onset of differentiation, cell morphology changes have been observed to take place in myoblasts which align to fuse to each other to form myotubes. Myoblasts display fibroblast like morphology before initiation of differentiation. When they align with each other, they become elongated and spindle shaped ([77], [78]). Various proteins involved in actin cytoskeleton rearrangement, cell migration and adhesion play a critical role in regulating these changes in myoblast morphology. Non muscle myosin 2A and 2B, along with actin stress fibres, have been found to co localize near the cell membrane of aligned spindle shaped myoblasts [77]. Non muscle myosin 2A was found to preferentially interact with actin and to be responsible for myoblast elongation whereas non muscle myosin 2B was found to be crucial for the prevention of over elongation of myoblasts [77]. Kindlin-2, a focal

adhesion molecule, has also been shown to play a critical role in myocyte elongation and was found to be required for muscle differentiation [78]. Major reorganization in the actin and micro tubular cytoskeleton occurs during myoblast elongation and is required for myogenesis [79]. Actin filaments have been found to be present near the periphery of the aligned myoblasts [79], highlighting the importance of their function during myoblast differentiation.

Cell migration is another prerequisite for muscle differentiation. Fusion-competent myoblasts align and migrate towards each other to fuse and form myotubes. Hence, any defect in migration has been observed to reduce myogenesis ([80], [81], [82]). Migration of myoblasts towards each other has been observed to be influenced largely by the chemokines, growth factors and ECM proteins secreted by the neighbouring myoblasts. HGF, TGF-β, IGF-1 have been shown to be positive regulators of myoblast migration ([83], [84]). It has also been observed that during embryonic development, migrating muscle precursor cells target the development of organs such as limbs, tongue etc.[83]. Bandow et al, 2004 also observed that HGF increases migration and proliferation of myogenic cells in the mouse tongue by inducing the expression of MMP-9, a member of the zinc metalloproteinase family involved in degradation of extracellular matrix [83].

1.7.3 Recognition and cell-cell adhesion:

Recognition between fusion competent myoblasts is an important step towards facilitating their fusion. Changes in the membrane compositions of the fusing membranes, secretion of chemokines and growth factors, rearrangement of the actin cytoskeleton and expression of cell adhesion molecules such as cadherins are some of the changes that occur before fusion ([85], [86], [87]). High composition of amino phospholipids such as phosphotidylserine was observed on the cytoplasmic and external surface of cells ready to fuse suggesting a possible role for lipid composition in membrane fusion [87]. Changes in the composition and orientation of phospholipids on the plasma membrane allow for topological changes of the membrane allowing them to fuse with one another [87].

Cell-cell adhesion, after recognition, is also essential for membrane fusion. Gap junctions have been observed to form before membrane fusion take place [88]. Several adherens junction proteins such as N-cadherin, M-cadherin, β-catenin etc have been implicated in mediating events that occur immediately before membrane fusion. β-catenin forms a connection between the cadherins and the actin cytoskeleton. This cadherin-catenin

complex is mechanosensitive and is responsible for the rearrangement of the actin cytoskeleton that takes place during muscle differentiation [89]. The N- terminus of β-catenin has been shown to bind to actin through other adaptor and focal adhesion proteins such as α-catenin, vinculin, Arp2/3, VASP etc [89]. Signaling proteins downstream of the cadherins regulating actin cytoskeleton such as Rac 1 and Cdc42 have been found to be essential for myoblast fusion [90]. Rac1 and Cdc42 are Rho GTPases which are responsible for the formation of actin rich motile structures during migration and vesicle trafficking [90]. Conditional knockout of Rac1 and Cdc42 in mice showed that although early adhesion processes such as recruitment of α and β- catenins was not affected, accumulation of vinculin, VASP and actin at the fusion sites was significantly reduced and this affected membrane fusion [90]. It was also hypothesized that Rac1 and Cdc42 act independently with Arp2/3 recruitment at the contact sites being strongly reduced in Rac1 knockout mice. These Rac1 deficient mice showed a stronger defect in membrane fusion ability compared to mice with Cdc42 deficiency [90].

1.7.4 Myotube formation/membrane fusion:

The last step of myogenesis is formation of myotubes and myofibres where the fusion competent, mononucleated myoblasts fuse to become multinucleated myotubes which eventually mature in to muscle fibres. Myotubes express the genes required for formation and maintenance of skeletal muscles such as myosin heavy and light chain(MyHC and MyLC) , muscle creatine kinase (MCK), desmin and troponin T [56]. Skeletal muscle contraction is largely dependent on the ATPase activity of myosin heavy chain isoforms expressed in the cell [91]. Genetic analysis of Drosophila mutants having defects in muscle formation have been instrumental in elucidating the cellular and molecular steps that are involved in muscle fusion. However, a detailed understanding of mechanisms that regulate fusion is still lacking and vague. It is, however, known that during membrane fusion, extensive cytoskeletal rearrangements occur. Cytochalasin B and taxol, which are inhibitors of microtubule assembly, have been observed to inhibit fusion [92]. Endocytic recycling, which is the process of recruitment of membrane proteins initially taken up by endocytosis back to the cell membrane has also been shown to play a critical role in myoblast fusion [93]. Ultrastructure analysis of fusing myoblasts has revealed the formation of vesicles at the site of fusion. Myoferlin is a membrane protein expressed highly in fusing myoblasts and was found to be required during fusion. It has been observed

to bind to EHD-2, a part of the eps15 homology domain protein (EHD) family implicated in endocytic recycling- during fusion [93].

1.8 Role of actin cytoskeleton in membrane fusion:

The actin cytoskeleton regulates almost all upstream signaling events responsible for various biological processes such as cell proliferation, growth, motility, differentiation etc. Actin polymerization in myoblast fusion is critical since fusion competent cells migrate towards one another in order to fuse. For this migration step to occur successfully, they need to form actin rich motile structures (filopodia). In C2C12 murine myoblasts, it was observed that myoblast treatment with latrunculin A, an actin filament destabilizing agent, reduced myoblast fusion [94]. Also, in Drosophila, it was found that F- actin rich foci were formed at the site of fusing myoblasts indicating an important role for actin during fusion [94]. Actin polymerization occurs by the rapid assembly of F actin monomers at specific sub cellular locations in the cell. Actin monomers are generally stabilized by proteins such as profilin which prevents spontaneous assembly of F actin [95], though still making them readily available for nucleation, which forms the rate limiting step in actin assembly. Different proteins play specific roles in controlling the length and time of actin polymerisation thereby regulating its function. Nucleation, being the most critical step, is regulated by many proteins some of which form the Arp2/3 complex. This is a protein complex of seven polypeptides two of which are actin related proteins (Arp2 and Arp 3) [96]. Dimerization of this protein complex mimics the actin nucleus to which actin monomers are further added. However, it has been observed that the binding of actin monomers to the Arp2/3 complex alone is weak. Hence, nucleation promotion factors (NPF) are required to facilitate this process [95]. The most well studied NPFs belong to the WASp/WAVE/SCAR family of proteins which play two significant roles in nucleation [95]. Firstly, they bring the Arp2 and Arp3 components of the complex closer to increase nucleation efficiency. Secondly, they can themselves recruit actin monomers to facilitate the nucleation process [95].

The mammalian WASp/WAVE/SCAR family consists of Wiskott Aldrich Syndrome protein (WASp), Neural- WASP (N-WASP), WASp-family verprolin-homologous protein (WAVE) 1/SCAR1, WAVE 2 and WAVE 3 (Scar is a homolog of WAVE found in

Dictyostelium) [97]. WASp is mainly found in the hematopoietic cells whereas N-WASP expresses ubiquitiously although it is known to be abundantly expressed in the brain [97]. WASp and N-WASP have similar domain structure including a GTPase Binding Domain (GBD) present in both proteins. A Rho GTPase, Cdc42, was found to bind to the GBD and is also required for activating the auto inhibitory conformation of both proteins [97]. Actin polymerization assays have shown that both N-WASP and WASp induce polymerization of actin when over expressed in fibroblasts ([98], [99]). It was separately observed that Cdc42 and the Arp2/3 complex induce actin polymerisation in Xenopus extracts [100]. Rohatgi et al, 1999 showed that the binding of N-WASP C- terminal to the Arp2/3 complex activates actin polymerization to a significant extent in vitro. This interaction also links Cdc42 signals to the Arp2/3 complex and subsequent actin polymerisation in vitro and in Xenopus egg extracts [21]. N-WASP and WAVE proteins were also found to participate in the formation of actin rich motile structures-filopodia and lamellipodia that are induced by Cdc42 and Rac1 respectively ([101], [102]).

WASp and N-WASP possess common domain structure with a N-terminal WH1 domain (Ena –Vasp-Homology domain 1/EVH1), a basic region and a GTPase binding domain (GBD) [103]. The N-WASP and WASP C- terminus is responsible for their actin polymerization activity. The C terminus contains a verprolin homology domain (V) followed by a central (C) and acidic (A) domain. Together, the VCA region is responsible for interacting with actin monomers and Arp2/3 complex leading to its activation and a subsequent burst of actin polymerisation [103]. The N-terminus, on the other hand, interacts with several proteins which regulate its stability and activity. For e.g. the WH1 domain has been shown to bind to proline rich sequences of WASP interacting protein (WIP). This interaction has been found to be stable and responsible for suppressing the activity of N-WASP [103]. GBD is also known as the Cdc42/Rac-interactive binding region (CRIB). As the name suggests, Cdc42, a Rho GTPase, can bind to this region and has been found to be responsible for activating it by releasing the auto inhibited closed conformation [21]. There is also a proline rich domain (PRD) which has been shown to bind to a number of SH3 domain containing proteins such as Nck, Grb2, Toca-1, IRSp53, WISH, Crk etc [103] thereby influencing different downstream signaling pathways. N-WASP has been found to be required for myoblast fusion in mice [104]. Even though N-WASP was not found to be required during the initial stages of myogenesis, N-WASP$^{-/-}$ myoblasts failed to fuse and formed mono nucleated myotubes [104].

1.9 Research on Grb2 in muscle differentiation:

As described earlier, Grb2 can interact with the proline rich domain of N-WASP through its C-terminus SH3 domain [20]. It has been observed that Grb2 links N-WASP to Arp2/3 complex dependent actin polymerisation by activating it [20]. Grb2 has also been shown to link N-WASP to EGF [22] thereby indirectly linking receptor tyrosine kinase signaling to the actin cytoskeleton. However, the role of Grb2 in myoblast fusion has not been explored in details. Grb2, being an adaptor protein, is known to interact ,either directly or indirectly, with various growth factors such as HGF, EGF, TGF-β1, FGF-2. Most of these interactions is known to occur between the SH2 domain of Grb2 and tyrosine phosphorylated residues of the activated receptors. Many of these receptors are known to inhibit muscle differentiation by stimulating proliferation. Grb2 has also been shown to be involved in HGF induced myoblast proliferation in chicken skeletal myoblasts [49]. It was observed that in response to HGF i.e engagement of the HGF receptor, c-Met, coupling of c-Met to Grb2 is required for HGF- mediated inhibition of differentiation whereas binding of c-Met to Phosphotidylinositol 3-kinase (PI3K) increases differentiation of myoblasts [49]. Another growth factor, FGF-2 has been shown to induce ERK (MAPK) phosphorylation in quiescent satellite cells in vitro. This induction was found to be dependent on Grb2 and Protein kinase C(PKC) [105]. However, no independent or alternate mechanism of Grb2 mediated signaling in muscle differentiation has been illustrated thus far. Although Grb2 is known to play a critical role in cell proliferation, no pathway that defines its role in differentiation in the absence of growth factor activation has been elucidated so far. The process of muscle differentiation requires a balance between proliferation of myoblasts and the induction of differentiation or myogenic program. However, it is not clear as yet where Grb2, an important, multifunctional adaptor protein, fit into this proliferation/differentiation scheme.

Epithelial-to Mesenchymal Transition (EMT) is another biological process that also involves major rearrangement of the actin cytoskeleton when highly polarized epithelial cells undergo developmental switch and become increasingly motile and adopt a mesenchymal phenotype. Hence, actin nucleation promotion factors such as N-WASP and its interacting protein, Grb2 could be speculated to play a significant role during EMT. EMT, though widely studied as a hallmark of tumor progression, also occurs during physiological processes such as wound healing, embryonic morphogenesis etc. EMT shares

certain common cellular events with myogenesis such as changes in cell morphology, increased cell migration etc. In contrast to myogenesis, where cell-cell adhesion or cell-myotube fusion occurs, EMT involves decrease in cell-cell adhesion and increased migration and invasion which becomes a characteristic feature of mesenchymal cells[106].

1.10 Epithelial-To-Mesenchymal Transition (EMT):

Metastasis is the one of the main cause of majority of cancer related deaths. In most of the cancer types, deaths occur not due to primary tumours but more by the spread of the tumour cells from the primary sites of origin to distant organs and tissues to form secondary tumours [107]. Metastasis is a complex multistep process in which the primary tumour cells travel from the primary site of origin through the components of the extracellular matrix, enter the blood circulation by forming new blood vessels (tumour angiogenesis) and migrate to distant organs and tissues(extravasation) and start proliferation to form new secondary tumours [48]. Despite the extensive focus on the area of cancer research, different steps of metastasis and molecular and cellular mechanisms regulating it is still poorly understood. Most of the anti cancer drugs focus on controlling the proliferation of tumour cells which may not be exactly specific and effective. Hence, understanding the molecular mechanisms underlying cancer metastasis will help to design and develop more efficient anti cancer drugs and therapies.

Cancer metastasis is a multistep process largely divided in to invasion, intravasation, transport, extravasation and colonization [108]. Metastasis begins with the changes in the adhesive properties of potentially metastatic primary tumour cells making them more invasive and motile. Epithelial cancer cells in primary tumours are strongly associated with each other via different types of cellular junctions such as tight junctions, adherens junctions and desmosomes [108]. They are also connected to the underlying extracellular matrix through hemidesmosomes to maintain polarity. Finally, both the desmosomes and hemidesmosomes connect to the intermediate filament via cytokeratin specific to epithelial cells [108] . Tumour cells thus have to break these tight associations before they can invade in to the surrounding tissues as single cells [107]. To facilitate this type of invasion, epithelial cells may undergo a process termed as epithelial-to-mesenchymal (EMT) transition which can be stimulated by various internal and external cues such growth factors, cytokines and signaling pathways ([109], [110],[111]). EMT is not limited to cancer metastasis, in fact it was originally recognized as a key step in

morphogenesis during embryonic development [112]. Based on its context, EMT has been divided in to 3 types: Type 1 EMT involves embryonic development; Type 2 EMT includes wound healing and regeneration of tissues and type 3 EMT is associated with cancer progression ([113],[114],[115]). Although the three types of EMT involve a whole range of biological processes, some of the cellular and regulatory mechanisms in all of them are well conserved.

1.11 Molecular Mechanisms of EMT:

A characteristic feature of EMT is the functional loss of epithelial cell-cell adhesion molecule, E-Cadherin which maintains the intercellular junctions of epithelial cells. Many signaling pathways have been found to play critical roles in regulating E-Cadherin expression and EMT. Regulation has been observed at the transcription level, with various transcription factors acting as molecular switches in the EMT program. Some of these are Twist, Snail/Slug family, ZEB1, ZEB2 etc ([116], [117], [118]). Most of these transcription factors respond to different environmental stimulus and control E-Cadherin expression. More specifically, these repressors bind to DNA sequences located near the transcription start site called E-boxes which then recruit transcriptional co-repressors and histone deacetylases [119]. The most extensively studied transcriptional co-repressor of the E-Cadherin expression in EMT is Snail. This zinc finger transcription factor family was first identified in Drosophila during embryogenesis and later found to be important during mammalian EMT [112]. It has been shown to repress the expression of many other epithelial markers such as claudins, Occludins etc, along with E-Cadherin repression [120]. High levels of Snail have been observed in patients with breast cancer [121]. However, Snail does not work independently, other transcription repressors, such as Twist and ZEB1, work in conjunction to regulate EMT [122].

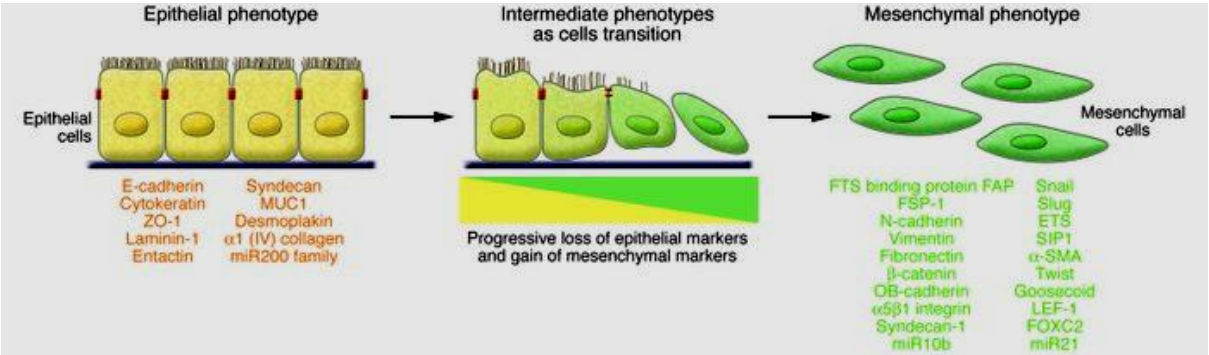


Fig 1.6 Basic mechanism of Epithelial-Mesenchymal Transition: EMT involves functional transition of polarised epithelial cells in to mobile and invasive mesenchymal cell types. The markers used to study epithelial cells and mesenchymal cells are listed above. Intermediate phenotype of EMT is the co localization of both the markers of EMT *[114]*

A variety of environmental stimuli have been observed to trigger EMT such as extracellular matrix (collagen), growth factors and cytokines such as transforming growth factor-β(TGF-β), Epidermal Growth Factor (EGF), Hepatocyte Growth Factor (HGF), tumor necrosis factor-α(TNF-α)/Nuclear Factor κB(NF-κB), Wnt, Notch etc [123]. The involvement of several different signaling mechanisms in inducing EMT stresses on its dynamic nature and the level of regulation and cross talk. The focus of the lab is on TGF-β induced EMT.

The transforming growth factor-β (TGF-β) belongs to a large super family of more than 40 different cytokines which control almost every aspect of cell function such as cell differentiation, apoptosis, proliferation, migration, stem cell maintenance, regulation of immune system etc [45]. Most of the members of the family are quite cell and developmental stage specific; however, the three isoforms of TGF-β, TGF-β1, TGF-β2 and TGF-β3 are more ubiquitously expressed and most extensively studied. TGF-β exists in an inactive form consisting of TGF-β associated with Latency Associated Proteins (LAPs) [45]. This complex of TGF-β and latency proteins associated with disulphide bonds attached to the extracellular matrix can be activated by various stimuli such as conformational changes of the proteins induced by thrombospondin-1 [124], cleavage mediated by proteases such as matrix metalloproteases (MMPs) [125], up regulation of the $\alpha_V\beta_6$ integrins in response to inflammation [126], low pH levels [127], irradiation releasing reactive oxygen species (ROS) [128], mechanical contraction of myofibroblasts [129] etc . Once active, TGF-β binds and activates its receptor, TGF-β receptor (TβR). Signal transduction can take place through three isoforms of TGF-βR- TβRI, TβRII and TβRIII, though signaling via TβRI and TβRII have been most extensively studied. Both the receptors belonging to the Ser/Thr protein kinase family consist of about 500 amino acids and made up of an extracellular N-terminal ligand binding domain, a transmembrane region and a C-terminal serine/threonine kinase domain [130]. On the membrane, the receptors exist as a heterotetrameric complex consisting of two TβRII which can bind to the ligand and two TβRI which cannot directly bind the ligand but can transduce the signal received from TβRII to downstream signaling molecules [131]. TβRI contains a unique sequence, SGSGSG, termed as the GS domain which is the site of

phosphorylation and activation by TβRII [130]. Binding of the active TGF-β to the extracellular ligand binding domain of both the receptors changes the conformation such that the cytoplasmic kinase domain of both come in close proximity of one another so that TβRII can phosphorylate and activate TβRI [130]. The SMAD family of proteins are the major effector proteins which transduce the signal from activated TβRI to downstream signaling proteins [132]. So far, about eight different mammalian Smad genes have been identified which are placed in three distinct groups: receptor activated Smads (R- Smads) which consist of Smads 1,2,3,5 and 8, a common mediator Smad (Co-Smad), Smad4 and inhibitory Smads (I-Smads) which includes Smad 6 and 7 [131]. Smads derive their name from their Drosophila homolog, Mad, and C.*elegans* homolog, Sma proteins. The mammalian Smads share structural similarities with their homologs with N- and C- terminal MAD homology domains, MH1 and MH2 respectively, connected by proline rich linker regions [131]. In the absence of TGF-β, Smads remain inactive by autoinhibition. TGF-β induces conformational changes to release the inhibition and allow the MH2 domain of R-Smads to interact with the active TGFβRI [133].

1.11.1 Smad dependent Signaling:

Smad dependent signaling begins with the binding of TGF-β to the TGFβRII followed by the phosphorylation and activation of TGFβRI which then undergoes conformational changes to make it more accessible for the binding of R-Smads (Smad2/Smad3) through their MH2 domain [134] further leading to their phosphorylation at the conserved SSXS C- terminal motif [135]. Phosphorylation of R-Smads causes the formation of heterotrimeric complexes of R-Smads and Co-Smad(Smad4) which can translocate to the nucleus to alter gene expression [136]. Smads act as transcription factors and along with various co-activators or co-repressors can regulate variety of physiological processes such as epithelial-to-mesenchymal transition (EMT) and epigenetic changes, indirectly, such as chromatin remodelling ([137], [138]). TGF-β signaling through the Smad proteins is regulated by a negative feedback loop controlled by the I-Smads, Smad 7, a major target protein of this pathway [139]. Under normal conditions, Smad 7 localizes to the nucleus, on TGF-β stimulation, however, it translocates to the plasma membrane and binds to the activated TGF-β receptor thereby not allowing the R-Smads to bind and terminates TGF-β downstream signaling ([140], [141]). Alternately, it can also interact with E3-ubiquitin ligases Smurf1 or Smurf 2 in the nucleus. After TGF-β stimulation Smad7-Smurf complex

can translocate to the membrane and cause proteasomal degradation of TGF-β receptors and inhibit the activation of downstream proteins. [135].

1.11.2 Smad Independent Signaling:

TGF-β has also been shown to activate other downstream proteins besides Smads. For eg. TGF-β has been found to activate MAPK signaling pathway in various types cells and tissues such as epithelial and endothelial cells, as well as in breast and colorectal cancer to promote migration and EMT ([142], [143], [144]). An overall model of TGF-β induction on Erk signaling has been shown to occur through direct autophosphorylation of TGFβRII or by Src at its tyrosine residues which can then phosphorylate TGFβRI again at its Tyrosine residues. This phosphorylation causes the formation of ShcA/Grb2/Sos complex which can then activate the proteins Ras/Raf/MAPK of the Erk signaling pathway ([145], [146]). TGF-β has also been found to activate JNK and p38MAPK pathway by activating MKK4 and MKK3/6 respectively ([147], [148]). Also, TGF-β has been shown to activate the PI3K/Akt cell survival pathway by phosphorylation of Akt to induce EMT and cell migration. In fact, the p85 subunit of PI3K has been shown to be constitutively bound to TβRII and on TGF-β form a complex with TβRI as well to activate Akt cell survival pathway ([149],[150]). The mammalian target of Rapamycin (mTOR) has also been observed to be activated by TGF-β stimulation and found to be critical for protein synthesis by regulating the phosphorylation of eukaryotic initiation factor 4E binding protein and EMT ([151], [152]). Finally, TGF-β stimulation has also been found to affect cytoskeletal rearrangement, cell polarity and migration by activation of Rho GTPases. TGF-β can activate RhoA and Cdc42/Rac1 pathway and cause formation of actin stress fibres and EMT ([153], [154]).

It is interesting to note that TGF-β can behave both as tumor suppressor and tumor initiator. Numerous studies have shown that TGF-β can behave as a tumor suppressor in various normal cells and tissues such as epithelial cells, endothelial cells, neuronal cells and cells of the immune system ([155], [156], [157], [158]). It has been primarily found to induce the expression of genes that control hyper proliferation in G1/S transition. Most of these genes include cyclins/cyclin dependent kinase (CDK) complexes regulating the cell cycle [159]. However, in tumor cells, upon genetic manipulations, it promotes a proinvasive and prometastatic phenotype.

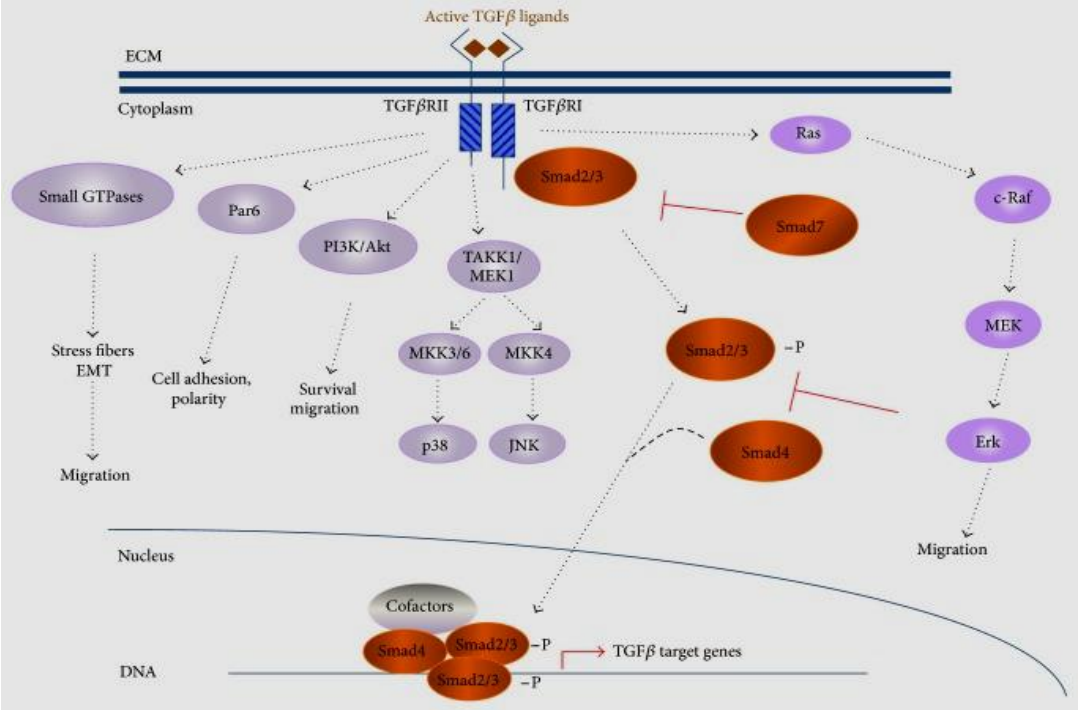


Fig 1.7 Smad dependent and independent TGF-β pathways: The canonical Smad dependent pathway begins with the phosphorylation of TGFβRI by binding of active TGF-β to the receptors. Phosphorylation of TGFβRI induces phosphorylation and binding of R-Smads (Smad2/3) causing further binding of Smad 4. This heterotrimeric complex translocates to the nucleus to alter gene expression. TGF-β has been shown to activate proteins besides Smads such asMek/ Erks, PI3K/Akt, JNK, p38MAPK, Rho GTPases to modulate biological processes such as EMT, cell migration, stress fibre formation, survival etc [45]

1.12 TGF-β induced epithelial-to-mesenchymal transition (EMT):

EMT has been shown to play a vital role in morphogenesis required for embryonic development. For the past few years, it has been studied extensively for its role in tumor progression. EMT involves the induction of organized, reversible transcriptional program in which the tightly connected epithelial cells lose their polarity and differentiate in to disorganized and highly motile mesenchymal cells. This process is marked by the down regulation of certain proteins of the tight junctions linking the epithelial cells such as E-Cadherin, occluding, claudin, β-catenin etc. This is also supported by cellular changes such as changes in cell polarity, reorganization of actin cytoskeleton, increase in cell migration, formation of actin stress fibres etc. At the same time, cells acquire mesenchymal characteristics such as expression of marker proteins such as N-Cadherin, vimentin, α-smooth muscle actin, fibronectin along with certain morphological changes such as spindle shape, fibroblast like phenotype [160].On a molecular level, TGF-β induces the expression of transcription factors, known as master regulators of EMT, such as Snail, Slug, ZEB1and

ZEB2 whereas the basic helix loop helix (bHLH) protein Twist can be upregulated by EGFR or Wnt signaling ([161], [162], [118, 163]). TGF-β/Smad 2 complex can also promote EMT phenotype by epigenetic silencing of epithelial markers such as E-Cadherin, claudin-4, cingulin etc . Smad 2 has also been found to regulate the binding activity of DNA methyltransferase (DNMT 1) and its subsequent methylation of the promoter regions of the corresponding genes [138]. There has been a lot of cross talk observed between TGF-β and other signaling pathways such as Erk signaling. It has been observed that Smads are required for EGF induced EMT and vice versa. In fact, it has been further reported that TGF-β and MAPK pathway can co-operatively induce EMT [164]. Alternately, microRNAs – miR-200 and miR- 205 have been found to inhibit transcription factors ZEB1 and ZEB2 to suppress EMT and maintain epithelial phenotype. Down regulation of these micro RNAs has been reported in breast cancers to facilitate invasion and metastasis [165]. Also, TGF-β has been found to suppress the expression of another non coding RNA, miR-203 to upregulate its target gene SLUG and induce EMT [166]. It can be clearly concluded that TGF-β signaling controls a wide network of inter connected pathways and regulates EMT, invasion and tumor progression of cancer cells

1.13 Research on GRB2 in EMT:

Over the past couple of decades, role of GRB2 in EMT and tumor progression has been extensively studied with most focus being on breast tumors. GRB2 has been found to be highly expressed in tumors, especially breast tumors [48]. Galliher et al, 2007 identified a novel $\alpha_V\beta_3$ integrin/Src/Y284/TβRII signaling axis after stimulation by TGF-β in mammary epithelial cells (MECs) regulating their invasion and metastatic properties. They demonstrated that, in the presence of TGF-β, Src kinase phosphorylated TβRII at Tyr284 in vivo and in vitro with the help of point mutants [145]. They also showed that this tyrosine phosphorylated residue was required for the activation of p38-MAPK through the intermediate binding of SH2 domain containing proteins such as GRB2 and Shc to Tyr284. The importance of $\alpha_V\beta_3$ integrins and Src kinase in activating the TGF-β signaling pathway in mammary epithelial cells was established [145] as was validated with patient samples wherein in about 70% of human breast cancers high levels of Src kinase was observed [145]. Galliher-Beckley et al, 2008 stressed on the importance of binding of GRB2 to Tyr284 of TβRII, on TGF-β stimulation, in mammary cell EMT observed NMuMG cells [46]. GRB2/TβRII complex was found to be required for the activation of p38MAPK in NMuMG cells by knocking down the expression of Grb2 and using TβRII mutant which cannot bind to

Grb2 (Y284F-TβRII) [46]. Also, Lee et al, 2007 showed that TβRI can phosphorylate ShcA, on TGF-β stimulation, which can then associate with GRB2/Sos complex to activate MAPK signaling pathway [146]. Specifically, phosphorylation of $p52^{ShcA}$ isoforms of ShcA was found to induce Erk signaling on TGF-β stimulation [146].

Lung cancer is still one of the major causes of deaths due to cancer. Even today, it is often diagnosed at later stages with ineffective chemotherapies [167]. Various receptor tyrosine kinases such as EGFR, HGFR/c-Met along with their downstream signaling molecules such as GRB2, MAPK,PI3K have been found to be over expressed in lung cancer [167]. The c-Met receptor has been specifically found to be over expressed in non small cell lung cancer cells (NSCLC). Several studies have shown that in lung cancer cells isolated from either NSCLC or small cell lung cancer (SCLC) cells, the Tyr1003 residue of c-Met receptor was found to be phosphorylated which has been found to be required for its function. GRB2 has also been shown to bind to another tyrosine residue Tyr1349[167]. However, its role in receptor tyrosine kinase or Ser/Thr kinase activated signaling such as TGF-β in NSCLC or SCLC has still not been elucidated so far. Studying its function in these cancer cell lines will help to correlate it to patient lung tumor samples and will aid in developing therapeutic strategies to treat lung cancer.

OBJECTIVES

The focus of this thesis is to understand the cellular and molecular details of the role of Grb2, an adaptor protein, in mammalian myogenic differentiation and Epithelial-to-Mesenchymal transition (EMT) using cell culture techniques. Studying biological processes such as EMT and muscle differentiation using established secondary cell lines such as C2C12 (mouse myoblast cell line) and A549 (NSCLC cell line) has become more convenient and reliable. Though in vitro systems may not replicate exact physiological scenario, using secondary cell lines have advantages such as: 1) controlling the cellular environment in cell culture systems is easier, 2) manipulations of cellular components is possible.

Grb2 is a crucial adaptor protein known to link the growth factor activated receptor tyrosine kinases to many downstream signaling pathways that regulate many biological processes including myogenesis and EMT. Growth factors such as HGF and EGF have been known to promote myoblast proliferation and inhibit muscle differentiation. The balance between proliferation and differentiation requires the efficient recruitment of adaptor proteins such as Grb2. Besides being a critical downstream intermediate in various oncogenic signaling pathways, Grb2 mediated signaling has also been implicated in pathogenesis of many human cancers. EMT, characterized by the transition of polarized epithelial phenotype to elongated mesenchymal phenotype to facilitate invasion and metastasis, is significantly induced by TGF-β in many cancer cell lines. TGF-β induced EMT has been shown to activate Grb2 mediated signaling pathways such as Erk1/2 and p38 MAPK in breast cancer cell lines. The three main objectives of my study were:

1. **Characterize the role of Grb2 in muscle differentiation:** Grb2 mediated signaling has been shown to play important role in cell proliferation. Balance between myoblast proliferation and differentiation is required for myogenesis. The role of Grb2 in skeletal muscle differentiation will be studied using C2C12, a muscle myoblast cell line capable of undergoing differentiation under low serum conditions. Firstly, the expression level of Grb2 in C2C12 cells undergoing differentiation will be analyzed. Then, the expression of Grb2 will be knocked down using RNA interference and exogenously expressed in C2C12 cells. Also, the role of the three domains of Grb2 (N- SH3, SH2 and C-SH3) during muscle differentiation will be studied.
2. **Characterize the role of Grb2 in the formation of EGF induced vinculin patch assembly:** Cell-matrix adhesion is a crucial aspect of myoblast migration and

alignment during muscle differentiation. Vinculin is a cytoplasmic focal adhesion protein known to localize to integrin mediated cell-ECM adhesions. Localization of vinculin at the focal adhesion was studied in C2C12 cells. Effect of EGF stimulation on the vinculin patch formation in Grb2 knocked down and over expressing cells will also be studied.

3. **Characterize the role of Grb2 in TGF-β induced EMT:** TGF-β is a well known and potent inducer of EMT. To study the role of Grb2 in TGF-β induced EMT in NSCLC cell line, A549, the expression level of Grb2 in the presence of TGF-β will be analyzed. Then the effect of Grb2 knock down and over expression on TGF-β induced EMT will be studied. Finally, the domains and the mechanism responsible for Grb2 mediated signaling in TGF-β induced EMT will be characterized.

Chapter 2: MATERIALS AND METHODS

2.1 Materials

2.1.1 Plasmids and Commercial Vectors

For DNA subcloning experiments, vectors pUC19 (Invitrogen) and pUC18 (Invitrogen) were used. For expression of proteins in mammalian cells, vector pFIV-H1/U6-copGFP (Open Biosystem, CO, USA) or pEFCG Puro E and pEFCG YIP plasmids were used. For knockdown, pEFCG Puro E and pLJM EGFP was used. Table 2.1 summarizes the commercial vectors used and their applications.

Table 2.1: Expression vectors used in the study

Vector	Resistance	Expression Host	Used in
pUC19	Amp	*E.coli*	DNA subcloning
pUC18	Amp	*E.coli*	DNA subcloning
pEFCG puro	Amp	Bacteria/Mammalian cells	Grb2 knockdown in C2C12 cells
pCopW1GFP pCopRFP	Amp	Bacteria/Mammalian cells	Grb2 overexpression in mammalian cells
pEFCG YIP	Amp	Bacteria/Mammalian cells	Grb2 overexpression in C2C12 cells
pLJM EGFP	Amp	Bacteria/Mammalian cells	Grb2 knockdown and overexpression in A549 cells

2.1.2 Bacterial strains

The bacterial strain, *Escherichia coli* (*E. coli*) DH5α, was used for plasmids amplification and DNA subcloning, *E.coli*(Stabl3) was used to amplify shRNAs. *E.coli* ER2925 cells were used to clone sh-Grb2 in lentiviral vector.

2.1.3 Mammalian cell lines

HEK293T (Human Embryonic Kidney 293 cells) and NSCLC cell line, A549 was from American Type Culture Collection (ATCC, USA). The mouse myoblast cell line C2C12 was a gift from Prof. Ravi Kambadur (School of Biological Science, Nanyang Technological University).

2.1.4 Cell culture reagent and Plasticware

Dulbecco's Minimal Eagles medium (DMEM) – (Hyclone) was used for maintenance of mammalian cells. Cell culture media additives were purchased from Hyclone or Gibco. Plasticwares were bought from Costar, Corning, Nunc and Eppendorf.

2.1.5 Antibodies

The antibodies used in this study are listed below (Table 2.2 & 2.3) and their respective dilutions used for the Western blot experiments are specified. The secondary antibodies are conjugated with Horseradish peroxidase (HRP).

2.1.5.1 Primary antibodies

Table 2.2: Primary antibodies and the dilutions used for Western blot analysis and immunoflouroscence (IF).

Antibody	Company	Dilution for IF	Dilution for WB	Blocking conditions
MyHC	Developmental Studies Hybridoma Bank, IOWA, USA	1:50	1:100	3% milk
Rabbit Anti-MyoD polyclonal antibody	Santa Cruz	-	1:400	5% milk
Mouse Anti-Vinculin monoclonal antibody	Sigma	1:50	-	-
Mouse Anti- Paxillin monoclonal antibody	BD biosciences	1:25	1:500	3%milk
Mouse Anti- Myc	Clontech	-	1:10000	3% milk

monoclonal antibody	Laboratories, CA, USA			
Mouse Anti-GAPDH monoclonal antibody	Ambion, TX, USA	-	1:10000	3% milk
Rabbit Anti-Grb2 (C-23) polyclonal antibody	Santa Cruz	1:25	1:500	3% milk
Mouse Anti Grb2(C-7) monoclonal antibody	Santa Cruz	-	1:1000	3% milk
Goat Anti Snail polyclonal antibody	Santa Cruz		1:400	3% milk
Mouse Anti E-Cadherin monoclonal antibody	BD	1:25	1:500	3%milk
Mouse Anti vimentin monoclonal antibody	BD	1:25	1:500	3%milk

2.1.5.2 Secondary Antibody

Table 2.3: Secondary antibodies and the dilutions used for Western blot analysis and immunoflouroscence.

Antibody	**Source**	**Dilution for IF**	**Dilution for WB**
Anti-mouse IgG-HRP	Sigma	-	1:10000
Anti-rabbit IgG-HRP	Sigma	-	1:10000
Donkey anti-goat IgG-HRP	Santa Cruz	-	1:5000
Alexa Fluor 594 Phalloidin	Molecular Probes	1:100	-
Anti-mouse Alexa Fluor 594	Molecular Probes	1:500	-
Anti-mouse Alexa Fluor 488	Molecular Probes	1:500	-
Donkey Anti-goat Alexa Fluor 488	Santa Cruz	1:200	-

2.1.6 Bacterial culture media

2.1.6.1 Luria-Bertani (LB) broth

1% bacto-tryptone, 0.5% bacto-yeast extract and 0.5% NaCl was dissolved in distilled water and autoclaved.

2.1.6.2 LB agar plates

2% of bacto-agar was added to LB broth and autoclaved. Medium was left at room temperature to cool below 60°C and 25 ml of medium was poured into each plate and left to solidify.

2.1.7 Enzymes and kits

Restriction endonucleases	Fermentas New England Biolabs (NEB)
T4 DNA ligase and buffer	Fermentas
Plasmid DNA isolation from *E. coli* cells	Tiangen Miniprep Kit, Tiangen Biotech
RNA isolation from mammalian cells	Trizol RNA isolation protocol, Invitrogen
DNA extraction and purification from agarose gels	Tiangen Gel Extraction Kit, Tiangen Biotech
Bradford assay	Bio-Rad DC protein assays
Western blot developing	Millipore Immobilon Western, Millipore Corp Super Signal West Pico, Thermo scientific
Microporation kit and instrument	Invitrogen

2.1.8 Polymerase chain reaction (PCR)

DNA polymerase	KAPA HiFi, KAPA biosystems Biotools DNA polymerase, Biotools
dNTPs (dTTP, dATP, dGTP and dCTP) mixture	Invitrogen
Quantitative Real time PCR	SYBR Green/ROX, Fermentas qPCR master mix, Fermentas

2.1.9 Chemicals and reagents

DNA work	
DNA loading ladder	New England Biolabs (NEB)
6X DNA loading buffer	New England Biolabs (NEB)
Ampicillin, Kanamycin	Sigma-Aldrich
Protein work	
Phenylmethylsulfonyl fluoride (PMSF), protease inhibitor	Sigma-Aldrich
Dithiothreitol (DTT)	Biorad
Protein molecular weight marker	New England Biolabs (NEB)
Nitrocellulose membrane	Biorad

2.1.10 General buffers and solutions

2.1.10.1 DNA subcloning experiments

2.1.10.1.1 50X Tris-acetate-EDTA (TAE)

40 mM Tris-acetate (pH 8.3) was made in 1.0 mM EDTA. The 1X TAE buffer was used in both making agrose gel and as a running buffer.

2.1.10.1.2 Agarose gel

1% agarose was made by suspending 10 g of agarose in 1000 ml of 1X TAE buffer and microwaved for 10 min. The agarose solution can be maintained in liquid form at 60°C by storing in an oven.

