THE EFFECT OF CDK1 MEDIATED GOLGI VESICULATION ON MITOTIC PROGRESSION IN MAMMALIAN CELLS

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Table of Contents

Acknowledgements i

Table of Contents iii

List of Figures vi

List of Tables viii

List of Abbreviations ix

Summary xi

Chapter 1: Introduction 1

1.1 Cell division; the basis for cell multiplication and life on earth 1

1.2 The Golgi apparatus 2

1.2.1 Golgi structure and inheritance 2

1.2.2 Golgi ribbon undergoes severing during G2 phase 4

1.2.3 The mechanism of Golgi vesiculation 7

1.2.3.1 The reason for Golgi vesiculation 7

1.2.3.2 The role of GM130 in COPI- dependent Golgi vesiculation 8

Chapter 2: Materials and methods 12

2.1 Cell line 12

2.2 Reagents

2.2.1 Solutions 12

2.2.2 Drugs 12

2.2.3 Antibodies 12

2.3 Cell culture 14

2.3.1 Culture conditions 14

2.4 Molecular biology 15

2.4.1 E.coli strain used and plasmid amplification strategy 15

2.4.2 Plasmid construction 15

2.4.3 ShRNA 16

2.5 Plasmid transfection 16

2.6 Microscopy 17

2.6.1 Sample preparation for live imaging 17

2.6.2 Sample preparation for immunofluorescence 17

2.6.3 Image acquisition 18

2.6.4 Image analysis 18

2.6.4.1 Quantification of fluorescence intensity 18

Chapter 3: Results 20

3.1 The mammalian Golgi apparatus undergoes dynamic changes during mitosis 20

3.2 Purvalanol A treatment affects Golgi vesiculation and mitotic progression 22

3.3 Purvalanol A treatment abolished GM130 phosphorylation 24

3.4 Over expression of GM130 does not affect Golgi dynamics or mitosis 27

3.5 ShRNA against GM130 successfully depleted GM130 29

3.6 Depletion of GM130 does not affect Mitotic progression 30

3.7 GM130 over expression does not affect mitotic progression or Golgi vesiculation 31

Chapter 4: Discussion 34

4.1 Golgi dynamics during mitosis is dependent on cdk 1 34

4.2 Perturbing GM130 does not modulate Golgi vesiculation or mitosis 36

Chapter 5: Conclusion 43

Chapter 6: References 44

List of figures

Figure 1.1	The Golgi undergoes dramatic morphological changes during mitosis	4
Figure 1.2	COP 1 vesicle tethering under interphase and mitotic conditions	9
Figure 3.1	Golgi undergoes dynamic changes during mitosis	21
Figure 3.2	Golgi vesiculation and mitotic progression were affected in purvalanol A treated cells	23
Figure 3.3	Phosphorylation of GM130 S25 was abolished in purvalanol-A treated cells	25
Figure 3.4	Golgi dynamics was unaffected by GM130 over expression	28
Figure 3.5	ShRNA against rat GM130 depleted endogenous GM130 efficiently	30
Figure 3.6	Depletion of GM130 did not affect mitotic progression	31
Figure 3.7	Over expression of S25A GM130-tomato in cells depleted of GM130 does not affect Golgi vesiculation	33
Figure 4.1	Schematic representation of the hypothesised regulation of mitotic Golgi vesiculation by GM130	39

List of tables

Table 1.1	List of kinases involved in regulating Golgi structure and function	6
Table 2.1A	Primary antibodies used in this study	13
Table 2.1B	Secondary antibodies used in this study	13

List of abbreviations

BARS	Brefeldin A (BFA) Adenosine Diphosphate–Ribosylated Substrate
CDK	Cyclin Dependent Kinase
CGN	Cis Golgi Network
COP1	Coat Promoter 1
DNA	Deoxyribonucleic acid
EDTA	Ethylene Di amine Tetra acetic Acid
ERK	Extracellular signal Regulated Kinase
ER	Endoplasmic reticulum
F12K	Kaighn's modified F12
GalT	Galactosyltransferase
GFP	Green fluorescent Protein
GRASP65	Golgi Reassembly Stacking Protein
HELA	Henrietta Lacks
HBBS	Hank's balanced salt solution
KDa	Kilo Dalton
LB	Luria Bertani
MEK 1	Mitogen Activated Kinase 1
MGC	Mitotic Golgi Clusters
MTOC	Microtubule Organizing Centre
NC	Negative Control
NRK	Normal Rat Kidney
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
RNA	Ribo Nucleic Acid
ShRNA	Short Hairpin RNA
STE	Sodium Tris EDTA
TGN	Trans Golgi Network

Summary

The mammalian Golgi consists of hundreds of Golgi stacks interlinked to form a single ribbon structure in the peri-nuclear area. During mitosis, the Golgi undergoes two sequential fragmentation steps to break from ribbon to individual stacks, then from stacks to vesicles and tubules. The first fragmentation step is mediated by phosphorylation of the Golgi matrix protein GRASP65 and it has been shown to be essential for G2-M transition. To understand if the second, vesiculating step might be involved in mitotic progression, we looked at GM130, a Golgi matrix protein mitotically phosphorylated by Cdk1 and thought to be essential for the mitotic Golgi vesiculation. When NRK cells were treated with the Cdk1 inhibitor purvalanol A, Golgi vesiculation was blocked and organisation of the mitotic spindle was affected and mitotic progression was delayed. Over expression of a GFP fusion to a GM130 phosphorylation mutant (S25A) had no apparent effect on Golgi vesiculation and mitotic progression. Also, depletion of GM130 showed no effect on cell cycle progression. Further down, over expression of the mutant GM130 (S25A) in the background of the depletion showed no apparent defects in Golgi vesiculation or mitotic progression. Our work suggests while Cdk1 based phosphorylation is essential for mitotic Golgi vesiculation and mitotic progression, cells might have redundant downstream pathways that ensure that Golgi vesiculation proceeds in spite of inactivation of any single component.

1. Introduction

1.1 Cell division; the basis for cell multiplication and life on earth

All living organisms have the same basic functional unit that makes them up; the cell. Nearly 150 years after Rudolph Virchow famously proposed that all cells arise from pre existing cells(Tan and Brown 2006), we have made great progress in furthering his theory. However, we are still in the dark about the finer details of the mechanism that allows a cell to generate daughter cells. The cell reproduces by firstly duplicating its contents, segregating them and then redrawing its boundaries. In the process, it passes though a carefully regulated cycle of events called the cell cycle.