2.1.10.2 Western blots

2.1.10.2.1 10% SDS

10 g of SDS was dissolved in 100 ml of deionised water.

2.1.10.2.2 10% Ammonium persulphate (APS)

1 g of ammonium persulphate was dissolved in 10 ml of deionised water.

2.1.10.2.3 5X Tris-glycine electrophoresis buffer

0.125 M Tris, 0.96 M glycine, 0.5% SDS were made in dd H_2O, the pH of the solution was adjusted to 8.3.

2.1.10.2.4 Membrane transfer buffer

25 mM Tris (pH 8.3), 192 mM glycine, 0.1% SDS and 20% methanol were mixed.

2.1.10.2.5 2X SDS-PAGE gel-loading buffer

2X buffer had 100 mM Tris-HCl (pH 6.8), 200 mM dithiothreitol (DTT), 4% SDS, 0.2% Bromophenol blue and 20% glycerol. The solution was stored at 4°C.

2.1.10.2.6 1M Tris buffer

121.12 g of Tris was dissolved in deionised water and the pH of the solution was adjusted to 8.0 using HCl. The final volume was topped up to 1 litre.

2.1.10.2.7 5M NaCl

292 g of NaCl was dissolved in 1 litre of deionised water.

2.1.10.2.8 1X Tris buffered saline (TBS)

TBS had 20 mM Tris and 500 mM NaCl, the pH was adjusted to 7.5.

2.1.10.2.9 Blocking solution for immunoblot

5% or 3% of non-fat milk powder was dissolved in 1X Phosphate buffered saline (PBS) to make the blocking solution.

2.1.10.2.10 Wash solution for immunoblot

To make the wash solution, 5 ml of 10% Triton-X was added to 995ml of 1X PBS.

2.1.11 Tissue culture

2.1.11.1 Freezing media

The freezing media was prepared by adding 10% of dimethyl sulfoxide (DMSO) in FBS and was filter sterilized through a 0.2µm filter.

2.1.11.2 Lysis buffer for mammalian cells (RIPA buffer)

• 50 mM Tris–HCl, pH 7.5,

• 200 mM NaCl,

• 1% Triton X-100,

• 0.1% SDS,

• 0.5% sodium deoxycholate,

• 10% glycerol,

• 1 mM EDTA,

• 1mM phenylmethylsulphonyl fluoride (PMSF),

• Protease inhibitor cocktail containing 10 µg/ml of leupeptin, pepstatin, and aprotinin each.

2.1.11.3 Transfection reagent

2 mg/ml solution of Polyethyleneimine (PEI, linear) (Sigma) was prepared in deionised water. PEI solution was then filter sterilized by passing through a 0.2µm filter. 5µg of PEI per µg of plasmid DNA was used for transfection in mammalian cells.

2.2 Methods

2.2.1 Escherichia coli cells

2.2.1.1 Growth conditions and maintenance of *E. coli* cells

E. coli was either cultured in LB broth or maintained on LB agar plates at 37°C. *E. coli* transformed with plasmids containing ampicillin or kanamycin resistance marker were cultured in LB-broth or grown on LB agar plates supplemented with 75 µg/ml ampicillin or 25µg/ml kanamycin.

2.2.1.2 Glycerol stock of *E. coli*

For long term preservation *E. coli* were maintained in glycerol stock. A single colony of the *E. coli* strain was inoculated in 5 ml LB medium and cultured until mid log phase. 200 µl of 80% glycerol solution was mixed with 800 µl of the bacterial culture and then transferred to a cryo-vial and stored at -80°C.

2.2.1.3 Preparation of *E. coli* competent cells ($CaCl_2$ method)

A bacterial colony was picked from LB plate and inoculated in 5 ml of LB medium and grown overnight at 37°C. The culture was diluted to an OD_{600} of 0.05 in 50 ml of LB and incubated at 37°C until the OD_{600} was approximately 0.5. The culture was then transferred to sterile centrifuge falcon tubes and stored in ice until further use. The cells were centrifuged at 4470 x *g* at 4°C for 10 min. The supernatant was discarded and the pellet was resuspended in 25 ml of ice cold sterile 100 mM $MgCl_2$. The cells were then centrifuged at 4470 x *g* for 10 min at 4°C. The supernatant was discarded and the cell pellet was resuspended in 5 ml of sterile ice cold 100 mM $CaCl_2$ and incubated on ice for 2 hrs to make the cells competent. 2 ml of sterile ice cold 50% glycerol was added to the cell suspension. The cells were later

stored at -80°C as 200µl aliquots in eppendorf tubes. The cells and the reagents were always maintained at 4°C.

2.2.1.4 Transformation of *E. coli* cells

Frozen competent cells in eppendorf tubes were first thawed on ice. For DNA amplification, 100 ng of DNA was added to 50 µl of competent cells. For subcloning of plasmids, 20 µl of ligation reaction was added to 100 µl of competent cells. The tubes were incubated on ice for 20 min and heat shocked at 42°C in a water bath for 90 seconds. They were then placed on ice immediately for 30 min. Cells were plated on LB agar plates containing the selective antibiotic and incubated at 37°C overnight.

2.2.1.5 Isolation of plasmid DNA from *E. coli*

A single bacterial colony was inoculated in 5 ml of LB containing antibiotic and grown overnight at 37°C. The cells were spun at 4470 x *g* at 4°C for 5 min. The plasmid DNA was isolated from the pellet according to the manual provided in the plasmid purification kit.

2.2.2 Mammalian cell culture methods

2.2.2.1 Growth and maintenance of mammalian cells

Mouse C2C12 cells were maintained in growth media, GM (DMEM + 10% FBS and 100 IU/ml penicillin and 100 IU/ml streptomycin) at 37^{o}C in a 5% CO_2 humidified atmosphere.

2.2.2.2 Passaging and trypsinizing of mammalian cells

Mammalian cells were washed once with 1X PBS and detached from the culture flask by incubating them in 1ml of 1X trypsin EDTA solution (Gibco 10X) for 2 min at 37^{o}C. Trypsinization of cells were stopped by adding 4 ml of pre-warmed DMEM media supplemented with 10% FBS, subsequently cells were transferred at the desired density to a new tissue culture flask. The cells were then maintained at 37°C in a 5% CO_2 incubator.

2.2.2.3 Preparation of mammalian cell stocks

Cells were first trypsinised and then resuspended in the appropriate growth media followed by centrifuging at 400 x *g* at 4 mins. The supernatant was discarded and the cell pellet was resuspended with freezing medium. The cell suspension was then aliquoted into cryogenic storage vials and stored at -80°C in an isopropanol cryobox, that had a controlled freezing rate, overnight. The frozen cells were then transferred to a liquid nitrogen tank.

2.2.2.4 Thawing of cells

Cryo-vials were taken from liquid nitrogen and re-suspended in 4.0 ml of DMEM medium supplemented with 10% FBS until the frozen medium had melted. The thawed cells were then transferred to 20 ml of fresh DMEM medium with 10% FBS and transferred in to culture flask. Growth medium was replaced after 24 hrs with fresh DMEM+10% FBS and 1% Penicillin and Streptomycin.

2.2.2.5 Transfection of mammalian cells

One day before transfection, cells were seeded in 6-well plates so that the cells would be 60-80% confluent at the time of transfection. 4.0µg of DNA was diluted in 90µl of serum-free medium (SFM). In a separate eppendorf tube, 10µl of PEI (20 µg) was diluted in 90µl SFM. Subsequently, the diluted DNA was added into the PEI solution. The DNA/PEI mixture was then allowed to stand for 10 min at room temperature after which this reaction mixture was added drop-wise onto the wells containing cells. The cells were incubated for 12 hrs at 37°C in the 5% CO_2-humidified incubator before changing the media to new GM. After 36 hrs of incubation at 37°C in a 5% CO_2-humidified incubator, cells were ready for further analysis.

2.2.2.6 Lentivirus preparation and infection

HEK 293T cells were transfected with lentiviral plasmid (pLJM- linker) expressing human Grb2 specific shRNA or exogenously expressing his tagged Grb2 gene (pLJM-Grb2) along with the empty vector control using polyethylene imine (PEI) transfection method. 293T cells were grown in complete DMEM media up to 80-90 % confluency in a 10 cm culture dish. Prior to transfection, the cells were washed with 10 ml of serum free media. The cells were then incubated in CO_2 incubator for 5 min. The following plasmids were used for each transfection: VSVG (3.75 µg), REV (3.125µg), pDNL (6.125µg) and plasmid DNA (18.75µg). The volume was made up to 5ml using serum free media and the solution was filtered through a 0.2µm filter. 49.8µlts of PEI (2 mg/ml) was used for transfection and made up to 5 ml using serum free media and the mix was filtered using 0.2µm filter. The two mixes were added together and incubated at RT for 20 mins. This transfection mixture was then added to the cells in a dropwise manner. Cells were then incubated for 4 hrs in CO_2 incubator before changing to fresh complete DMEM media. After 24 hrs of transfection in 293T cells, the media (containing the virus) was passed through a 0.45 µm filter. In a falcon tube, a mixture of complete DMEM media and polybrene (5mg/ml) in the ratio 0.5 ml: 2µlts was made respectively. This mixture was first added to the cells to be infected (A549 cells)

previously seeded up to 40% confluency. 0.5ml of virus suspension was then added to the cells. This infection was repeated again after 24 hrs.

2.2.2.7 Microporation of C2C12 cells

C2C12 cells were microporated using the Neon Transfection System (Invitrogen, CA, USA) according to manufacturer's instructions. Briefly, 5 x 10^6 cells in 100 µl of resuspension buffer were mixed with 10 µg of plasmid DNA and subjected to three 10ms pulses at 1650 V. We were able to achieve 80-90% transfection efficiency with this method. Transfected cells were incubated for 36 hrs in growth medium before analysis.

2.2.2.8 Mammalian cell lysis

Adherent cells were trypsinised and resuspended in the appropriate growth media. The number of cells and cell viability were determined using the hemocytometer and Trypan Blue exclusion. 2 x 10^6 cells were centrifuged at 400 x g for 4 mins and washed with PBS. The supernatant was discarded and the cell pellet was resuspended in 50µl of lysis buffer. Cells were left on ice for 1 hr and unlysed cells were removed by spinning down at 14.1k x g for 10 mins. 50 µl (equal amounts) of 2X loading dye and 2 µl (40mM) of 1M DTT were added to the lysate. Protein concentration was quantified using Bradford (Biorad) protein assay method. The lysate was boiled at 100°C for 5 mins and stored at -20°C until further use.

2.2.2.9 Coating of coverslips

For immunofluorescence and localization experiments, C2C12 cells were seeded on to 22 mm glass coverslip that had been coated with silane as described here: glass coverslips were washed with acetone, air-dried and treated with 1% (3-Aminopropyl) triethoxysilane (Sigma) solution in acetone for 2 mins. The treated coverslips were then washed with distilled water twice. After that coverslips were allowed to dry and were sterilized under UV light.

2.2.3 Cell based assays

2.2.3.1 Differentiation Assay

C2C12 cells were grown in GM (Growth media, DMEM + 10% FBS) till confluence and switched to differentiation medium (DM, DMEM supplemented with 2% horse serum (HS)). The cells were observed for the next 72 hrs for differentiation into myotubes.

2.2.3.2 Measurement of fusion index

Fusion index was calculated as the ratio of the nuclei number in myotubes (cells having more than two nuclei) to the total nuclei number in a field. Six random 10x fields were selected for

each experimental condition. Three independent experiments were carried out for any set of conditions. Statistical significance analysis was performed using one-way Anova and $P<0.05$ was considered statistically significant.

2.2.3.3 Immunofluorescence

C2C12 cells grown on coated coverslips were fixed, permeabilized and blocked with 1%BSA in PBS. The cells were then incubated with appropriate primary antibodies in 1% BSA in PBS for 3 hrs at room temperature. The cells were rinsed twice with 1 x PBS and then incubated with Alexa488-conjugated (green) rabbit anti-mouse (Molecular Probes; 1:1000) as well as with Alexa568-conjugated (red) phalloidin (Molecular Probes) in PBS for 1 hrs at room temperature. Fluorescence images were acquired using Olympus IX51 fitted with Cool $SNAP^{HQ}$ camera and analysed using Metamorph software (Molecular Devices).

2.2.3.4 Cell spreading assay

Bovine fibronectin was diluted with 0.1M sodium bicarbonate buffer (pH 9.1) to a final concentration of 1.40 µg/ml. 100µL of fibronectin was added to the wells of 96-well plate and incubated at 37^oC for 1.5 hrs. After coating, each well was blocked with 100µl of 0.1% (w/v) BSA at 37^oC and washed once with serum-free DMEM. Cells were trypsinized and allowed to recover in serum-free DMEM for half an hour. Cells were then seeded at a density of 2×10^5 cells/ml, and incubated at 37^oC in a CO_2 humidified incubator. Samples were viewed at 10 mins, 20 mins and 30 mins time intervals (magnification X40). MetaMorph software was used to calculate the mean surface area of each population.

2.2.3.5 Migration Assay

Transfected C2C12 cells were seeded at a density of 3×10^5 in 6 well plates and were grown in DMEM supplemented with 10% heat inactivated FBS, 100 IU/ml penicillin and 100 IU/ml streptomycin at 37^oC in a 5% CO_2 humidified atmosphere. Monolayer of the transfected cells was scratched using a sterile 200µl yellow pipette tip; Time lapse was set up to observe the cell migration using the Olympus microscope IX51 fitted with Photometrics Cool Snap HQ2 camera over a period of 12 hrs. In each experiment at least 12 randomly selected regions were monitored. Size of the scratch was measured using Metamorph software. The minimal separation between the opposing cell fronts was measured. Wound distance was measured by calculating the width of wound.

2.2.3.6 Proliferation Assay

Cells were seeded at a density of 7.5 X 10^3 cells/well in a 24 well plate in triplicates for a period of four days. After every 24 hrs, the cells were trypsinized and resuspended in 1ml of complete media and 10 µL of each sample was added on a hemocytometer slide and the cells were counted. An average of the triplicate for each sample was used to plot the graph of proliferation rate (the ratio of the cell count on day2, day3 or day4 to day 1) of each sample.

2.2.3.7 MTT (3-(4,5-Dimethylthiazol-2-yl)-2-5diphenyltetrazolium bromide) Assay

Cells were seeded at a density of 7.5 X 10^3 cells/well in a 96 well plate in triplicates and incubate for two days at 37° C incubator with 5% CO_2. On the third day, 20 µL of 5mg/mL MTT was added to each well. One set of wells with MTT but no cell was included as a control. The plate was covered with aluminium foil and incubated for 3.5 hrs at 37° C. Then, the media was removed carefully and 150 µL of MTT solvent was added to each well. The plate was again wrapped in aluminium foil and the cells were agitated on orbital shaker for 15 mins. The purple coloured formazan formed was measured by measuring the absorbance at 590nm with a reference filter of 620nm using a plate reader.

2.2.3.8 Induction of EMT by TGF-β stimulation

A549 cells were seeded at a density of 2 X 10^5 in 60mm plates and were grown in DMEM supplemented with 10% heat inactivated FBS, 100 IU/ml penicillin and 100 IU/ml streptomycin at 37° C in a 5% CO_2 humidified atmosphere. Upon reaching 25% confluency, the cells were washed with 1X PBS and serum starved for 12 hrs. After 12 hrs, 5 ng/ml of TGF-β was added to the cells which were then incubated for a period of 48 hrs. A set of non stimulated control was also maintained.

2.2.3.9 Matrigel Invasion Assay

Firstly, Matrigel invasion inserts (Becton Dickinson) of 8.0µm pore size were coated with matrigel diluted at 1:10 with serum free media and was allowed to gel overnight at 37° C incubator with 5% $C0_2$. Next day, A549 cells were trypsinized and seeded at a density of 2.5 X 10^5 cells in 200 µL serum free media in the upper chamber of matrigel coated invasion inserts. The well below was filled with 600 µL of complete media and the cells were incubated in the cell culture incubator for around 40 hrs. Cells were then removed from the upper chamber and the chamber was washed twice with 1X PBS. The cells invaded in the insert were fixed with 3.7% formaldehyde for 2 minutes, washed twice with 1X PBS and

stained with 1% crystal violet in methanol for 15 minutes at room temperature. Excess stain was removed by washing the inserts with 1X PBS. Cells that did not invade through the matrigel were removed by a cotton swab. Only those cells that have made it to the bottom side of the membrane now remained and were visualized under a 10X objective lens of an inverted microscope.

2.2.4.0 Statistics

All the statistical data was generated from at least three independent experiments. Statistical analysis was performed using two-tailed unpaired student's t-test to compare the difference between groups. Significance is indicated by stars *p ≤0.05, **p≤0.01, ***p≤0.001.

2.2.5.0 DNA methodology

2.2.5.1 Agarose gel electrophoresis

0.5 µg/ml of ethidium bromide was added to 1% agarose in 1X TAE and the gel were left to set. 6X DNA loading buffer was added to the samples and loaded into the wells of the gel, submerged in 1X TAE. DNA was resolved by running the gel at 110V for 50 mins. DNA was visualized under the long wavelength UV light.

2.2.5.2 DNA extraction from agarose gel

The DNA sample resolved in the agarose gel was first visualized under UV light. The bands of desired molecular weight were excised with a scalpel and placed in an eppendorf tube. The DNA was extracted according to the manual provided in the gel extraction kit.

2.2.5.3 DNA subcloning

1. Purified plasmid DNA was incubated with restriction enzymes at 37^{o}C for 2 hrs. For restriction enzymes having star activity such as BamHI and *Eco*RI, restriction digestion was performed only up to 1.5 hrs.

2. Restriction enzyme digested DNA were resolved using agarose gel electrophoresis.

3. DNA fragments of interest were excised and DNA is purified and extracted from the gel using gel extraction kit.

4. Purified insert DNA is then ligated with purified digested vector DNA using T4 DNA ligase and the ligation reaction mixture is made according to the instructions supplied by the manufacturer.

5. The ligation reaction mixture is incubated at 24°C for 3 hrs.

6. The ligation reaction mixture is then introduced into competent *E. coli* cells by following the transformation protocol for *E. coli* (Secondtion 2.2.1.4).

2.2.5.4 Verification of recombinant plasmid DNA constructs

The recombinant plasmid DNA was verified by checking with restriction enzymes that cut at restriction sites that are unique. The digested DNA was then resolved by agarose gel electrophoresis. The bands obtained in the gel were checked to verify for the presence of the correct DNA fragment.

2.2.5.5 Polymerase chain reaction (PCR)

Polymerase chain reaction was performed to specifically amplify DNA from very small amount of DNA All PCR reactions were performed using the Peltier Thermal Cycle PTC-100. A 50 µl reaction using BIOTOOLS system was set up as follows:

- 5.0µl of 5x BIOTOOLS Reaction Buffer
- 5.0µl of 2 mM dNTP mix
- 1.0µl of 5' primer (10 µM)
- 1.0µl of 3' primer (10 µM)
- 0.5µl of Template DNA
- 0.5µl of BIOTOOLS DNA Polymerase (1 U/µL)
- Deionised water (topped up to 50 µl)

The cycle conditions used were:

- Initial Denaturation: 95ºC for 10 mins
- Denaturation: 95ºC for 1 min
- Annealing: 55 ºC for 1 min
- Extension: 72ºC for 30 second per kb (30 cycles)
- Final Extension: 72ºC for 5 min

The PCR reaction was cycled 30 times for optimal amplification of the target fragment. The PCR products were analysed by Agarose gel electrophoresis.

2.2.5.6 Reverse transcription of RNA to cDNA

Total RNA from the cell samples was isolated using Trizol reagent according to the manufacturer's protocol. 1-5µg of RNA was added with 1µl of DEPC-treated water and 1µl of Random primers. The mixture was incubated at 70°C for 5 min and incubated on ice immediately for 5 min. A reaction mixture prepared as follows was added to the RNA and incubated at 37°C for 5 min.

Reaction mixture:

- 6 µl of 5X reaction buffer
- 3µl of 0.1M DTT
- 0.5 µl of 25mM dNTPs
- 9 µl of DEPC-treated water

The tube was transferred to the ice and incubated for 5 min. 0.5 µl of Reverse Transcriptase was added to the tube and it was incubated for 60 min at 42°C. The Reverse Transcriptase was then heat inactivated at 70°C for 5 min. The tube containing the cDNA was stored at 4°C and used for RT-PCR.

2.2.5.7 Real-time PCR (RT-PCR)

25 µl Real-time PCR reaction was set up as follows:

- 12.5µl of SYBR Green Master Mix
- 2.5µl of cDNA (Diluted 5X)
- 2.5µl of diluted primers (Forward + Reverse, 50nm in 25µl)
- 7.5µl of DNAase/RNAase free water.

Each reaction was performed in triplicates and the primers used are listed in Appendix IV. The RT-PCR was performed on 7500 Real-Time PCR system (Applied Biosystems) using the comparative C_T method and the relative quantification of WASP expression was calculated using the formulae below:

$Ct_{\text{target gene}} - Ct_{\text{Endogenous control}} = \Delta Ct$
$Ct_{\text{target gene}} - Ct_{\text{Reference sample}} = \Delta\Delta Ct$

The standard deviation was calculated using the following formula: $S=\sqrt{(S_1^2 + S_2^2)}$. Mitochondrial Ribosomal Protein L27 (MRPL27) was used as the loading control.

2.2.5.6 Site directed mutagenesis (SDM)

Overlap extension technique was used to generate the mutation of interest at specific locations in the target DNA (Grb2 gene). The oligonucleotide pair sequences (forward and reverse) used for making the point mutations in the three domains of Grb2 has been provided

in Appendix III. Cloning of human Grb2 shRNA was also done using overlap extension PCR with pLKO plasmid as the template for U6 promoter and the primer sequences for the shRNA as provided in Appendix II.

2.2.5.7 Overlap extension PCR

In the overlap extension PCR, as illustrated in figure 2, two halves of the gene of interest are first amplified separately with an outer flanking primer and a mutagenesis primer each (primers a and b, primers c and d). The two mutagenesis primers are complementary to each other. The PCR products from the two PCR reactions (AB and CD) are purified and mixed together to be used as template for the third PCR reaction. In the third PCR reaction, the two outer flanking primers (primers a and d) are used to amplify the final full length PCR product (AD). Finally, the end product obtained is the gene of interest bearing the desired mutation.

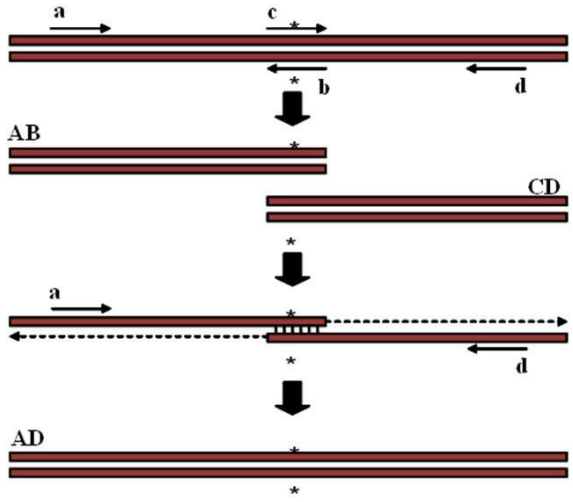


Figure 2: Schematic diagram of the overlap extension PCR

b and c are the mutagenesis primers while a and d are flanking primers. AD is the final PCR product which contains the desired mutation.

2.2.5.8 DNA precipitation

For 20µl of DNA mix, 2.22 µl of 3M Sodium acetate (1/10 of DNA mix) and 44.44 µl of 100% ethanol (2X of DNA and NaOAc mix) was added. The mixture was incubated at -20°C for 2 hrs or more and centrifuged at 4470 x *g* for 10 mins. The resulting pellet was washed with 70% ethanol and spun down for 10 mins. The pellet was air-dried and dissolved in appropriate amount of deionised water or 1X Tris-EDTA buffer according to the downstream application.

2.2.6.0 SDS-PAGE electrophoresis

2.2.6.1 Sample preparation for SDS-PAGE electrophoresis

Bradford assay was performed to measure the amount of protein in samples. The protein sample was first mixed with 2X loading buffer (*Section 2.1.10.2.5***)** and heated at 100 ℃ for about 5 min. 25 µg of the protein sample was used to run the SDS-PAGE.

2.2.6.2 SDS-PAGE gel preparation

A 10% SDS-PAGE gel was prepared as follows:

Resolving gel:

Components	**ml**
1.5 M Tris-HCl, pH 8.8	1.3
10% SDS	0.05
30% Acrylamide/Bis-acrylamide	1.7
10% ammonium persulfate (APS)	0.05
TEMED	0.002
H_2O	1.9

Stacking gel:

Components	**ml**
1.0 M Tris-HCl, pH 6.8	0.38
10% SDS	0.03
30% Acrylamide/Bis-acrylamide	0.5
10% ammonium persulfate (APS)	0.03
TEMED	0.003
H_2O	2.1

Proteins in samples were resolved by loading the appropriate amounts in the SDS-PAGE gel and the gel was run at 90V for 120mins, until the dye front has migrated past the end of the gel. The gel was then further processed either for the staining of proteins or Western blot analysis.

2.2.6.3 Western blot analysis

Proteins were resolved by 10% SDS-PAGE gel and transferred onto nitrocellulose membrane. They were then probed overnight with the appropriate primary antibody. The probing is followed by three washes with 0.1% Triton-1 x PBS. The blot was probed with the secondondary antibody for 1 hr. The probing was followed by three washes with 0.1% Triton-1 x PBS. The secondary antibody is

conjugated with horse-radish peroxidase (HRP) and detected with Millipore Immobilon Western (Millipore) reagent for weaker antibodies and Super Signal West Pico (Thermo Scientific) detection reagent for stronger antibodies.

Chapter3: RESULTS

Role of Growth Factor Receptor- bound Protein 2(Grb2) in Myogenesis

Adaptor proteins play an important role in controlling various signaling mechanisms by relaying the signals from the activated growth factor receptors to downstream target proteins. Almost all the cellular processes such as proliferation, differentiation, migration, survival depend on the activation of intracellular signaling pathways in response to various environmental stimuli such as growth factors, cytokines etc [168]. Myogenesis is one such process which, along with co-ordinated cellular and molecular changes, is also regulated by cell-cell, cell-matrix interactions and several extracellular secreted factors [56]. Growth factors such as Hepatocyte Growth Factor (HGF), Fibroblast Growth Factor (FGF), Transforming Growth Factor-β (TGF-β), Insulin-like Growth Factor (IGF) and Epidermal Growth Factor (EGF) have been found to be crucial in regulating the balance between proliferation and differentiation of myoblasts ([56], [169]). Adaptor protein Grb2 has been found to be important in these growth factor induced myoblast proliferation or differentiation [49]. Grb2 is a SH2 domain containing adaptor protein flanked by N- terminal and C-terminal SH3 domains [4]. The SH2 domain has been shown to interact with the tyrosine phosphorylated residues of activated growth factors receptors such as EGFR [4] and play a critical role in cell proliferation in many cell types by activating the downstream MAPK signaling pathway. In chicken skeletal myoblasts, Grb2 was shown to inhibit differentiation by preferentially binding to c-Met, Hepatocyte Growth Factor Receptor [49]. However, no specific mechanism or characteristic role of Grb2 in muscle differentiation has been studied so far. This project aims at identifying the specific role of Grb2 in muscle myoblast cell line C2C12.

C2C12 is a muscle myoblast cell line obtained from serial passaging of satellite cells derived from the thigh muscle of C3H mice after injury [170]. It proliferates rapidly under high serum conditions and differentiates under low serum conditions expressing muscle specific markers. It is a commonly used model cell line to study in vitro muscle differentiation and mechanisms.

3.1. Expression of Grb2 remains relatively constant during muscle differentiation.

Mouse myoblast cell line C2C12 was cultured in growth media (DMEM+10%Fetal Bovine Serum+1%Penicillin-Streptomycin) till around 80-90% confluency. At 90% confluency the media was changed to low serum differentiation media (DMEM+2% Horse serum+1%Penicillin Streptomycin) and the cells were allowed to differentiate for 3-4 days. The media was replaced with fresh differentiation media after 48 hrs. On day0 and day1 of differentiation, the myoblasts cells underwent changes in the cell morphology and became spindle shaped and aligned in a particular direction. By day 2 of differentiation the C2C12 cells began to fuse with one another and form smaller primary myotubes. By the third and the fourth day of differentiation, the myoblasts started forming bigger secondary myotubes and almost 70% of the cells had fused in to myotubes with many nuclei in one myotube (Fig 3.1.1(A, B)). Protein extracts from each day of differentiation, including day 0, at 24 hrs interval was isolated and the lysates were subjected to western blot analysis and probed for Grb2 to determine its expression level. To monitor the process of differentiation, specific muscle markers such as MyoD (Myogenic Determinant) and MyHC (Myosin Heavy Chain) were also analyzed by western blot. MyoD is an early differentiation marker with its peak of expression observed by day 1 of differentiation whereas MyHC is a late differentiation marker with its highest expression seen on day 4 of differentiation. Expression of Grb2 was found to increase after the addition of differentiation media with its expression being relatively constant during the four days of differentiation (Fig 3.1.1 (C)). GAPDH was used as the loading control.

Localization of Grb2 in C2C12 cells was visualized by immunostaining for Grb2 from day 0 to day 4 of differentiation. C2C12 cells were seeded on silane coated coverslips and the cells were fixed and immunostained every 24 hr interval from day 0 in growth media to day1-4 in differentiation media. The process of muscle differentiation was monitored by staining for Myosin Heavy Chain (MyHC) as the terminal differentiation marker. DAPI was used to stain the nucleus. Grb2 was found to be present in the nucleus on day 0 in the growth media (Fig3.1.2). Merging with DAPI showed its co localization in the nucleus. It was also found to be present in the cytoplasm with no specific localization. On day 1 of differentiation, it was found to have moved outside the nucleus with its localization more at the perinuclear region. From day 2 to day 4 of differentiation, it was found to be present specifically more in the fusing myotubes compared to the non fused myoblasts. In the myotubes, Grb2 was found to be present in the cytoplasm. MyHC staining showed MyHC positive myotubes on the 3rd and the 4th day of differentiation (seen in yellow after overlaying

it with Grb2 stained cells). Hence, localization of Grb2 was found to be consistent with its expression indicating the relatively constant presence of Grb2 during differentiation.

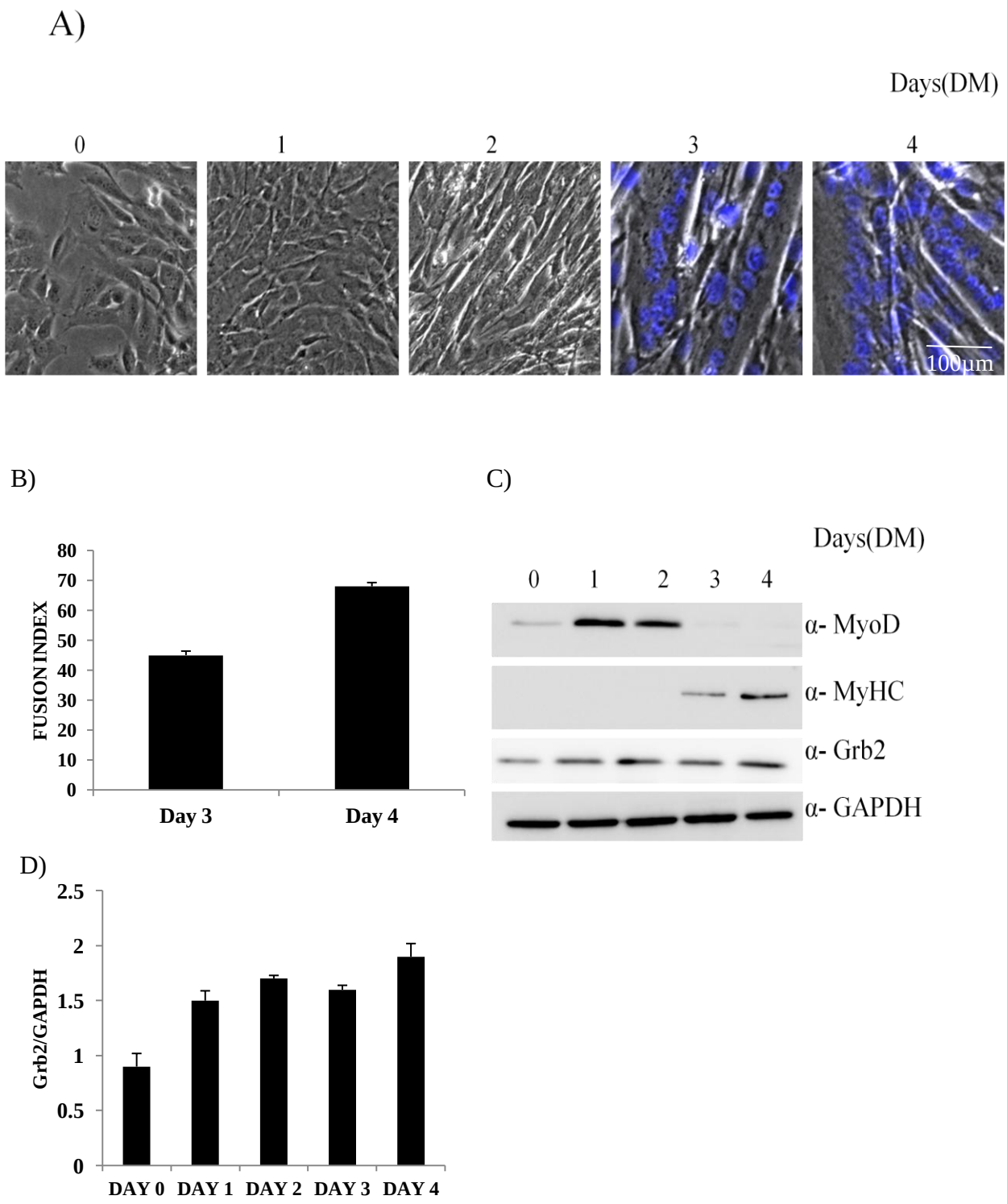


Fig 3.1.1: Expression of Grb2 remains relatively constant during myogenesis in C2C12 cells: A) C2C12 cells at around 90% confluency were induced to undergo differentiation by changing the growth media to differentiation media (DMEM+2%Horse serum) and images were taken at 24 hr intervals for up to 4 days as indicated. B) Fusion index is calculated as the ratio of number of nuclei in the myotubes to the total number of nuclei in a particular field. Fusion index for day3 and day4 of differentiation was calculated using Image J. C)

C2C12 cell lysates were collected at these 24 hr intervals and immunoblotted for Grb2 to determine its expression levels. MyoD and MyHC were used as early and late differentiation markers respectively and GAPDH was used as the loading control**.** D) Densitometric analysis of Grb2 levels was carried out.

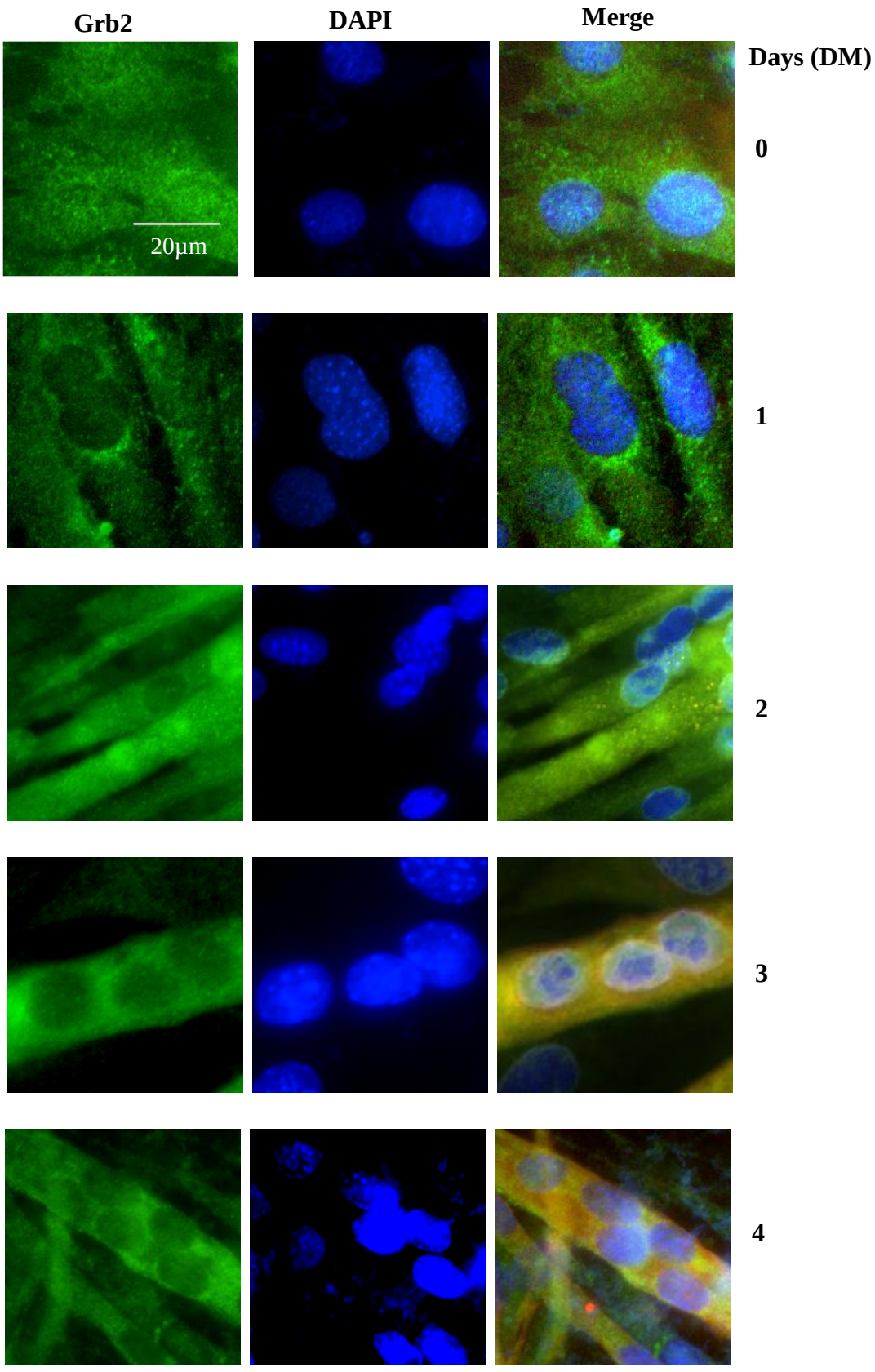


Fig 3.1.2: Localization of Grb2 during myogenesis in C2C12 cells: E) Localization of Grb2 during differentiation in C2C12 cells was observed by immunostaining for Grb2 (green), MyHC (Red, yellow in merge) was used as the marker for late differentiation on day 2, day 3 and day 4. DAPI (blue) was used to stain the nucleus**.**

3.2 Expression of Grb2 remains stable in the presence of Epidermal Growth Factor (EGF)

Grb2 has been shown to bind directly to activated Epidermal Growth Factor Receptor (EGFR) through its SH2 domain [171] to further interact with Sos and activate the Ras MAPK signaling pathway. Recently, EGF has been shown to inhibit human primary myoblast differentiation [169]. Hence expression and localization of Grb2 during differentiation in the presence of EGF was studied.

C2C12 cells were cultured in growth media till they reached 80-90% confluency. At around 90% confluency, the media was changed to differentiation media with EGF (50ng/mL) and the cells were allowed to differentiate for 48 hrs which marks the beginning of terminal differentiation and the expression of late differentiation marker MyHC. A set of control cells without the addition of EGF was differentiated simultaneously. As reported, it was found that differentiation was less in C2C12 cells differentiated in the presence of EGF. Analysis of protein extracts, obtained from each day of differentiation on Immunoblotting, showed that MyoD levels were less on day 1 of differentiation in the presence of EGF as compared to the control. By day 2 of differentiation MyHC was present in the control cells. In the cells treated with EGF, MyHC was not observed. However, the level of Grb2 was still found to be almost the same in the EGF treated and non treated cells with a slight decrease in the treated compared to the non treated cells (Fig 3.2.1 (A)).

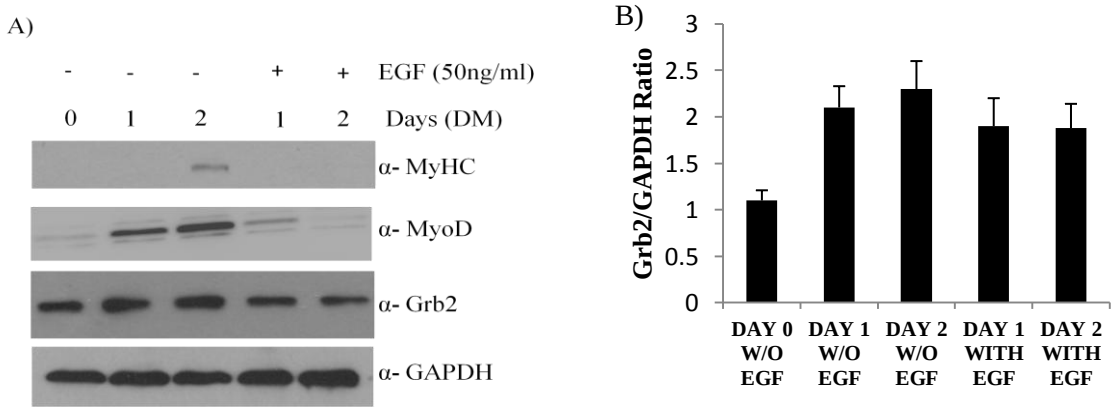


Fig 3.2.1: Expression of Grb2 remains unchanged during differentiation in C2C12 cells in the presence of EGF: A) C2C12 cells were induced to differentiate in differentiation media (DMEM+2%Horse serum) in the

presence and absence of EGF(50ng/mL). Cells were lysed after 24 hr intervals from Day0 to Day 2 of differentiation and subjected to immunoblotting against Grb2 to detect its expression. MyoD and MyHC were used as the early and late differentiation markers respectively and GAPDH was used as the loading control. B) Densitometric analysis of Grb2 levels in the presence and absence of EGF during differentiation was carried out.

Immunostaining of Grb2 in the EGF treated C2C12 cells during differentiation was carried out to study its localization during differentiation. It was observed, as reported in the literature, that the number of myotubes formed in the EGF treated cells was much lower than the untreated ones. Immunostaining for MyHC showed that MyHC signal was quite reduced on the 2nd and the 3rd day of differentiation (as observed by the reduced intensity of the yellow color overlaid on Grb2 stained cells). During differentiation, in the presence of EGF (day1 and day2), it was observed that Grb2 localized mainly in the cytoplasm. It was also found to be localized near the plasma membrane of the myotubes in EGF stimulated cells on the 1st day of differentiation (Fig 3.2.2). By day 2 of differentiation in EGF stimulated cells, Grb2 was found to be present more in the cytoplasm of the myotubes and not near the plasma membrane. The number of myotubes formed was quite less compared to the control cells. This localization pattern was observed in majority of the myotubes formed in EGF stimulated C2C12 cells.

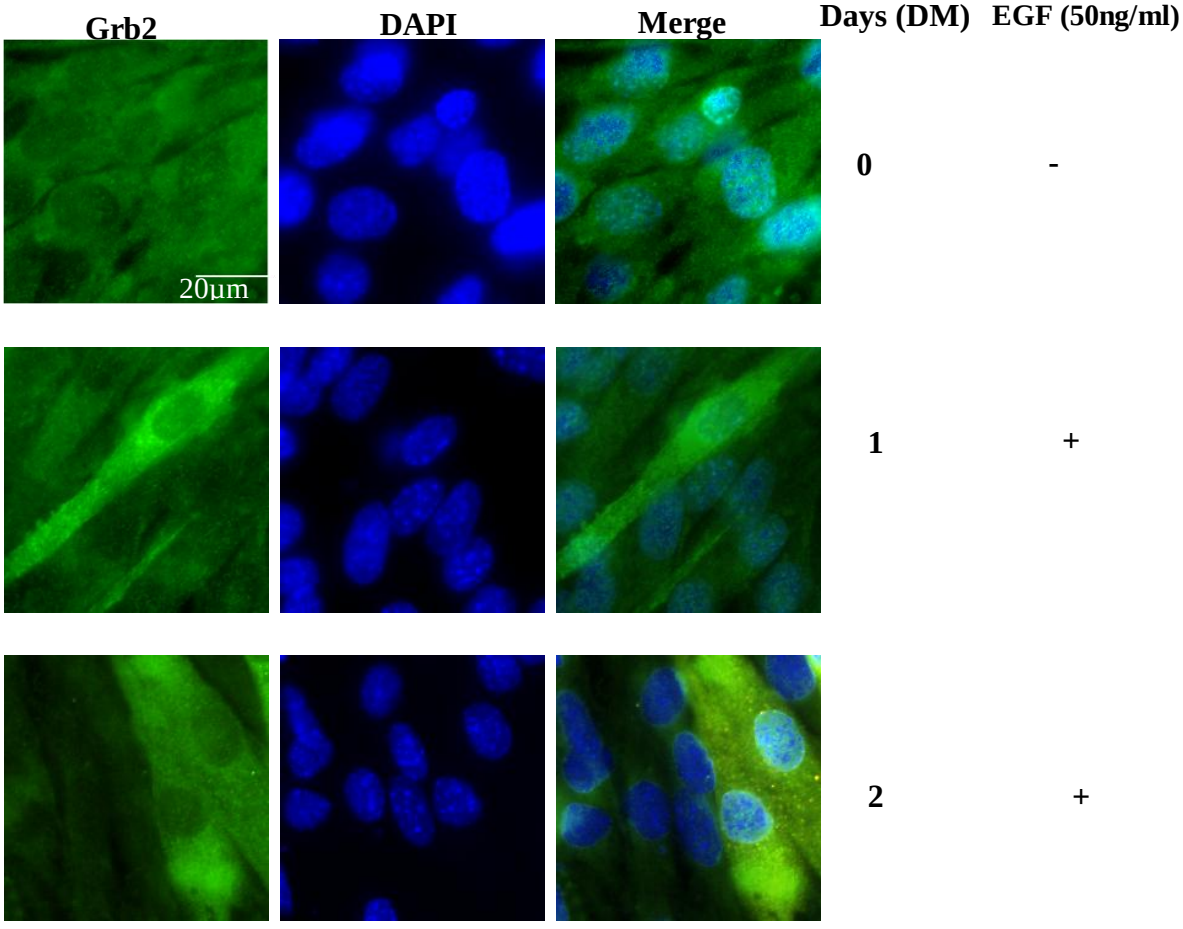


Fig 3.2.2: Localization of Grb2 changes during differentiation in C2C12 cells in the presence of EGF: B) Localization of Grb2 during differentiation in the presence of EGF (100ng/m) was visualized by immunostaining for Grb2 (green), DAPI(blue) in the absence (Day 0) and presence of EGF(Day 1 and Day 2) respectively. Myosin heavy chain was also stained for and can be visualized as yellow in the merge image of Day 2.

3.3 Knockdown of Grb2 expression increases myotube formation in C2C12 cells:

Level of Grb2 during differentiation was found to be relatively constant as observed in Fig 3.1(C). This suggests that it probably plays either a stimulatory or inhibitory role with respect to muscle differentiation in C2C12 cells. To elucidate its role further, C2C12 myoblasts were microporated with either empty (control) vector or sh-Grb2 plasmids following the conditions specified by the manufacturers. Efficiency of transfection was found to be around 90%. The cells were then split and seeded for differentiation. Upon reaching around 80% confluency, the growth media was changed to differentiation media and the cells were allowed to differentiate for three days. At the end of three days, the cells were fixed with 3.7% formaldehyde and immunostained for Myosin Heavy Chain (MyHC). DAPI was used to stain the nucleus. C2C12 cells microporated with sh-Grb2 were found to form myotubes faster than the control (Fig 3.3(C)). Also, the myotubes formed were found to be thicker in sh-Grb2 C2C12 cells compared to the control. Fusion index was calculated as the ratio of the number of nuclei present in the myotubes to the total number of nuclei in a particular field. Fusion index calculated from three sets of experiments showed that the fusion index of sh-Grb2 microporated cells was significantly higher than the control(Fig 3.3(D)).Protein extracts of the control and sh-Grb2 microporated cells were also immunoblotted with Grb2 and GAPDH (loading control) to check the knockdown efficiency of Grb2 (Fig 3.3(A)). Knockdown efficiency was calculated using Image J software and found to be significant (around 70%) (Fig3.3 (B)).

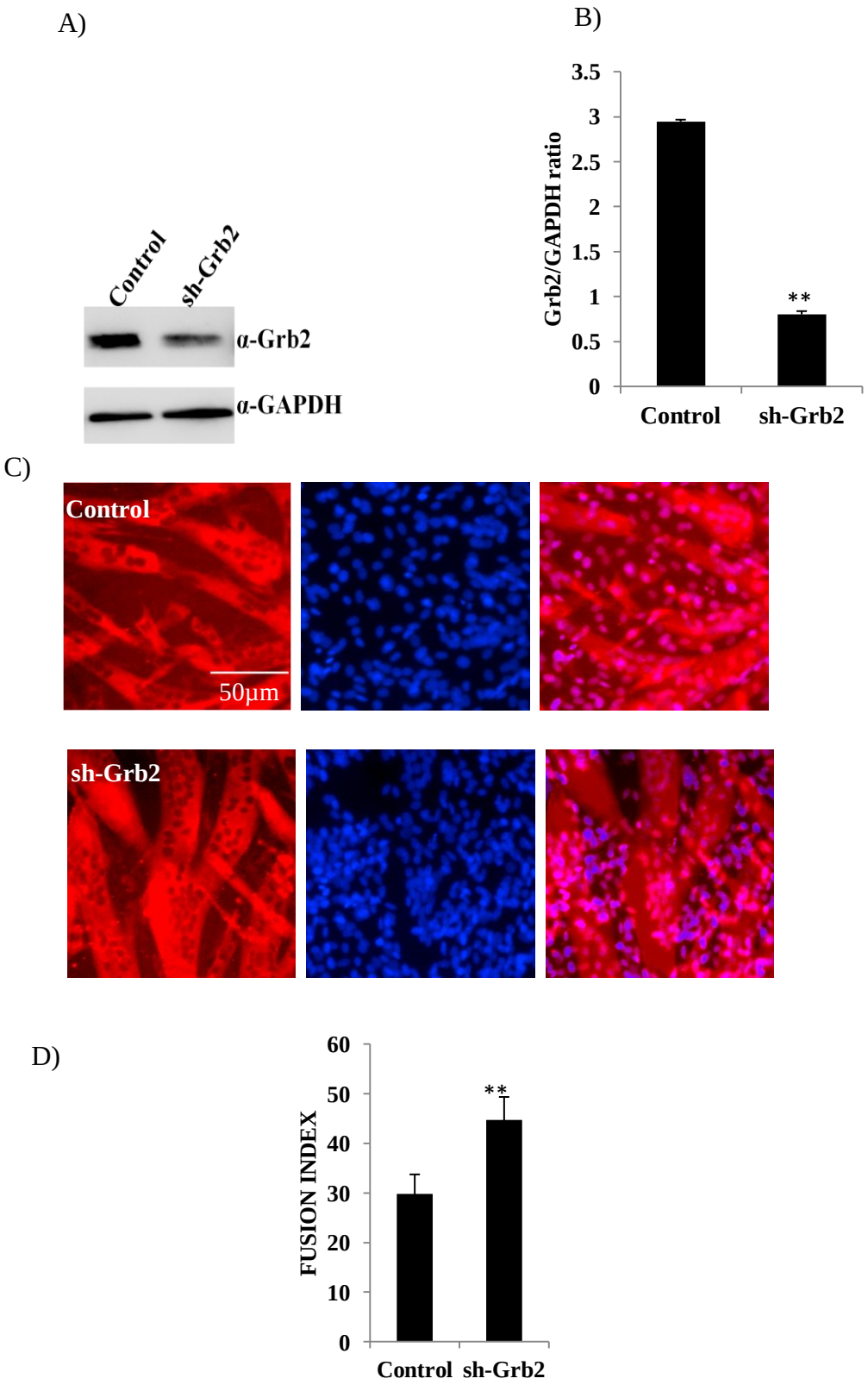


Fig 3.3 Knockdown of Grb2 increases myogenic differentiation in C2C12 cells: C2C12 cells were microporated with control and sh-Grb2 plasmids using conditions specified by the manufacturers. The microporated control and knockdown cells were lysed and immunoblotted for Grb2 to determine the knockdown

efficiency. GAPDH was used as the loading control. B) Knockdown efficiency was calculated using Image J software. Three independent set of experiments were used to calculate significance. C) 2.5X 10^6 cells/ well was seeded for each sample. At 90% confluency the cells were induced to differentiate in differentiation media(DMEM+2% Horse serum), After 3 days of differentiation, the cells were immunostained for myosin heavy chain (red). DAPI (blue) was used to stain the nucleus. Images were acquired and merged using the MetaVue software. D) Fusion index was calculated at the end of 3 days as the ratio of the number of nuclei in the myotubes to the total number of nuclei in a field. Measurement of 9 fields per set of data was carried out using the Image J software. Experiment was carried out in three independent sets to determine the significance. (*$p<0.05$,**$p<0.01$, ***$p<0.001$)

3.4 Knockdown of Grb2 increases the expression of myogenic markers

Differentiation of myoblast begins with the expression of specific set of transcription factors known as the myogenic regulatory factors (MRFs) followed by the expression of muscle specific marker proteins such as myosin heavy chain. MyoD is an MRF which is expressed at the beginning of differentiation [52]. Myosin heavy chain is a muscle specific terminal differentiation marker required for muscle specific activities such as contraction [91]. To study the effect of knockdown of Grb2 on the expression of these muscle markers, control and sh-Grb2 microporated C2C12 cells were allowed to differentiate for 3 days and lysates were collected at each day of differentiation and subjected to western blotting. Antibodies against Grb2, MyoD and MyHC were used to study their expression levels. It was observed that the expression of MyoD was higher in the sh-Grb2 containing C2C12 cells compared to the control cells on the 2^{nd} day of differentiation. Also the expression of MyHC was also detected earlier in sh-Grb2 containing cells (slightly at Day1) compared to the control suggesting that terminal differentiation occurs faster in Grb2 knockdown cells compared to the control. The expression of MyoD was also found to be higher at day 2 and day 3 of differentiation in Grb2 knockdown cells compared to the control (Fig 3.4).

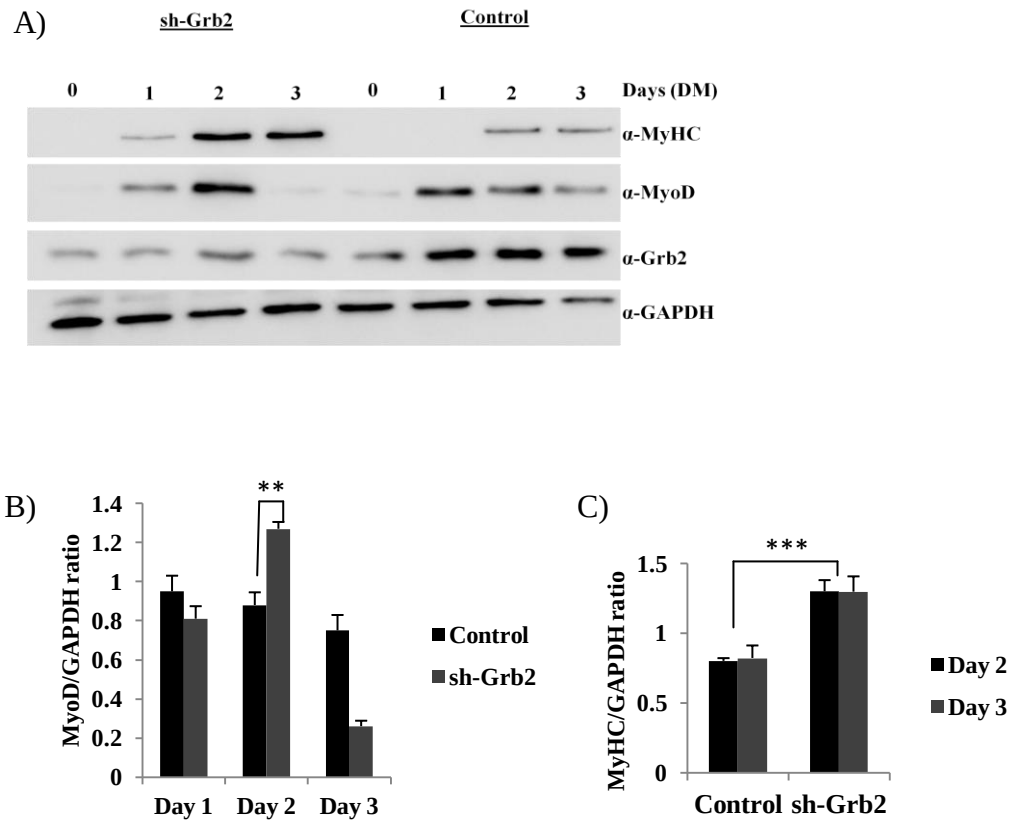


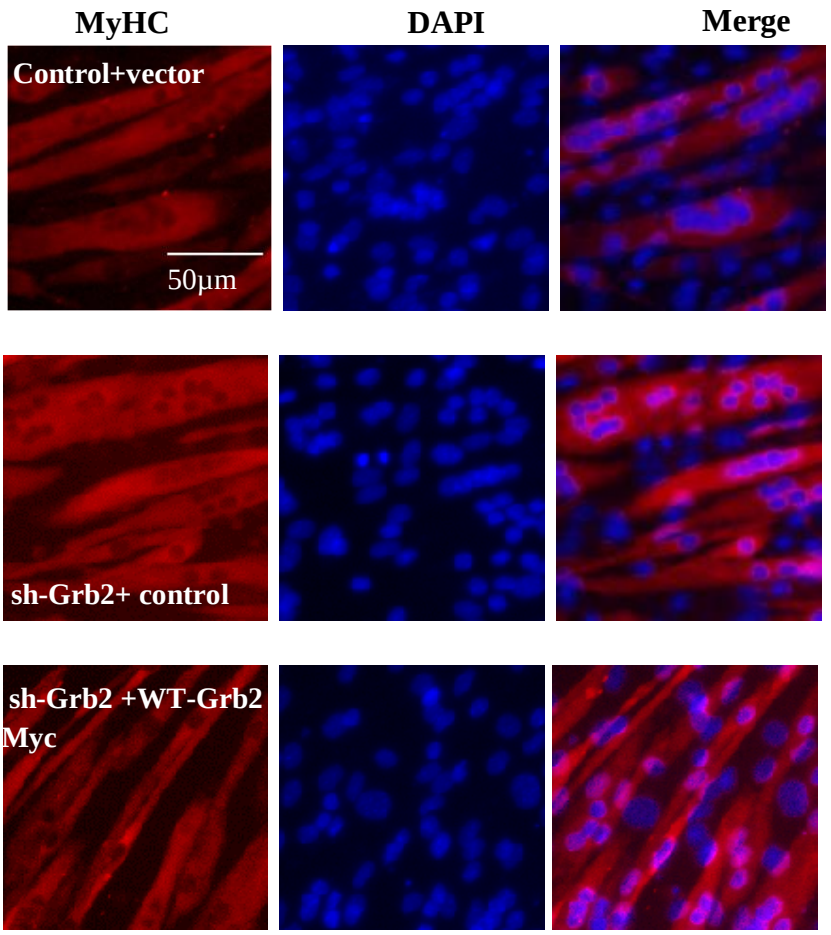
Fig 3.4 Knockdown of Grb2 increases the expression of myogenic markers: A) C2C12 cells were differentiated for 3 days and protein extracts were collected at each day and subjected to western blot analysis and probed for MyoD, MyHC and Grb2 to study their expression. GAPDH was used as the loading control. B) Densitometric analysis of the MyoD and MyHC expression levels (*p<0.05, **p< 0.01, ***p<0.001)

3.5 Reconstitution of Grb2 knockdown in C2C12 cells by exogenous expression of Grb2 can rescue the phenotypic defect

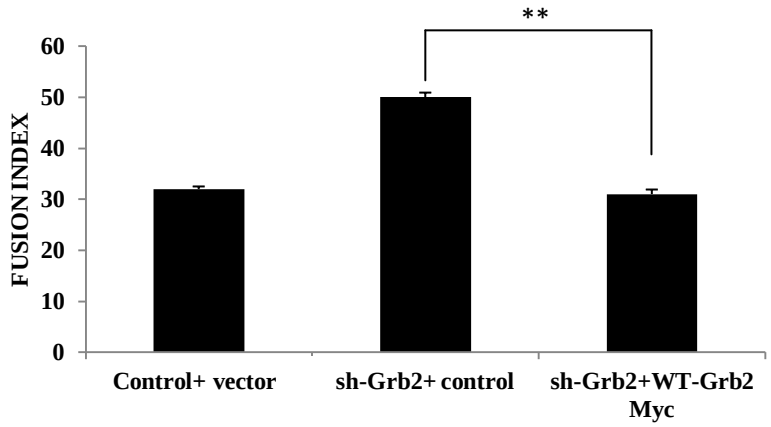
Knockdown of Grb2 was found to increase the myotube formation in C2C12, as observed in Fig 3.3 (C, D), suggesting an inhibitory role of Grb2 in muscle differentiation in C2C12 cells. However, sometimes shRNA constructs, even though designed specifically for the target gene, can target other non specific mRNAs [172]. To address this issue, a phenotype rescue experiment was carried out by using a mouse specific shRNA construct resistant to the human Grb2 gene (WT-Grb2 Myc). For this purpose, first, control and sh-Grb2 microporated cells were selected with puromycin (1.5µg/ml) and maintained stably in growth media. Second, to the Grb2 knock down stable cells, control and WT-Grb2 Myc plasmids were microporated. After around 30-40 hrs of microporation, the cells were seeded for differentiation. Additional control was maintained by microporating control plasmid in to the control stable cells. At around 90%

confluency, differentiation media was added and the cells were allowed to undergo differentiation for 3 days. At the end of three days, the cells were fixed and immunostained for MyHC to calculate the fusion index. It was observed that Grb2 knockdown C2C12 cells microporated with WT-Grb2 Myc had rescued the phenotypic defect (increased muscle differentiation) observed in the Grb2 knockdown C2C12 cells (Fig 3.5 (A)). The fusion index of these cells was also found to be lower compared to the Grb2 knockdown cells and similar to the control (Fig 3.5(B)) Also, protein extracts were collected by adding differentiation media and subjected to Immunoblotting against Grb2 to detect its expression level in rescued C2C12 cells (Fig 3.5(C)). GAPDH was used as the loading control.

A)



B)



C)

D)

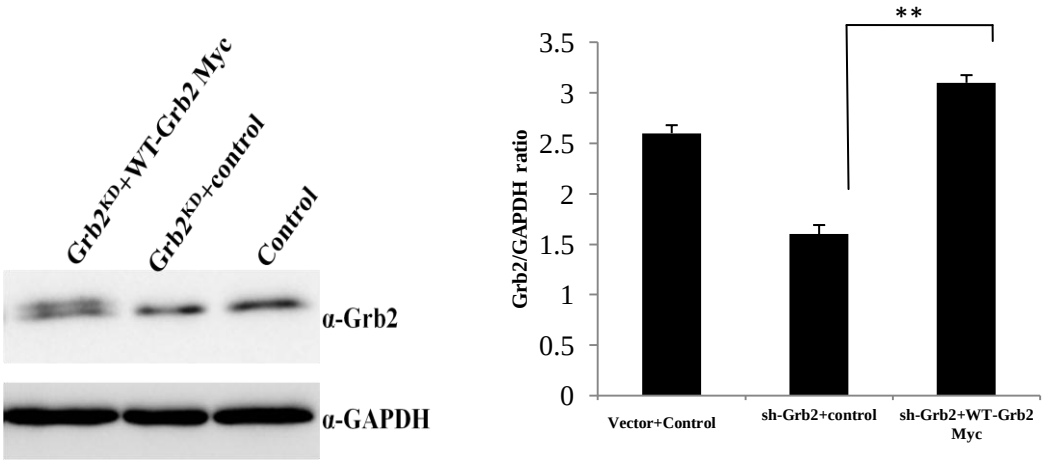
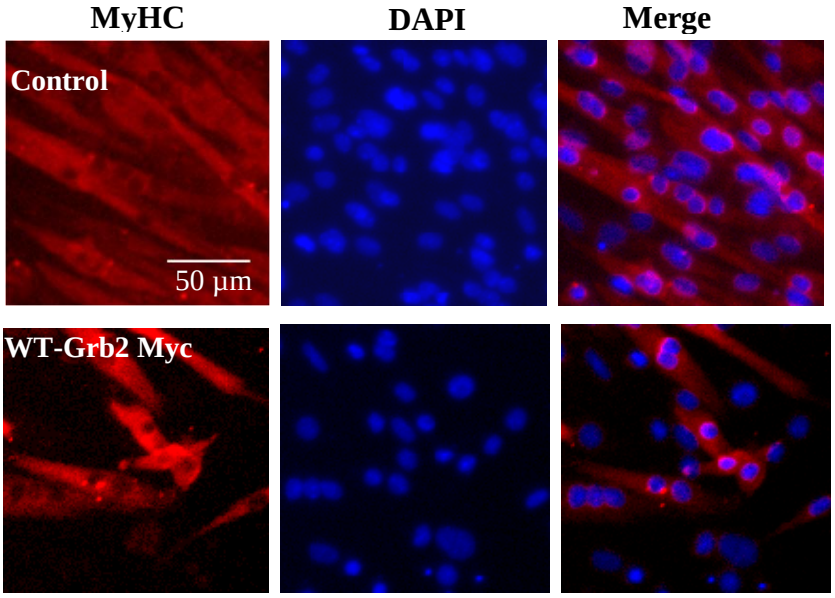


Fig 3.5 Reconstitution of Grb2 knockdown in C2C12 cells by exogenous expression of Grb2 can rescue the phenotypic defect: A) Control and sh-Grb2 containing stable C2C12 cells were microporated with WT-Grb2 Myc plasmid. After, 36 hrs of microporation the cells were split and seeded with appropriate controls. At 90% confluency the cells were induced to differentiate for 3 days after which they were fixed and immunostained for MyHC. DAPI was used to stain the nucleus. B) Fusion index was calculated as the ratio of the number of nuclei in the myotubes to the total number of nuclei in a particular field. A total of 9 fields per experiment were chosen for calculations. A set of three independent experiments was done to calculate the significance value. C) Protein extracts from the above samples were also lysed and subjected to western blotting and antibody against Grb2 was used to determine expression level of Grb2 in the rescued cells (*p<0.05,**p<0.01,***p<0.001)

3.6 Overexpression of Grb2 reduces muscle differentiation in C2C12 cells

Knocking down the expression of Grb2 enhanced myogenesis (Fig 3.3 (C, D) suggesting that Grb2 plays an inhibitory role in C2C12 muscle differentiation. Thus, it was hypothesized that the exogenous expression of Grb2 may reduce or inhibit muscle differentiation in C2C12 cells. To test the hypothesis, control and Epstein-Barr virus (EBV) vector containing shRNA resistant full length Grb2 gene tagged with Myc at the C-terminal (WT-Grb2 Myc) plasmids were microporated in C2C12 cells. After 36hrs of microporation, the C2C12 cells were split and seeded for differentiation in equal cell number. At 90% confluency, the media was changed to differentiation media and the cells were allowed to differentiate for 3 days after which they were fixed and immunostained for myosin heavy chain to calculate the fusion index. Fusion index values indicated that the number of myotubes formed in Grb2 over expressing cells was significantly lower than the control ((Fig 3.6 (A, B)) suggesting that over expression of Grb2 reduces muscle differentiation in C2C12 cells. The overexpression of Grb2 was verified by western blot analysis (Fig 3.6 C).

A)



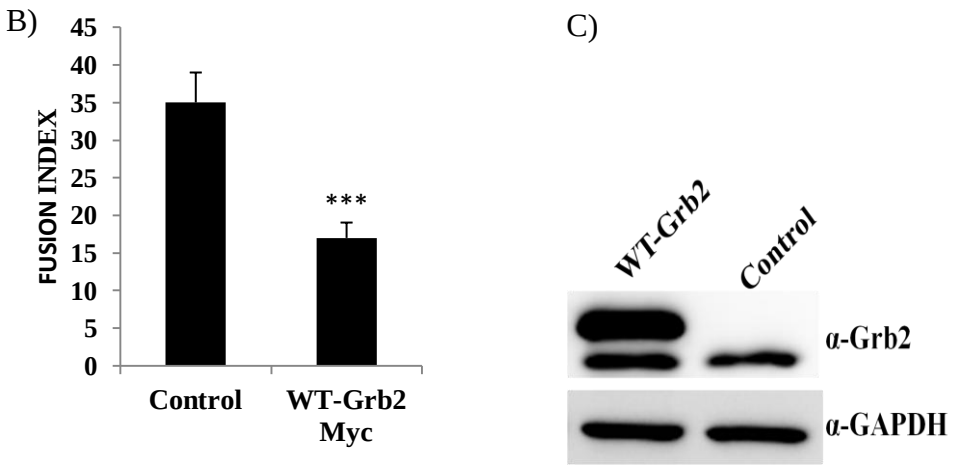


Fig 3.6: Over expression of Grb2 reduces myotube formation in C2C12 cells: A) Control and WT-Grb2 plasmids were microporated in C2C12 cells. After 36 hrs of microporation the cells were split and seeded with appropriate controls. At 90% confluency the cells were induced to differentiate for 3 days after which they were fixed and immunostained for MyHC. DAPI was used to stain the nucleus. B) Fusion index was calculated as the ratio of the number of nuclei in the myotubes to the total number of nuclei in a particular field. A total of 9 fields per experiment were chosen for calculations. A set of three independent experiments was done to calculate the significance value. C) Protein extracts from the above samples were also lysed and subjected to western blotting and antibody against Grb2 was used to determine expression level of Grb2 in the rescued cells (*p<0.05,**p<0.01,***p<0.001)

3.7 Overexpression of Grb2 reduces the expression of myogenic markers

Expression of muscle specific genes such as the MRFs and MyHC is regulated throughout the process of muscle differentiation and their expression is an indication of the progress of myogenesis. MyoD is one of the early differentiation markers expressed within the first two days of differentiation whereas as Myosin Heavy Chain (MyHC) is a terminal differentiation marker produced for the skeletal muscle maintenance and expressed by the end of the differentiation process. To study the effect of Grb2 over expression on the protein levels of the myogenic markers such as MyoD and MyHC, the control and Grb2 over expressing C2C12 cells were seeded from day 0 to day 3 of differentiation in equal numbers. After reaching 90% confluency, the cells were trypsinized to collect the day 0 lysate and remaining cells were made to undergo differentiation. At every 24 hr interval, the cells were trypsinized and the lysates were collected. The protein extracts from day 0 to day 3 were subjected to western blot analysis and probed using antibodies against MyoD

and MyHC. It was found that even though the early stages of differentiation occurred normally in both control and Grb2 over expressing cells, the expression of MyHC was considerably lower in Grb2 over expressing cells compared to the control (Fig 3.7 (A,B)). This suggests that may be the terminal differentiation stages are affected in Grb2 over expressing cells compared to the control.

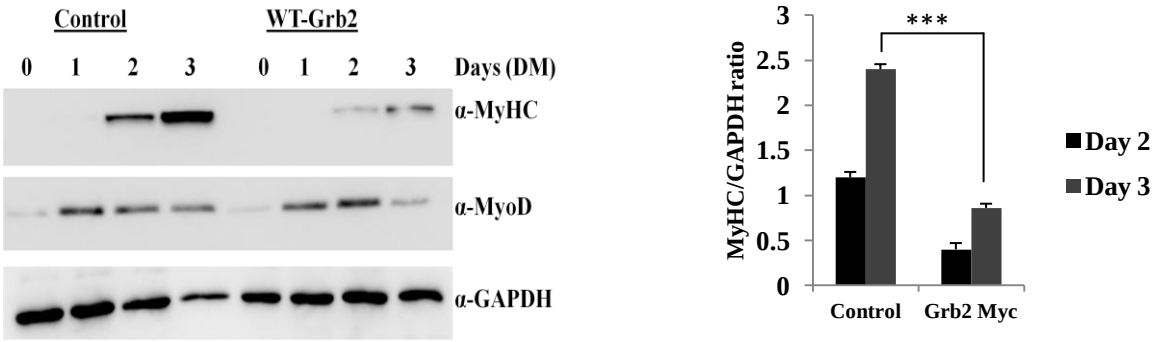


Fig 3.7 Over expression of Grb2 reduces the expression of myogenic markers: A**)** C2C12 cells were differentiated for 3 days and protein extracts were collected at each day and subjected to western blot analysis and probed for MyoD and MyHC to study their expression. GAPDH was used as the loading control. B) Densitometric analysis of MyHC expression level (*p<0.05, **p<0.01, ***p<0.001)

3.8. Grb2^{KD} C2C12 myoblast cells proliferate faster than control

Results from Grb2 knockdown and overexpression studies have established that Grb2 inhibits muscle differentiation in C2C12 cells. The next step was to study the stages of muscle differentiation that Grb2 may probably affect. Muscle differentiation is a series of co-ordinated cellular and molecular changes acting on environmental stimulus [173]. Grb2 has been extensively studied for its role in proliferation by activation of Ras proteins in many cell types. It has been shown to be recruited to the plasma membrane by activated EGFR along with Sos, which being a Gunanine nucleotide exchange factor hydrolyzes Ras, a GTPase and activates MAPK signaling cascade which has been shown to control cell growth in many cell types [24].

For that purpose, a proliferation assay was carried out where 7500 cells of control, sh-Grb2 and WT-Grb2 Myc microporated C2C12 cells were seeded on a 24 well plate for a period of four days and each day after 24 hrs intervals the cells were trypsinized and counted. Interestingly, it was found that the proliferation rate was higher in Grb2 knock down cells compared to the control. However, not much significant difference was observed between the Grb2 over expressing cells and the control (Fig 3.8.1(A)).

Also, MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was carried out to determine the metabolic activity of the proliferating cells. In this assay, 7500 cells/ 100 µL of control, sh-Grb2 and WT-Grb2 Myc microporated C2C12 cells were seeded in a 96 well plate in triplicates. After 48 hrs of incubation at 37° C, 20 µL of 5mg/ml of MTT reagent was added to each well One set of well for each sample was kept as control containing MTT but no cells. The cells were incubated at 37° C for 3.5 hrs. Media was then removed carefully form the wells and 150 µL of MTT solvent was added to the cells. The plate was covered with tin foil to avoid exposure to light and agitated on a shaker for 15 mins. At the end of 15 mins, the cells were measured for their absorbance at 590nm with a reference wavelength measurement at 620nm [174]. It was observed that after 48 hrs the absorbance of Grb2 knock down C2C12 cells was higher than the control. Again, not much difference was observed between the Grb2 over expressing cells and the control (Fig 3.8.1(B)).

Next, the levels of cyclin D in the C2C12 cells were checked. Cyclin D is an important protein involved in the control of cell cycle. Cyclin D1 can specifically bind and activate cyclin dependent kinases such as CDK4/CDK6 and together as c complex, regulate the completion of DNA replication and cell division [175]. Hence, cyclin D1 expression gives an indication about the number of proliferating cells since its expression is higher in proliferating cells compared to differentiating cells. Hence control, Grb2 knockdown and Grb2 overexpressing cells were grown to in a 6 well up to around 90% confluency without allowing differentiation to begin, lysed and the protein extracts were immunoblotted using antibodies against Cyclin D1 and Grb2 to check the expression level in control, Grb2 knockdown and Grb2 overexpressing cells. It was observed that the level of Cyclin D1 was slightly higher in the Grb2 knock down cells compare to the control in co- relation with the results obtained for the proliferation assay and MTT assay (Fig 3.8.1(C)). However, again, not much difference was obtained between the Cyclin D1 expression of control and Grb2 overexpressing cells.

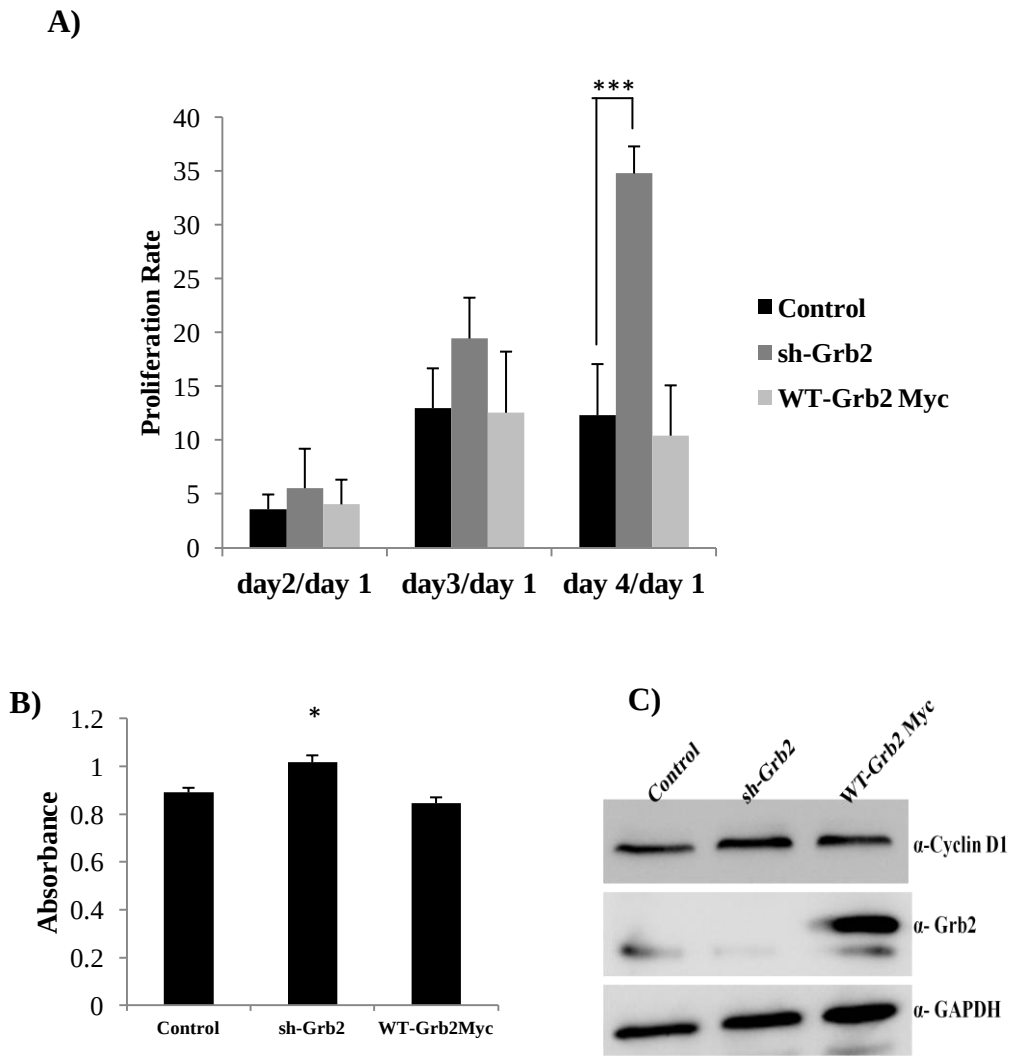
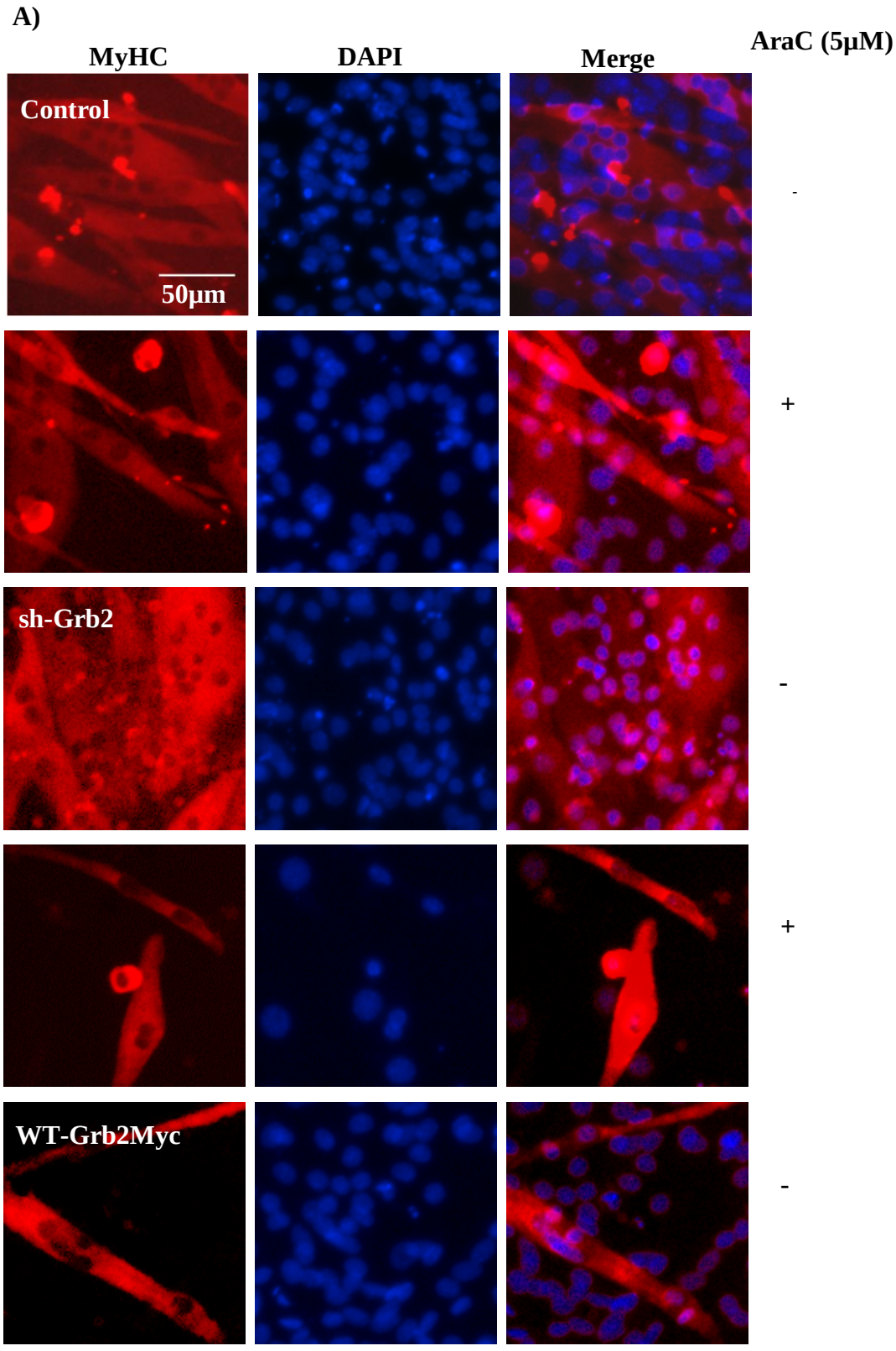


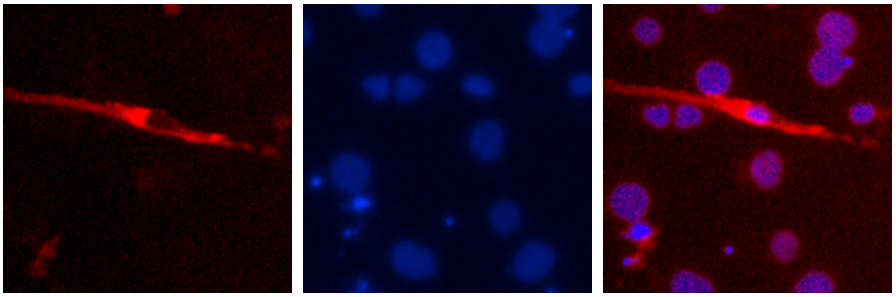
Fig 3.8: Grb2 knock down C2C12 myoblast cells proliferate faster than control: Control, Grb2 knock down and Grb2 over expressing C2C12 cells were seeded in triplicates at a cell number of 7500 cells/ ml in a 24 well plate and incubated at 37° C for a period of 4 days. After 24 hr interval, the cells were trypsinized and counted. The proliferation rate was calculated by dividing the cell number obtained by dividing the day2, day3 and day 4 of proliferation numbers of the sample by day 1. A set of three independent experiments was carried out. B) MTT assay was carried out to determine the metabolic activity of the proliferating cells. Control, sh-Grb2 and WT-Grb2 Myc microporated C2C12 cells were seeded in triplicates at a cell number of 7500 cells/ 100µL in a 96 well plate. After 48 hrs of incubation at 37 ◦ C, 20 µL of 5 mg/ml of MTT reagent was added to each well with one set as control containing MTT but no cells. The cells were incubated at 37° C for a period of 3.5 hrs. Then the media with MTT was carefully removed from the cells and 150 µL of MTT solvent was added to the cells, plate was covered with tin foil and agitated on a shaker for 15 mins. Absorbance was read at 590nm with a reference wavelength at 620nm. C) Cyclin D1 levels were checked by lysing the cells before differentiation and subjecting them to western blot analysis and probed against Cyclin D1 and Grb2 expression.(*p<0.05,**p<0.01,***p<0.001)

3.9 Increase in proliferation may affect differentiation of Grb2^{KD} C2C12 myoblasts

Earlier we observed that the proliferation of Grb2 knockdown cells was significantly higher than the control cells. There was no significant difference between the proliferation rate of the Grb2 overexpressing cells and the control. Based on this result, we wanted to investigate whether the increase in proliferation of Grb2 knock down C2C12 cells had any effect on the differentiation capacity of the Grb2 knock down myoblasts. It is known that proliferation and differentiation of myoblasts are inter-related mechanisms. In fact, proliferation is known to be inversely related to differentiation. Myoblasts are known to be proliferating muscle progenitor cells which under differentiation conditions, divide much less and undergo differentiation [176]. However, it was noticed that for Grb2 knockdown C2C12 cells, both the proliferation rate and the fusion index were significantly higher than the control. So we decided to study the differentiation capacity of Grb2 knock down and Grb2 over expressing C2C12 cells in the presence of a proliferation inhibitor such as AraC. AraC (Arabinosylcytosine, 1-β-D-Arabinofuranosylcytosine) is a selective inhibitor of DNA synthesis that does not interfere with transcription in mammalian cells [177]. This deoxycytidine analog incorporates in to the cytidine sites of DNA strand and terminates the elongation of DNA at its incorporation site. Since Grb2 knockdown cells were found to proliferate faster than the control, hence double the original cell number for control, Grb2 knockdown and Gr2 over expressing cells were seeded on a 6 well plate. After about 12 hrs, when confluency of around 90% was reached, the media was changed from growth media to differentiation media containing 5 µM AraC (DMEM+2% Horse Serum+5 µM AraC). A set of controls for control, grb2 knockdown and Grb2 over expressing cells was also maintained. Cells were allowed to differentiate for 3 days, then fixed and immunostained using antibodies against MyHC (late differentiation marker). It was observed that, as expected, the ratio of the number of myotubes in AraC treated control, Grb2 knockdown and Grb2 over expressing C2C12 cells was much lower than the untreated cells. The myotubes formed in the Grb2 knockdown cells treated with AraC reduced drastically compared to the control (treated cells) and Grb2 knockdown (untreated cells) which indicate that the rate of proliferation does affect the differentiation efficiency in Grb2 knockdown cells (Fig 3.9 (A, B)). It was observed that the number of myotubes in Grb2 over expressing C2C12 cells also reduced when compared to the untreated cells, but the reduction was not very significant. It was

observed that the nuclei in Grb2 knockdown and Grb2 over expressing C2C12 cells was comparatively bigger in size than their untreated counterparts and treated control cells.