The eukaryotic cell cycle has been best studied in yeasts and mammalian cells. It consists of two phases, the interphase and the mitotic phase (M-phase). The interphase in turn is made up of three phases; the first gap phase (G1-phase), synthesis phase (S-phase), and second gap phase (G2-phase). The interphase, seemingly a period of rest for the cells is actually a period of intense activity wherein the cell readies itself for division by synthesizing proteins required for growth, duplicating its genetic material and other organelles such as the centrosomes, the Golgi among others. The mitotic phase of the cell cycle is visually much more dynamic with the cell undergoing rapid changes in morphology. Through a set of well orchestrated processes, the cell segregates its chromosomes to opposite ends with the physical force required for the above essential process being provided by one of the cytoskeletal components of the cell; the microtubules. The other organelles in the cell such as the centrosome,

the ER, the Golgi among others would now have segregated through distinct mechanisms. Following this, the cell undergoes a series of dramatic changes in its morphology that ultimately result in the division of the cell into two. The story ends differently depending on the cell type (Balasubramanian, Bi et al. 2004) but in essence, a barrier is brought in between the two nascent cells, and cell division is complete.

1.2 The Golgi Apparatus

1.2.1 Golgi structure and inheritance

The Golgi apparatus is one of the most fascinating organelles in the eukaryotic cell. Though first identified in 1898 by the Italian physician Camillo Golgi, many of its functions remain among the great mysteries of the cell. In most eukaryotic cells, the Golgi exists as a network of tubules and vesicles that are arranged into stacks of flattened cisternae. Newly synthesized proteins from the ER are received at the cis Golgi network (CGN), modified posttranslationally as they traverse the Golgi stack to reach the trans Golgi network (TGN), where they are sorted for delivery to their ultimate target (Mellman and Simons 1992)

In the plant cell and also in lower animal cells, the Golgi exist as individual stacks that are dispersed throughout the cytoplasm. In mammalian cells however, the Golgi apparatus often displays a juxtanuclear or pericentriolar localization wherein the individual stacks of the Golgi are interconnected to yield a Golgi ribbon. It has previously been shown that the presence of the Golgi at the pericentriolar area is dependent on the microtubules and is mediated by the action of the Rho GTPases (Nobes

and Hall 1999). Perturbation of the microtubules using either a depolymerising agent (nocodazole) or a stabilizing agent (taxol) has affected Golgi structure and localization (Sandoval, Bonifacino et al. 1984; Turner and Tartakoff 1989; Corthesy-Theulaz, Pauloin et al. 1992; Cole, Sciaky et al. 1996; Thyberg and Moskalewski 1999). The pericentriolar localization of the Golgi apparatus in mammalian cells has led scientists to speculate about the possible link between the Golgi and the centrosomes, the Microtubule Organizing Centre of the cell (MTOC). The affinity of the Golgi apparatus for microtubules can be attributed to the fact that the microtubules tend to associate with the MTOC in a minus end directed manner. The localization of the Golgi apparatus at the crucial position could therefore be interpreted as a controlling position for monitoring and possibly directing a number of cellular events.

The mechanism that ensures the inheritance of the Golgi to both the daughter cells appears to be cell type-dependent, and perhaps reflects functional requirement of Golgi during mitosis. In plants and many single-celled organism, the Golgi is inherited as intact, individual stacks into the daughter cells (Nebenfuhr, Frohlick et al. 2000). However, in mammalian cells where protein secretion ceases during mitosis, the Golgi undergoes sequential fragmentations from ribbons to dispersed stacks in G2, and from stacks to tubules and vesicles later in metaphase. Such extensive fragmentation steps are believed to ensure Golgi partition to both the daughter cells in a precise manner (Lucocq, Pryde et al. 1987; Lucocq and Warren 1987; Lucocq, Berger et al. 1989) .

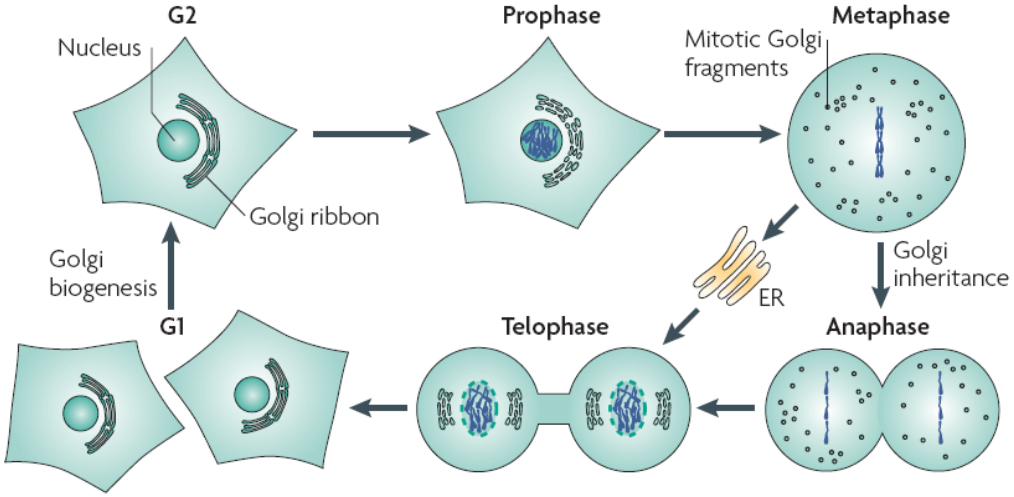


Figure 1.1. **The Golgi undergoes dramatic morphological changes during mitosis.** During prophase, the tubular connections between the individual Golgi stacks are lost and the Golgi ribbon is broken down into individual stacks that remain close to the nucleus. Between prophase and metaphase, the Golgi undergoes further fragmentation whereby the individual cisternae are converted into small ~50–70 nm vesicles, and larger vesicular and tubular elements. These mitotic Golgi fragments either exist as discrete units or they might fuse with the endoplasmic reticulum (ER). During telophase, the Golgi fragments fuse with each other to initiate the reformation of new Golgi stacks that ultimately connect to form a Golgi ribbon in each daughter cell. Figure reproduced from: (Lowe and Barr 2007)

1.2.2 Golgi ribbon undergoes severing during G2 phase

It was shown that in mammalian cells, the initial fragmentation event converts the intact Golgi ribbon into isolated Golgi stacks or group of stacks that while fragmented, remain clustered around the nucleus (Colanzi, Carcedo et al. 2007; Feinstein and Linstedt 2007). When this fragementationfragmentation step was blocked, by inhibiting the fission-inducing protein BARS or the kinase MEK 1 (Colanzi, Carcedo et al. 2007; Feinstein and Linstedt 2007), the cells were arrested in G2 phase. Supporting this, it was shown that the fragmentation of the Golgi

apparatus was essential for G2/M transition and entry into mitosis (Sutterlin, Hsu et al. 2002). By targeting the Golgi matrix protein GRASP65 using specific inhibitory peptides and antibodies, they showed that blocking the Golgi fragmentation process at G2 prevented cells from entering mitosis. However, Itit remains unknown why this initial Golgi fragmentation is essential for mitotic progression. It is possible that the fragmentation process is designed to ensure that both the daughter cells inherit the organelle. It is also speculated that given the proximity between the Golgi and the centrosome, Golgi breakdown might be required for the correct maturation and separation of the centrosomes as failure in this fragmentation might physically prevent the MTOC reorganization during mitosis thereby leading to mitotic failure (Colanzi and Corda 2007)

The molecular mechanism that is behind the first Golgi fragmentation event has been extensively studied. The fission inducing protein BARS was shown to be necessary for the process although BARS on its own is insufficient to induce fragmentation. Subsequently it was shown that another Golgi matrix protein GRASP65 might also be involved. GRASP 65 is a coiled coil protein that forms homodimers and it has been thought that GRASP65 dimers located on adjacent Golgi stacks could help to link them together to form the ribbon structure(Preisinger, Koİˆrner et al. 2005; Wang, Satoh et al. 2005). GRASP65 carries out a number of different functions and each of them seem to be mediated by specific phosphorylation of specific sites.