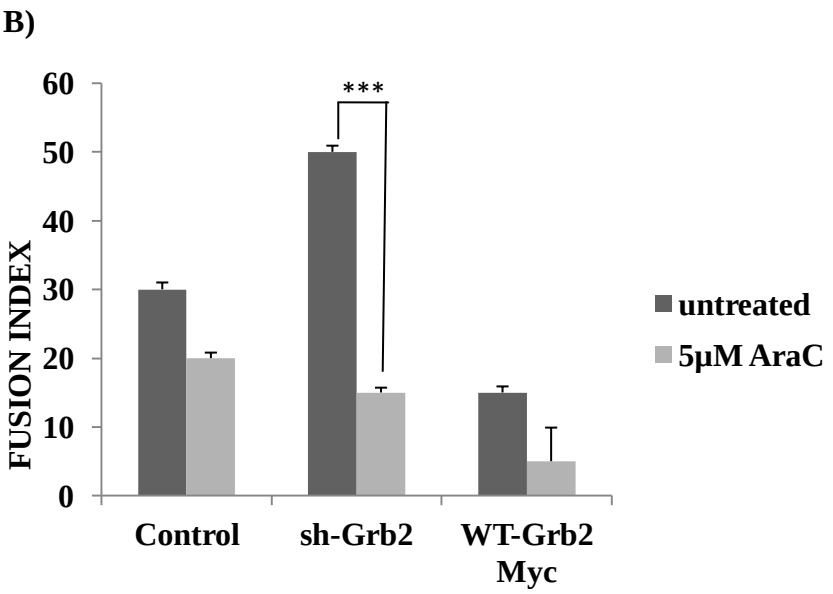


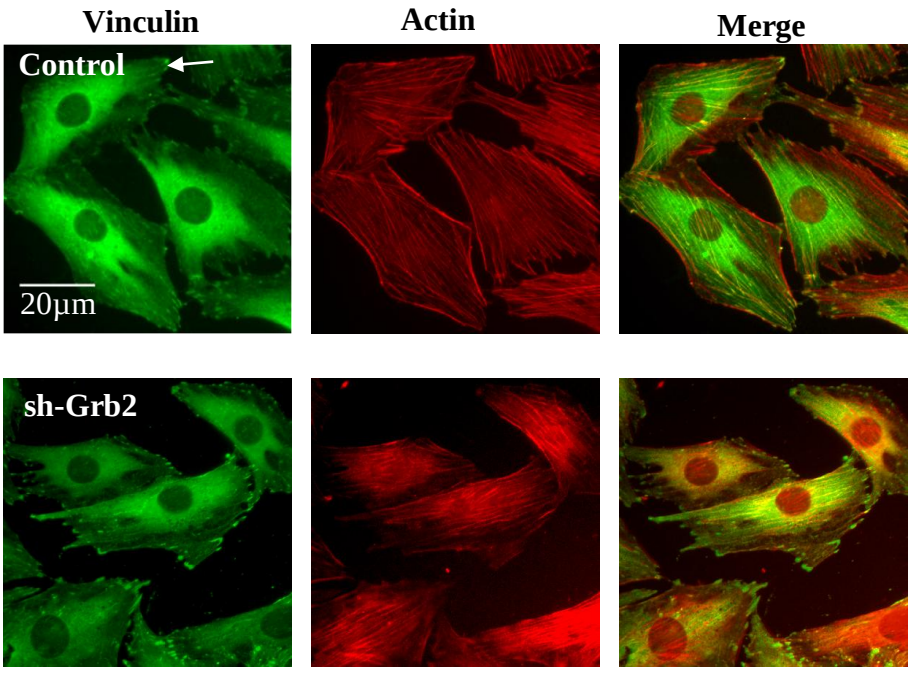
Fig 3.9 Increase in proliferation may affect differentiation of Grb2 knockdown C2C12 myoblasts: A) Control, Grb2 knockdown and Grb2 overexpressing C2C12 cells were seeded at double the cell number previously used for differentiation assay. After 12 hrs at around 90% confluency, the cells were allowed to differentiate in the presence or absence of 5µM AraC. After 3 days, the differentiated cells were fixed and immunostained using antibodies against MyHC (late differentiation marker). DAPI was used to stain the nucleus. B) Fusion index was calculated, in the presence or absence of AraC, as the ratio of the nuclei present in the myotube to the total number of nuclei present in a field. A total of 9 fields per experiment were quantified using Image J (*p<0.05, **p<0.01, ***p<0.001)

3.10 Grb2 inhibits vinculin patch assembly formation

Integrin mediated cell-matrix adhesion also results in the activation and interaction of complex of proteins which form a part of the focal adhesion complex. This transient assembly of proteins plays important roles in cell adhesion and migration. Vinculin is a cytoplasmic focal adhesion protein which has been shown to play important role in regulation of actin cytoskeleton and cell spreading [74]. Vinculin is a part of focal adhesions and forms streaks or patches at the leading edge of the cell [74]. Vinculin is a

commonly used marker protein to study focal adhesions in particular and cell adhesion in general. For this purpose, vector, psh-Grb2 and pGrb2 Myc microporated C2C12 cells were seeded on silane coated coverslips and grown till they achieved around 40% confluency so that individual cells could spread well and display vinculin streaks on staining. At around 40% confluency, the cells were fixed and immunostained using antibodies against Vinculin. Focal adhesions have been found to regulate actin cytoskeleton and localize at the periphery of actin stress fibres. Hence, phalloidin was used to stain actin. Merging the two images showed co localization of vinculin with actin. Quantification of the number of vinculin patches was done manually by counting the number of separate and individual streaks around the periphery of the cell. It was observed that the number of vinculin patches for Grb2 knockdown cells (40 patches/cell) was significantly higher (35%) compared to the control (25 patches/cell) whereas the number of vinculin patches was lesser in the Grb2 overexpressing cells (15 patches/ cell) compared to the control (around 40%) (Fig 3.10(A, B)). This observation correlates with the cell spreading data wherein Grb2 knockdown cells were found to spread more and also show higher number of vinculin patches

A)



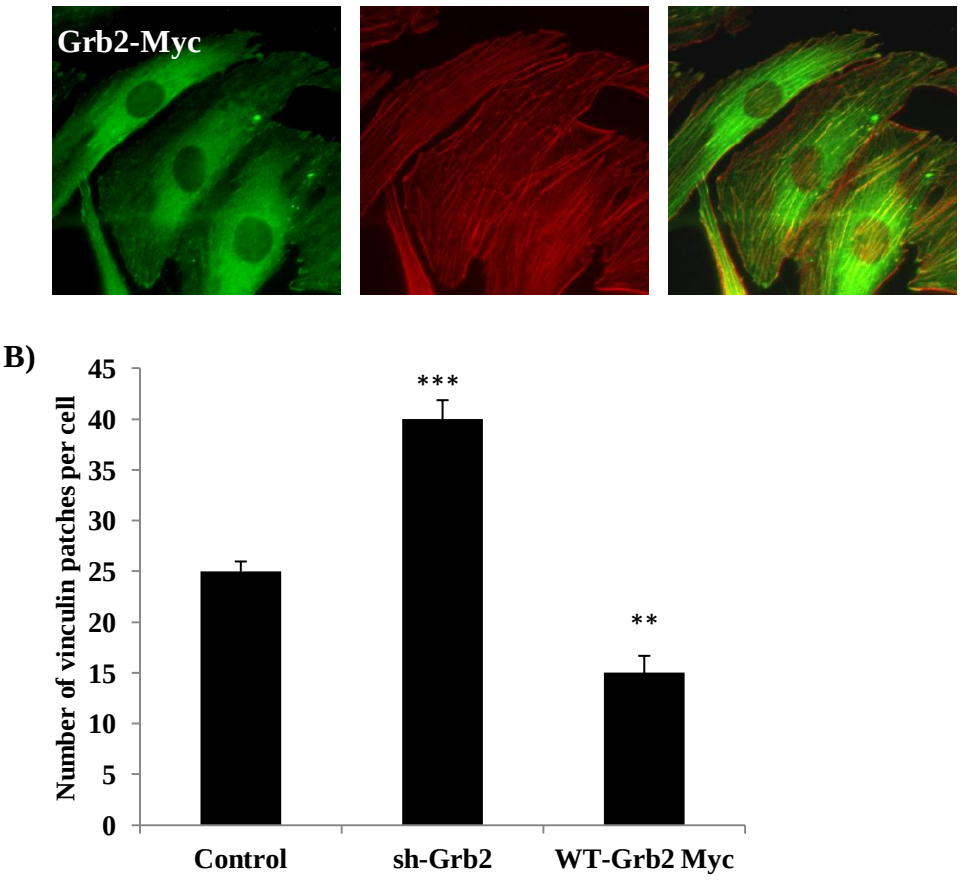
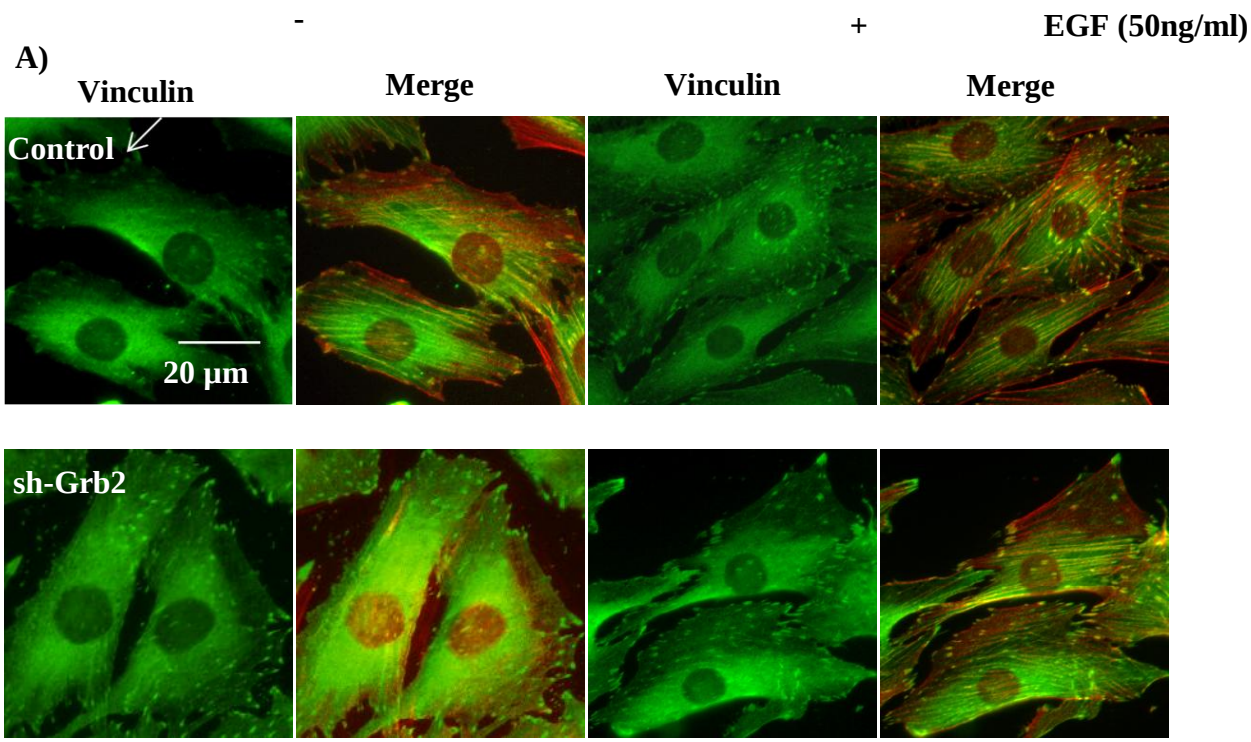


Fig 3.10 Grb2 inhibits vinculin patch assembly formation: A) Vector, psh-Grb2 and pGrb2 Myc microporated C2C12 cells were seeded on silane coated coverslips and grown up to 40% confluency, fixed and immunostained using antibodies against vinculin(Green) to detect vinculin patches. Phalloidin (Red) was used to stain actin. B) The number of vinculin patches per cell was quantified manually. A total of 25 cells per sample were counted for each experiment. Three independent experiments were carried out for determining the p value (*p<0.05,**p<0.01,***p<0.001).

3.11 Grb2 inhibits EGF induced vinculin patch assembly in C2C12 cells

Epidermal Growth Factor (EGF) has been shown to induce the expression of vinculin and β1 integrin in 3T3 fibroblasts [178]. EGF has also been shown to regulate cell-cell adhesion through the interaction of E-Cadherin with the actin cytoskeleton as well as proteins of the focal adhesions such as vinculin [179]. Also, Grb2 has been shown to directly bind to activated EGFR through its SH2 domain [4]. Earlier work in our lab has shown that EGF induced vinculin patch assembly in mouse embryonic fibroblasts (MEFs). Hence, to study the role of Grb2 in EGF induced vinculin patch assembly in C2C12 myoblasts, the control vector, psh-Grb2 and pGrb2 Myc microporated C2C12 cells on silane coated cover slips and grew till they reached around 25-30% confluency. At this confluency, the cells were serum starved for a period of 12 hrs. After 12 hrs, the cells were

treated with 50 ng/mL of EGF for 30 mins. A set of untreated controls was also maintained. After 30 mins of incubation at 37 ° C, the cells were fixed and immunostained using antibodies against vinculin. Phalloidin was used to stain actin. The number of vinculin patches per cell was counted manually. It was observed that the number of vinculin patches per cell increased quite significantly after EGF treatment in control C2C12 cells. In Grb2 knockdown C2C12 cells, the number of vinculin patches was more before EGF treatment and the number did not increase significantly after EGF treatment. Also, in Grb2 over expressing cells, the number of vinculin patches per cell was low before EGF treatment and did not increase significantly after EGF treatment as well (Fig 3.11 (A, B)). This indicates that Grb2 may inhibit EGF induced vinculin patch assembly in C2C12 cells. This suggests that the activated EGFR could be preventing Grb2 from inhibiting vinculin patch assembly formation further in Grb2 knockdown cells. In Grb2 overexpressing C2C12 cells the activated EGFR may not be sufficient enough to prevent the excess of Grb2 from inhibiting vinculin patch assembly.



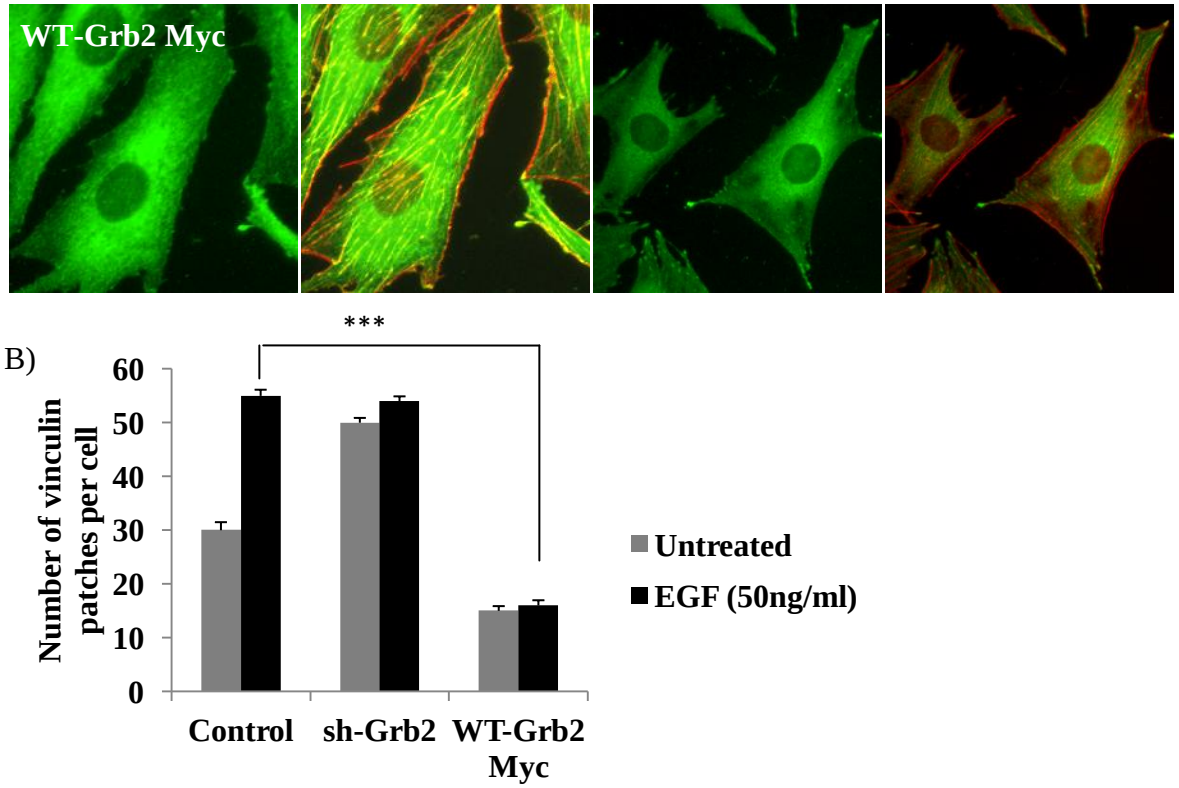
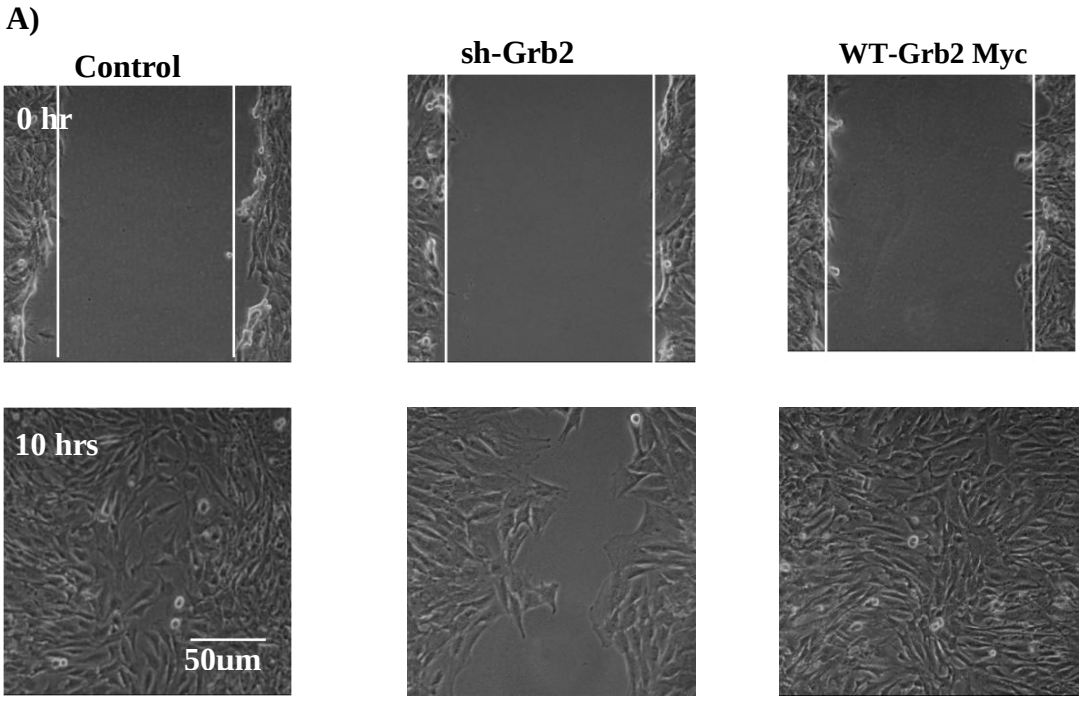


Fig 3.11 Grb2 inhibits EGF induced vinculin patch assembly: A) Control, sh-Grb2 and WT-Grb2 Myc microporated C2C12 cells were seeded on silane coated coverslips and grown up to 25-30% confluency. At 30% confluency, the cells were serum starved for a period of 12 hrs. After 12 hrs, 50 ng/mL EGF was added to the cells and cells were incubated at 37°C for 30 mins. After 30 mins, the cells were fixed and immunostained using antibodies against Vinculin. Phalloidin was used to stain actin. B) The number of vinculin patches per cell was calculated manually. A total of 25 cells per sample were counted for each experiment. Experiments were carried out in three independent sets. (*p<0.05,**p<0.01,***p<0.001).

3.12 Grb2 promotes cell migration in C2C12 myoblasts

Cell migration is an important process during the differentiation of myoblasts. For the proliferating myoblasts to fuse and differentiate efficiently, they must migrate and form stable cell-cell contacts [82]. Since Grb2 is closely associated with factors controlling the components of the focal adhesion complex assembly which also play important roles during cell migration, the effect of Grb2 knockdown and overexpression on the migration of C2C12 myoblasts was studied. Hence, a cell migration assay was carried out in which the control, sh-Grb2 and WT-Grb2 Myc microporated C2C12 cells were seeded in a 6 well plate. After the cells reached 100% confluency, a wound or a scratch was made on the cell monolayer with a white tip in triplicates. Images were taken at 0 hr time point under a 10X objective lens and the cells were incubated at 37 °C till the gap was found to close. This time point was optimized at 10 hrs for C2C12 cells. At 10 hrs, images were again captured

using a 10X objective lens. The gap width or the distance between the scratch at 0 hrs and 10 hrs was quantified using Image J. Cell migration was considered to be inversely proportional to the gap width. More the gap width lesser was the cell migration. Accordingly, it was observed that the gap width of the Grb2 knockdown cells was more than the control at 10 hrs which indicated that the cell migration was slower in Grb2 knock down cells compared to the control (Fig 3.12 A). Also, the gap between the Grb2 over expressing cells closed much faster than the control indicating the Grb2 over expressing cells migrate faster than the control. The calculation for gap width was done by measuring 10 random distances between the gap in all the samples at 0 hr and 10 hrs time points. 9 points per field at each time point was measured. Gap width value was found to be significantly more (about 70%) for Grb2 knockdown cells compared to control whereas for Grb2 over expressing cells it was found to be significantly lesser (around 75%) than the control (Fig 3.12 B). This suggests that Grb2 may promote cell migration in C2C12 myoblasts.



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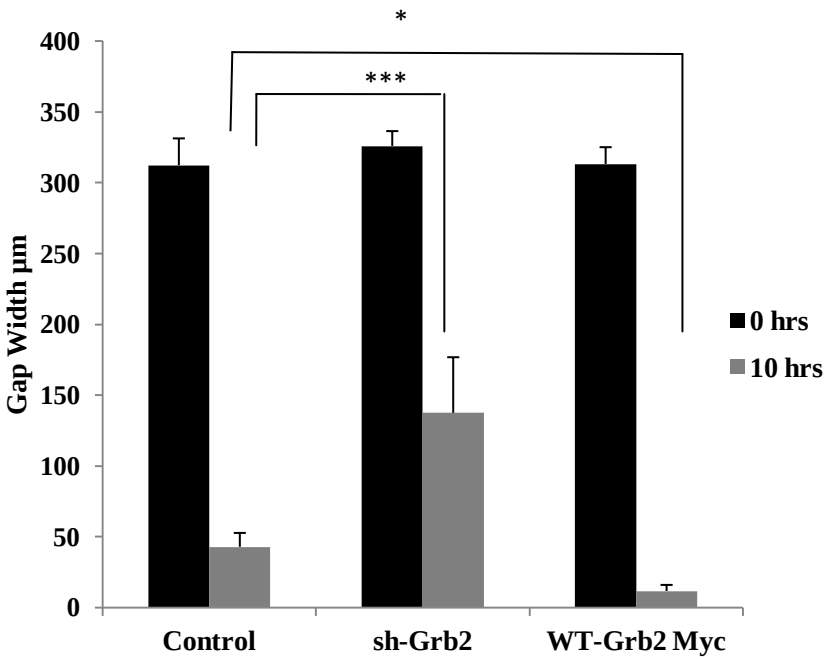
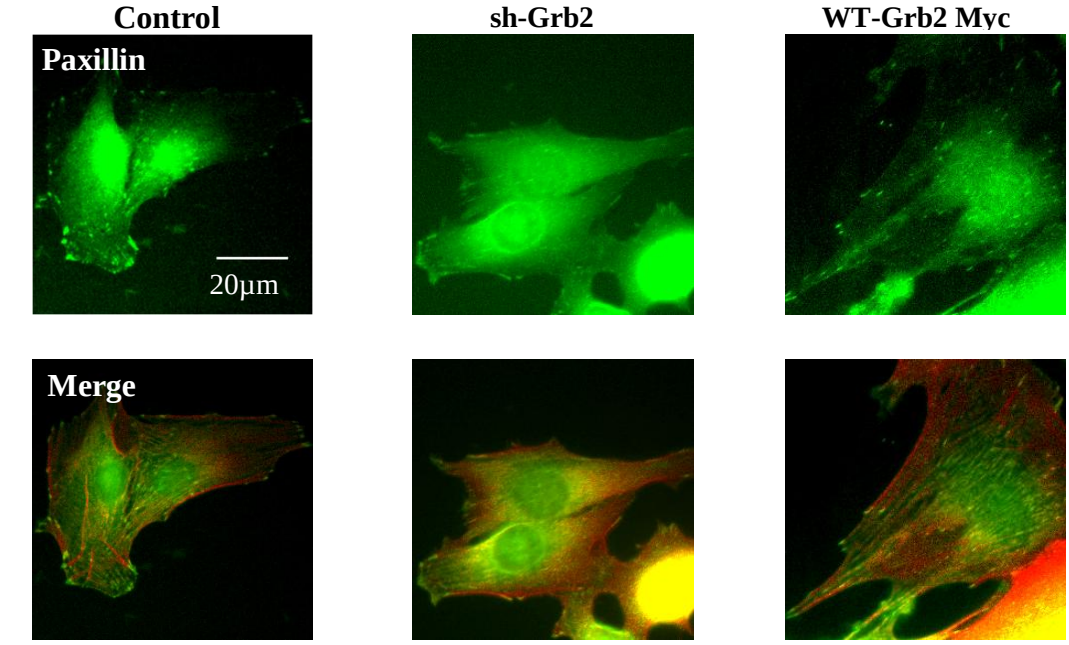


Fig 3.12 Grb2 promotes cell migration in C2C12 myoblasts: A) Control, Grb2 knockdown and Grb2 over expressing C2C12 cells were seeded on a 6 well plate at $2X10^5$ cell number. After reaching 100% confluency a scratch was made on the monolayer and images of the gap was taken under 10X objective lens at 0 hr time point. The cells were incubated at $37^{\circ}C$ for a period of 10 hrs by when the gap of the control cells has almost closed. At 10 hr time point, image of the gap was again captured using 10X objective lens. B) The gap width was measured by selecting 10 random distances between the gaps at both the time points of control, Grb2 knock down and Grb2 over expressing cells and measuring their length using Image J. Average length was calculated for every time point. A total of 9 fields for every time point and every cell sample were considered for quantification of each experiment. Three independent experiments was carried out to calculate significance. (*p<0.05,**p<0.01,***p<0.001)

3.13 Grb2 did not affect the distribution of paxillin at the focal adhesion of C2C12 myoblasts:

Paxillin is another scaffold protein found at the focal adhesion sites. However, it has been linked to the migratory capacities of highly metastatic cells through its phosphorylation [180]. Overexpression of Paxillin and its phosphorylated form was found to increase the migration of metastatic human osteosarcoma cell lines [180]. Since Grb2 was found to increase migration in C2C12 myoblasts, the effect of Grb2 knockdown and overexpression on endogenous Paxillin localized on the focal adhesion complex was studied. Control, Grb2 knockdown and Grb2 overexpressing cells were seeded on silane

coated coverslips and grown up to 40% confluency. Cells were then fixed and immunostained for endogenous Paxillin using antibodies against Paxillin. The number of Paxillin streaks per cell was quantified manually. It was observed that the number of Paxillin streaks per cell was not significantly different for Grb2 knockdown or Grb2 over expressing cells. Pro or anti migratory effect on Paxillin by Grb2 could not be concluded by staining for endogenous Paxillin (Fig 3.13 (A,B)).



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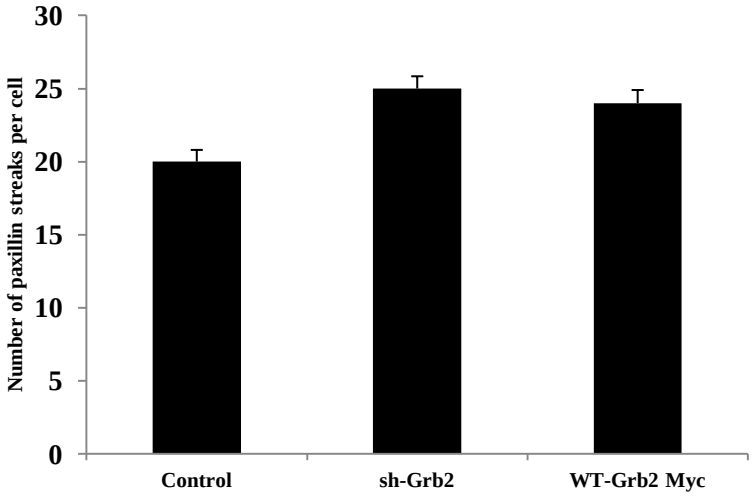


Fig 3.13 Grb2 did not affect the distribution of paxillin at the focal adhesion of C2C12 myoblasts: A) Control, Grb2 knockdown and Grb2 over expressing C2C12 cells were seeded on silane coated coverslips and grown up to 40% confluency. Cells were then fixed and immunostained using antibodies against Paxillin (green). Phalloidin was used to stain actin. Images were taken under 40X oil immersion objective lens. B) The number of Paxillin streaks per cell was quantified manually. A total of 25 cells were quantified for each experiment. (*p<0.05,**p<0.01,***p<0.001)

Grb2 is an adaptor protein consisting of SH3-SH2-SH3 domains all three of which have been shown to interact with wide variety of proteins. Both N and C terminal SH3 domains are known to bind GEF, Sos with a strong affinity [3]. The C-SH3 domain has been shown to bind to N-WASP at its proline rich domain [20]. Several other proteins can also bind to the SH3 domains such as Abl, dynamin, Shc, GAB1 etc [3]. SH2 domain has been shown to bind to the phosphorylated tyrosine residues of receptors of many growth factors such as EGF, FGF, c-Met etc, [4]. Hence studying the domain of Grb2 responsible for inhibition of differentiation in C2C12 myoblasts will help in identifying the proteins and possibly the mechanisms involved in the inhibition of differentiation mediated by Grb2. Independent point mutations in the three domains of Grb2 were used and their role in myogenic differentiation was characterized (Fig 3.15).

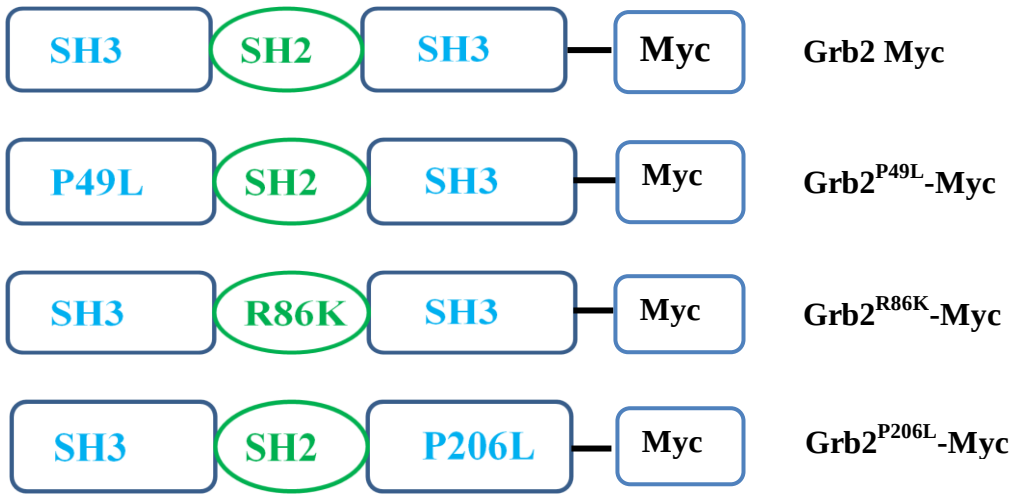


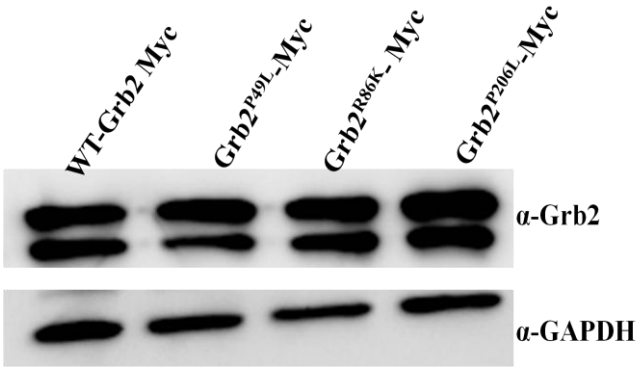
Fig 3.14 Grb2 domain structure and mutants used in the study

The R86K (Arginine to Lysine) mutation in the SH2 domain has been shown to affect the interaction of Grb2 to tyrosine phosphorylated residues [9]. The proline to leucine mutations in the N and C- terminal domains have been shown to be associated with loss of phenotype functions in Drosophila and affect interactions with SH3 domain containing proteins [9]. The P206L mutation has been shown to affect the interaction of Grb2 with N-WASP at its proline rich domain [20].

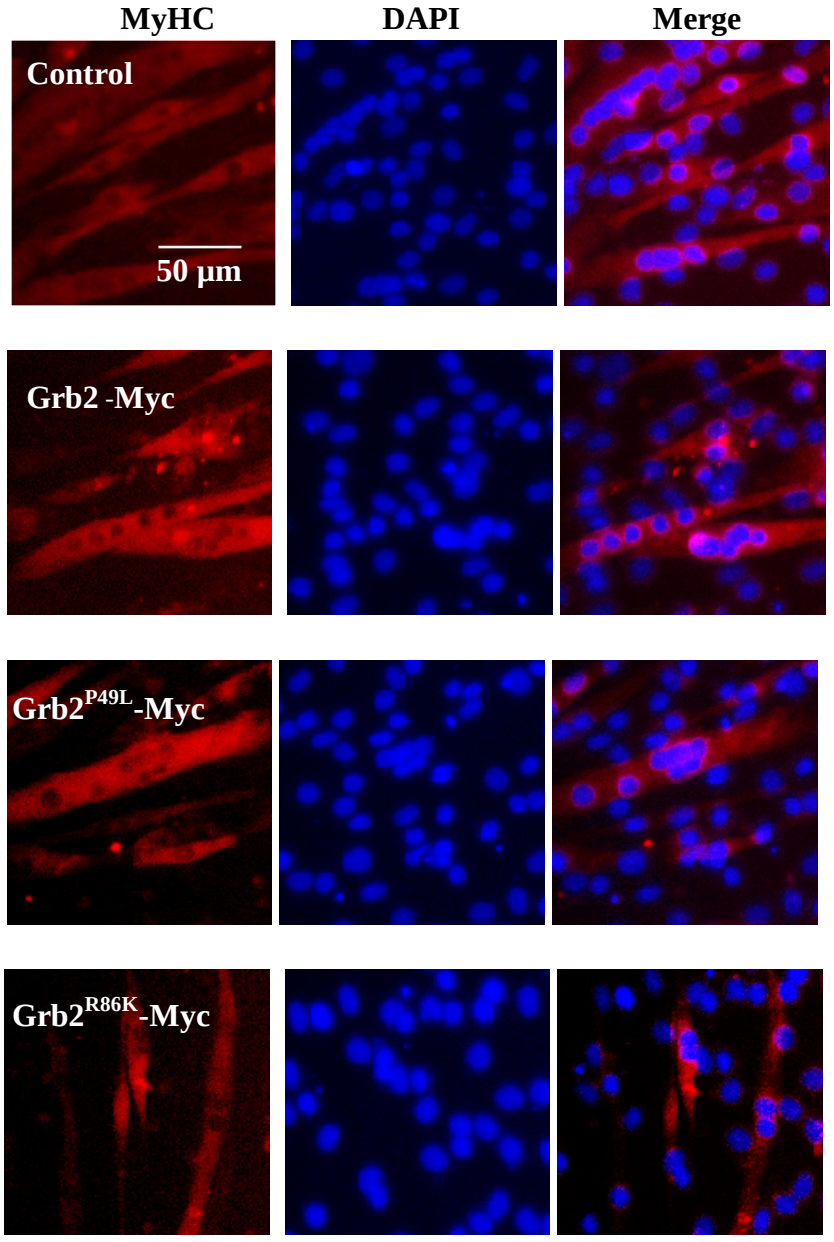
3.14 C-terminal SH3 domain of Grb2 is critical for Grb2 mediated inhibition of C2C12 differentiation.