It has been shown that GRASP65 phosphorylation at S277 is essential for G2/M progression (Yoshimura, Yoshioka et al. 2005). In addition, it was

shown that the kinases MEK 1 and Plk1 might also be involved in the G2 Golgi fragmentation.

Table 1.1. List of kinases involved in regulating Golgi structure and function. Table modified from: (Lowe and Barr 2007)

Kinases	**Interaction partners**	**Substrates**	**Proposed function of substrate**
CDK1	Cyclin B	GM130	Golgi membrane and vesicle tethering
		GRASP65	Membrane tethering and cisternal stacking
		RAB1	Golgi membrane and vesicle tethering
		p47	Membrane fusion
		NIR2	Phospholipid transfer
PLK1	GRASP65	GRASP65	Membrane tethering and cisternal stacking
	RAB1	RAB1	Golgi membrane and vesicle tethering
PLK3	Giantin MEK1 ERK2	Unknown	Not applicable
MEK1	PLK3	Unknown	Not applicable
ERK1/2	PLK3	GRASP55	Cisternal stacking
ERK1c	Unknown	Unknown	Not applicable
Unknown	Unknown	Golgin-84	Golgi-membrane and vesicle tethering

The effector of MEK1 on the Golgi , ERK1c was discovered and it was demonstrated that depletion of ERK 1c reduces Golgi fragmentation (Shaul and Seger 2006). The target for ERK1c is speculated to be GRASP55 as it was shown to be phosphorylated by ERK1/2 at T222 or T225 and failure to do so delayed mitosis (Feinstein and Linstedt 2007). Though the multiple kinases and pathways detailed above might function together to mediate G2 specific Golgi fragmentation, their precise regulation and function remain to be identified.

1.2.3 The mechanism of Golgi vesiculation

1.2.3.1 The reason for Golgi vesiculation

The reason as to why the Golgi has to undergo further fragmentation is not clear. Accurate Golgi inheritance is possible with the individual stacks generated by the initial fragmentation process and has been demonstrated to be the case in many organisms such as plants. The second fragmentation event might be essential to ensure a more accurate inheritance or it might also have a role in regulating mitosis by releasing factors that are sequestered in the Golgi during interphase. It is also possible that the Golgi vesiculation might have effects on influencing the rapid changes in the cytoskeleton that occurs during mitosis.

The second fragmentation step of the Golgi, however, has been the source of a considerable debate in the community (Shima, Haldar et al. 1997; Shima, Cabrera-Poch et al. 1998). At the onset of mitosis, the isolated Golgi stacks undergo further fragmentation or vesiculation to produce a dispersed array of tubulovesicular clusters also known as the Mitotic Golgi Clusters (MGC) (Lucocq and Warren 1987; Lucocq, Berger et al. 1989; Warren, Levine et al. 1995; Shima, Haldar et al. 1997). The MGCs contains most of the Golgi resident enzymes except p115 (Lowe, Gonatas et al. 2000). The changes in the Golgi morphology are concomitant with an elevation in Cdk 1 levels. When the cell reaches prometaphase, the MGCs relocate and now surround the newly formed mitotic spindle (Shima et al. 1998, Whitehead & Rattner 1998, Jokitalo et al. 2001). The MGCs undergo still further separation just before

metaphase. Part of the MGC remains associated with the spindle while the other part is distributed to the cell cortex presumably by the mitotic spindle (Shima, Cabrera-Poch et al. 1998)

The molecular mechanism behind the mitotic Golgi vesiculation has been studied. It is known that the mitotic disassembly of Golgi stacks proceeds via two distinct, concurrent fragmentation pathways. The first pathway also known as the COPI-dependent pathway proceeds because COPI vesicles continue to bud from the Golgi stack but due to restrictions in mitotic transport, they are unable to tether and fuse with their target membrane (Misteli &Warren 1994, Nakamura et al. 1997). This pathway is thought to contribute to about 65% of the mitotic Golgi vesiculation (Misteli and Warren 1994; Misteli and Warren 1995; SÃ¶nnichsen, Watson et al. 1996). The second pathway which is also known as the COPI-independent pathway converts the flattened cisternal cores into a heterogeneous array of tubulovesicular elements via unknown mechanisms (Misteli and Warren 1995).

1.2.3.2 The role of GM130 in COPI- dependent Golgi vesiculation

A molecular explanation for the COPI dependent mitotic Golgi vesiculation has been proposed. p115 is a homodimeric vesicle-tethering protein that is required for intra-Golgi (Waters, Clary et al. 1992; Seemann, Jokitalo et al. 2000) and ER-Golgi transport(Allan, Moyer et al. 2000; Moyer, Allan et al. 2001). p115 brings the Golgi membrane and the vesicle membrane together in interphase by binding to two Golgins, GM130 and Giantin through its two arms.

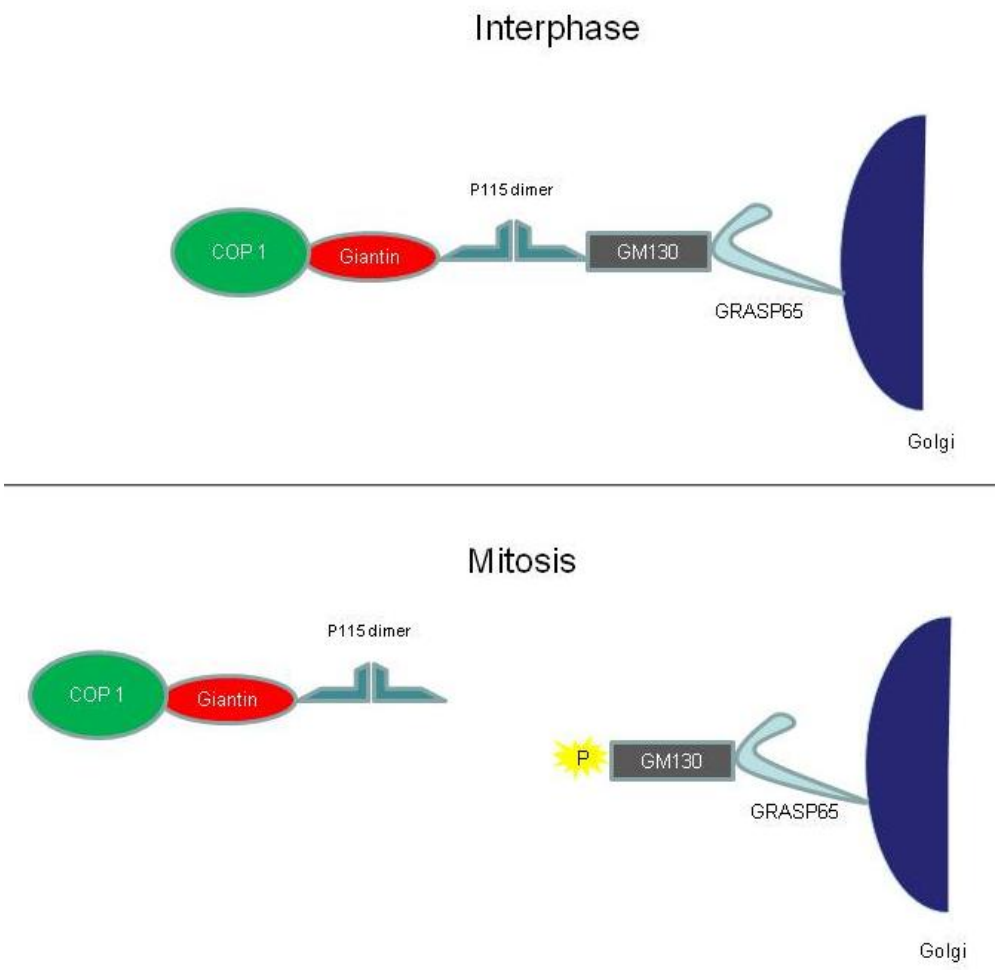


Figure 1.2. **COPI vesicle tethering under interphase and mitotic conditions.** During interphase, p115 dimers link giantin on the COPI vesicles to GM130 on the Golgi membrane. During mitosis, GM130 is phosphorylated at S25 and this precludes p115 binding to GM130 thereby preventing COPI vesicle tethering to the Golgi.

GM130 and Giantin are long, rod-like fibrous proteins due to an extensive coiled-coil domain structure typical of Golgins (Linstedt and Hauri 1993; Nakamura, Rabouille et al. 1995). GM130 consists of 986 amino acids and has 6 coiled coil domains. It has an N terminal region that binds to p115. Its C terminal was shown to interact with another Golgi structural

protein GRASP65. Giantin, on the other hand, is present both on the Golgi membrane and on the surface of the COPI vesicles (Nakamura, Rabouille et al. 1995; SÃ¶nnichsen, Watson et al. 1996; MartÃ-nez-MenÃ¡rguez, Prekeris et al. 2001).

During mitosis, the N-terminal domain of GM130, comprising the p115 binding site, is phosphorylated on serine 25 by CDK1/Cyclin B, thereby inhibiting p115 binding(Nakamura, Lowe et al. 1997; Lowe, Rabouille et al. 1998). Although p115 can still bind Giantin, it is no longer able to cross-link to GM130. As a result, COPI vesicles accumulate, as they are unable to tether and fuse, and intra-Golgi transport is inhibited (Collins and Warren 1992; Stuart, Mackay et al. 1993).

Supporting this, GM130 was shown to be phosphorylated *in vivo* during prophase at the onset of Golgi fragmentation, using an antibody that specifically recognizes pS25 GM130 (Lowe, Gonatas et al. 2000). GM130 remains phosphorylated until telophase, when it is dephosphorylated. GM130 phosphorylation and dephosphorylation is synchronous with p115 dissociation and reassociation with Golgi membranes in addition to Golgi fragmentation and reassembly (Lowe, Gonatas et al. 2000).

More recently, it was shown that depletion of GM130 in HeLa cells causes centrosomal and spindle abnormalities (Kodani and SuÌˆtterlin 2008). Hence GM130 appears to play a major role in linking the Golgi structureal dynamics to the progression of mitosis. Based on the above model that has been proposed to explain GM130 mediated Golgi vesiculation, the use of a phosphorylation deficient mutant (S25A) is an ideal method to perturb the vesiculation process and observe the effects on mitotic

progression. The lack of a phosphorylation site at S25 will presumably allow the COPI vesicles to continue to dock and fuse with the Golgi thereby preventing mitotic Golgi vesiculation.

2. Materials and Methods

2.1 Cell line

The cell line used in the study is the adherent Normal Rat Kidney Epithelial (NRK) cells usually designated as NRK-52E.

2.2 Reagents

2.2.1 Solutions

0.05% trypsin (See appendix for composition)

STE (See appendix for composition)

2.2.2 Drugs

The Cdk inhibitor purvalanol A (Tocris Bioscience) used in this study was stored as 15mM aliquots at -20^{0}C and the working concentration was 15 uM. Purvalanol A was shown to act as a competitive inhibitor of ATP binding to Cdk (Villerbu, Gaben et al. 2002).

2.2.3 Antibodies

The primary and secondary antibodies used in this study are listed in Table 2.1A and 2.1B, respectively.

Table 2.1A: Primary antibodies used in this study

Product	Source	Fixation	Dilution
Monoclonal GM130	Translucent Labs	PFA	1:1000
Polyclonal Rabbit GM130	Dr. Graham Warren	PFA	1:1000
pS25 GM130	Santa Cruz	PFA	1:50

Table 2.1B: Secondary antibodies used in this study

Product	Source	Fixation	Dilution
Anti-mouse conjugated with Alexa Fluor 488	Molecular Probes	PFA	1:300
Anti-mouse conjugated with Alexa Fluor 563	Molecular Probes	PFA	1:300
Anti-rabbit conjugated with Alexa Fluor 488	Molecular Probes	PFA	1:300
Anti-rabbit conjugated with Alexa Fluor 563	Molecular Probes	PFA	1:300

2.3 Cell culture

2.3.1 Culture conditions

NRK cells were maintained in Kaighn's modified F12 (F12K; Sigma) medium supplemented with 10% FBS, 1 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (GIBCO) at 37°C and 5% CO_2. Cells were maintained on 100 or 60 mm culture dishes. For microscopic imaging, cells were cultured on a custom made 35mm glass chamber as described previously (Mckenna and Wang, 1989). Briefly, a 35 mm hole was drilled through a plate of acrylic plastic. A layer of vacuum grease was then laid around the hole and the plate was autoclaved. The hole was then covered by a sterilized large glass cover slip to form a culture chamber.

To subculture, cells were rinsed with STE (~5 ml for 100 mm dish and ~2 ml for 60 mm dish) briefly. Cells were then treated with 2 ml of 0.05% trypsin for 1-5 minutes at room temperature. When the majority of cells were seen to have detached from the culture dish, the complete medium was added and cells were vigorously detached. An appropriate number of cells were transferred into culture dishes containing fresh medium.