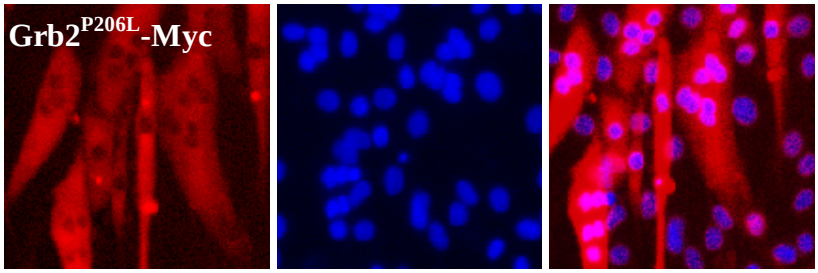
Grb2 was found to inhibit differentiation of C2C12 myoblasts (Fig 3.3 C, D). To identify which of the three domains of Grb2 is critical for the inhibition of differentiation, $Grb2^{P49L}$-Myc, $Grb2^{R86K}$-Myc, $Grb2^{P206L}$-Myc and WT-Grb2 Myc constructs were microporated in C2C12 cells. Initially, 10 µg of each plasmid was used for the microporation. However, it was observed that the expression level of the $Grb2^{P206L}$-Myc construct was quite low compared to the WT-Grb2 Myc construct. The other two point mutant constructs expressed as well as the wild type. Hence, any difference in the differentiation experiment could be attributed to difference in expression as well and the results would be inconclusive. Hence, the expression level of $Grb2^{P206L}$-Myc construct was adjusted to that of the wild type by testing the expression of different amounts of the point mutant. Finally, 15 µg of $Grb2^{P206L}$-Myc plasmid was found to express as well as the wild type and the other two mutants. Plasmid DNA for the wild type and the other two point mutants was then adjusted to 15 µg by adding 5 µg of empty vector plasmid. The cells were then seeded for differentiation. At around 90% confluency the media was changed to differentiation media and the cells were allowed to differentiate for 3 days after which they were fixed and immunostained using antibodies against MyHC (late differentiation marker). Fusion index was calculated as the ratio of the number of nuclei in myotubes to the total number of nuclei in a field. It was observed that the number of myotubes formed by the cells microporated with $Grb2^{P206L}$-Myc mutant was significantly higher than the Grb2 over expressing (wild type) C2C12 cells indicating that the mutation abolished the ability of Grb2 to inhibit myogenic differentiation. The fusion index of $Grb2^{P49L}$-Myc and $Grb2^{R86K}$-Myc mutant was comparable to that of Grb2 overexpressing cells suggesting that mutations in the N-SH3 and SH2 domains are not able to abolish the inhibition of differentiation mediated by Grb2. The inability of the $Grb2^{P206L}$-Myc mutant to inhibit differentiation was not due to low expression as checked by subjecting the protein lysates to western blot analysis and probing for Grb2 (Fig 3.15 (B,C)). Exogenous Grb2 can be observed at a slightly higher molecular weight compared to endogenous Grb2 expression (Fig 3.15 A).

A)


WT-Grb2 Myc
Grb2P49L-Myc
Grb2R86K- Myc
Grb2P206L-Myc
α-Grb2
α-GAPDH

B)


MyHC
DAPI
Merge
Control
50 µm
Grb2 -Myc
Grb2P49L-Myc
Grb2R86K-Myc



C)

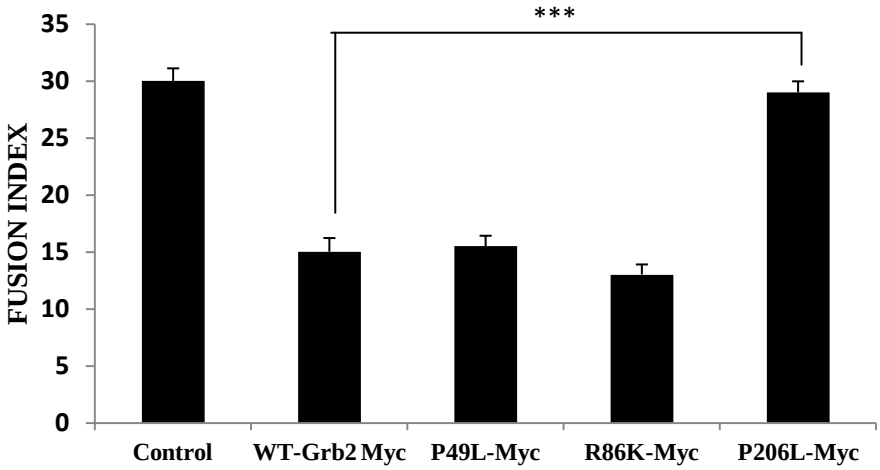


Fig 3.15 The C-terminal SH3 domain of Grb2 is critical for regulating the inhibition of differentiation mediated by Grb2 in C2C12 myoblasts: A) Control, pGrb2 Myc, pGrb2^{P49L}-Myc, pGrb2^{R86K}-Myc and pGrb2^{P206L}-Myc constructs were microporated in C2C12 cells. After 48 hrs the cells were split, lysed and the protein extracts were subjected to western blot analysis to probe against Grb2 to determine the expression levels of the mutants. B) Cells were also seeded for differentiation. After reaching 90% confluency, the cells were allowed to differentiate for 3 days. After 3 days, the cells were fixed and immunostained with antibodies against MyHC (late differentiation marker). DAPI was used to stain the nucleus. Images were taken under a 10X objective lens. C) The fusion index was calculated as the ratio of the number of nuclei in myotubes to the total number of nuclei using Image J software. A total of 9 fields per experiment were quantified. Three independent set of experiments were carried out to calculate significance (* $p<0.05$. **$p<0.01$, ***$p<0.001$)

3.15 Overexpression of Grb2^{P206L} mutant does not inhibit the expression of muscle markers observed in Grb2 over expressing cells

The expression of muscle specific marker proteins is critical to monitor the progression of the muscle differentiation process. It was observed that knocking down the expression of Grb2 lead to increase in the expression of the terminal differentiation marker,

MyHC (Fig 3.4 A) whereas over expressing Grb2 in C2C12 cells reduced the expression of MyHC (Fig 3.9 A). This observation supported the hypothesis that Grb2 inhibits muscle differentiation in C2C12, especially terminal differentiation. The differentiation defect in Grb2 over expressing cells was not observed in the P206L mutant of Grb2 which suggests that Grb2 may be inhibiting the terminal muscle differentiation process through its C-terminal SH3 domain. To study the effect of mutants on the expression of muscle specific markers, protein lysates from each day of differentiation was subjected to western blot analysis and probed using antibodies against MyHC and MyoD. GAPDH was used as the loading control. We found that the expression of the late differentiation marker, MyHC was more in the P206L mutant of Grb2 compared to the Grb2 over expressing cells supporting the observation that the inhibition of differentiation by Grb2 may be occurring through its C-terminal SH3 domain (Fig 3.16 A). The expression of MyHC in the Grb2^{P49L} and Grb2^{R86K} mutants remained almost similar to that observed in Grb2over expressing cells. The expression of MyoD remained almost constant in all the mutants (Fig 3.16 (A, C)).

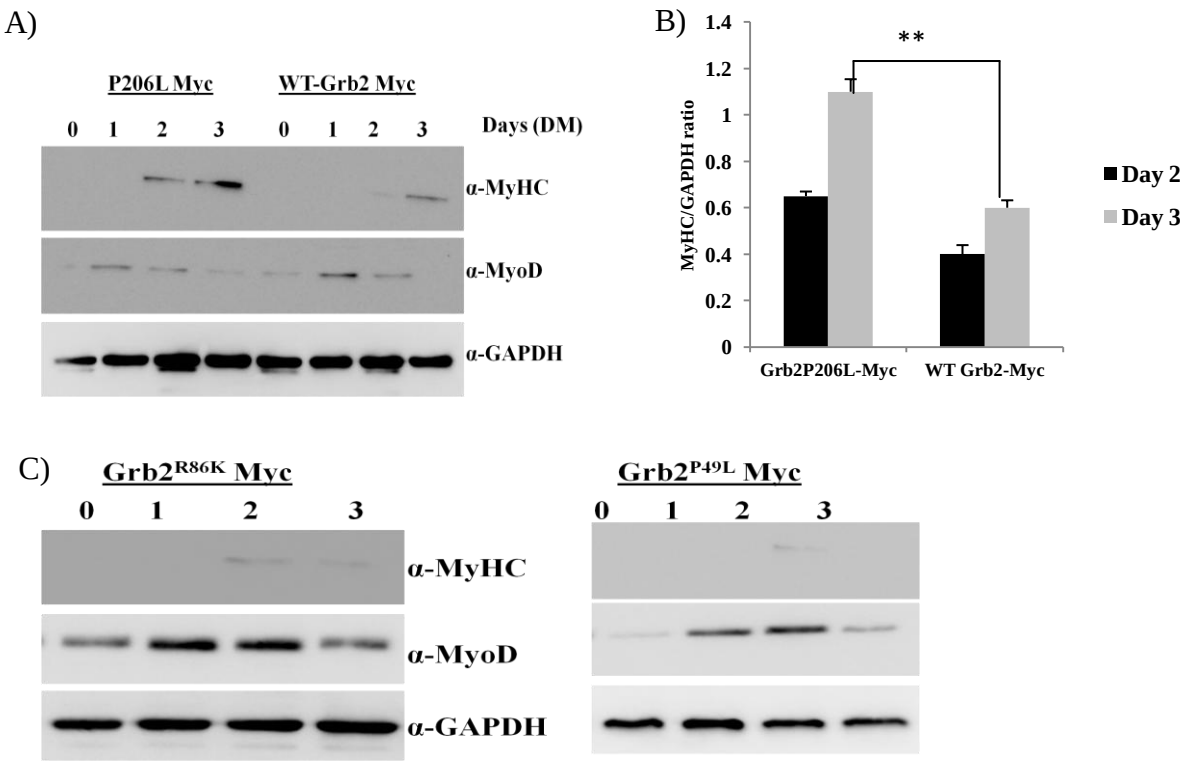


Fig 3.16 Over expression of Grb2^{P206L} mutant does not inhibit the expression of muscle markers observed in Grb2 over expressing cells: A), C) WT Grb2 Myc, Grb2^{P49L}-Myc, Grb2^{R86K}-Myc and Grb2^{P206L}-Myc constructs were microporated in C2C12 cells which were then differentiated for 3 days and protein extracts were collected at each day and subjected to western blot analysis and probed for MyoD and MyHC to study their

expression. B) Densitometric analysis of western blot to quantify the level of MyHC in WT Grb2-Myc and Grb2^{P206L}-Myc microporated cells (*p<0.05, **p<0.01, ***p<0.001)

3.16 Grb2^{P206L} mutant does not inhibit proliferation of C2C12 cells

Grb2 has been shown to be involved in normal cell proliferation in many cell types. We also found that Grb2 knockdown C2C12 cells proliferate faster than the control (Fig 3.9 A), although no significant difference was observed for the Grb2 overexpressing cells. However, the proliferation rate of the Grb2 over expressing was still lower than the control. We wanted to study the effect of the Grb2 mutations on the proliferation rate of C2C12 cells. For that purpose, we carried out a proliferation assay where 7500 cells of control, point mutants and WT-Grb2 Myc microporated C2C12 cells were seeded on a 24 well plate for a period of four days and each day after 24 hrs intervals the cells were trypsinized and counted. Interestingly, it was found that the proliferation rate was higher in the Grb2^{P206L} mutant cells compared to the wild type Grb2 cells and slightly higher than the control (Fig 3.17). However, no significant increase was observed between the proliferation rate of Grb2 overexpressing cells and Grb2^{P49L} and Grb2^{R86K} mutants.

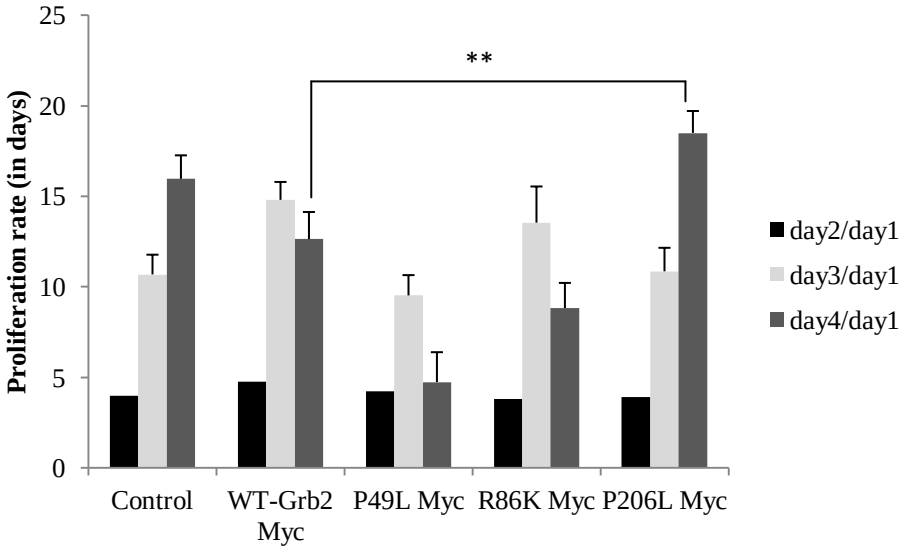
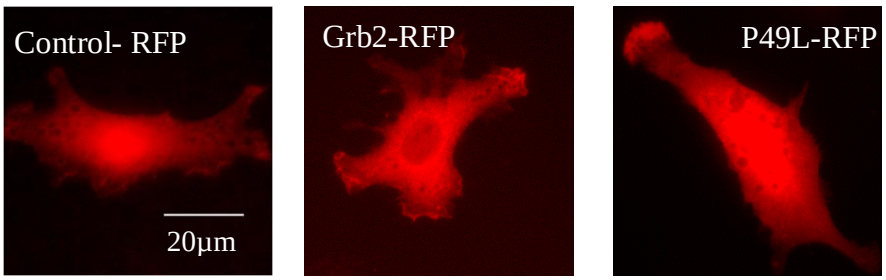


Fig 3.17 Grb2^{P206L} mutant does not inhibit proliferation of C2C12 cells: Control, point mutants and Grb2 overexpressing C2C12 cells were seeded in triplicates at a cell number of 7500 cells/ ml in a 24 well plate and incubated at 37° C for a period of 4 days. After 24 hr interval, the cells were trypsinized and counted. The proliferation rate was calculated by dividing the cell number obtained by dividing the day2, day3 and day 4 of proliferation numbers of the sample by day 1. A set of three independent experiments was carried out (*p<0.05,**p<0.01,***p<0.001)

3.17 Wild type Grb2 localizes in the cytoplasm along with Grb2^{R86K} and Grb2^{P206L} mutant whereas Grb2^{P49L} mutant mostly localizes in the nucleus

Grb2 has been shown to localize in the cytoplasm under resting conditions. Upon stimulation by growth factors, it is known to localize to the plasma membrane along with its GEF partner, Sos 1.[9]. Localization of Grb2 in myoblasts, in particular, before or during the process of differentiation is not well characterized. Understanding the dynamics of Grb2 localization in C2C12 myoblasts will definitely provide a clearer picture in to its role during myogenesis. In order to follow the localization of Grb2 visually, a RFP fusion protein of wild type Grb2 was made. Also, in order to study the localization of individual domains of Grb2, RFP tagged constructs of Grb2^{P49L}, Grb2^{R86K} and Grb2^{P206L} were also made. The expression of all the constructs used was checked for their appropriate molecular weight by western blotting using Grb2 antibody specifically and the molecular weight was found to be around 50KDa.

Wild type Grb2-RFP and the three mutants were transfected in C2C12 cells seeded on silane coated coverslips. The cells were grown for around 36 hrs following transfection, after which they were fixed and mounted on slides to be, visualized under 40X oil immersion lens. The localization of wild type Grb2 was found to be in the cytoplasm, as expected. The Grb2^{R86K} mutant and Grb2^{P206L} mutant were also found to be present in the cytoplasm exclusively. The Grb2^{R86K}-RFP mutant was observed to be present in the form of patches or dots throughout the cytoplasm. However, the Grb2^{P49L}-RFP mutant was found to be localized in the nucleus as well along with plasma membrane. This could possibly be explained by the fact that the N-terminal SH3 domain of Grb2 has been shown to bind to Sos 1 with very high affinity and in the absence of the binding, as in case of Grb2^{P49L}-RFP mutant, Grb2 may localize to the nucleus.



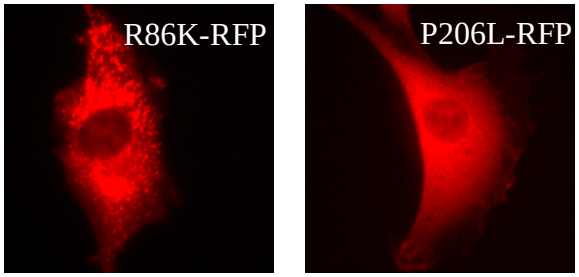
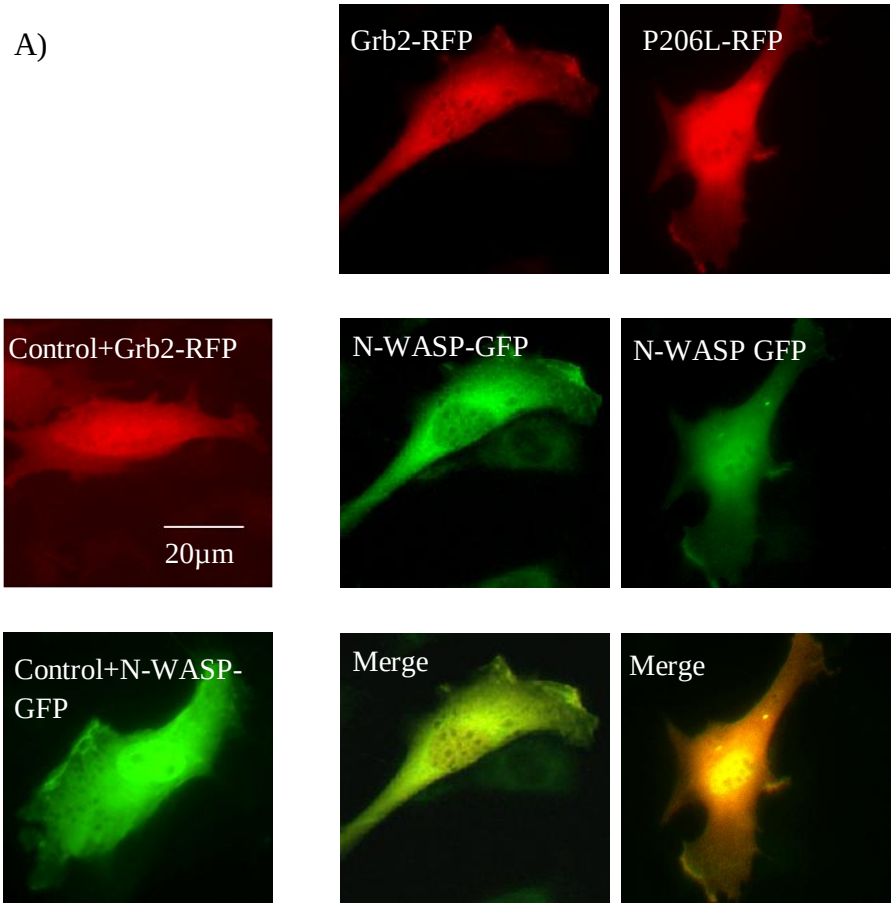


Fig 3.18 Wild type Grb2 localizes in the cytoplasm along with Grb2^{R86K} and Grb2^{P206L} mutant, but not Grb2^{P49L} mutant which mostly localizes in the nucleus: C2C12 cells were grown on a silane coated coverslip and at 40% confluency, they were transfected with WT-Grb2-RFP, Grb2^{P49L}- RFP, Grb2^{R86K}-RFP and Grb2^{P206L}-RFP plasmids. After 36 hrs, the cells were fixed, washed and visualized under 40X oil immersion lens. Around 30 cells were viewed for each sample.

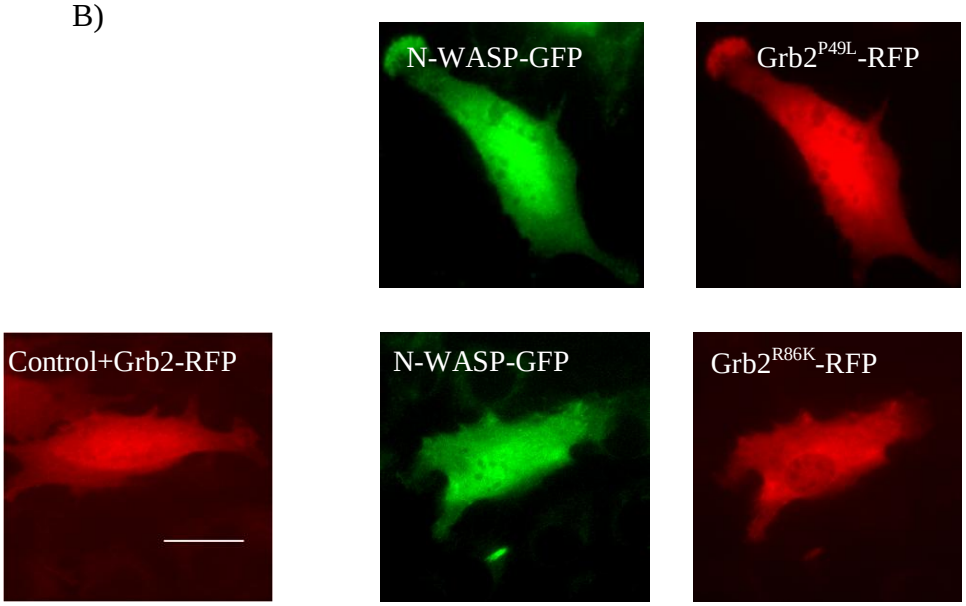
3.18 Co-localization of Grb2, not Grb2^{P206L} mutant, may cause N-WASP GFP to localize in the cytoplasm in C2C12 myoblasts:

The Grb2^{P206L} mutant did not inhibit muscle differentiation of C2C12 cells. Grb2^{P206L}mutant has been previously found to inhibit the interaction of Grb2 and N-WASP [9]. To identify if the difference in the co-localization of Grb2 and its mutant with N-WASP could be the reason for the differentiation defect, Grb2-RFP or Grb2^{P206L}-RFP constructs were co-transfected with N-WASP-GFP. N-WASP GFP has been found to localize in the nucleus in many cell types [181]. After 36 hrs of incubation the cells were fixed, washed and mounted on a slide to be observed under the 40X oil immersion lens. We found strong co-localization of Grb2-RFP with N-WASP GFP. Interestingly, in the presence of wild type Grb2, N-WASP GFP was found to localize outside the nucleus suggesting that Grb2 may cause N-WASP GFP to localize in the cytoplasm. However, both Grb2^{P206L} RFP and N-WASP GFP were found to localize in the nucleus suggesting that the difference in the localization of N-WASP GFP in the presence of WT-Grb2 and Grb2^{P206L} may be responsible for the difference in myogenesis potential observed between the wild type and its mutant (Fig 3.19 A). Grb2^{R86K}-RFP too did not co localize with N-WASP GFP whereas Grb2^{P49L}-RFP localized in the nucleus even without the co-transfection with N-WASP GFP (Fig 3.19 B).

A)


Grb2-RFP
P206L-RFP
Control+Grb2-RFP
20µm
N-WASP-GFP
N-WASP GFP
Control+N-WASP-GFP
Merge
Merge

B)


N-WASP-GFP
Grb2P49L-RFP
Control+Grb2-RFP
N-WASP-GFP
Grb2R86K-RFP

C)

Change in N-WASP GFP localization	**No of cells showing cellular localization (n=25)**	**% of cells showing cellular localization**	**No of cells showing non nuclear localization (n=25)**	**% of cells showing non nuclear localization**
N-WASP GFP + Vector	19±2	**76**±8	6±1	**24**±4
N-WASP GFP+ Grb2 RFP	5±3	**20**±12	20±2	**80**±8
N-WASP GFP+ $Grb2^{P49L}$ RFP	17±2	68±8	8±4	32±16
N-WASP GFP+ $Grb2^{R86K}$ RFP	16±1	64±4	9±2	36±8
N-WASP GFP+ $Grb2^{P206L}$ RFP	18±1	72±4	7±3	28±12

3.19 Co-localization of Grb2, not Grb2P206L, may cause N-WASP-GFP to localize in the cytoplasm of C2C12 myoblasts: A) N-WASP GFP was co transfected with Grb2-RFP and $Grb2^{P206L}$-RFP, The cells were then fixed, washed and observed under 40X oil immersion lens. About 30 cells were viewed for each sample. B) N-WASP GFP was also co transfected with $Grb2^{P49L}$ RFP and $Grb2^{R86K}$ RFP. The fixed cells were washed and

observed under 40X immersion oil lens. (Scale bar 20 µm). C) The data was also quantified for 25 cells for 3 independent experiments.

3.19 N-WASP expression in the Grb2 overexpressing cells can rescue the differentiation inhibition mediated by Grb2

Grb2 overexpression was found to inhibit muscle differentiation in C2C12 cells. Also, the $Grb2^{P206L}$ mutant, which cannot bind to N-WASP, did not inhibit differentiation as compared to the WT-Grb2 cells. N-WASP has been reported to be required for muscle cell fusion in mice [104]. The next step was to examine whether expression of exogenous N-WASP could rescue the inhibition in differentiation mediated by Grb2. For that purpose, firstly, empty vector and WT-Grb2 plasmids were microporated and stably expressed in C2C12 cells. Then, control and full length N-WASP plasmids were again microporated each in the control and Grb2 overexpressing cells. Now, control, Control+N-WASP, Grb2+ control and Grb2+N-WASP expressing C2C12 cells were set for differenetiation for 3 days and later fixed and immunostained with MyHC (terminal differentiation marker) and DAPI. Fusion index was caluculated using Image J software. As reported, the fusion index of N-WASP overexpressing cells was higher than the control indicating that N-WASP is required for muscle differentiation. The fusion index of Grb2 overexpressing cells was lower than the control, as expected. On expression of exogenous N-WASP in Grb2 overexpressing cells, the fusion index increased significantly compared to that of Grb2 overexpressing cells (Figure 3.20(A,B)). This indicates that expression of exogenous N-WASP in Grb2 overexpressing cells can rescue the inhibition of differentiation mediated by Grb2.

This suggests that N-WASP being downstream of Grb2, may be mislocalized or inhibited in the presence of excess of Grb2. However, overexpression of exogenous N-WASP may be able to rescue the inhibition caused by Grb2 and increases the differentiation potential of the cells back to that of control cells.

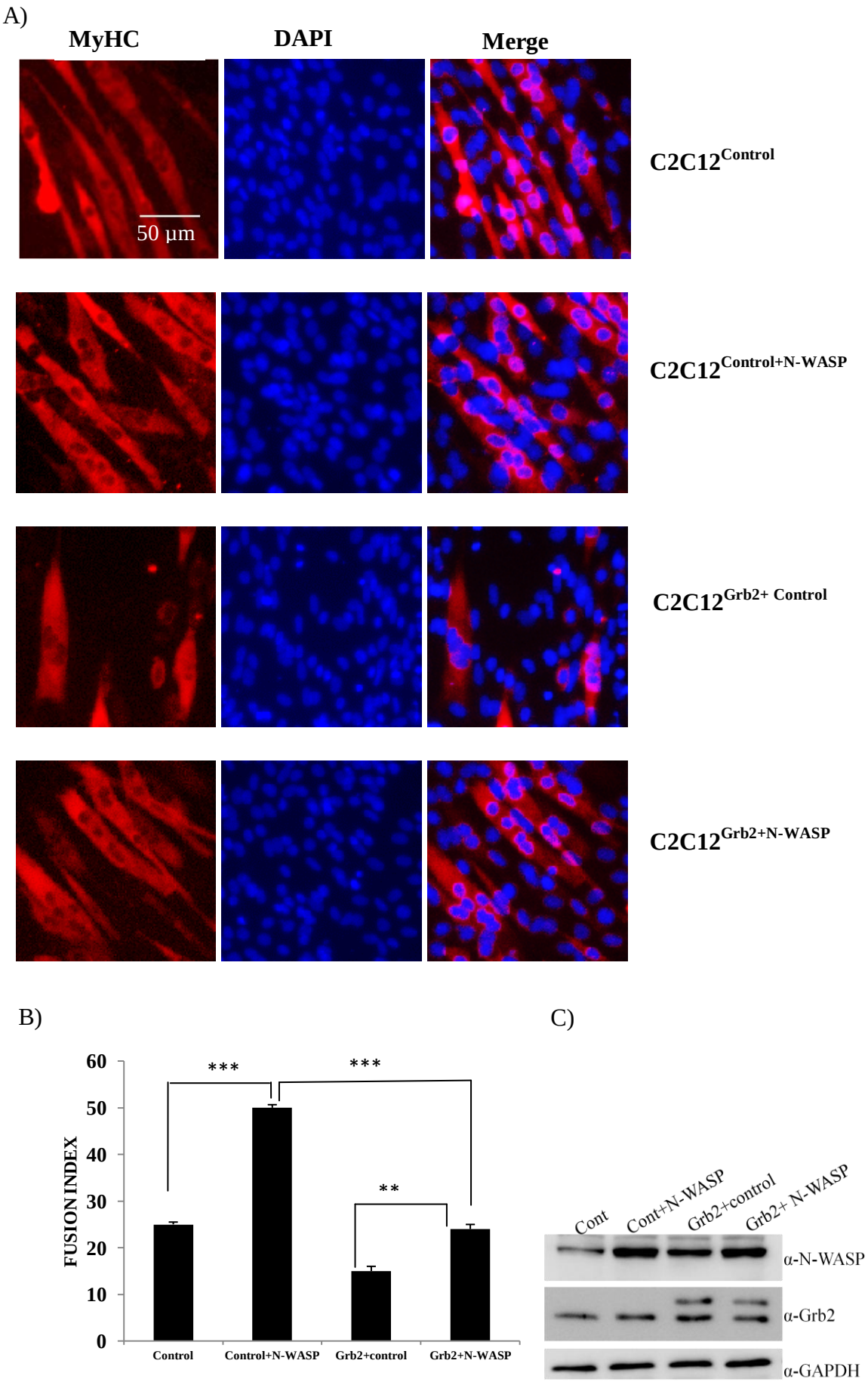


Figure 3.22 N-WASP expression in the Grb2 overexpressing cells can rescue the differentiation inhibition mediated by Grb2: A),C) Control, N-WASP, Grb2 and Grb2+N-WASP constructs were

microporated in C2C12 cells. After 48 hrs the cells were split, lysed and the protein extracts were subjected to western blot analysis to probe against Grb2 to determine the expression levels. A) Cells were also seeded for differentiation. After reaching 90% confluency, the cells were allowed to differentiate for 3 days. After 3 days, the cells were fixed and immunostained with antibodies against MyHC (late differentiation marker). DAPI was used to stain the nucleus. Images were taken under a 10X objective lens. B) The fusion index was calculated as the ratio of the number of nuclei in myotubes to the total number of nuclei using Image J software. A total of 9 fields per experiment were quantified. Three independent set of experiments were carried out to calculate significance (* $p<0.05$. **$p<0.01$, ***$p<0.001$)

Chapter 4: RESULTS

Role of GRB2 in TGF-β induced Epithelial-to-Mesenchymal transition in non small cell lung cancer cells A549:

Epithelial-to-Mesenchymal transition (EMT) is a well orchestrated biological process in which highly polarized epithelial cells, which are strongly attached to the extracellular matrix, are induced to lose their polarity by various environmental stimuli and assume mesenchymal cell like phenotype, enhanced migratory capabilities and invasiveness and expression of mesenchymal markers [114]. EMT has been found to be important in normal biological processes such as embryonic development, tissue repair, organ fibrosis etc [114] Interestingly, EMT is also considered as one of the important mechanism of initiation and progression of tumors in epithelial cells [182]. The acquisition of invasive phenotype, during EMT, finally has been proposed to lead to the onset of later stages of metastasis and cancer with life threatening consequences in majority of cancer types. Although EMT involves a series of changes in the genetic, cellular and biochemical characteristics of epithelial cells, a hallmark of EMT is the reduction in the expression of E-Cadherin, an important cell- cell adhesion protein present in the adherens junction of epithelial cells [112]. Many *in vivo* and *in vitro* studies have shown that, after acquiring mesenchymal phenotype, marker proteins such as vimentin, N-Cadherin, α-Smooth muscle actin, fibronectin also are highly expressed [114]. EMT has been found to be induced by various growth factors associated with the tumor microenvironment such as HGF, EGF, PDGF and TGF-β. These growth factors have been shown to induce the activation of various transcription factors required for EMT such as Snail/Slug family, ZEB1, Twist etc [114].

TGF-β has been shown to induce EMT in many types of cancer cells [183], though it has also been found to play a dual role in suppressing epithelial cell proliferation and primary tumorigenesis [114]. Two possible signaling mechanisms have been shown to be activated during TGF-β induced EMT. The first of the two involves the activation of Smad proteins which have been shown to directly bind activated TGF-β receptor I and induce EMT and cell motility [184]. Many studies supporting the theory of the involvement of Smad independent signaling pathway during TGF-β induced EMT has been observed in recent years. It has been observed that TGF-β induced EMT can activate p38 MAPK signaling pathway and requires the activation of αVβ6 integrins in NMuMG mouse mammary epithelial cells [185]. In human breast cancer, receptor tyrosine kinase epidermal growth

factor receptor (EGFR) has been found to be over expressed. This over expression has been shown to activate downstream mitogenic signaling pathways through the over expression of adaptor protein GRB2 [186]. Involvement of GRB2 has also been shown in the activation of downstream p38 MAPK and Erk/ MAPK signaling pathways induced by TGF-β in mammary epithelial cells ([46], [146]). However, the role of GRB2 in lung cancer has not been studied much. Lung cancer is the cause of 30-40% of deaths due to cancer worldwide. Hence, understanding the mechanism involving GRB2, which has been found to be highly expressed in lung tumors as well, will help in solving the complexity of EMT program controlling tumorigenesis a bit further and also make designing therapeutic target drugs a little easier.

The cell line used for study in this project is A549, which is a human alveolar (lung) basal epithelial cell line derived from the cancerous lung tissue in the explanted tumor of 58 yr old caucausian male [187]. It is non metastatic adenocarcinoma and a non-small cell lung cancer cell line (NSCLC). About 85-90% of the lung cancers known are non-small cell lung cancers with poor prognosis (www.cancer.org/cancer/lungcancer).

4.1 Expression of GRB2 increases after stimulation with TGF-β in a dose dependent manner in A549 cells

Cell response to TGF-β has been shown to be dependent on the ligand concentration to which they are exposed [188]. The signal received on the plasma membrane is interpreted and transmitted to the nucleus where the activation of target transcription factors is known to take place. Hence, to investigate the optimal TGF-β concentration required to induce EMT in A549 cells, three different concentrations of TGF-β as provided in the literature was used. A549 cells were seeded at a cell number of $2x10^5$ cells/ 60 mm dish. At around 20-30% confluency, the cells were washed with 1X PBS and serum starved for a period of 12 hrs in DMEM. After 12 hrs, 2, 5 and 10 ng/ml of TGF-β was added to the cells along with one unstimulated control. The cells were incubated at 37°C with 5% CO_2 for a period of 48 hrs. After 48 hrs, the cells were scraped, lysed and the protein extracts were subjected to immunoblotting with antibodies against E-Cadherin (epithelial marker) and vimentin (mesenchymal marker).

It was observed that the reduction of E-Cadherin expression highest at 5 and 10ng/ml of TGF-β stimulation with still a slight expression of E-Cadherin observed at 2 ng/ml of TGF compared to the unstimulated control (Fig 4.1). The expression of mesenchymal marker, vimentin, was found to have increased in all the three different

concentrations of TGF-β with the maximum increase observed at 5 ng/ml of TGF-β. Hence, both the concentrations of 5 and 10 ng/ml of TGF-β were found to be sufficient to induce EMT. Interestingly, however, it was observed that the increase in the expression of GRB2 increased gradually from 2 to 5ng/ml of TGF-β but decreased at 10 ng/ml of TGF-β. The reason for this observation was not investigated further and could be many. But from hereafter, all the experiments were carried out with the TGF-β concentration at 5 ng/ml at which EMT was found to occur and GRB2 expression was the highest.

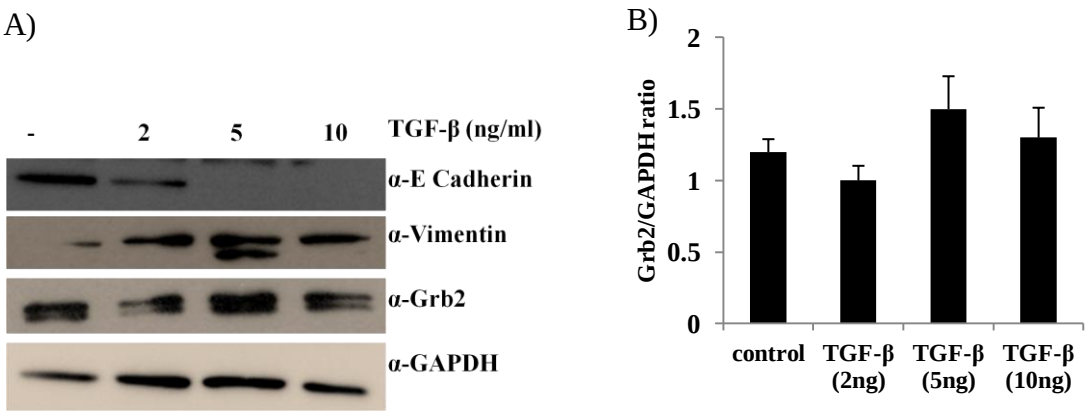


Fig 4.1 Expression of Grb2 increases after stimulation with TGF-β in a dose dependent manner: A) A549 cells were seeded in 60mm dish at $2X10^5$ cells/ dish. At 20-30% confluency the cells, were serum starved in DMEM for 12 hrs. After 12 hrs, TGF-β at 2, 5 and 10 ng/mL concentration was added to the cells with one control dish without the addition of TGF-β. After 48 hrs at 37° C, the cells were scraped, lysed and the protein extracts were subjected to western blotting and probed against antibodies for E-Cadherin, vimentin and Grb2. GAPDH was used as the loading control. B) Densitometric analysis of Grb2 levels was carried out.

4.2 Expression of GRB2 increases considerably and the localization of GRB2 changes after TGF-β stimulation

GRB2 levels have been found to increase in human breast cancer tissues correlating with the over expression of Epidermal Growth Factor Receptor (EGFR) [186]. Hence, the expression and localization of GRB2 during TGF-β induced EMT in A549 cells was further investigated. Once the concentration of TGF-β optimal for EMT was established, the expression and localization of GRB2 after TGF-β stimulation at 5ng/ml was studied. It was observed that the expression of GRB2 increased significantly after TGF-β stimulation with 5ng/ml. A549 cells were seeded at $2X10^5$ cells/ 60 mm dish. At 25% confluency, the cells were serum starved for a period of 12 hrs. After 12 hrs, 5 ng/ml of TGF-β was added to the cells. A control without the TGF-β was also maintained. After 48 hrs of incubation at 37° C, the cells were visualized for changes in their morphology and then scraped and lysed to

detect the GRB2 expression. Protein extracts from the scraped cells was immunoblotted with antibodies against GRB2, E-Cadherin (epithelial marker) and Vimentin (mesenchymal marker). GAPDH was used as the loading control.

Morphologically, it was observed that the after around 24 -27 hrs of TGF-β stimulation, the epithelial A549 cells started losing their cell- cell contacts, became elongated and assumed more mesenchymal and spindle shaped phenotype. At the end of 48 hrs, most of the A549 cells displayed mesenchymal phenotype (Fig 4.2.1 A). Western blot of the protein extracts prepared from these lysates showed that there was a drastic reduction in the expression of epithelial marker, E-Cadherin and increase in the level of mesenchymal marker, vimentin compared to the control indicating that epithelial-to-mesenchymal transition had taken place. Also the expression of GRB2 was found to have increased drastically in TGF-β treated cells compared to the control (Fig 4.2.1 B). This indicates that the expression of GRB2 increases after TGF-β stimulation in A549 cells and GRB2 may be required for EMT.

Localization of GRB2 was also studied during EMT. GRB2 is known to localize in the cytoplasm in most of the cell types. However, it has been reported that, in both normal and tumor breast tissue, GRB2 localizes both in the cytoplasm and the nucleus [186]. Immunostaining for GRB2 was carried out in A549 cells under TGF-β induced EMT. A549 cells were seeded on coverslips and grown under the above mentioned conditions of EMT induction. After 48 hrs, the cells were fixed and stained antibodies against GRB2 and actin. E-Cadherin staining was also carried out to monitor the morphological changes during EMT. DAPI was used to stain the nucleus. It was observed that E-Cadherin localized mainly at the cell-cell junctions in the control cells whereas in TGF-β treated cells, it mainly was present in the cytoplasm at a very low intensity (Fig 4.2.2 A). It was also observed that the actin filaments in the control cells are more diffused. On TGF-β stimulation, the actin filaments are present more in the form of stress fibres. This is supported by the data that TGF-β stimulation has been shown to activate Rho A dependent stress fibre formation [154]. It was also observed that the localization of GRB2 changed during EMT. In the control cells, GRB2 was found to be present mostly in the nucleus as observed when merged with DAPI (Fig 4.2.2 B). This observation correlates with the localization observed in the human breast cancer tissue. GRB2 localization was observed to be in the form of dots like structures. On TGF-β stimulation, GRB2 was observed to move out of the nucleus as dots near or close to the plasma membrane. This may suggest that in the absence of TGF-β stimulation, GRB2 might

play a role in the nucleus. On activation by TGF-β, it may move out of the nucleus to interact with proteins of the signaling pathway activated by TGF-β induced EMT.