2.4 Molecular biology

2.4.1 E.coli strain used and plasmid amplification strategy

The E.coli XL1 Blue was used for maintenance and amplification of the plasmids. Electrocompetent XL1-Blue cells were transformed with 200-500 ng of the individual plasmid using MicroPulser Electroporator (Bio-Rad Laboratories, Hercules, CA, USA) at 2.5 V. The cells were then recovered in Super Optimal broth with Catabolite repression (SOC) for 1hr at 37˚C with shaking at 250 revolutions per minute (rpm) in Unitron-Plus incubator shaker (Infors, Bottmingen, Switzerland), and incubated on Luria-Bertani Broth (LB) agar containing appropriate concentration of the antibiotic for antibiotic selection for 14 hrs at 37 ˚C in MIR-262 incubator (SANYO Biomedical, Wood Dale, IL, USA). Resultant colonies were picked and cultured in 2×yeast extract tryptone (YT) medium containing appropriate concentration of the antibiotic for 14 hrs at 37 ˚C with shaking at 250 rpm in Unitron-Plus incubator shaker. Purification of plasmid DNA was performed using either High-Speed Plasmid Mini Kit (Geneaid, Taiwan) or QIAprep Spin Miniprep Kit (QIAGEN) and plasmid Maxiprep kit (QIAGEN) according to manufacturers' instructions.

2.4.2 Plasmid construction

pCDNA Rat GM130 FL and GM130 S25A mutant cDNA constructs were a kind gift from Dr. Martin Lowe (Manchester University). The cDNA was

removed from the pcDNA vector using the EcoR1 and BamH1 restriction enzymes and cloned into the corresponding sites in pEGFP C2 vector (Clontech). Subsequently, PTdTomato-GM130 FL WT and S25A were constructed by substituting the GFP with TdTomato using the restriction enzymes Nhe1 and BsrG1.

2.4.3 ShRNA

SureSilencing shRNA plasmids (SABiosciences, Washington, DC.USA) targeting rat GM130 were purchased for the knockdown experiments. Two shRNA plasmids targeting rat GM130, shRNA2 -GFP and shRNA 4 –GFP recognizing the GM130 sequence 'TGAGGAAGTTCAGGGCAAGAT' and 'AACAACTGCAGGTTCACATT' respectively were used. The negative control, shRNA NC- GFP, contained the scrambled artificial insert sequence 'GGAATCTCATTCGATGCATAC '. The GFP in the shRNA plasmids allowed the identification of transfected cell under the microscope.

2.5 Plasmid transfection

For GalT –GFP, GFP-GM130 FL and GFP-S25A mutant over expression experiments, 0.5 µg of the respective plasmid was transfected into NRK cells using Effectene® (Qiagen, Hilden, Germany) as per the manufacturer's instructions. Briefly, NRK cells at a density of 5×10^4 cells/ml were plated in glass chambers and incubated for 24 hrs to

reached 40–60% confluence. Effectene reagent was diluted in F12k medium containing 1% FBS. The cells were transiently transfected with 0.3-0.8 µg of plasmid DNA with Effectene in accordance to the manufacturer's instruction for 6-6.5 hrs. The Effectene-DNA complex mixture was removed and cells were cultured for an additional of 40-42 hrs in F12K medium supplemented with 10% FBS before live-cell imaging or immunofluorescence was carried out.

For the GM130 ShRNA transfection, cells were grown on a cover slip chamber to ~50% confluency and were transfected with 1 µg each of ShRNA2 and ShRNA 4 targeting rat GM130 using lipofectamine 2000 transfection reagent according to manufacturer's instructions. The cells were prepared for live imaging or for immunofluorescence analysis 72 hours post transfection.

2.6 Microscopy

2.6.1 Sample preparation for live-cell imaging

For live-cell imaging, the chamber containing the cells was overlaid with mineral oil (Sigma-Aldrich) to avoid evaporation of medium.

2.6.2 Sample preparation for immunofluorescence staining

Cells were rinsed twice with warm PBS and then fixed with either of two fixative reagents, 4% paraformaldehyde (EM Sciences) for 10 min at room temperature or 100% methanol for 10 minutes at -20^{0}C. Cells were then washed with PBS three times for 5 minutes and permeabilized with 0.2%

Triton X-100 in PBS for 2-5 minutes. After washing with PBS three times for 5 min, cells were blocked for 1 hour with 3% BSA in PBS, and then incubated with primary antibodies for 45-60 minutes at 37°C. After rinsing three times with PBS (5-10 minutes), the cells were incubated with the appropriate secondary antibodies for 45 minutes at 37°C.

2.6.3 Image acquisition

For live-cell imaging, cells were maintained at 37°C in a custom made incubator built on top of an Axiovert 200 M inverted microscope (Carl Zeiss) and viewed with a 100×, NA1.30, Plan-NEOFLUAR lens. All images were acquired with cooled charge - coupled device camera (CoolSNAP$_{HQ}$, Roper Scientific) using MetaView imaging software (Universal Imaging). Immunofluorescence stained samples were visualized using either the Axiovert 200 M inverted microscope (100×, NA1.30, Plan-NEOFLUAR lens) or a LSM 510 Meta inverted confocal microscope system (100×, NA 1.25 Achroplan lens or a 100×, NA 1.4 Plan-Apochromat lens).

2.6.4 Image analysis

2.6.4.1 Quantification of fluorescence intensity

Fluorescence intensity was measured with MetaMorph (Universal Imaging) and ImageJ (NIH) software. Briefly, the z stacks obtained at different time points were subjected to sum slice intensity projection along

the z axis. Following this, regions of interest were selected and the average or integrated intensities were obtained. The intensity of the background was also obtained. The results were logged into Microsoft Excel® and subjected to further analyses.

3. Results

3.1 The Golgi apparatus in mammalian cells undergoes dynamic changes during mitosis

The dynamics of the Golgi was observed using GFP fusions of a Golgi resident enzyme GalT (Galactosyltransferase) (Fig. 3.1). It was seen that during the interphase, the Golgi exhibited a pericentriolar ribbon like architecture. In early prophase, the Golgi no longer resembled the ribbon structure and instead was found as disconnected masses. The Golgi blobs however were still able to localize to the pericentriolar region. Upon the onset of mitosis, the individual Golgi blobs appeared to be stable until nuclear envelope breakdown. Concomitant with NE breakdown, the Golgi blobs started disintegrating into smaller fragments. (Fig. 3.1, time 00:00). At metaphase (Fig. 3.1, time 08:13), the Golgi was reduced to small dispersed dots over a haze, which represent mitotic Golgi clusters and ER.

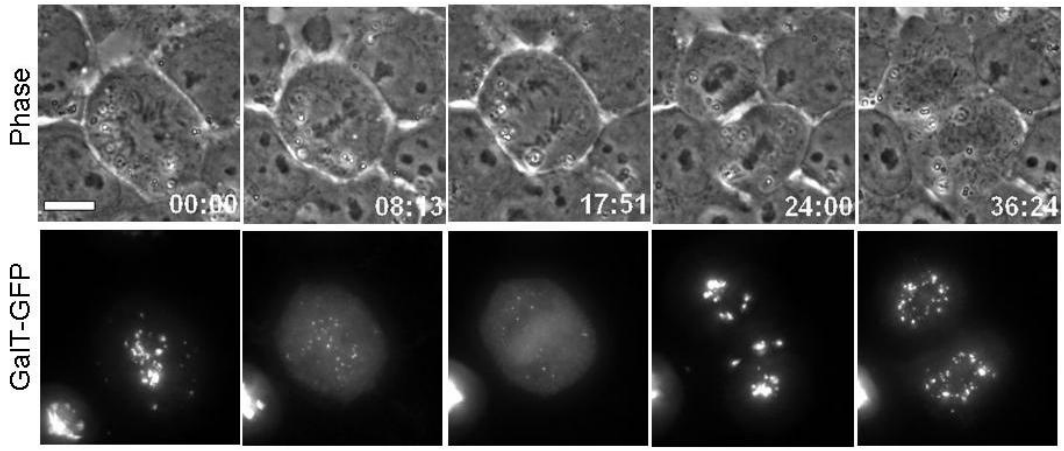


Figure 3.1 **Golgi underwent dynamic changes during mitosis.** Cells were transfected with the Golgi resident enzyme GalT (Galactosyl Transferase) fused to GFP and subjected to live imaging. Z stacks were obtained at intervals of two minutes and reconstructed using maximum intensity projection. During mitosis, the Golgi starts vesiculating during prophase and is reduced to a haze by around metaphase. Following chromosome segregation during anaphase, the Golgi starts reappearing as spots that further coalesce into larger blobs finally reforming the Golgi ribbon during telophase. Scale bar represents 5 μm. Time elapsed is shown.

After anaphase onset, when the chromosomes started separating, the Golgi reappeared slowly (Figure 3.1 time 17.51). At first they were seen as bright spots throughout the cell. Soon, these small spots started to coalesce and at the same time move towards the newly formed nucleus presumably close to the MTOC. During cytokinesis two fractions of the Golgi were observed on either side of the nucleus with one fraction in close proximity to the nascent midbody. This suggests that the membrane-fusion events that are necessary to complete cytokinesis are preceded by the Golgi reformation.

3.2 Purvalanol A treatment affects Golgi vesiculation and mitotic progression

Our preliminary approach to perturb the Golgi vesiculation process was to use a Cdk 1 inhibitor. It was previously shown that the Cdk 1 inhibitor, purvalanol A effectively inhibited Cdk activity specifically (Villerbu, Gaben et al. 2002) . In our experiments, cells transiently transfected with GalT-GFP were treated with Purvalanol A (15 µM) and immediately visualized. In dividing cells, we observed a dramatic change in Golgi dynamics. The Golgi network though fragmented, was still able to retain its pericentriolar localization throughout the process of mitosis and was inherited intact to the daughter cells. Moreover, the cells also displayed mitotic delay and defects (Fig 3.2). Most cells were unable to form a functional metaphase plate. In cases where a metaphase plate was formed, the chromosome congression and segregation were severely affected and the time taken to complete mitosis was much longer than that in control cells (Fig 3.2, time 76:13)

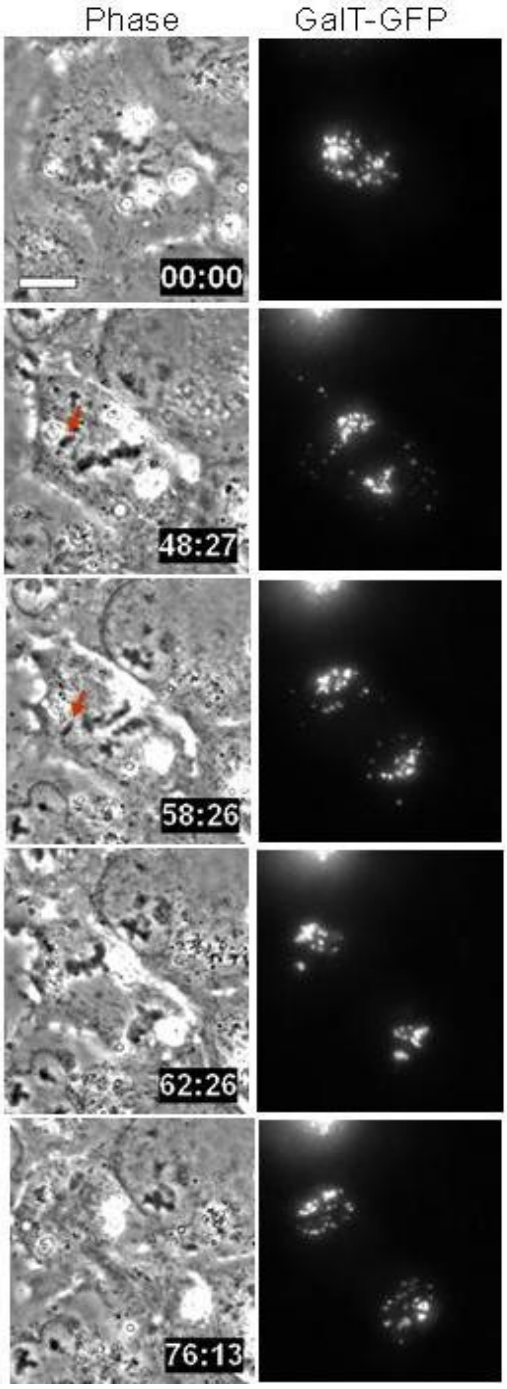
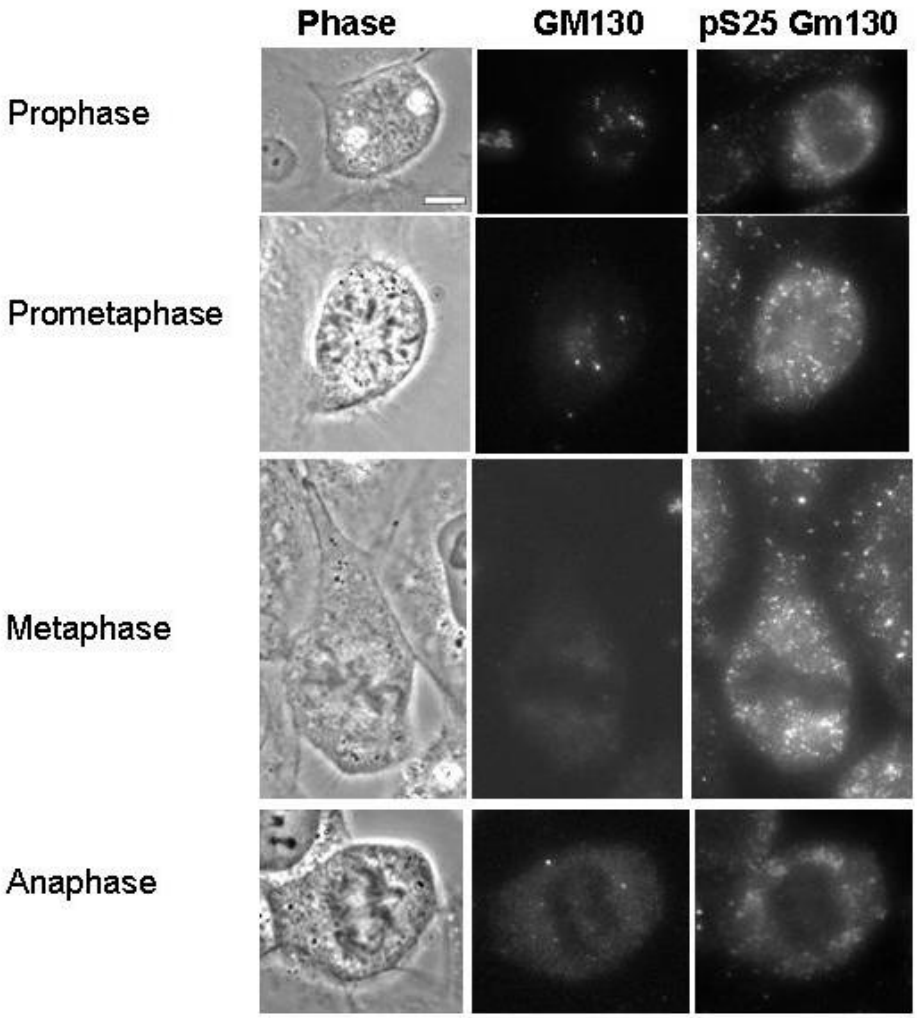