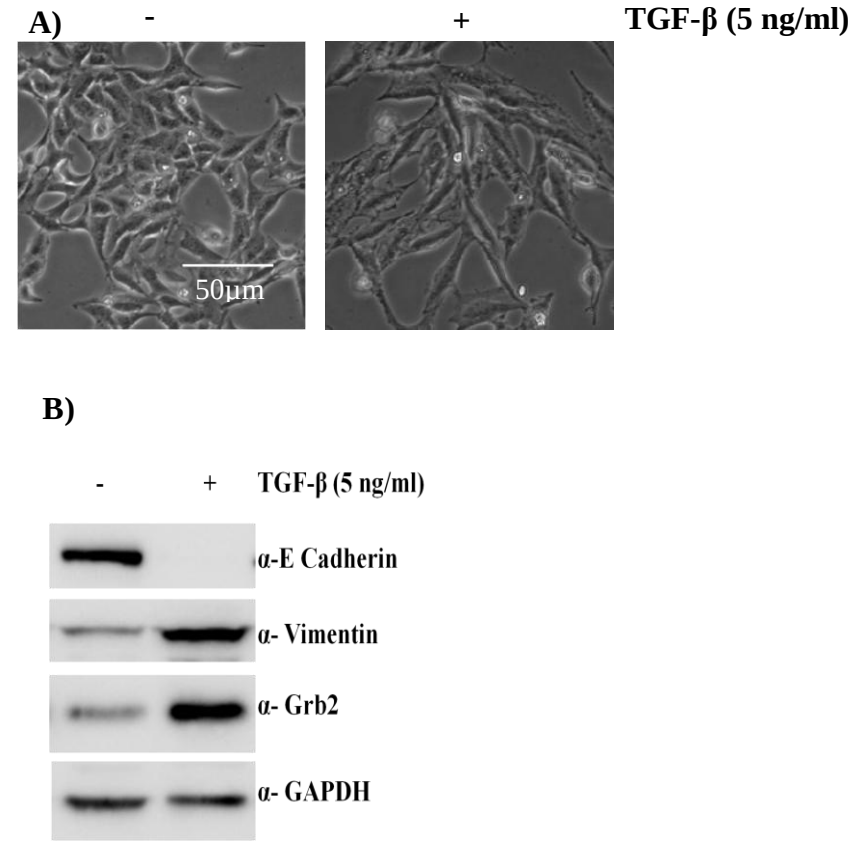
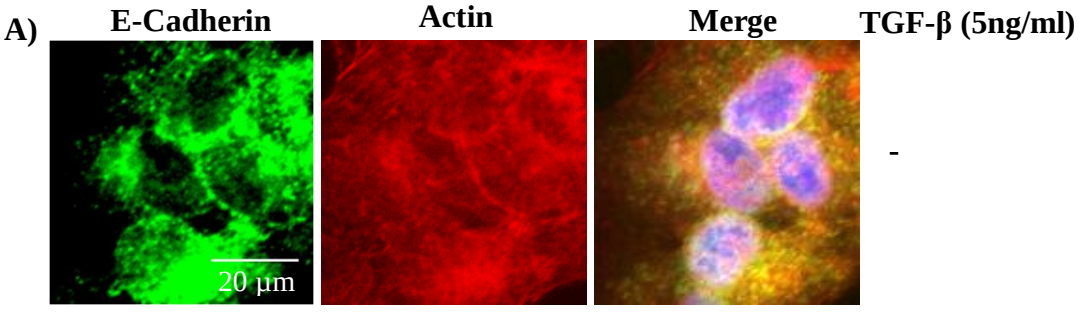


Fig 4.2.1 Expression of GRB2 increased and the localization of GRB2 changed after TGF-β stimulation: A) A549 cells were seeded at $3X10^5$ cells/ 60 mm dish. After the cells reached 20-30% confluency, they were serum starved for a period of 12 hrs. After 12 hrs, 5 ng/ml of TGF-β was added to the cells along with a control without the addition of TGF-β. Cells were visualized after 48 hrs of incubation at 37° C. B) Cells were scraped, lysed and the protein extracts were subjected to western blot analysis and probed against antibodies for Grb2, E-Cadherin(epithelial marker), vimentin (mesenchymal marker) and GAPDH (loading control).



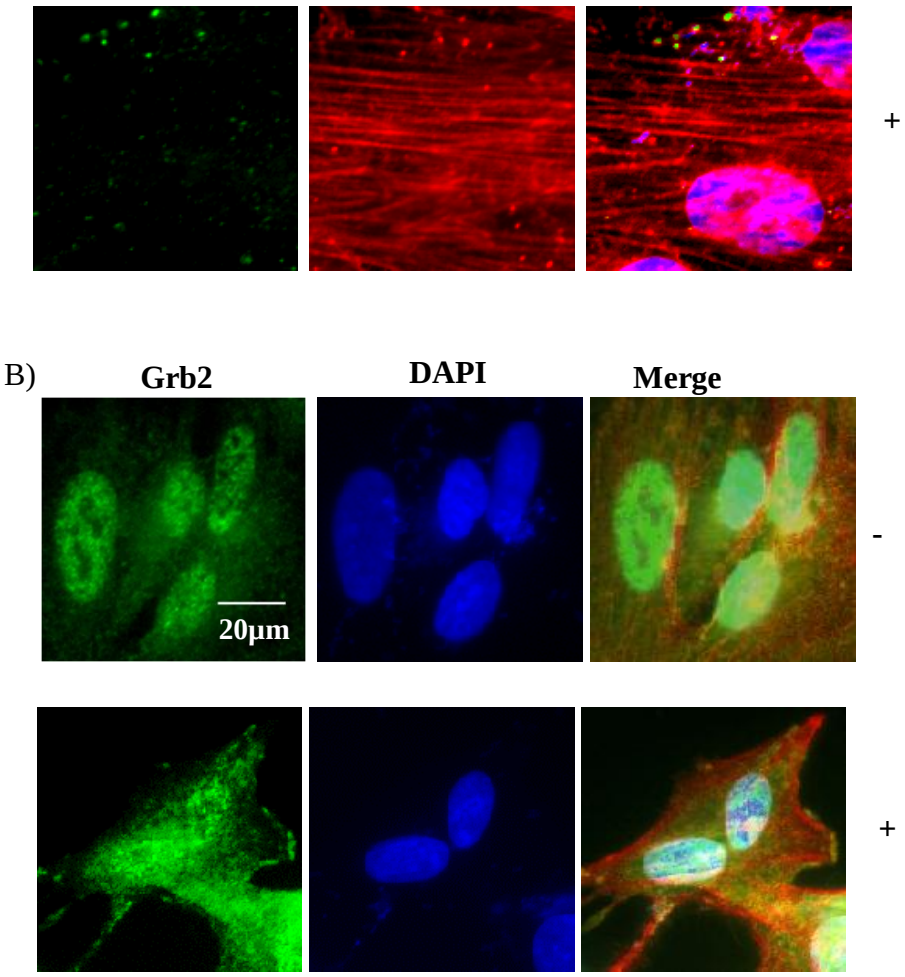
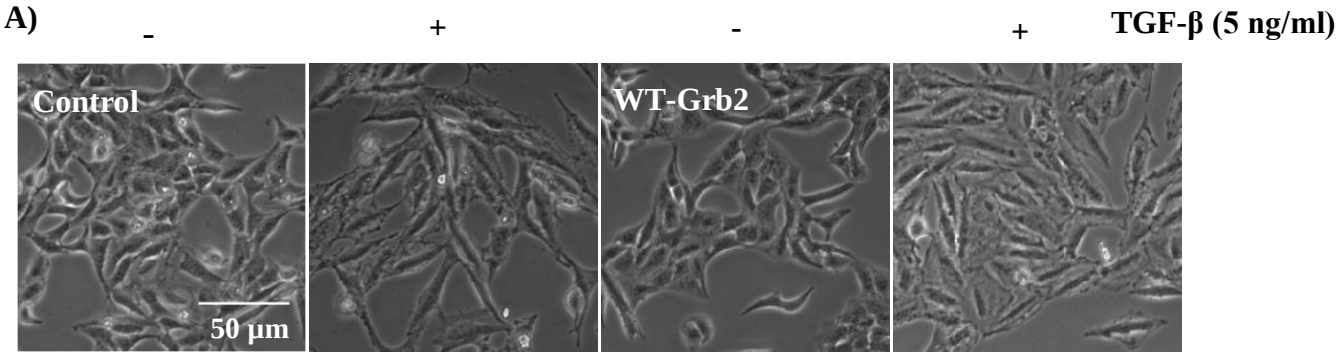


Fig 4.2.2 Localization of GRB2 after TGF-β stimulation in A549 cells: A) A549 cells were seeded on a coverslip at a cell number of $2X10^5$ cells/ 6 well plate. At 25% confluency, the cells were serum starved for a period of 12 hrs. After 12 hrs, 5 ng/ml of TGF-β was added in the cells with one well as a control without the addition of TGF-β. After 48 hrs, cells were fixed and immunostained against E-Cadherin (epithelial marker-Green), actin (Red). DAPI was used to stain the nucleus (blue). Images were taken under 40X lens. Scale bar is as represented. B) Untreated and TGF-β treated A549 cells were also stained for GRB2 to study its localization. Following the above mentioned conditions, the cells were fixed and immunostained for GRB2 using antibodies against GRB2 (green), actin (red) and DAPI (blue).

4.3 Overexpression of GRB2 in A549 cells after TGF-β stimulation leads to loss of E-Cadherin expression and increase in vimentin expression:

GRB2 was found to be over expressed in A549 cells after TGF-β stimulation. Hence, the effect of GRB2 overexpression on Epithelial-to-Mesenchymal transition in A549 cells and other aspects of physiological changes that occur during EMT in tumor cells such as cell migration, expression of focal adhesion proteins etc were studied. For that purpose, third generation lentivirus containing the over expression plasmid (pLJM-Grb2 His)-WT *GRB2* was produced in 293T cells after which two consecutive infections of the virus along with a

control (empty) vector was carried out in A549 cells. The infection efficiency was found to be around 90%. A round of puromycin selection with 2 µgm/ml of puromycin was done to kill off all the uninfected cells and the level of GRB2 overexpression was verified using western blot analysis. GRB2 over expressing A549 cells were now seeded at a cell number of $2X10^5$ cells in a 60 mm dish. After reaching 30% confluency the cells were serum starved for a period of 12 hrs. After 12 hrs, 5 ng/ml of TGF-β was added to both the control and the GRB2 over expressing A549 cells. The cells were allowed to undergo EMT for 48 hrs at 37° C. After 48 hrs the cells were lysed and the protein extracts were subjected to western blot analysis to determine the expression levels of E-Cadherin (epithelial marker) and vimentin (mesenchymal marker). GAPDH was used as the loading control. It was observed that the expression of E-Cadherin was much lower in the GRB2 over expressing cells than the control cells before the TGF-β stimulation. After induction by TGF-β, the reduction in the expression of E-Cadherin in GRB2 over expressing A549 cells was much more drastic than the control cells after TGF-β stimulation. Also, the increase in vimentin expression was more in GRB2 over expressing A549 cells compared to the control cells after TGF-β stimulation after 48 hrs (Fig 4.3 B). This suggests that GRB2 overexpression accelerates EMT in A549 cells. Morphologically, GRB2 over expressing cells after TGF-β stimulation also showed more separation and elongation compared to the control cells after 48 hrs (Fig 4.3 A). Also, more number of GRB2 over expressing cells was found to have undergone EMT compared to the control.



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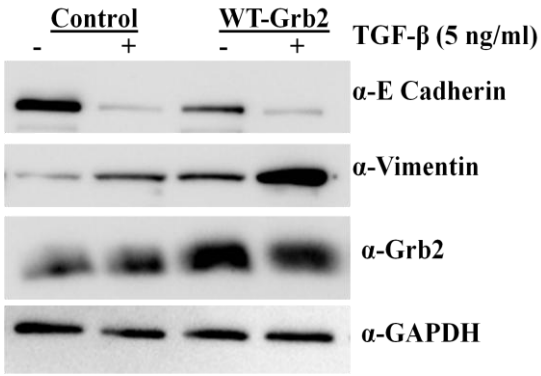


Fig 4.3 Overexpression of GRB2 in A549 cells after TGF-β stimulation leads to loss of E-Cadherin expression and increase in vimentin levels: A) Control and GRB2 over expressing stable A549 cells were seeded at $2X10^5$ cells in a 60 mm dish. At 25% confluency the cells were serum starved for 12 hrs. After 12 hrs, 5ng/ml of TGF-β was added to the control and GRB2 overexpressing cell. One set of control (unstimulated) was maintained for both control and GRB2 overexpressing cells. After 48 hrs of EMT induction, images were captured using a 10X objective lens to study morphological changes after TGF-β stimulation. B) Cells were then scraped, lysed and the protein extracts were subjected to western blot analysis and probed using antibodies against E-Cadherin (epithelial marker) and Vimentin (mesenchymal marker). GAPDH was used as the loading control.

4.4 GRB2 overexpression leads to reduction in Cell-cell adhesion

One of the hallmarks of tumor progression is the loss of an important cell-adhesion molecule, E-Cadherin, present in the adherens and tight junctions of epithelial cells. E-Cadherin is responsible for maintaining the epithelial cell morphology and quiescence [112]. The extracellular domains of E-Cadherin of adjacent molecules bind to each other by homotypic interactions whereas the intracellular domains can interact with the actin cytoskeleton through α-catenins/β-catenins [189]. Reduction in the expression level of E-Cadherin in GRB2 over expressing A549 cells indicates that there may be a defect in the localization of E-Cadherin at the cell-cell junction as well. Hence, immunostaining of the control and GRB2 over expressing A549 cells before and after TGF-β stimulation was done using antibodies against E-Cadherin. It was observed that the localization of E-Cadherin at the cell-cell junction in GRB2 over expressing cells was not significantly less compared to the control before the addition of TGF-β. The cell morphology was observed to be slightly elongated in GRB2 over expressing untreated A549 cells compared to the control. After TGF-β stimulation, E-Cadherin appeared to localize as small dot like structures inside the cell. The intensity of the dot shaped E-Cadherin localized structures was of lower intensity in GRB2 over expressing TGF-β treated cells compared to the treated control cells. This

indicates that the reduction of E-Cadherin expression results in the decrease of E-Cadherin localization at the cell-cell junction in A549 cells after TGF-β stimulation. The decrease in E-Cadherin localization in the GRB2 over expressing cells, before TGF-β stimulation, may not be evident probably because change in localization may be transient. Hence further detailed study needs to be performed.

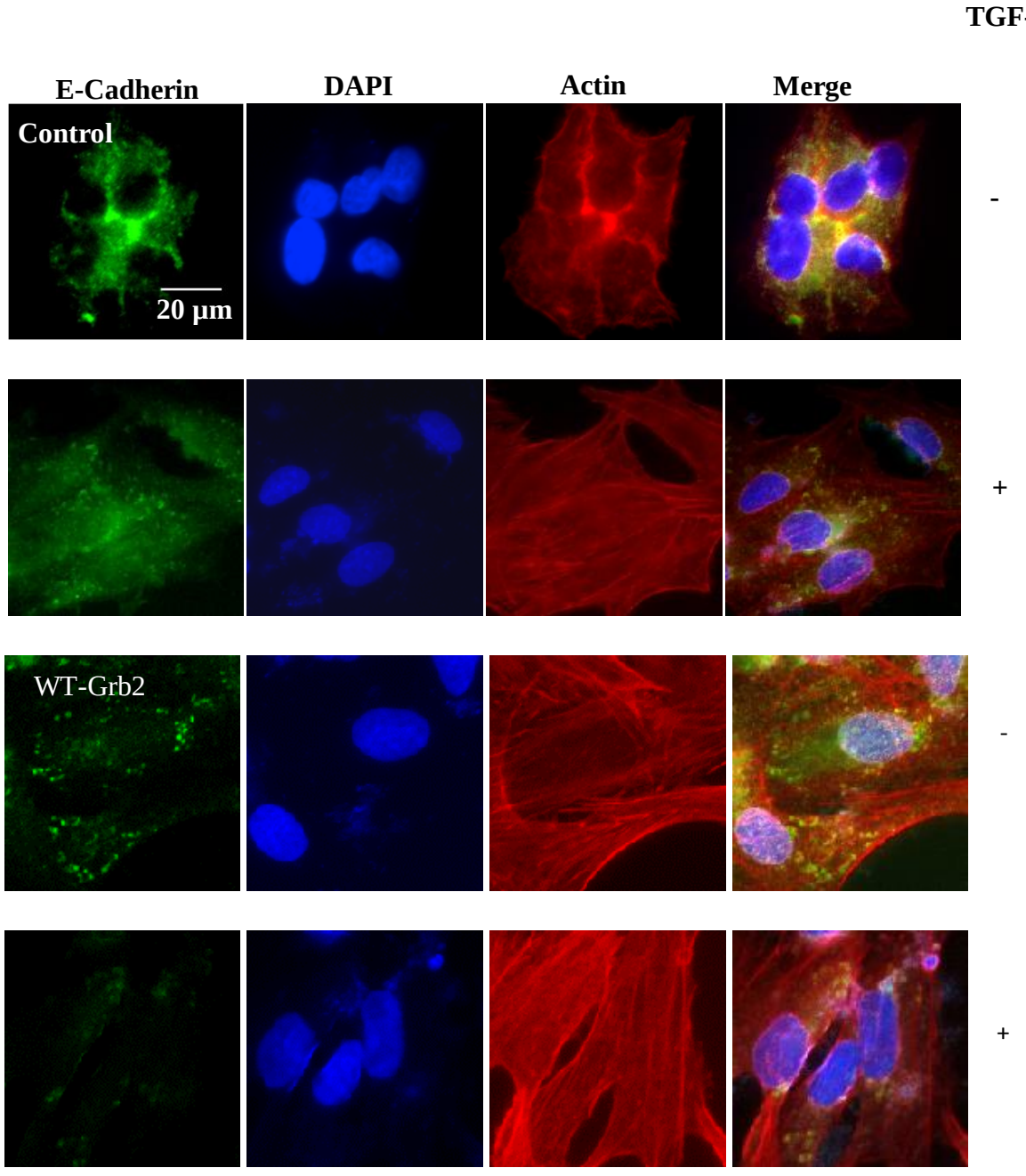


Fig 4.4 GRB2 overexpression leads to reduction in Cell-cell adhesion: Control and GRB2 over expressing A549 cells were seeded on coverslips at a cell number of $2X10^5$ per 60 mm dish. At 25% confluency, the cells were serum starved for 12 hrs. After 12 hrs, 5ng/ml of TGF-β was added to one well of control and GRB2 over expressing cells. Other set of each was kept untreated (control). After 48 hrs of induction of EMT, the cells were

fixed and immunostained using antibodies against E-Cadherin (green) and actin (red). Images were taken under a 40X oil immersion lens.

4.5 Overexpression of GRB2 in A549 cells leads to significant reduction in the level of E-Cadherin expression compared to the control:

The drastic reduction in the expression of E-Cadherin in GRB2 over expressing cells before TGF-β stimulation at 48 hrs was considered to be an important observation in relation to EMT since regulation of E-Cadherin expression is one of the master regulatory mechanism in controlling EMT in tumor cells. Hence, we sought to investigate whether EMT occured faster i.e the changes in the E-Cadherin and vimentin expression could be observed earlier in GRB2 over expressing cells. For that purpose, control and GRB2 over expressing A549 cells were induced to undergo EMT following the above conditions for 24 and 48 hrs. Cells were scraped and lysed at 24 hrs and 48 hrs and the protein extracts were subjected to western blot analysis and probed using antibodies against E-Cadherin and vimentin. GAPDH was used as the loading control. It was observed that even though there were no significant morphological changes between the control and the GRB2 over expressing A549 cells at 24 hrs, however, there was noticeable difference in the expression of E-Cadherin between the control and GRB2 over expressing A549 cells at 24 hr time point indicating that even though EMT may not be faster due to GRB2 over expression, per se, but the reduction in the level of E-Cadherin expression after TGF-β stimulation at 24 hrs is surely more in GRB2 over expressing cells compared to the control and may partly contribute to the process of EMT in A549 cells.

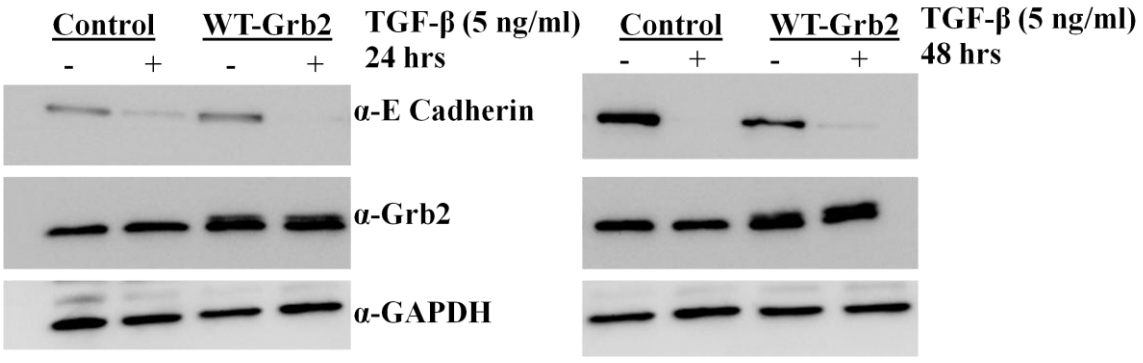


Fig 4.5 Over expression of GRB2 in A549 cells leads to significant reduction in the level of E-Cadherin expression compared to the control: Control and GRB2 over expressing A549 cells were seeded at a cell number of $2X10^5$ cells in 60 mm dish. After 12 hrs of serum starvation at 25% confluency, 5ng/ml TGF-β was added the control and GRB2 over expressing cells. The cells were scraped and lysed at 24 hrs and 48 hrs and

subjected to western blot analysis and probed using antibodies against E-Cadherin and GRB2. GAPDH was used as the loading control.

4.6 GRB2 knockdown does not cause any significant change in the E-Cadherin expression compared to the control, during epithelial-to-mesenchymal transition, in A549 cells

GRB2 over expression in A549 cells was found to accelerate EMT (Fig 4.3 B), it was hypothesized that reduction in GRB2 expression may inhibit EMT in A549 cells. For that purpose, the expression of GRB2 was knocked down by RNA interference using lentivirus vector. Lentivirus constructs of control and GRB2 knock down plasmids were transfected in HEK 293T cells to produce control and GRB2 knock down lentivirus particles which were then infected in A549 cells by two consecutive rounds of infections. The cells were then passed through one round of puromycin selection with 2µg/ml of puromycin to kill all the uninfected cells. Knockdown efficiency was verified by running the protein extracts through western blotting and probing against GRB2 and GAPDH (loading control) after which the control and the GRB2 knockdown A549 cells were maintained stably.

Control and GRB2 knockdown cells were then seeded for induction of EMT at a cell number of $2X10^5$ cells. After reaching 25% confluency, the cells were serum starved for 12 hrs after which 5 ng/ml of TGF-β was added to the cells to induce EMT and the cells were incubated for 48 hrs at 37°C. A set of untreated controls was also maintained. Morphologically after 48 hrs, GRB2 knockdown cells looked quite similar with or without TGF-β stimulation (Fig 4.6 A). However, the cells did not appear as much separated as the control TGF-β treated cells. The cells were further scraped, lysed and western blotted and probed against E-Cadherin (epithelial marker) and vimentin (mesenchymal marker). However, the expression of the E-Cadherin did not show any significant difference compared to the control indicating that the knockdown of GRB2 did not have much effect on E-Cadherin expression of A549 cells (Fig 4.6 B). However, expression of vimentin was found to be slightly lower in $GRB2^{KD}$ treated cells compared to the control.

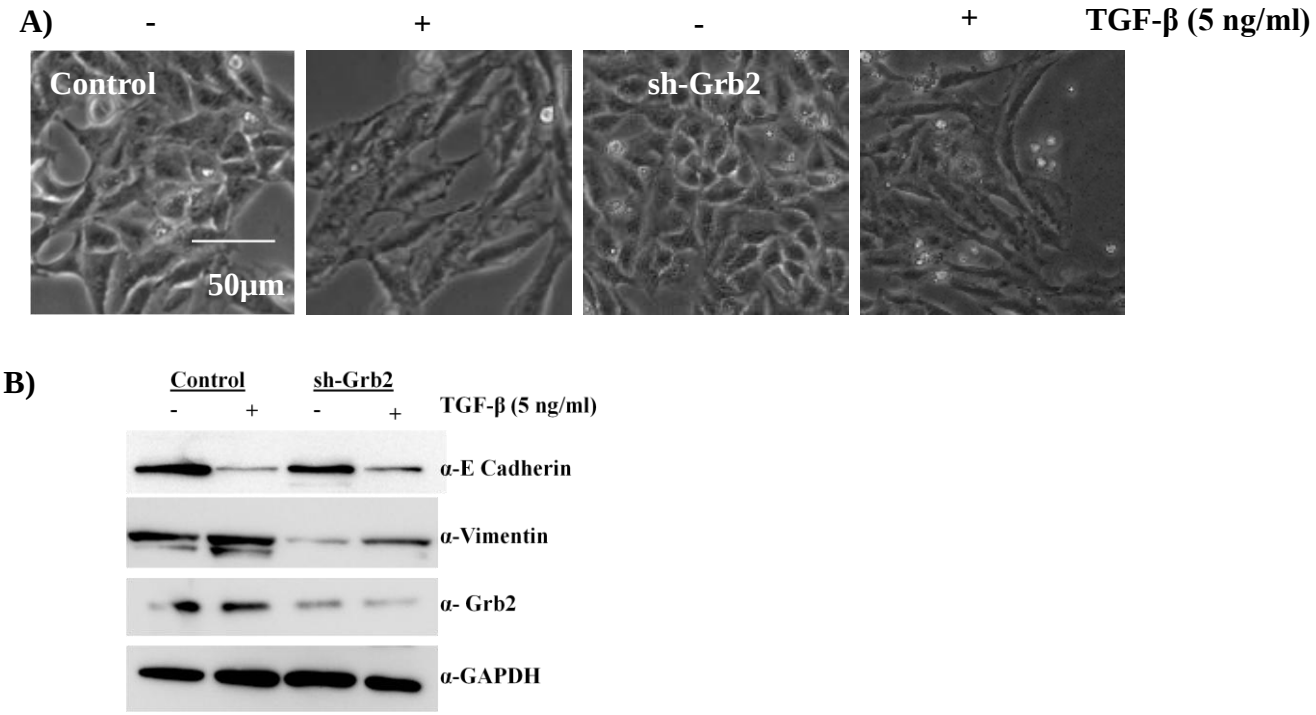


Fig 4.6 GRB2 knockdown does not cause any significant change in the E-Cadherin expression compared to the control, during epithelial-to-mesenchymal transition, in A549 cells: A) Control and GRB2 knock down A549 cells were seeded at a cell number of $2X10^5$ cells in a 60 mm dish. At 25% confluency, the cells were serum starved for 12 hrs. After 12 hrs, 5 ng/ml of TGF-β was added in each well. A set of controls were maintained. After 48 hrs of incubation, the cells were visualized under 10X objective lens. B) Cells were also scraped, lysed and protein extracts were subjected to western blot analysis to probe for E-Cadherin, Vimentin and GRB2. GAPDH was used as the loading control.

4.7 GRB2 knockdown does not affect E-Cadherin localization after TGF-β stimulation in A549 cells:

Loss of E-Cadherin localization at the cell-cell junction forms one of the crucial steps in tumor progression [190]. In GRB2 over expressing A549 cells, decrease in E-Cadherin localization at cell-cell junction was observed. Hence, we sought to study the effect of GRB2 knock down on the E-Cadherin localization in A549 cells during EMT. For that purpose, we seeded the control and the GRB2 knock down A549 cells on coverslips and serum starved them at 25% confluency. Then 5ng/ml TGF-β was added to the cells and EMT was induced for 48 hrs. Cells were then fixed and immunostained using antibodies against E-Cadherin. Actin was stained using phalloidin. Images were captured under 40X immersion oil lens. It was observed that before TGF-β stimulation, the localization of E-Cadherin in GRB2 knock down cells was quite similar to the control at the cell-cell junction. But, after TGF-β

stimulation, unlike the control cells, the localization or the intensity of E-Cadherin did not reduce in to dot like structures in the cytoplasm. In fact they appeared to be localized at the cell-cell junction as well suggesting that knocking down the expression of GRB2 causes accumulation of E-Cadherin at the cell-cell junctions, like the control. The cells also looked smaller in size with distinct presence of cortical actin and less of actin stress fibres like the control.

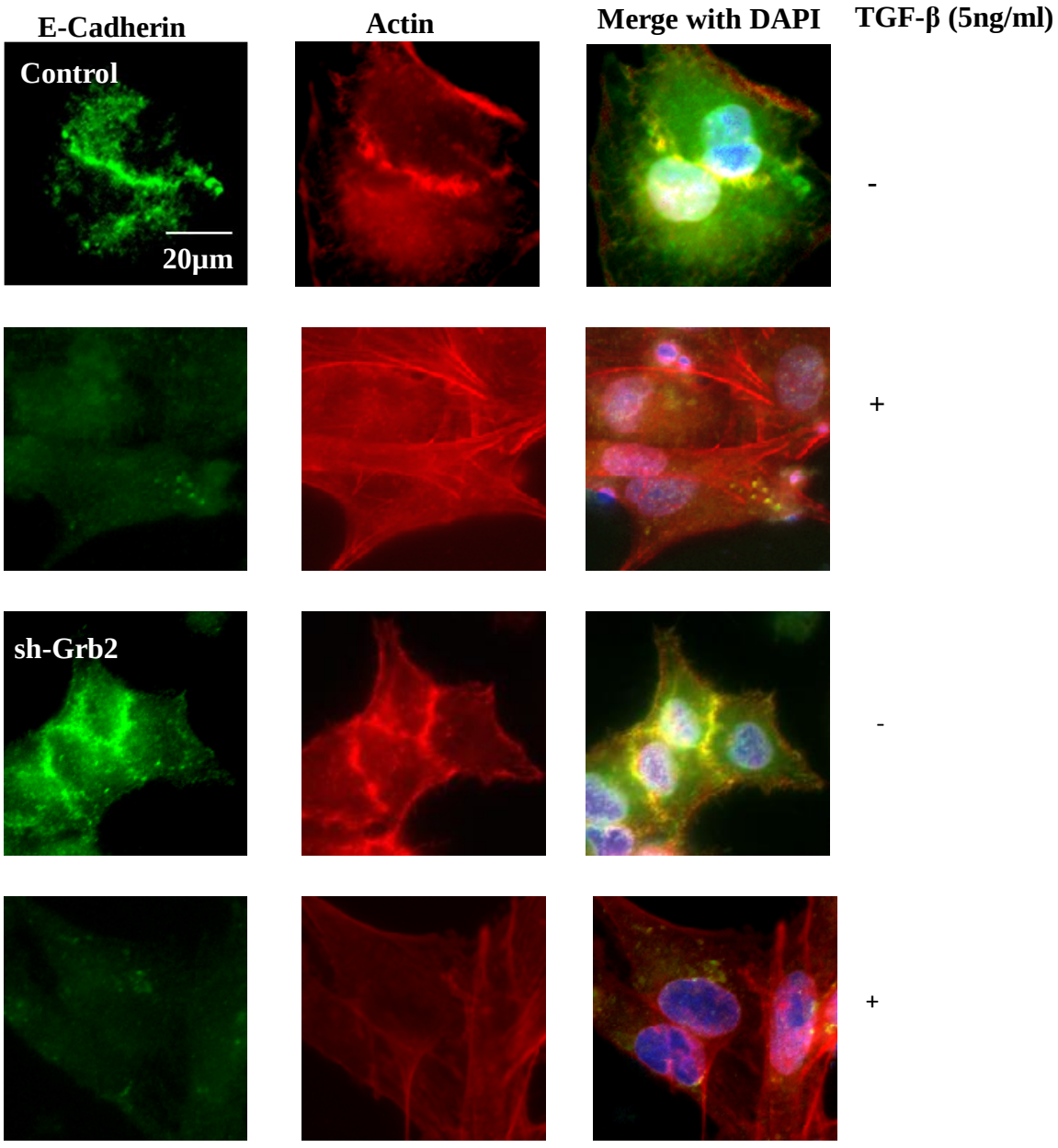
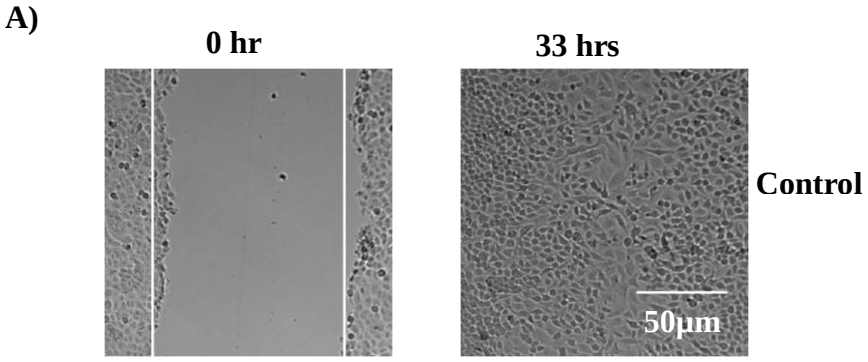


Fig 4.7 GRB2 knockdown does not affect E-Cadherin localization after TGF-β stimulation in A549 cells: Control and GRB2 knockdown A549 cells were seeded on coverslips and at 25% confluency, serum starved for 12 hrs and induced to undergo EMT after addition of 5ng/ml TGF-β. After 48 hrs of incubation, the

cells were fixed and immunostained with antibodies against E-Cadherin. Phalloidin was used to stain actin. Images were taken under 40X oil immersion lens.

4.8 GRB2 enhances cell migration in A549 cells

Cell migration is also an important characteristic feature of tumor cells during EMT. Epithelial cancer cells need to acquire migratory and invasive capabilities to metastasize to secondary sites of tumor growth [191]. Proteins interacting with the actin cytoskeleton have been found to be up regulated during the migration of tumour cells during EMT. GRB2 has been found to interact with proteins belonging to the WASP family of actin cytoskeleton regulators such as WASP and N-WASP at their proline rich domains [48]. To study the role of GRB2 in migration capacities of A549 cells, a scratch assay was performed with the control, GRB2 knock down and GRB2 over expressing A549 cells. Control, GRB2 knockdown and GRB2 over expressing A549 cells were seeded on a 6 well plate and grown until they reached 100% confluency. At 100% confluency, a scratch or a wound was made on the A549 cell monolayer in triplicates and the cells were set up for time lapse imaging using a live cell imaging microscope for a period of 48 hrs with images being captured under a 10X microscope at regular intervals of 30 mins. The images were analyzed and the distance or the gap width was calculated using Image J software. It was observed that at 33 hrs, the gap of the scratch made on control cells had closed. Taking 33 hrs as the reference time point, it was seen the GRB2 over expressing cells migrated faster and closed the gap before 33 hrs as that of the control whereas the GRB2 knockdown cells migrated much slower than the control (Fig 4.8 A). Gap width is considered to be inversely proportional to cell migration meaning shorter the gap width faster is the migration. This indicates that GRB2 over expressing cell migrated faster and GRB2 knock down cells migrated slower than the control suggesting that GRB2 enhances migratory capacity of A549 cells (Fig 4.8 B).



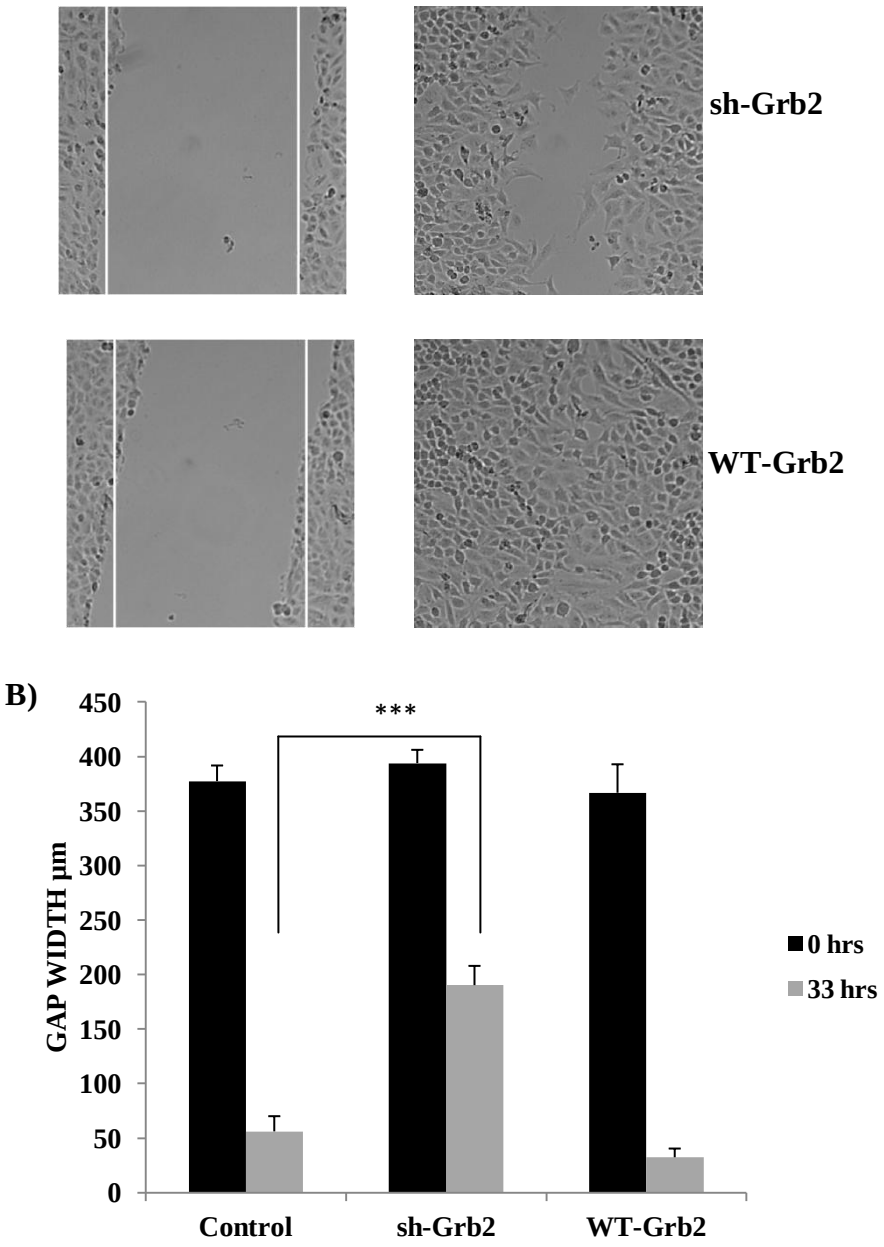
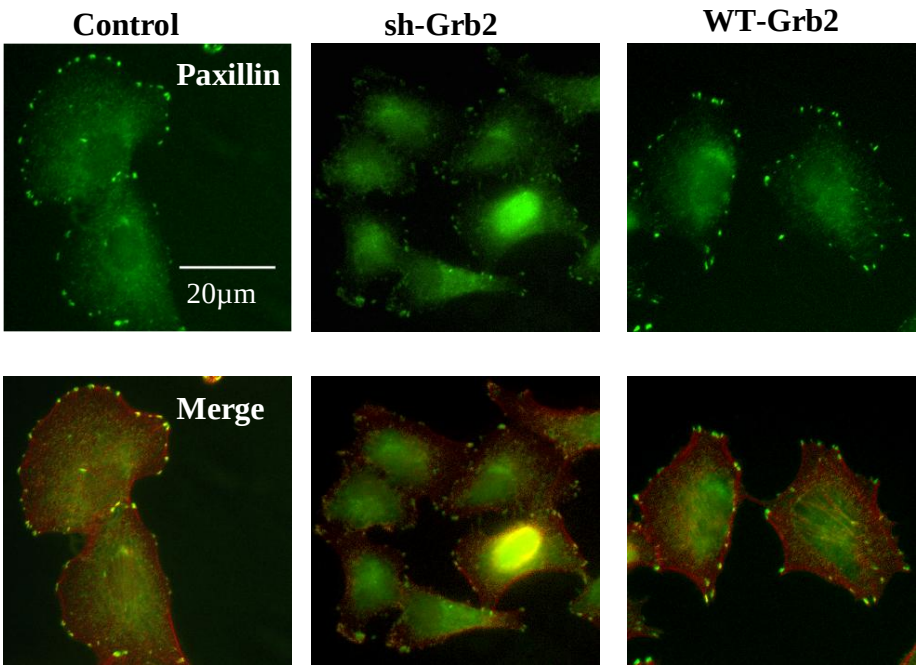


Fig 4.8 GRB2 enhances cell migration in A549 cells: A) Control, GRB2 knockdown and GRB2 over expressing A549 cells were seeded on a 6 well plate and grown up to 100% confluency. At 100% confluency, a scratch or a gap was made on the monolayer and the cells were set for time lapse imaging in a chamber at 37◦C with 5% CO2 for a period of 48 hrs with live cell images being taken at 30 mins interval under a 10X objective lens. The time point at which the gap of the control cells was found to close was taken as the reference time point (33 hrs). Images from the 0 hr and 33 hrs time points were considered for calculations. B) Gap width was measured as the distance between a scratch and calculated using Image J software. A total of 6 fields per time point per cell sample were considered for an experiment. Three independent experiments were performed to calculate the significance value. (*p<0.05, **p<0.01,***p<0.001)

4.9 Reduction in the GRB2 expression reduces Paxillin distribution at the focal adhesions in A549 cells

Paxillin distribution at the focal adhesion complex formation point has been considered as an indication of migration in many highly metastatic cell lines as the expression of the protein and its phosphorylated form has been found to increase in these cell lines [190]. We found that the migration of GRB2 knockdown A549 cells was much lower than the control. To study the effect of GRB2 knockdown and GRB2 over expression on the Paxillin localization in A549 cells, we seeded the control, GRB2 knock down and GRB2 over expressing A549 cells on silane coated coverslips, grew them up to 40 % confluency, fixed and immunostained using antibody against Paxillin. Phalloidin was used to stain actin. It was observed that the number of Paxillin streaks per cell was significantly reduced in GRB2 knock down cells compared to the control (Fig 4.9 A). However, not much difference was observed between the number of Paxillin streaks in the control and the GRB2 over expressing A549 cells.