Figure 3. **Golgi vesiculation and mitotic progression were affected in cells treated with Purvalanol A**. Cells were transfected with the Golgi resident enzyme GalT (Galactosyl Transferase) fused to GFP. The Cdk1 inhibitor purvalanol A was added to the cells at the concentration of15uM and the cells were imaged immediately. Upon purvalanol A treatment, the Golgi did not undergo normal mitotic dynamics. It failed to form the haze and instead was inherited intact to both the daughter cells. Not only was Golgi vesiculation blocked, but cells exhibited mitotic defects (Arrow indicates an unattached chromosome even after initiation of chromosome segregation) and delays (time elapsed is shown). Scale bar represents 5 μm.

3.3 Purvalanol A treatment abolished GM130 phosphorylation

In order to examine whether phosphorylation of the Golgi matrix protein GM130 was affected by purvalanol A treatment, we analyzed the phosphorylation state of GM130 using an antibody that specifically recognized GM130 phosphorylated at S25 (Shah, Patel et al. 2005). Cells were grown to about 70% confluence upon when, 15 uM purvalanol A was added. After an incubation period of 30 minutes, the cells were processed for immunofluorescence. We found that treatment of cells with purvalanol A not only prevented golgi vesiculation as visualized by GM130 staining but also abolished the mitotic phosphorylation of the Golgi matrix protein GM130 as visualized by pS25 staining. While in control mitotic cells the Golgi haze was brightly stained with the pS25 GM130 antibody, this staining was not observed in Purvalanol A treated mitotic cells (fig3.3A and 3.3B). To quantify the staining at different mitotic stages, 5 cells from each of the different cell cycle stage were visualized and the fluorescence intensity was quantified (Fig. 3.3C). It was observed that the fluorescence intensity increased 1.5 times when control cells entered mitosis whereas the increase was reduced to about 0.5 fold in purvalanol A treated cells. The marginal increase in fluorescence in purvalanol A treated cells could be due to non-specific interaction of the pS25 GM130 antibody to other Cdk 1 phosphorylated targets during mitosis.

A


Phase
GM130
pS25 Gm130
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Metaphase
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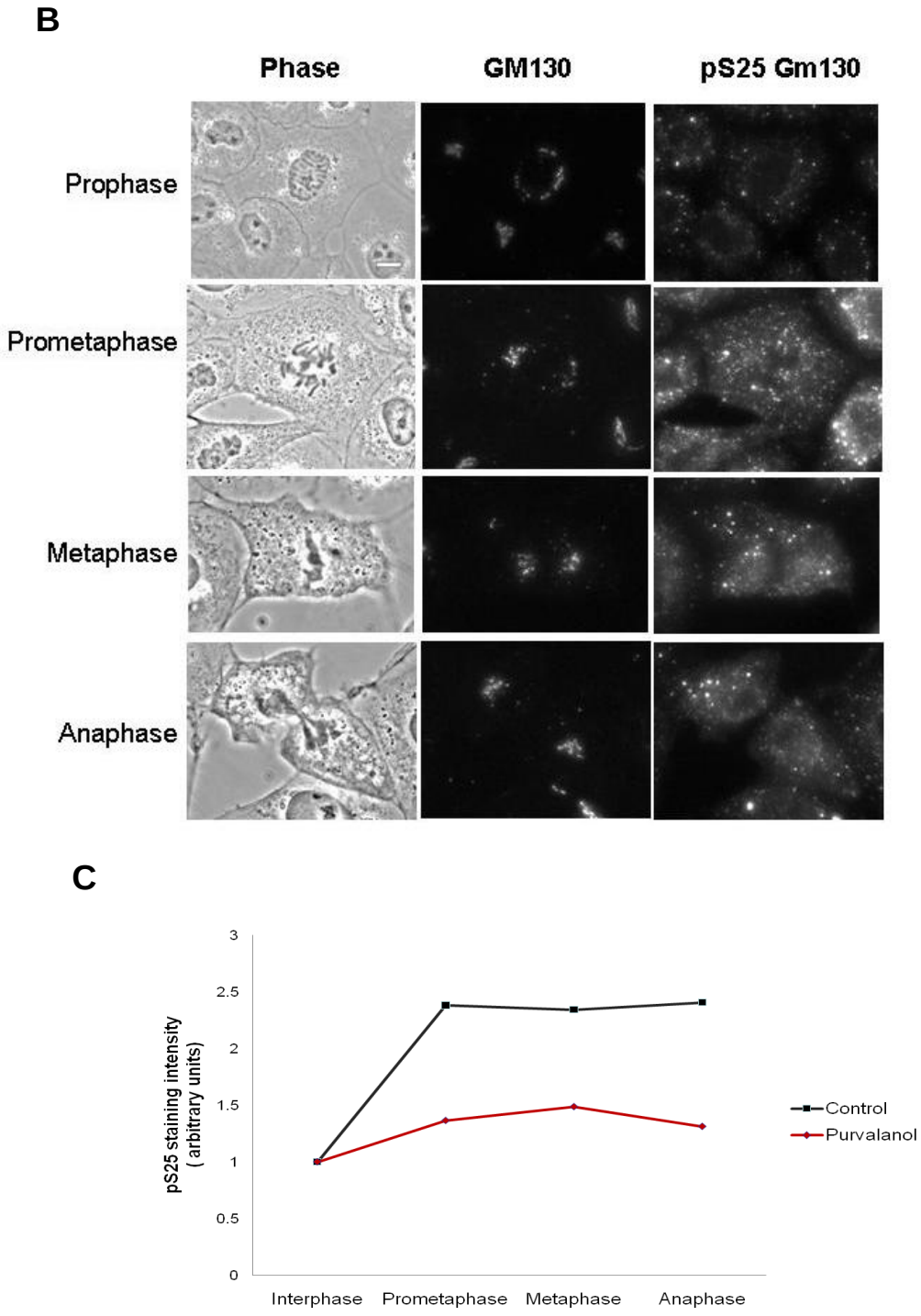