A)



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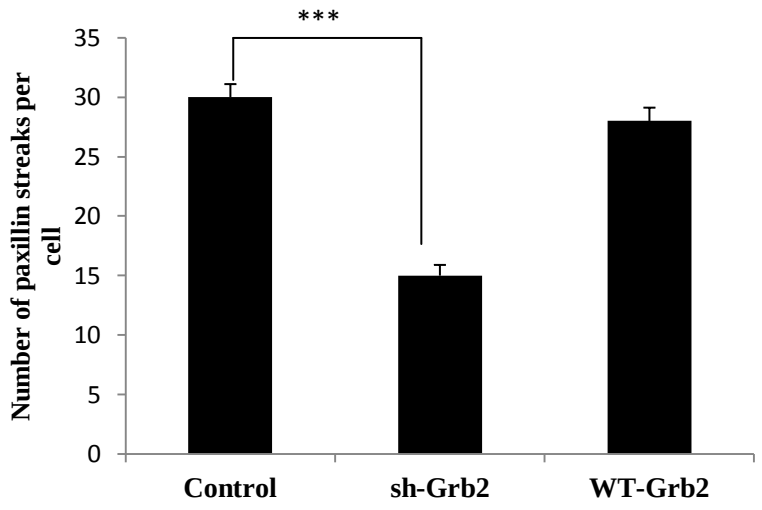
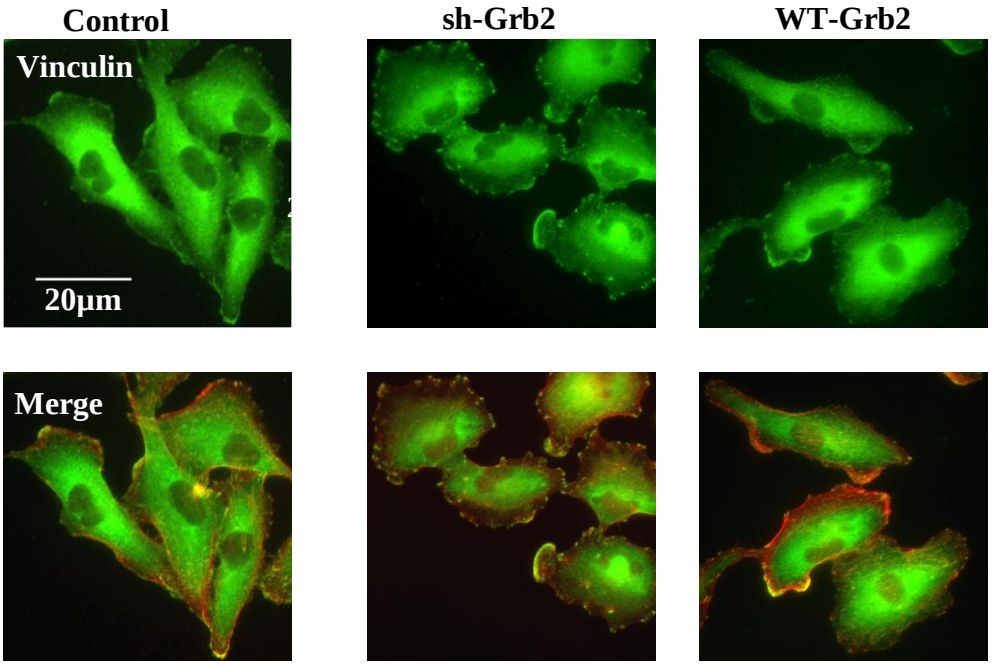


Fig 4.9 Reduction in the GRB2 expression reduces Paxillin distribution at the focal adhesions in A549 cells: A) control, GRB2 knockdown and GRB2 over expressing cells were seeded on silane coated coverslips and grown up to 40% confluency. Cells were then fixed and immunostained using antibody against Paxillin. Images were observed under 40X oil immersion lens. B) The number of Paxillin streaks per cell was calculated manually. A total of 25 cells per cell were considered for an experiment.

4.10 GRB2 reduces the localization of vinculin at the focal adhesions in A549 cells

Vinculin is another important cytoskeletal focal adhesion protein which has been found to play important role in cell adhesion and migration. During the initial cell attachment, vinculin has been found to localize at the focal complexes at dots which later mature in to focal adhesions and regulate cell adhesion and migration of many cell types. [74] Hence to study the role of GRB2 in adhesion of A549 cells, the control, GRB2 knockdown and GRB2 over expressing cells were seeded on silane coated coverslips, grown up to 40% confluency and immunostained using antibodies against vinculin. The quantification of the number of vinculin patches per cell was done manually. A total of 30 cells per sample were quantified for each experiment. Vinculin patches in A549 cells appear as small dot like structures unlike in myoblasts, where they localize as larger streaks. It was observed that the number of vinculin patches per cell was significantly reduced in GRB2 over expressing cells compared to the control whereas it was found to have increased for GRB2 knockdown A549 cells compared to the control (Fig 4.10 A). This suggests that the vinculin localization and hence the focal adhesion assembly is inhibited by GRB2 in A549 cells. This correlates with the observation that GRB2 over expressing A549 cells migrate faster and hence may be undergo EMT at a faster rate compared to the control.

A)



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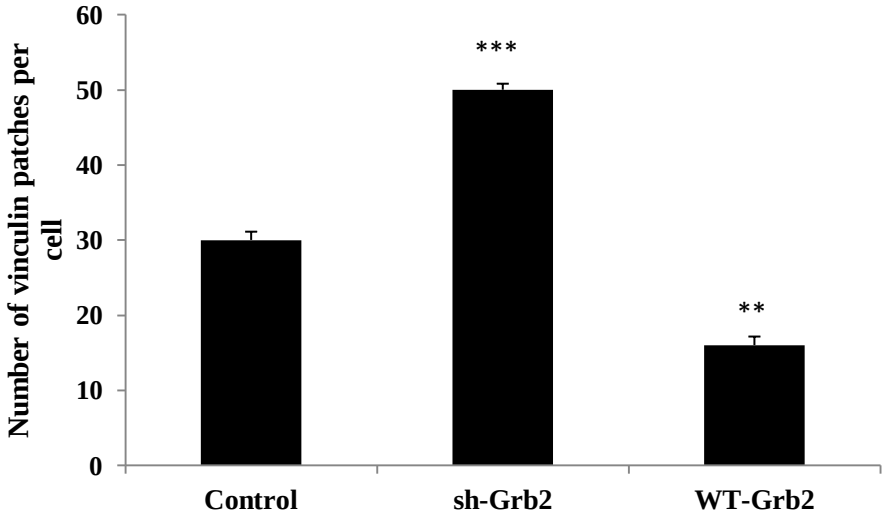


Fig 4.10 GRB2 reduces the localization of vinculin at the focal adhesions in A549 cells: A) Control, GRB2 knockdown and Grb2 over expressing A549 cells were seeded on silane coated coverslips, grown up to 40% confluency, fixed and immunostained using antibodies against vinculin. Phalloidin was used to stain actin. The images were taken under 40X oil immersion lens. B) The number of vinculin patches per cell was calculated manually. 25 cells per sample were counted for one experiment. Three independent experiments were carried out to calculate significance (*p<0.05, **p<0.01, ***p<0.001)

4.11 Focal adhesion kinase (FAK) localization at the focal adhesion sites increases with the increase of GRB2 expression after TGF-β stimulation

Focal adhesion kinase **(**FAK) is an important focal adhesion molecule which has been shown to be over expressed in various types of tumors with increased metastasis and poor prognosis [48] Upon binding of integrin to extracellular matrix proteins such as fibronectin, FAK has been found to co localize at the integrin receptors and form an important point of regulation during cell migration. FAK function is regulated by tyrosine phosphorylation at several of its residues. One of the phosphotyrosine residues, Y925, has been found to have strong affinity to the SH2 domain of GRB2 [48]. Studies have shown that phosphorylation on this residue can lead to de regulation of E-Cadherin expression and lead to epithelial-mesenchymal-transition[48]. However, the exact role of GRB2 in this mechanism has not been established but highly suspected. Hence, to study the effect of GRB2 over expression on FAK localization after TGF-β stimulation in A549 cells was carried out to gain an insight in to the co localization features of the two proteins.

For that purpose, control and GRB2 over expressing cells were seeded on silane coated coverslips and grown till 25% confluency, after which they were serum starved for 12 hrs. After 12 hrs, 5ng/ml of TGF-β was added to control and GRB2 over expressing cells and TGF-β stimulation was done for 24 hrs. After 24 hrs, the cells were fixed and immunostained using antibodies against GRB2 and FAK. The images of co localization of GRB2 and FAK were taken under 40X oil immersion lens. It was observed that before TGF-β stimulation, in the control cells, GRB2 and FAK were not found to colocalize with each other and had quite dispersed localization. However, after TGF-β stimulation, they were found to colocalize at many areas in the cytoplasm. However, FAK was found to form streaks close to the cell membrane after TGF-β stimulation and GRB2 was not found to still colocalize with the streaks. However, in GRB2 over expressing cells, even before TGF-β stimulation, FAK and GRB2 were found to colocalize and in some areas even close to the plasma membrane. However, after TGF-β stimulation, both GRB2 and FAK were found to co localize specifically near the plasma membrane indicating that after TGF-β stimulation, during EMT, both the proteins may interact and cause further downstream changes observed during EMT.

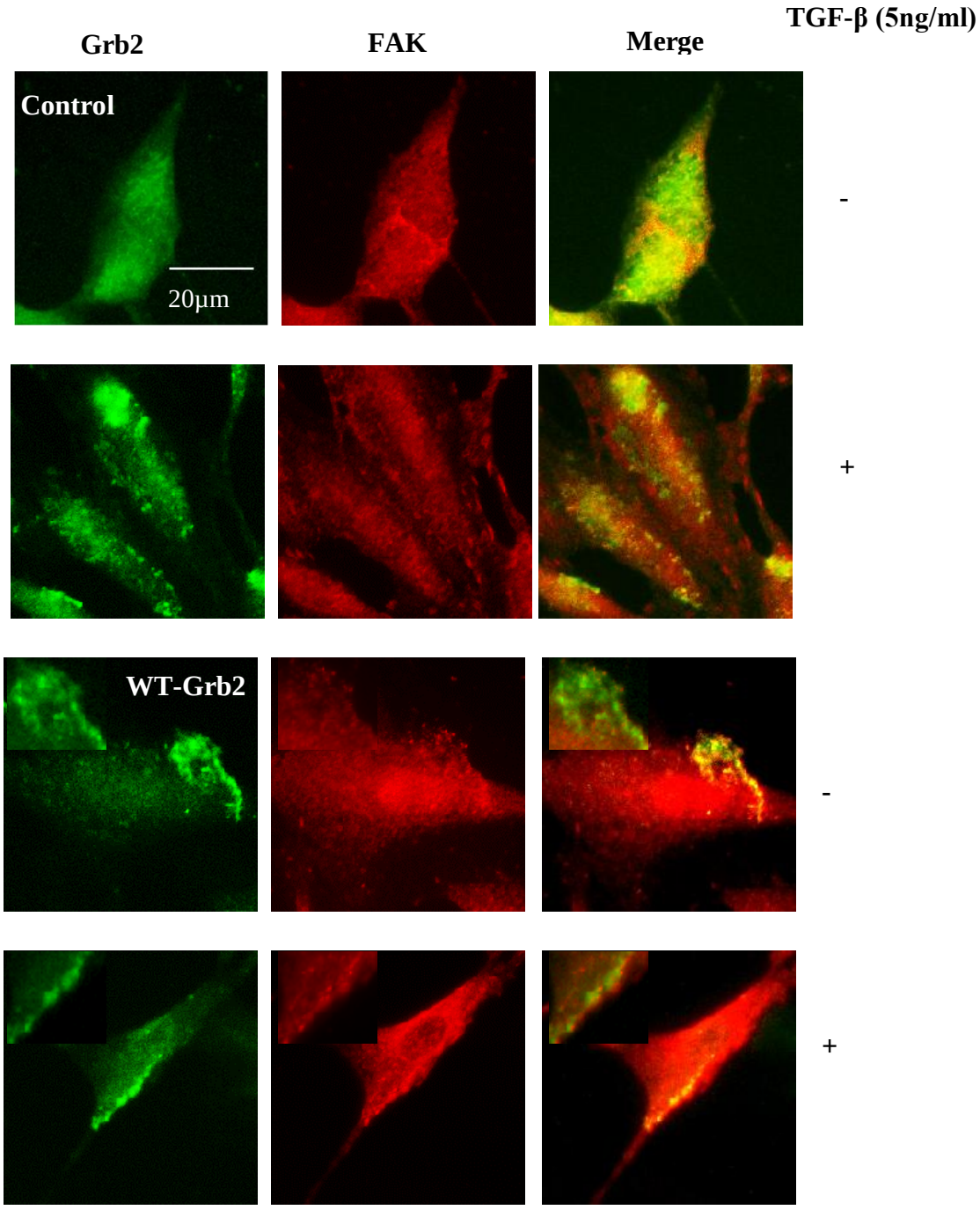
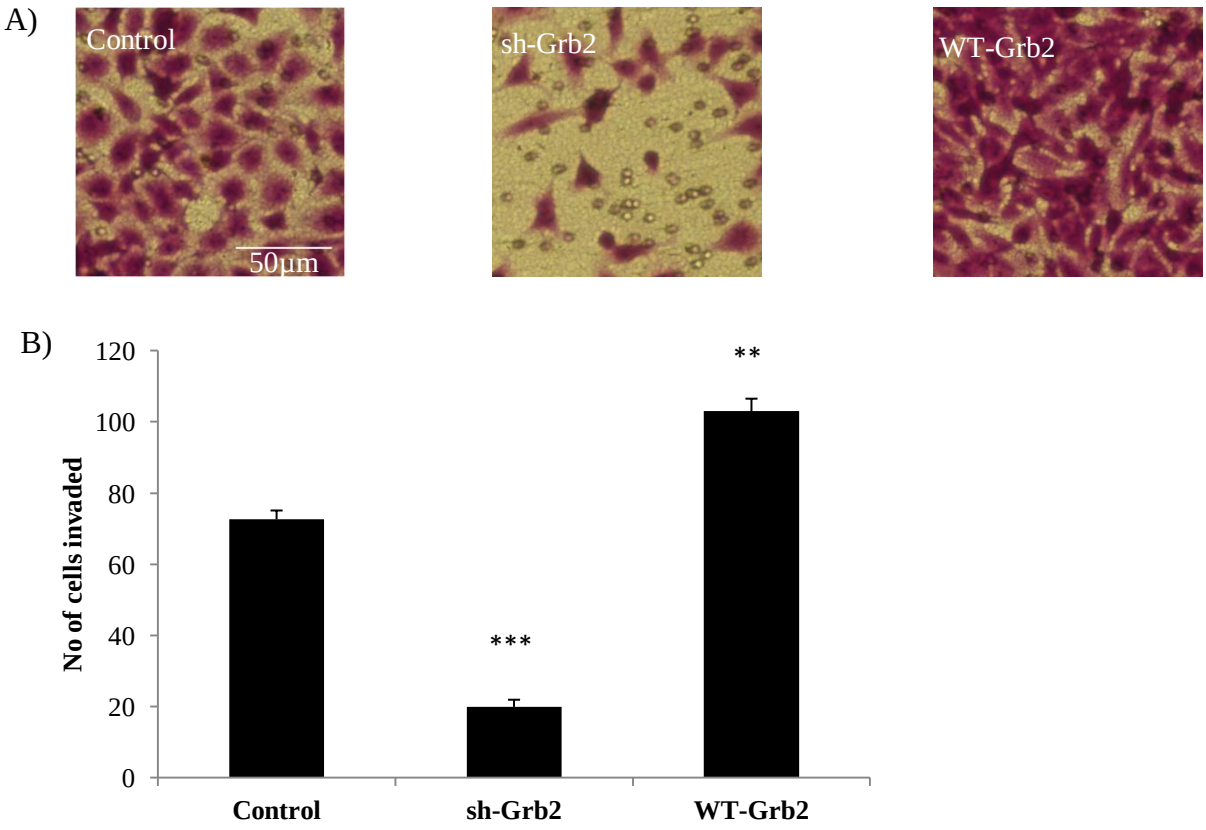


Fig 4.11 Focal adhesion kinase (FAK) localization at the focal adhesion sites increases with the increase of GRB2 expression after TGF-β stimulation: Control and GRB2 over expressing A549 cells were seeded on silane coated coverslips and grown up to 25% confluency. The cells were then serum starved for 12 hrs after which 5 ng/ml of TGF-β was added to the cells and were stimulated for 24 hrs. After 24 hrs the cells were fixed and immunostained using antibodies against GRB2 (green) and FAK (red). The images were taken under 40X oil immersion lens.

4.12 Knockdown of GRB2 suppresses cell invasion in A549 cells

Tumor invasion is a critical step in the cancer progression and EMT. The ability of cancer cells to break the ECM barrier and invade the surrounding tissues and enter the blood circulation is what gives them the advantage of metastasizing to distant organs with extraordinary speed. Since GRB2 was found to be required for EMT in A549 cells, the effect of GRB2 on the invasive properties of A549 cells was studied by carrying out a matrigel invasion assay. Matrigel coated invasion assay inserts of 8.0 µm pore size were seated with 2 X 10^5 cells per 200 µL of serum free media on the upper chamber of the insert and the well below was filled with complete media containing 10%FBS. The cells were allowed to invade the matrigel towards the chemokines for about 40 hrs in the cell culture incubator at 37°C with 5% $C0_2$. After that, the cells that had invaded to the lower side of the insert were fixed and stained with crystal violet and observed under a 10X objective lens and quantified. We found that the GRB2 knockdown cells invaded the matrigel much lesser compared to the control. Also, the invasion by the GRB2 over expressing cells was higher than the control indicating that GRB2 may enhance the migratory and invasive properties of A549 cells.



4.12 Knockdown of GRB2 suppresses cell invasion in A549 cells: A) Matrigel coated invasion assay inserts were seeded with 2X10^5 cells per 200µL of serum free media on the upper chamber of the insert. The well

below was filled with complete media containing 20% FBS. The cells were incubated in the cell culture incubator for 40 hrs after which the invaded cells were fixed, washed and stained with crystal violet and observed under a 10X objective lens. B) 4 fields per sample were counted to quantify the number of cells invaded. Three independent set of experiments were carried out to calculate significance.

4.13 Expression of Snail mRNA and protein level increases on GRB2 overexpression in A549 cells

Repression of E-Cadherin expression during EMT has been shown to be strictly regulated by different mechanisms. The control of E-Cadherin levels by SNAIL/SLUG family of transcription factors has been found to be quite critical. It is known that Snail directly represses E-Cadherin expression in many epidermal cell types. It is also known to induce EMT and fibroblastic phenotype in many epithelial cell lines [161]. However, the involvement of GRB2 in this regulation has not been shown so far. We earlier observed that GRB2 overexpression caused reduction in the E-Cadherin levels even before TGF-β stimulation. Hence we wanted to study if the regulators of E-Cadherin expression such as Snail or Slug were up regulated on GRB2 overexpression in A549 cells. Protein lysates from control and GRB2 overexpressing A549 cells, before and after TGF-β treatment, were collected and subjected to western blot analysis to probe against antibodies against Snail and Slug. Slug expression could not be detected. Snail, being an inducer of EMT, was found to be expressed only in the TGF-β treated control cells and not in the untreated cells. However, we found that the expression of Snail protein occurred in GRB2 overexpressing cells even before TGF-β stimulation and the level increased slightly after TGF-β stimulation in GRB2 overexpressing cells (Figure 4.13B). Also, RT-PCR analysis of the cDNA reverse transcribed from the total RNA isolated from control and GRB2 overexpressing cells, before and after TGF-β stimulation quantified that Snail mRNA levels were significantly more in GRB2 overexpressing cells before TGF-β stimulation compared to the control (Figure 4.13A). This indicates that regulation by transcription factor Snail could be one of the mechanisms for the reduction in E-Cadherin expression on GRB2 overexpression in A549 cells

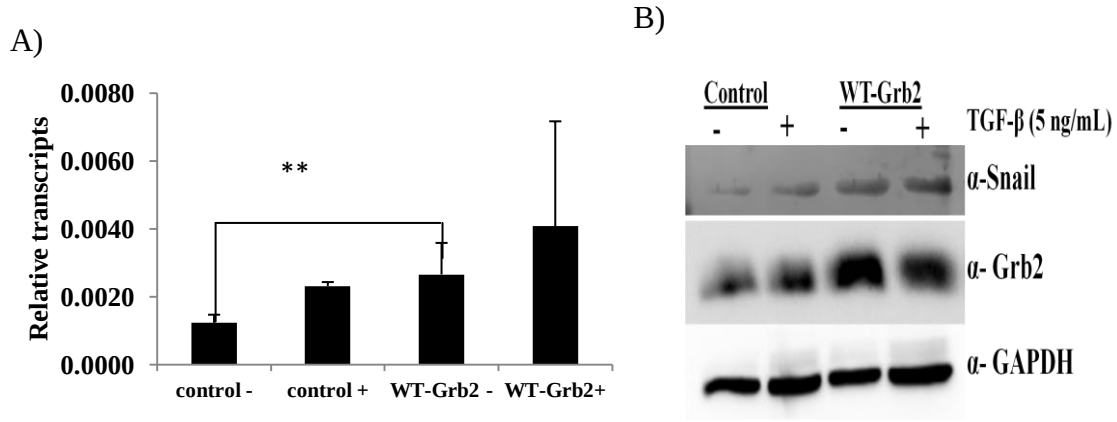


Figure 4.13 Expression of Snail mRNA and protein increases on GRB2 overexpression in A549 cells: A) RNA was extracted from the samples by Trizol reagent, followed by cDNA preparation and RT-PCR using Snail primers. MRPL was used as the control. Cq measurement was done for two independent experiments and the average Cq values of the control and GRB2 overexpressing samples was plotted B) Protein extracts from control and GRB2 over expressing cells, before and after TGF-β treatment, were subjected to western blot analysis and probed using antibodies against Snail and GRB2. GAPDH was used as the loading control (*p<0.05, **p<0.01, ***p<0.001)

The domain of GRB2 that may be responsible for accelerating EMT in A549 cells was studied by using the point mutants used in the previous study. Wild type *GRB2* and the three mutants ($GRB2^{P49L}$, $GRB2^{R86K}$ and $GRB2^{P206L}$) used are his tagged in this study.

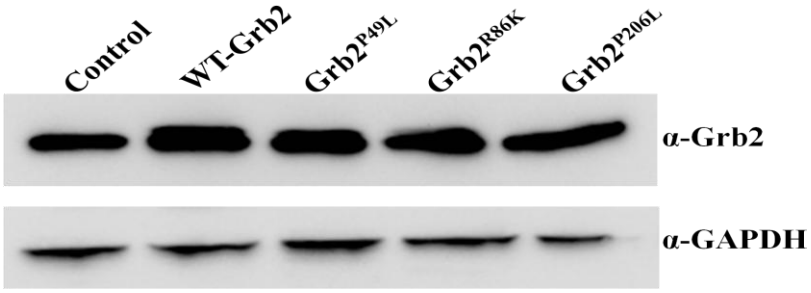


Fig 4.14 Expression of GRB2 and its point mutants in A549 cells. : Protein extracts of WT-*GRB2*, and the point mutants were subjected to western blot analysis and probed using antibodies against GRB2 and GAPDH.

4.14 Overexpression of the three mutants of GRB2-$GRB2^{P49L}$, $GRB2^{R86K}$ and $GRB2^{P206L}$, did not reduce level of E-Cadherin expression, before TGF-β stimulation.

To characterize the role of individual domains of GRB2 in EMT, the point mutants of *GRB2*- P49L, R86K and P206L plasmids were infected in A549 cells as lentivirus, selected and set up for EMT by seeding $2X10^5$ cells in 60mm plate. After reaching 25% confluency the cells were serum starved for 12 hrs and then treated with 5ng/ml TGF-β for 48 hrs after which they were lysed and subjected to western blot analysis and probed using

antibodies against E-Cadherin (epithelial marker) and vimentin (mesenchymal marker). All the three mutants were observed to induce EMT since loss of E-Cadherin expression was observed in all the three mutants after TGF-β stimulation. All the three mutants of GRB2 , individually, did not reduce E-Cadherin expression, before TGF-β stimulation, as observed in wild type GRB2 expressing cells indicating that all the three domains of GRB2 may be playing a role in the regulation of E-Cadherin expression mediated by GRB2. GRB2 has been shown to interact with the TGFβRII on TGF-β stimulation in mammary epithelial cell line NMuMG [46]. But the domain responsible for doing so has not been characterized so far.

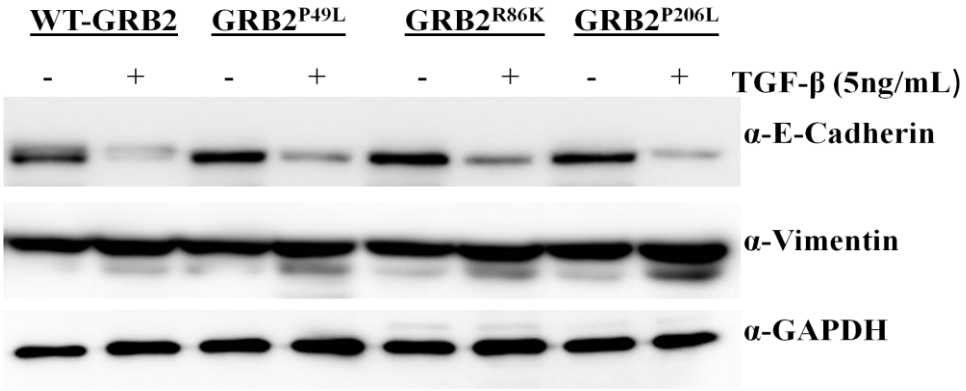
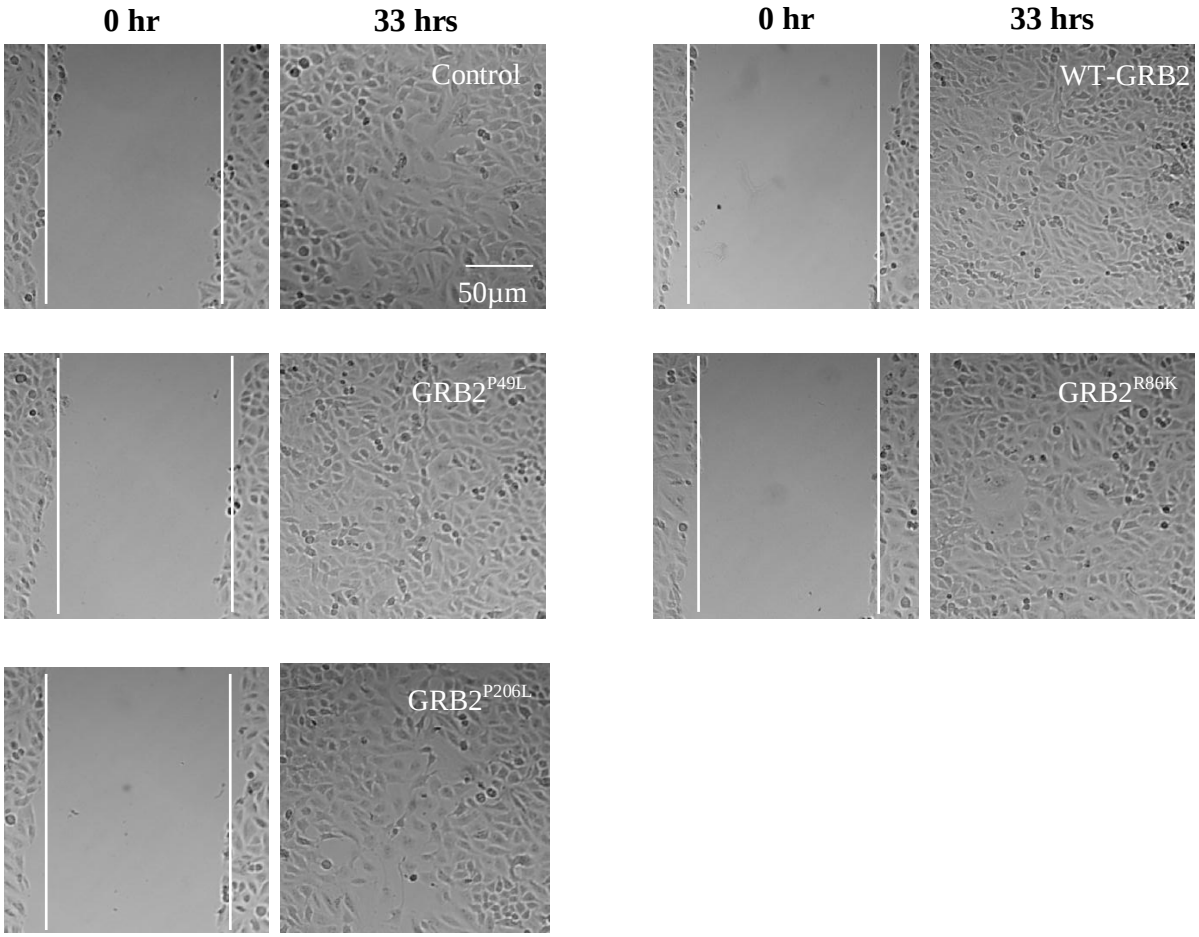


Fig 4.15 Overexpression of the three mutants of GRB2-GRB2^{P49L}, GRB2^{R86K} and GRB2^{P206L}, did not reduce level of E-Cadherin expression, before TGF-β stimulation: Wild type GRB2, GRB2^{P49L}, GRB2^{R86K} and GRB2^{P206L} mutant of Grb2 were infected as lentivirus particles on A549 cells, selected with puromycin and set up for induction of EMT by seeding 2X10^5 cells in 60mm dish and serum starving at 25% confluency. Then the cells were treated with 5ng/ml of TGF-β and incubated for 48 hrs. Cell was then lysed and the protein extracts were subjected to western blot analysis by probing using antibodies against E-Cadherin and vimentin. GAPDH was used as the loading control.

4.15 Point mutants of GRB2 migrate in a similar manner as the wild type A549 cells

Cancer cells acquire enhanced migratory abilities to spread to distant organs and tissues at a faster rate compared to the normal cells. Increased migration is also a characteristic feature of cells undergoing EMT [191]. GRB2 was observed to increase migration in A549 cells (Fig 4.8). To identify the domain of GRB2 critical for increase in migration, wound healing assay was carried out with the wild type GRB2 and its point mutants. Wild type GRB2, GRB2^{P49L}, GRB2^{R86K} and GRB2^{P206L} overexpressing A549 cells were seeded on a 6 well plate and grown till they reached 100% confluency. A scratch was made on the monolayer of the cell in triplicates for each cell sample using the end of a white tip. The cells were then set for live cell imaging with images being captured every 30 minutes

for a period of 48 hrs at the same specified stage positions under 10X objective lens. The captured images were then analyzed for the specific time point at which the gap of the control cells was found to close. The time point was fixed at 33 hrs and the gap closure of all the other cell samples at 0 hr and 33 hrs was measured and calculated using Image J software. Gap width is considered to be inversely proportional to the cell migration capacity of the cell. It was observed that $GRB2^{P49L}$ and $GRB2^{R86K}$ mutants had similar gap width as the wild type GRB2 cells with all of them being more than the control (Fig 4.16 A). $GRB2^{P206L}$ mutant, however, showed slight increase in gap width compared to the wild type GRB2 cells. This suggests that the N-SH3 and SH2 domains of GRB2 probably do not individually affect the capacity of GRB2 to increase migration of A549 cells whereas the C-SH3 domain may affect migration of A549 cells mediated by GRB2.



B)

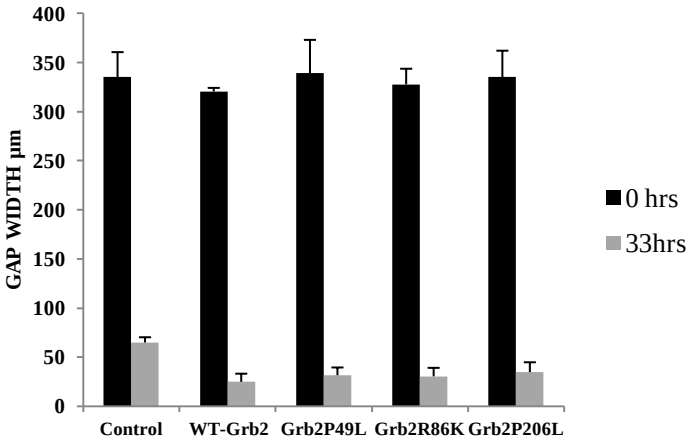
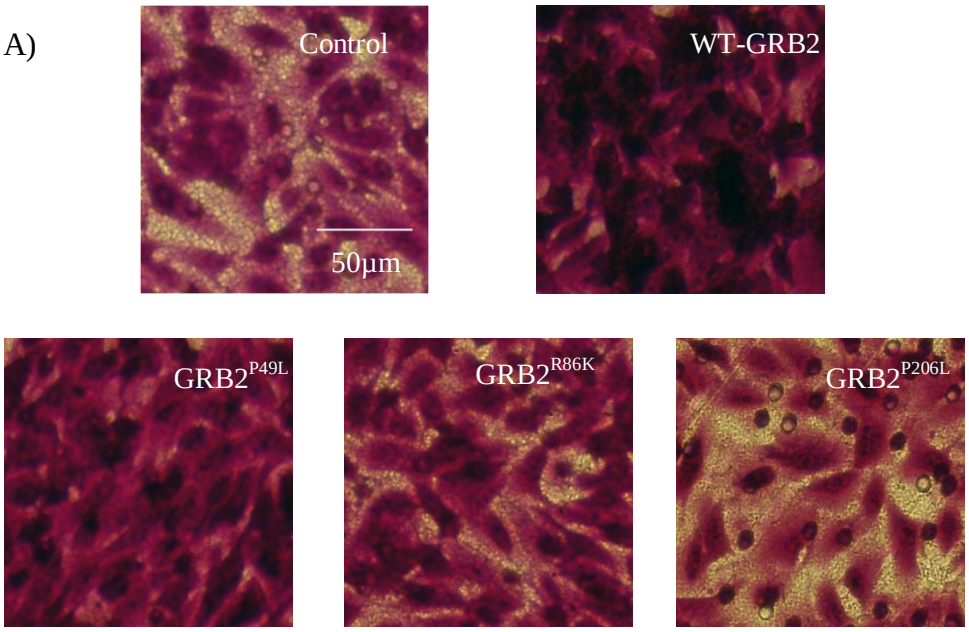


Fig 4.16 Point mutants of GRB2 migrate in a similar manner as the wild type GRB2 over expressing A549 cells: A) Control, WT- GRB2, $GRB2^{P49L}$, $GRB2^{R86K}$ and $GRB2^{P206L}$ mutants were seeded on a 6 well plate and grown up to 100% confluency. At 100% confluency, a scratch or a gap was made on the monolayer and the cells were set for time lapse imaging in a chamber at 37°C with 5% CO2 for a period of 48 hrs with live cell images being taken at 30 mins interval under a 10X objective lens. The time point at which the gap of the control cells was found to close was taken as the reference time point (33 hrs). Images from the 0 hr and 33 hrs time points were considered for calculations. B) Gap width was measured as the distance between a scratch and calculated using Image J software. A total of 6 fields per time point per cell sample were considered for an experiment. Three independent experiments were performed to calculate the significance value.

4.16 C-terminal SH3 domain of GRB2 is critical for GRB2 mediated increase in invasion of A549 cells

Tumor **i**nvasion is an important ability of the cancer cells to invade through the extracellular matrix of the neighboring tissues and metastasize to distant sites to cause secondary tumors. Cells undergoing EMT have also shown to have enhanced invasive capacities. GRB2 was found to increase matrigel invasion in A549 cells (Fig 4.12). To study which domain of GRB2 is required for increasing invasion in A549 cells, matrigel invasion assay was carried out with WT- GRB2 and point mutant over expressing cells. Matrigel coated invasion assay inserts of 8.0 µm pore size were seated with 2 X 10^5 cells per 200 µL of serum free media on the upper chamber of the insert and the well below was filled with complete media containing 10%FBS. The cells were allowed to invade the matrigel towards the chemokines for about 40 hrs in the cell culture incubator at 37°C with 5% $C0_2$. After that, the cells that had invaded to the lower side of the insert were fixed and stained with crystal violet and observed under a 10X objective lens and quantified. It was observed that the number of cells invaded were significantly more in wild type GRB2 and $GRB2^{P49L}$ mutant compared to the control (Fig 4.17 A). Invasion in $GRB2^{R86K}$ mutant expressing cells was

slightly more than the control. However, invasion in GRB2^{P206L} mutant expressing A549 cells was very similar to the control. The number of cells that invaded the matrigel was quantified by counting the cells stained with crystal violet in a particular field. 9 fields per sample were considered for quantification. The experiment was repeated in triplicates to calculate significance value. GRB2^{P206L} mutant is known to interact with N-WASP which has been known to be required for invasion [192]. This suggests that the C-terminal SH3 domain plays a critical role in regulating the increase in the invasive capacity of A549 cells mediated by Grb2.



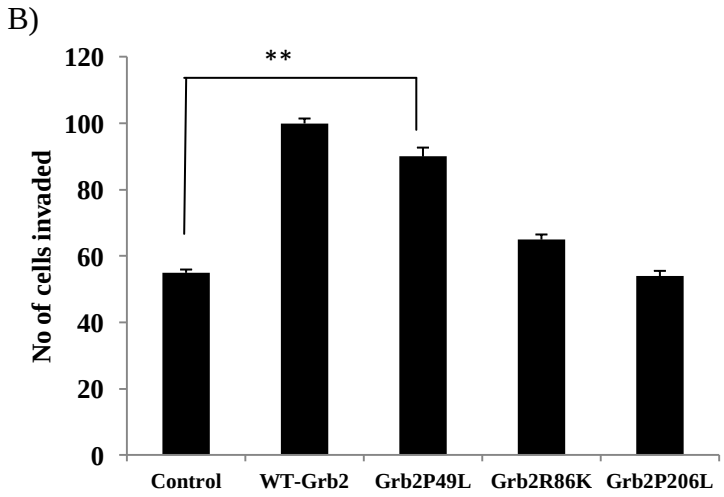


Fig 4.17 C-terminal SH3 domain of GRB2 is critical for GRB2 mediated increase in invasion of A549 cells
A) Matrigel coated invasion assay inserts were seeded with 2X10^5 cells per 200µL of serum free media on the

upper chamber of the insert. The well below was filled with complete media containing 20% FBS. The cells were incubated in the cell culture incubator for 40 hrs after which the invaded cells were fixed, washed and stained with crystal violet and observed under a 10X objective lens. B) 4 fields per sample were counted to quantify the number of cells invaded. Three independent set of experiments were carried out to calculate significance (*$p<0.05$, **$p<0.01$, ***$p<0.001$)

Chapter 5:DISCUSSION

Role of Grb2 in myogenic differentiation in C2C12 cells

Skeletal muscle regeneration or muscle differentiation is a complex phenomenon which requires many regulatory mechanisms simultaneously and co-coordinately controlling the cellular and molecular changes starting from satellite cell activation to terminal differentiation [173]. Adult skeletal muscle has ability to regenerate itself after injury or normal day to day wear and tear. The process of muscle regeneration in adults is quite similar to the embryonic muscle development [193]. Since muscle differentiation is a highly orchestrated multi step process, it requires many factors such as satellite cells, growth factors, cell-matrix and cell-cell interactions, expression of muscle specific transcription factors and muscle specific genes etc to regulate the entire process [193]. Muscle differentiation begins with the activation of quiescent satellite cells due to an external stimulus followed by their entry in to the cell cycle, proliferation and expression of muscle specific transcription factors such as MyoD, myogenin etc. After proliferation, the satellite cells, now known as myoblasts, differentiate and form primary myotubes. Some of the satellite cells do not differentiate and revert back to the quiescent stage to maintain the satellite cell pool [193]. Primary myotubes can also fuse with the existing or damaged myotubes to form secondary myotubes. Myotubes, at this point, express muscle specific structural proteins such as Myosin light and heavy chains ,troponin etc to maintain the muscle cell characteristics [55].

Various secreted growth factors have been shown to play important role in satellite cell proliferation and differentiation [193]. Growth factors such as HGF, FGF,TGF-β, EGF have been proposed to play specific roles during muscle differentiation [193]. Most of these growth factors have been shown to induce proliferation and inhibit differentiation of myoblasts ([49], [169]). This is probably because differentiation occurs at low serum conditions in the absence or low concentrations of the growth factors or mitogens. Grb2 is an adaptor protein shown to interact, either directly or indirectly, with most of these growth factor receptors on their activation through its SH2 domain [4]. Grb2, being an adaptor, has been shown to be involved in various downstream signaling pathways many of which have also been shown to play important roles during muscle differentiation [194]. Leshem et al, 2002 proposed a role of Grb2 in HGF induce myoblast proliferation and activation of HGF receptor, c-Met, in chicken skeletal muscle cells [49]. However, no specific or clear

mechanism involving Grb2 has been identified so far. Elucidating a specific role of Grb2 in regulation of muscle differentiation of mouse myoblasts will definitely give a better understanding regarding the complex regulation of the process of myogenesis and help in designing target drugs against various muscle related disorders.

5.1 Expression of Grb2 remains relatively constant during differentiation

It was observed, during differentiation of mouse myoblasts C2C12, that the expression level of Grb2 remained almost constant throughout differentiation with increase within 24 hrs of addition of differentiation media. It has been observed that Grb2 plays an important role in Ras signaling pathway only when it localizes to the plasma membrane [19] and binds to the GEF, Sos 1. Grb2 has been found to localize in the cytoplasm and nucleus as well, both in normal and tumor tissue samples [186]. Its activation is mostly dependent on the upstream receptor kinases such as EGFR, FGFR, c-Met which depend on specific environmental stimuli [4]. Growth factors too play an important role in myoblast proliferation and differentiation [193]. EGF has been shown to inhibit human myoblast differentiation [169]. It has been shown that EGFR levels reduce within 24 hrs of induction of differentiation and EGFR silencing triggers myoblast differentiation and fusion [169]. Grb2 has been shown to bind directly to the activated EGFR and form a complex with GEF, Sos-1 and control Ras signaling pathway [10]. However, the observation that Grb2 level increases slightly at day 1 of differentiation and remains almost constant during the four days of differentiation suggests that its expression during differentiation may still be different from its activated or bound form. It may indicate that since the activation of various downstream signaling pathways depends on Grb2, the cell needs to maintain a threshold level of Grb2 for its normal functioning and that any role it may play in either activation or inhibition of muscle differentiation may be indirectly through other proteins required for myogenesis.

Furthermore, the localization of Grb2 during differentiation was studied to get a better understanding about its function. It was observed that at day 0 before addition of differentiation media Grb2 localized both in the cytoplasm and the nucleus. Though Grb2's function has been widely studied in the cytoplasm and characterized to be present near the plasma membrane on activation in complex with Sos and other proteins, it has also been reported to be present in the nucleus [186]. No role of Grb2 in the nucleus is known so far. On the first day of differentiation, it is found to be localized more at the

perinuclear region and almost outside the nucleus which indicates that Grb2, during differentiation, localizes to the cytoplasm to interact with proteins important in muscle differentiation. From the 2^{nd} to the 4^{th} day of differentiation, Grb2 remains in the cytoplasm of the myoblasts and eventually the myotubes. In fact, the intensity of Grb2 staining was found to be more in the myotubes than in the adjacent non fusing cells. This indicates that Grb2 certainly is present in the myotubes during differentiation either to positively or negatively regulate the process.

Since the function of Grb2, as an adaptor protein, is dependent on its recruitment to the plasma membrane by activated EGF receptor and interaction with GTPase, Sos-1, we tried to study the effect of EGF stimulation on Grb2 expression and localization during differentiation. EGF recently has been shown to inhibit human myoblast differentiation [169]. It was observed that, on addition of EGF (50ng/ml) with the differentiation media, it was observed that differentiation C2C12 myoblasts was slower with lesser expression of MyoD (early differentiation marker) almost no expression of MyHC (late differentiation marker) by the 2^{nd} day of differentiation. However, there was slight decrease in the expression of Grb2 when compared to the cells without EGF stimulation. This suggests that Grb2 expression is not completely dependent on the presence of activated EGFR. It may possibly have other interactions in myoblast cells during differentiation. The localization of Grb2 was found to be quite similar to that observed in non stimulated cells with a slight difference in their localization on day2 of differentiation with a small fraction of Grb2 localizing near the plasma membrane.

5.2 Grb2 negatively regulates muscle differentiation in C2C12 cells

The role of Grb2 was still ambiguous from its expression and localization patterns in C2C12 cells. To understand its role better, knock down of the expression of Grb2 in the myoblasts was carried out using RNA interference. It was found that the differentiation of myoblasts in Grb2 knock down cells was faster compared to the control. The myotubes formed in the Grb2 knock down cells contained more nuclei than the control. This indicates that Grb2 negatively regulates muscle differentiation in C2C12 cells. Complete knockout of Grb2 has been found to be embryonically lethal [195]. In vivo, the interaction of Grb2 with c-Met has been found to be required for development of muscles [196]. According to the previous studies, we did observe that partially reducing the expression of Grb2 in C2C12 cells increased myogenic differentiation significantly

with increase in the expression of myogenic markers especially Myosin Heavy Chain which is a late differentiation marker. Subsequently, the over expression of Grb2 in C2C12 cells drastically reduced differentiation compared to the control strengthening the observation that Grb2 inhibits myogenic differentiation.

5.3 Grb2 may negatively regulate proliferation of C2C12 myoblasts

Proliferation and differentiation are two inter related mechanisms. It has been observed when differentiation need to occur, the proliferating muscle satellite cells exit the cell cycle and undergo differentiation and expression of terminal muscle genes and markers [55]. It has been observed that differentiation requires the removal of mitogens or growth factors which have been shown to increase cell proliferation. It has been observed that many growth factors such as HGF, EGF down regulate differentiation by increasing proliferation through the activation of growth factor receptors [49]. Grb2 has been shown to be involved in the inhibition of differentiation in HGF induced myoblast proliferation. However, its mechanism or role in proliferation of myoblasts has not been characterized as yet. Grb2 has however been known to play important role in cell proliferation and growth through the activation of Ras/MAPK pathway. We found that knocking down the expression of Grb2 in C2C12 myoblasts significantly increased the proliferation rate and over expressing Grb2 reduced proliferation rate but not significantly. Those observation can be explained by the fact that effect of Grb2 in cell growth and proliferation is dependent on other factors as well such as over expression of growth factor bound receptors or over expression of other components of the Ras/MAPK pathway such as Ras itself. It has been observed that without the over expression of any one of these factors Grb2 may in fact inhibit cell proliferation [197]. Hence the observation that Grb2 knock down in C2C12 cells increases cell proliferation indicates that may be other factors such as Ras or growth factors receptors are up regulated which still can activate the Ras/MAPK signaling pathway and increase cell proliferation. This is reiterated by the fact that during myoblast proliferation, mitogens such as EGF and HGF are present in excess and can activate their cognate receptors. However, in relation with the inhibition of differentiation by Grb2, it can indicate that proliferation may have a partial role to play in the increase of differentiation.

It was also hypothesized that proliferation rate of Grb2 may directly control differentiation in C2C12 cells based on the fact that inhibiting the proliferation of Grb2 by

using a proliferation inhibitor such as AraC drastically reduced the differentiation rate of the Grb2 knock down cells suggesting that the cells that could not proliferate could not exit the cell cycle and undergo differentiation. However, the reduction of proliferation in Grb2 over expressing cells did not cause as much drastic effect on differentiation potential also suggesting may be some other regulatory mechanism involving Grb2 may be controlling its inhibition of differentiation properties.

5.4 Grb2 affects migration properties of C2C12 cells

Cell adhesion and migration are important features controlling the proliferation and differentiation of myoblasts through the regulation of the actin cytoskeleton. It is widely known that during differentiation, a lot of rearrangement in the actin cytoskeleton takes place which is regulated by various factors controlling the actin nucleation machinery[198]. Rearrangement of the actin cytoskeleton is regulated by a family of actin nucleation promotion factors such as WASP family of proteins [199]. WASP and N-WASP both have been shown to interact directly with the actin cytoskeleton through the Arp2/3 complex and have been shown to be required for myoblast fusion ([104] , [200]). Both the proteins have also been shown to play important roles in cell migration through the formation of actin rich motile structures such as filopodia [101]. Also, Grb2 has been shown to bind directly to the proline rich domain of N-WASP through its C-terminal SH3 domain linking actin cytoskeleton to receptor tyrosine kinase signaling [20].

It was observed that reducing the expressing of Grb2 in C2C12 cells resulted in decrease in migration which is somewhat expected since cells that appear to attach more strongly to a substrate such as extracellular matrix has been observed to migrate slower and vice versa. Grb2 over expressing cells were found to migrate faster than the control. Cell migration result indicates that Grb2 may be inhibiting factors and proteins which regulate cell migration. Grb2 has been known to activate N-WASP from its auto inhibited conformation. In vitro experiments have shown that Grb2 increases N-WASP activity during Arp2/3 polymerisation by preferentially binding to its monomeric forms by protein based, cell independent assays [20]. It may be possible that the scenario in cell lines may be slightly different. Over expression of Grb2 in C2C12 cells may be inhibiting the conformational change in N-WASP which may be affecting its function in cell migration. However, this is just a hypothesis and many confirmatory experiments will be required to prove the same. Alternatively, Grb2 may be inhibiting some other protein entirely which

may be required for cell adhesion and migration since it's an adaptor protein and has been shown to interact with many different proteins simultaneously through its SH3 and SH2 domains.

Nonetheless, in regards to its effect on myogenic differentiation, the inability of Grb2 to assist in cell migration in C2C12 cells indicates that improper migration of Grb2 overexpressing cells may be affecting the myoblast alignment and elongation that is required for it to interact with each other and initiate the process of fusion. In the absence of proper cell migration, the later process of differentiation may be greatly affected which may be the reason for the inhibition of differentiation by Grb2.

Markers of focal adhesion such as vinculin and Paxillin were also found to be affected by Grb2 which reiterates the possibility that Grb2 may play an important role in controlling the aspects of cell spreading and adhesion in C2C12 myoblasts. Vinculin is an important protein of the focal adhesion complex which is a multi protein complex system whose continuous assembly and disassembly has been shown to control cell adhesion and migration. Vinculin is a commonly used marker protein used to study focal adhesion complex assembly. Vinculin has been shown to directly bind to the Arp2/3 complex linking the focal adhesions to the actin cytoskeleton [74]. It was observed that the localization of vinculin at the cell periphery in the form of streaks or patches was less in the Grb2 over expressing cells compared to the control. This observation was in support of the cell migration result which suggests that for proper cell migration, assembly and disassembly of focal adhesion complexes at the cell periphery is required. In the absence of proper assembly of focal adhesion complex, the myoblasts cannot align and differentiate. Another focal adhesion protein called Paxillin was also studied. Paxillin also is a part of the focal adhesion complex and has been shown to play an important role during migration as observed in case of some highly metastatic cell lines [180]. Knockdown the expression of Paxillin has been found to reduce cell motility [180]. However, the expression of paxillin was found to be quite low as well as the localization of Paxillin at the focal adhesions near the cell periphery was not very conclusive. Hence the pro or anti migratory function of Paxillin in Grb2 mediated inhibition of cell migration could not be concluded.

Interestingly, it was observed that for Grb2 knockdown cells, even though the migration rate was lower, the differentiation rate was much faster than the

control. This is quite unexpected since, ideally, the myoblasts that migrate slower may generally form lesser cell-cell contact and may therefore differentiate slower. However, it has been previously reported that inhibition of cell migration can lead to increased cell fusion [82]. One mechanism could be through the changes in cell-cell adhesion affecting the expression and the localization of cell adhesion molecules. Due to increased migration, stable cell-cell contacts may be affected which is required for proper alignment of myoblasts for differentiation.

5.5 Interaction of Grb2 with N-WASP may be responsible for inhibition of differentiation in myoblasts

Interaction of Grb2 through its two SH3 and one SH2 domains play important roles in its downstream signaling mechanisms. The SH2 domain of Grb2 has been found to bind to the tyrosine phosphorylated residues of various upstream growth factor receptors such as EGFR, PDGFR , FGFR etc and lead to the activation of various downstream signaling mechanisms [4]. Binding of Grb2 to activated EGFR at Tyr 1068 gas been shown to lead to its association with Sos, translocation to the plasma membrane and activation of Ras and its downstream signaling pathways [42]. The N and the C-terminal SH3 domains have been shown to number a number of proline rich proteins such as dynamin, N-WASP, Abl, Shc, synapsin etc [3]. However, the strongest binding affin ity of Grb2 is with GEF, Sos. It has been reported that Grb2 exists in a complex with Sos in the cytoplasm in its resting state and on growth factor receptor activation translocates to the plasma membrane to activate Ras/MAPK signaling. However this may not be entirely true since Grb2 has also been shown to participate in various other pathways independent of Sos. In fact excess of Grb2 has been shown to inhibit Ras signaling indicating that compartmentalization of Grb2 is required where it may be complexed to and interacting with other proteins [3]. Sos has been shown to have stronger affinity for the N-terminal SH3 domain of Grb2 compared to the C-terminal domain. The C-terminal SH3 domain of Grb2 has been found to interact with the proline rich domain of N-WASP and affect the N-WASP induced Arp2/3 activity and link the actin cytoskeleton to the receptor tyrosine kinase signaling [20].

Three point mutants of Grb2 were used in the study. The three point mutations were made at specific residues (reported to interact) in the 3 domains of Grb2. The N-SH3 mutant used was P49L (Proline⟶ Leucine substitution at the 49^{th} amino acid position).

This mutation has been reported to cause loss of function phenotype in Drosophila and also affect the binding of Grb2 to Sos [9]. The $Grb2^{R86K}$ mutant (Arginine → Lysine substitution at the 86^{th} amino acid position) was found to affect the interaction of Grb2 with the phosphotyrosine residues in general and the $Grb2^{P206L}$ mutant (Proline→ lysine substitution at the 206^{th} position) has been specifically found to inhibit interaction of Grb2 with N-WASP [20].

Grb2 has been known to interact with a number of proteins. The rationale was choosing the above three point mutants to study its mechanism was that mutant studies using the above three point mutations have been able to characterize important functions of Grb2 in various signaling pathways. Although Grb2 interacts with various proteins such as Shc, EGFR, synapsin, Sos, N-WASP, Abl to name a few, none of them have been specifically characterized for their role in muscle differentiation so far. The only role of Grb2 with respect to muscle differentiation has been shown to be in relation with c-Met (HGF receptor) with no specific importance on any of its domain involvement [49]. Among the known interactors of Grb2, N-WASP has been shown to be required for muscle differentiation in mice [104]

It was observed that, on differentiation, both the P49L and R86K mutants of Grb2 showed similar differentiation defect as the Grb2 over expressing C2C12 cells. This observation could not be attributed to poor expression. However, the P206L mutant of Grb2 was able to increase the myotube formation in C212 cells close to the control and significantly more than the wild type Grb2 overexpressing cells. This suggests that the P206L mutant of Grb2 which cannot bind to N-WASP can behave like the control indicating that the interaction of Grb2 with N-WASP may be responsible for inhibiting differentiation in C2C12 cells.

The $Grb2^{R86K}$ mutant was found to partially increase the differentiation in C2C12 cells as the myotube formation was found to be lower than the $Grb2^{P206L}$ mutant but higher than the $Grb2^{P49L}$ and wild type Grb2 overexpressing cells. This correlates with the proliferation defect observed in the Grb2 knockdown C2C12 cells. The R86K mutant, as mentioned, cannot interact with phosphotyrosine residues, in this case, could be the upstream growth factor receptor tyrosine residues, because of which the proliferation could be reduced and differentiation could be increased. But the effect may be partial, since the increase in differentiation is not as significantly high as the P206L mutation.

Hence, since Grb2 is an adaptor protein, it can interact with more than one protein at a particular time and affect more than one downstream signaling mechanism to control myogenic differentiation.

Localization of Grb2 has been observed to be in the cytoplasm in resting cells. Stimulation by growth factors may cause the protein to be translocated to the plasma membrane. It was observed that the localization of Grb2 was in the cytoplasm in C2C12 cells as well along with its two mutants- $Grb2^{R86K}$ and $Grb2^{P206L}$. However, the $Grb2^{P49L}$ mutant, which is known to inhibit the binding of Sos 1, was found to localize in the cytoplasm indicating that may be the binding of Grb2 to Sos1 keeps it in the cytoplasm. In the absence of it, it may serve some function in the nucleus. Since the P206L mutant of Grb2 was found to rescue the differentiation inhibition caused by the wild type Grb2 over expressing C2C12 cells, the colocalization of Grb2 and N-WASP was also studied. It was observed that the in the presence of Grb2, N-WASP GFP localized in the cytoplasm, although in the resting cells, it is known to localize in the nucleus. $Grb2^{P49L}$ and $Grb2^{R86K}$ mutant, when co transfected with N-WASP, no difference in the localization of N-WASP was observed. This suggests that may be the interaction of Grb2 with N-WASP causes it to localize in the cytoplasm in C2C12 cells. However, in the presence of $Grb2^{P206L}$ mutant, N-WASP was localized in the nucleus since the mutant is known to inhibit the interaction of Grb2 with N-WASP. This suggests that there is definitely a difference in the localization in N-WASP in the presence of WT-Grb2 and $Grb2^{P206L}$ and this difference in the localization of N-WASP may be partly responsible for the inhibition of differentiation mediated by Grb2.

Furthermore, exogenous expression of full length N-WASP in Grb2 overexpressing cells was carried out to examine whether the differentiation inhibition mediated by Grb2 is rescued by N-WASP. It was observed that the reduction in differentiation on Grb2 overexpression increased in the presence of WT-N-WASP bringing it close to the control. This indicates that Grb2 mediated inhibition of differentiation is indeed rescued by N-WASP. Grb2 is known to be present upstream of N-WASP regulating the stability of N-WASP [20]. It is possible that Grb2 may be inhibiting the localization of endogenous N-WASP, probably, near the cell-cell junction preventing muscle cell fusion. Presence of exogenous N-WASP in Grb2 overexpressing C2C12 cells must be rescuing the defect mediated by Grb2 and increasing differentiation potential comparable to the control. Hence, delineating the mechanism regulating the interaction

between Grb2 and N-WASP which probably, not only affects the localization of N-WASP but also the expression of downstream muscle markers controlling terminal differentiation will be the main focus of future studies.

Role of GRB2 in epithelial-to-mesenchymal transition (EMT) in A549 cells

Epithelial-to-mesenchymal transition (EMT) is an important physiological mechanism controlling not only the cancer metastasis and tumor progression but also normal physiological processes such as embryogenesis, tissue repair and organ development [114] EMT is an orchestrated series of molecular, genetic and cellular changes that take place when highly polarized epithelial cells undergo loss of polarity and lose cell-cell junctions, become elongated, more migratory and invasive and acquire mesenchymal phenotype. The reasons cancer cells undergo EMT is to metastasize from the primary site of tumor growth, move through the vascular system and infect distant organs and tissues [113]. Various factors have been shown to be responsible to inducing EMT in cells such as EGF, hypoxia, TGF-β etc. TGF-β is one of the potent inducer of epithelial-to mesenchymal transition in many cell types[201]. Interestingly, it is also known to act as tumor suppressor in many normal epithelial cell lines. Over expression of TGF-β in epidermis has been shown to reduce proliferation in keratinocytes and prevent tumor formation [201]. TGF-β has also been found to be over expressed in many tumor types. Hence the dual role of TGF-β as pro or anti cancer causing agent makes it an interesting candidate to study the changes and the downstream signaling mechanisms mediated by it

GRB2 has been found to be over expressed is many tumors such as breast cancer along with EGF receptor and some other components of the Ras signaling pathway [186]. Due to its proximity to many other growth factor receptors as well, it has been found to be over expressed in many metastatic and non metastatic cell lines. Since it is also known to interact with the proteins regulating the actin cytoskeleton, many of which have also been implicated in tumor growth, GRB2 SH2 domain antagonists have been shown to reduce cell migration and cancer metastasis in many animal models. Due to its wide range of interacting partners, it has been shown to be involved or affect many aspects of tumor progression such as cell adhesion, ECM remodelling, cell invasion, cell motility, tumor angiogenesis etc, [48] it becomes an interesting protein to be studied in EMT. In fact, studies on breast cancers have revealed that GRB2 is required for inducing EMT in mammary epithelial cells by TGF-β. Not much is known about the role of GRB2 in EMT in lung cancer except for the fact that over

expression of GRB2 has been observed in lung cancers as well. Hence the focus of this study is to identify the role of GRB2 in non metastatic non small cell lung cancer cell line such as A549.

5.6 Expression and localization of GRB2 in a549 cells was found to change after TGF-β stimulation

The expression and localization of GRB2 in A549 cells after TGF-β stimulation was studied. EMT was found to be induced after 48 hrs of incubation of the A549 cells in the presence of 5 ng/ml of TGF-β. EMT was verified by the decrease of epithelial marker, E-Cadherin and the up regulation of mesenchymal marker, vimentin. At the above mentioned conditions of EMT, it was observed that GRB2 expression had significantly increased in TGF-β treated cells compared to the control. Interestingly, we found that at slightly lower (2ng/ml) or higher (10 ng/ml) concentrations of TGF-β, the levels of GRB2 was less compared to the one observed at 5 ng/ml. This indicates that the effect of TGF-β on GRB2 expression may be dose dependent. This is an observable phenomenon wherein TGF-β concentration has been shown to affect Smad signaling kinetics [188].

Since GRB2 expression was consistently found to be higher at 5 ng/ml of TGF-β, it was used for all further experiments. The localization of GRB2 was also found to change after TGF-β stimulation in A549 cells. In the control cells, GRB2 was found to be present in the nucleus significantly along with also being dispersed in the cytoplasm. After TGF-β stimulation and induction of EMT, it was found to move out of the nucleus towards the cytoplasm and the plasma membrane in response to TGF-β. The localization changes of GRB2 indicated towards its active role in regulating changes induced during EMT. It has been previously reported that GRB2 localizes in the nucleus at a higher proportion in breast tumors [186] **.** Though no specific role of GRB2 in the nucleus has been characterized so far, it , may be to regulate, either positively or negatively, directly or indirectly, the expression of genes controlling the initiation of EMT. After TGF-β stimulation, it may be playing more important role in the cytoplasm which may make it to move out of the nucleus. TGF-β receptor has been shown to interact indirectly with GRB2 through Shc and other tyrosine kinases as well as directly through its phosphorylated tyrosine residue Tyr284 in mammary epithelial cells.[46]**.**

5.7 GRB2 is required for epithelial to mesenchymal transition in A549 cells

The next step was to knockdown or overexpresses GRB2 in A549 cells and study its effect on EMT. Overexpression of GRB2 in A549 cells caused the cells to undergo EMT faster than the control. Most importantly, the expression of E-Cadherin, epithelial marker was found to be significantly reduced in GRB2 ove expressing cell compared to the control, even before TGF-β stimulation indicating that increase in the expression of GRB2 causes de regulation in E-Cadherin and can cause the switch to EMT faster in the presence of TGF-β. Knocking down the expression of GRB2 did not cause much difference to the E-Cadherin expression and the EMT was similar to the control indicating that the changes due to reduction in the expression of GB2 may be compensated by other proteins that interact with GRB2. Since GRB2 can interact and form complex with a lot of proteins, it is highly likely that the downstream changes due to the absence of GRB2 may be compensated by other proteins.

5.8 GRB2 affects migratory and invasive properties of A549 cells

Cell migration is an important phenomenon during tumor metastasis in cancer cells. The epithelial cells during EMT display increased motility which helps them to invade the vascular system and metastasize to distant organs. The mesenchymal phenotype is linked with the capacity of cells to migrate to secondary organs and maintain stemness, allowing them to subsequently differentiate in to different cell types during development and initiation of metastasis [110]. Cell migration was found to be significantly affected in GRB2 knock down cells indicating that GRB2 is required by the A549 cells to migrate. This observation also strengthens the role of GRB2 in EMT in A549 cells supporting the fact the GRB2 increases cell motility in A549 cells. The other markers of cell migration and cell adhesion were found to be affected by GRB2 knockdown and overexpression in A549 cells as well. Expression of Paxillin and its phosphorylated form has been found to indicator of cell motility in highly metastatic cell lines [180]. We observed that the localization of Paxillin at the focal adhesions in the GRB2 knockdown A549 cells was significantly lesser than the control. However no significant difference in the localization of Paxillin between the Grb2 over expressing cells and the control was observed. GRB2 knockdown A549 cells, thus, migrated slower and expressed lesser Paxillin streaks at the focal adhesions indicating that cell migration is significantly affected in the GRB2 knockdown cells which may also be the

consequence of the E-Cadherin expression and localization still observed in these cells, unlike the GRB2 overexpressing A549, or for that matter, control cells.

Vinculin localization at the focal adhesions is a commonly used marker to study cell adhesions. It was observed that the number of vinculin patches per cell were reduced in GRB2 overexpressing cells and significantly more in the GRB2 knockdown cells compared to the control indicating that the focal adhesions are more in the GRB2 knockdown cells which also shown decreased cell motility. Hence, the decreased cell migration and increased focal adhesions may be the reason the GRB2 knockdown cells cannot lose cell polarity and less reduction of E-Cadherin expression is observed compared to the control and GRB2 over expressing A549 cells.

Tumor invasion is a critical step in metastasis and cancer progression. The ability of cancer cells to break down the ECM barrier and invade to distant organs gives them an advantage over normal cells to metastasize and spread cancer. The effect of GRB2 on the invasive property of A549 cells was studied by carrying out cell invasion assay. It was observed that the GRB2 knockdown reduced the number of cells invading the ECM barrier to a significant extent whereas GRB2 overexpressing cells increased the invasive capability of A549 cells indicating that GRB2 increases the invasive properties of A549 cells and may be responsible for inducing EMT faster in A549 cells.

5.9 GRB2 may localize with Focal Adhesion Kinase, after TGF-β stimulation and influence EMT

Focal Adhesion Kinase (FAK) is another focal adhesion protein which has been found to colocalize with the integrin receptors upon binding of integrin to extracellular matrix proteins such as fibronectin. Integrin/FAK pathway has been shown to activate many downstream signaling mechanisms required for cell adhesion and migration in many cell types [48]. FAK function is regulated by phosphorylation at various tyrosine residues in its sequence which can occur by stimuli such as activation by integrins as well. Phosphorylation of FAK forms docking sites for many proteins one of which is GRB2. GRB2 has been shown to bind to the phosphorylated Y925 residue of FAK [48] FAK has been found to be over expressed in many tumor types and interestingly, in colon cancer, specific phosphorylation at Y925 residue of FAK has also been observed indicating some co relation between FAK and GRB2 interaction and localization during cancer [48]

We tried to study the localization of GRB2 and FAK before and after TGF-β stimulation. We found that before TGF-β stimulation in the control cells, GRB2 and FAK localization was dispersed throughout the cytoplasm with no specific co-localization. Also, after TGF-β stimulation, along with localizing in the cytoplasm FAK was also found at the cell periphery as patches. Interestingly, GRB2 was specifically not found to localize at the cell periphery in these cells. In GRB2 overexpressing cells, before TGF-β stimulation, however, GRB2 and FAK were found to co localize at the cell periphery to some extent with GRB2 localization mostly at the cell membrane. Most importantly, after TGF-β stimulation, GRB2 and FAK were specifically found to co localize at the cell membrane indicating there is some interaction taking place between them after activation by TGF-β which may also be regulating the function of GRB2 during EMT.

5.10 Transcription factor, Snail, may be responsible for the loss of E-Cadherin expression observed in GRB2 overexpressing A549 cells

Transcription factor Snail is a known inducer of EMT and known to directly reduce E-Cadherin expression in many epithelial cell types. We wanted to study if GRB2 may be involved in the regulation of E-Cadherin expression mediated by Snail. We checked for the expression of Snail mRNA and protein levels in GRB2 over expressing cells before and after TGF-β stimulation. We found that even before TGF-β stimulation, Snail expression was observed in GRB2 overexpressing cells as compared to the control. This indicates that Snail is up regulated in GRB2 overexpressing A549 cells, both at the transcription and protein level and may be responsible for the loss of E-Cadherin expression observed in GRB2 overexpressing cells.

5.11 C-terminal SH3 domain of GRB2 is critical for increase in invasion of A549 cells mediated by GRB2

Invasion is an important biological process that tumor cells utilize to move through the extracellular matrix and invade to distant tissues and organs. Invasion has also been found to increase in cells undergoing EMT. GRB2 was found to significantly increase invasion of A549 cells. The three point mutants of GRB2 were then studied to identify the domain which is critical for the increase in invasion mediated by GRB2. It was found that the number of cells invading through the matrigel reduced quite significantly in $GRB2^{P206L}$ mutant expressing A549 cells and the quantification showed it to be similar to the control. $GRB2^{P49L}$ and $GRB2^{R86K}$ mutant showed no significant reduction in the invasion ability of

A549 cells when compared to the GRB2 overexpressing cells. This suggests that GRB2 causes increase in the invasive ability of A549 cells through its C-terminal domain.

So far, it was observed that GRB2 overexpressing A549 cells display a significant reduction in the level of E-Cadherin expression in non stimulated conditions, before TGF-β addition. Grb2 lies well upstream in intracellular signaling pathways. It has also been shown to interact with TGF-βRII and play a role in EMT in mammary epithelial cells [46]. However, no role of Grb2, absence of growth factor stimulation, has been reported so far. Here, reduction in E-Cadherin protein levels and increase in Snail mRNA and protein levels was observed in GRB2 overexpressing cells under non stimulated conditions suggesting that GRB2 may independently regulating the processes leading to EMT. Preliminary experiments on localization of GRB2 indicate that in GRB2 overexpressing cells, the localization of Grb2 is prominently more in the nucleus compared to the control. This corroborates the fact that GRB2 may directly be contributing to the regulation of E-Cadherin and Snail expression by localizing in the nucleus. Conventionally, Grb2 has been known to localize in the cytoplasm. However, it has recently been reported to be present in the nucleus as well of normal and tumor breast tissue sample [186]. Hence, there is a strong possibility of GRB2 playing an as yet unidentified role in nucleus in lung cancer cells. Presently, confirmatory experiments such as biochemical fractionation and localization studies are being done to validate the hypothesis.

Also, A549 lung adenocarcinoma cell line, though widely used to study EMT, has also been known for its heterogeneity and plasticity. It expresses certain mesenchymal markers such as vimentin and N-Cadherin, even before TGF-β stimulation making it a heterogenous cancer cell line. Hence, to validate the significant role of GRB2 observed in A549 cells, another homogenous NSCLC cell line H358, which does not express vimentin before TGF-β stimulation, is being optimized and studied.

FUTURE DIRECTIONS

1. **Generation of Grb2 hypomorphic alleles in mice to study the role of Grb2 in muscle development *in vivo:***

The mammalian Grb2 adaptor protein binds to the phosphorylated tyrosine residues of activated receptor tyrosine kinases (RTKs) and links it to the activation of Ras MAPK pathway through its binding to the Sos1 protein [26]. RTK-Grb2 signaling influences many biological processes *in vivo* during development. It was observed that $Grb2^{-/-}$ mice die early during development due to defect in endoderm differentiation [26] preventing the study of Grb2 later in the development. To overcome the severity of Grb2 null mutation, a hypomorphic allele of mouse Grb2 gene was generated by substituting the glutamate amino acid at 89^{th} position with lysine and intercrossing the mice heterozygous for the null and hypomorphic (E89K) alleles [26]. The resultant heterozygous E89K/Δ embryos survived but still showed several abnormalities such as defect in cardiovascular development making the study of Grb2 functions *in vivo* possible. These hypomorphic Grb2 mutant mice could be used to study muscle differentiation. Primary cells, if possible, could be isolated from these mice and the expression pattern and the inhibitory role of Grb2, as observed in C2C12 cells, could be verified in the primary myoblasts as well. Likewise, conditional knockout mice can also be used to study the role of Grb2 in muscle using a LoxP/Cre recombinase system and creating knockout specifically in the skeletal muscles using MLC-Cre or MyoD-Cre mice.

2. **Fluorescence Resonance Energy Transfer (FRET) to study the interaction between Grb2 and N-WASP during the differentiation inhibition in C2C12 myoblasts**

Grb2 is known to interact with the proline rich domain of N-WASP through its C-terminal SH3 domain [20] to stimulate N-WASP and increase actin polymerisation. In this study, it was observed that Grb2 inhibits muscle differentiation in C2C12 myoblasts probably through its interaction with N-WASP. It is also known that N-WASP is required for muscle fusion, especially terminal fusion, in mice [104]. The possible mechanism could be that the inhibition of differentiation by Grb2 could be through N-WASP. To study their interaction in myoblast, fluorescence Resonance Energy Transfer (FRET) could be used. FRET is a useful tool to study the

intermolecular interactions when proteins are present within 10nm distances of each other and the effects of conformational changes on these distances. Both Grb2 and N-WASP could be tagged to fluorescent proteins close enough in the wavelength spectrum but still having emission and excitation wavelength separated enough for the energy transfer to occur such as GFP and its spectrally distinct mutant BFP (Blue Fluorescent Protein). If, during terminal differentiation conditions, Grb2-BFP (donor) and N-WASP GFP (acceptor) physically interact, then increased intensity would be observed at the N-WASP GFP emission maximum when the complex is excited at the maximum absorbance wavelength of the donor. Failure of Grb2 and N-WASP to interact would result in no emission by N-WASP GFP. This study could give a closer idea regarding the mechanism of differentiation inhibition caused by Grb2 and mediated by N-WASP.

3. **To study the role of Grb2 in the nucleus of C2C12 and primary myoblasts:**

Grb2 has been earlier reported to be present in the nucleus of both normal and tumor human breast tissue [186] but its function in the nucleus has not been characterized so far. In this study, it was observed that Grb2 localizes in the nucleus of C2C12 myoblast before initiation of differentiation as well as in the nucleus of A549 NSCLC cell line before TGF-β stimulation. Role of Grb2 in the nucleus of C2C12 cells and primary myoblasts can be characterized which could give a better understanding of its localization and function at the beginning of differentiation. Isolation of nuclear fractions in particular and checking the expression pattern of Grb2 in C2C12 cells and primary myoblasts and immunohistochemical detection of Grb2 along with nuclear markers such as Ki67 and Proliferating Cell Nuclear Antigen (PCNA) could be carried out.

4. **To study the downstream signaling mechanism regulated by GRB2 in A549 cell, after TGF-β stimulation:**

GRB2 overexpression in A549 cells was found to accelerate the process of TGF-β induced Epithelial-to-Mesenchymal Transition (EMT) in A549 cells. Initiation of TGF-β induced EMT is correlated with activation of Smad dependent and independent signaling mechanisms [45]. GRB2 has been found to be involved in the Smad independent pathway with the formation of ternary complex of GRB2/Shc/Sos protein in mammary epithelial cells. Studying which of the

downstream signaling pathways-Erk/MAPK, p38MAPK or Smad pathway activated by GRB2 over expression in A549 NSCLC cells could give a better understanding regarding the mechanism of regulation by GRB2 during EMT.

5. **To study the interaction of TGF-β receptors with GRB2 in A549 cells:**

GRB2 has been shown to bind to activated receptor tyrosine kinases through its SH2 domain. Although TGF-β receptors are serine/threonine kinases, their activation and downstream signaling effector molecules show similarities to those activated by RTKs [46]. It has been reported that GRB2 can form complexes with phosphorylated Tyr284 residue of TGF-βRII, on TGF-β stimulation, in NMuMG breast cancer cells [46]. However, the domain of GRB2 responsible for this interaction has not been characterized yet. Deletion constructs of the three domains of GRB2 could be made and their interaction with the TGF-β receptors, in the presence or absence of TGF-β, could be studied by immunoprecipitation. In this study, we observed that changes in the E-Cadherin expression takes place in GRB2 over expressing cells before TGF-β stimulation in A549 cells. Hence, studying the interaction of GRB2 with TGF-β receptors before activation will give a better and probably novel understanding about the regulation of signaling pathways mediated by adaptor proteins such as GRB2.

6. **To study the mechanism linking the expression of E-Cadherin to transcription factor, Snail, mediated by GRB2:**

In this study, we observed that, in GRB2 overexpressing A549 cells where the reduction of E-Cadherin expression was observed, the level of transcription factor, Snail was high. Snail has been shown to repress E-Cadherin expression and induce EMT [161]. However, regulation of E-Cadherin expression by Snail has not been shown to be mediated by Grb2 so far. Snail, being a transcription factor, represses E-Cadherin expression by binding to specific elements in the E-Cadherin promoter. Hence, the role of Grb2 in Snail mediated repression of E-Cadherin expression could be studied at the DNA level by characterizing its promoter and locating specific DNA sequences which could be potential targets for Snail. Chromatin Immunoprecipitation (ChIP) could also be carried out to study interaction between Snail and Grb2 promoter.

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APPENDIX I :

List of plasmids cloned for this study:

pEFCGYIPEBx Grb2^{P49L} Myc	Grb2^{P49L} Myc under CMV promoter cloned into episomally replicating plasmid, selection marker- puromycin
pEFCGYIPEBx Grb2^{R86K} Myc	Grb2^{R86K} Myc under CMV promoter cloned into episomally replicating plasmid, selection marker- puromycin
pEFCGYIPEBx Grb2^{P206L} Myc	Grb2^{P206L} Myc under CMV promoter cloned into episomally replicating plasmid, selection marker- puromycin
pFIV cop CMV Grb2 RFP	Grb2 RFP cloned under CMV promoter, no selection marker
pFIV cop CMV Grb2^{P49L}-RFP	Grb2^{P49L}-RFP cloned under CMV promoter, no selection marker
pFIV cop CMV Grb2^{R86K}-RFP	Grb2^{R86K}-RFP cloned under CMV promoter, no selection marker
pFIV cop CMV Grb2^{P206L}-RFP	Grb2^{P206L}-RFP cloned under CMV promoter, no selection marker
pFIV cop CMV Grb2 GFP	Grb2 GFP cloned under CMV promoter, no selection marker
pFIV cop CMV Grb2^{P49L}-GFP	Grb2^{P49L}-GFP cloned under CMV promoter, no selection marker
pFIV cop CMV Grb2^{R86K}-GFP	Grb2^{R86K}-GFP cloned under CMV promoter, no selection marker
pFIV cop CMV Grb2^{P206L}-GFP	Grb2^{P206L}-GFP cloned under CMV promoter, no selection marker
pEFCG Puro mouse Grb2shRNA	Mouse specific Grb2shRNA under U6 promoter cloned into episomally replicating plasmid, selection marker- puromycin
pLJM Grb2 His	His tagged Grb2 cloned under CMV promoter Can be used to make lentivirus or transfected and selected
pLJM Grb2^{P49L} His	His tagged Grb2^{P49L} cloned under CMV promoter Can be used to make lentivirus or transfected and selected
pLJM Grb2^{R86K} His	His tagged Grb2^{R86K} cloned under CMV promoter Can be used to make lentivirus or transfected and selected

pLJM Grb2^{P206L} His	His tagged Grb2^{P206L} cloned under CMV promoter Can be used to make lentivirus or transfected and selected
pLJM human Grb2shRNA	Human specific Grb2 shRNA cloned under U6 promoter Can be used to make lentivirus or transfected and selected

Appendix II :

List of shRNAs used in this study:

sh RNA	**shRNA SEQUENCE (5' – 3')**
Mouse Grb2 shRNA	CCCTTGGAGAAAAGCCTTGTTTGaacatccgtgtccaggaaccaTTCAAGAGAtggttcctggacacggatgttTTTTTGGATCCG
Human Grb2 shRNA	hGrb2-R1 ataCTCGAGtatgtcccgcaggaatatctgCCGGTGTTTCGTCCTTTCCAC hGrb2-R2 cggaattcgtcgacCAAAAAcagatattcctgcgggacataCTCGAGtatgtcccgc

Appendix III :

List of mutant primers used in this study:

Grb2 mutants	**Mutant SEQUENCE (5' – 3')**
WT-Grb2	Forward Primer: 5'CATGCCATGGAAGCCATCGCCAAATATGAC'3 Reverse Primer:

	5’CGCGGATCCGACGTTCCGGTTCACG GGGGTGACATAATTGCGGGGAAAC’3
$Grb2^{P49L}$	Forward primer: 5’AAAGACGGCTTCATTCTC AAGAACTACATAGAA’3 Reverse Primer: 5’TTCTATGTAGTTCTTGAGAATGAAG CCGTCTTTT’3
$Grb2^{R86K}$	Forward Primer: 5’GGGGCCTTTCTTATCAAAGAGAGTG AGAGCGCT’3 Reverse Primer: 5’AGCGCTCTCACTCTTTTGATAAGAA AGGCCCC’3
$Grb2^{P206L}$	Forward Primer 5’CAGACCGGCATGTTTCTCCGCAATT ATGTCACC’3 Reverse Primer: 5’GGTGACATAATTGCGGAGAAACATG CCGGTCTG’3

Appendix IV :

List of RT-PCR primers used in this study:

Primer name	**Primer Sequence (5’ – 3’)**
Snail	Forward primer 5’-TCGGAAGCCTAACTACAGCGA-3’ Reverse Primer 5’-AGATGAGCATTGGCAGCGAG-3’
MRPL	Forward Primer

	5’CTGGTGGCTGGAATTGACCGCTA 3’ Reverse Primer 5’CAAGGGGATATCCACAGAGTACCTT G 3’

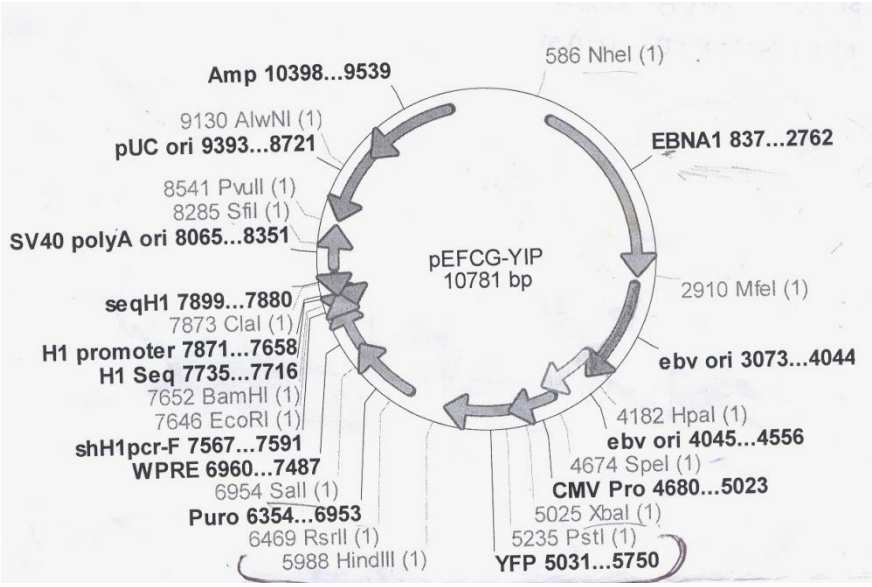
APPENDIX V

Vector Map of plasmids used in the study:

pEFCGYIP Grb2 Myc

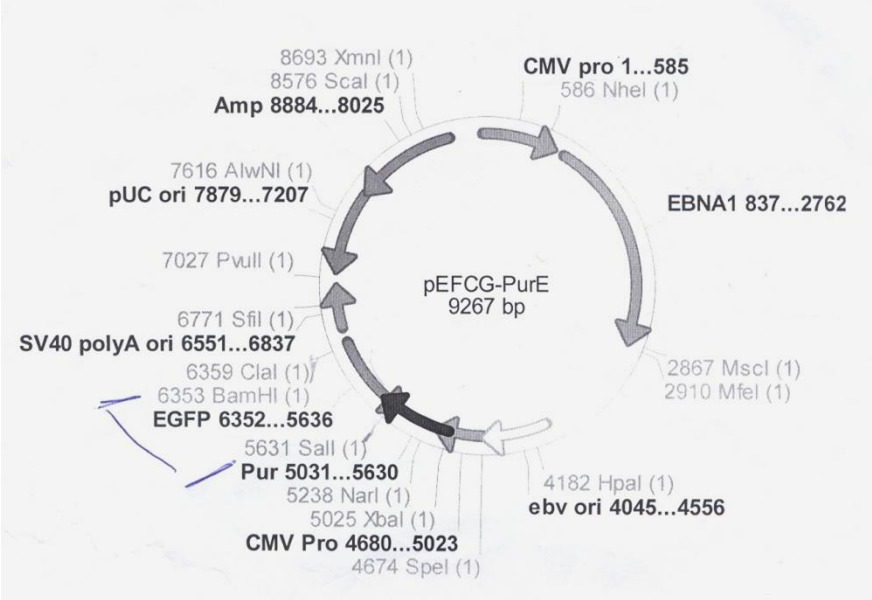
Grb2 Myc under CMV promoter

Used for over expression of Grb2 in C2C12 cells using microporation



pEFCG Puro E mouse Grb2 shRNA

Used for Knock down of Grb2 in C2C12 using microporation.



pLMJ Grb2 His/ human Grb2 shRNA

Used to make lentivirus and infection of Grb2 shRNA and Grb2His constructs in A549 cells