Figure 3.3: **Phosphorylation of GM130 S25 was abolished in Purvalanol A treated cells**. Cells were either not treated (A) or treated (B) with 15 uM purvalanol A and fixed with 4 % PFA after 30 minutes and stained for GM130 and pS25 GM130 (that specifically recognizes phosphorylated GM130). (A) In control cells GM130 underwent phosphorylation during prophase and retained the phosphorylation state till anaphase onset whereas (B) in cells treated with purvalanol A,

GM130 did not undergo substantial phosphorylation. The residual phosphorylation presumably reflects background staining (C) Quantification of the fluorescence intensity in control and Purvalanol A treated cells was performed at four different cell cycle stages and normalized to the interphase value. Scale bar represents 10 μm.

3.4 Over expression of GFP-GM130 FL and GFP-GM130 S25A mutant did not affect Golgi dynamics

In order to perturb mitotic Golgi vesiculation more specifically, we decided to focus on GM130, the target of Cdk 1 in the Golgi that was proposed to mediate Golgi vesiculation. To this end, we generated GFP fusion proteins to both the wild type and a phosphorylation deficient mutant (S25A) of GM130. Cells were transiently transfected with either GFP-GM130 FL WT or GFP-GM130 S25A and dividing cells were imaged (Fig 3.4). The results indicated that over expression of either GFP-GM130 full length or the phosphorylation deficient mutant S25A did not affect either Golgi vesiculation or mitotic progression (Fig. 3.4A and B). The Golgi exhibited normal kinetics, starting to vesiculate during early mitosis, forming the mitotic Golgi haze at metaphase and reappearing at telophase. The mitotic progression in the cells over expressing GM130 FL as well as the S25A mutant was analyzed and the duration for mitosis was comparable to control cells (Fig 3.4A, time 44:16, fig 3.4B, time 53:29)

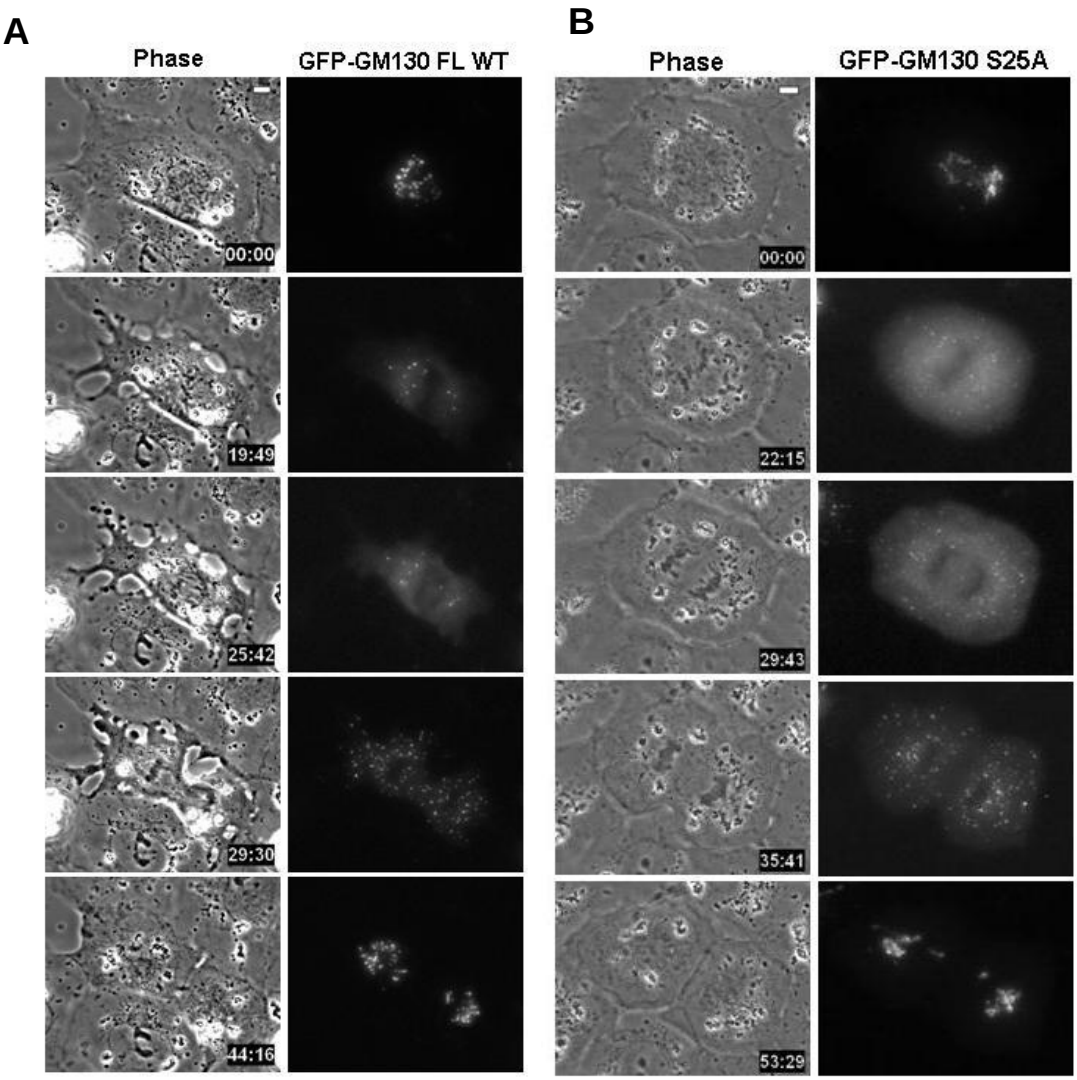


Figure 3.4: **Golgi dynamics was unaffected by GM130 over expression**. NRK cells were transfected with either (A) Full length GM130 or (B) S25A GM130 and followed by imaging. In both the cases, it was seen that over expression of either the full length or the phosphorylation deficient mutant of GM130 did not affect mitotic progression (Time elapsed is shown). Contrary to GalT dynamics, more spots of GFP-GM130 persisted through mitosis and this can be attributed to the fact that GM130 is a matrix protein and behaves differently from GalT which is a Golgi resident enzyme. Scale bar represents 5 μm.

3.5 ShRNA against GM130 successfully depleted GM130

The over expression of GM130 S25A mutant did not have any specific effects on mitotic Golgi vesiculation or mitotic progression. However, there was a possibility that the endogenous GM130 that was still capable of being phosphorylated might mitigate the effect of over expression of the S25A mutant. In order to exclude this possibility, we depleted endogenous GM130, by shRNAs designed specific to rat GM130. Altogether, 4 shRNAs (targeting 4 different regions in GM130) were tested. ShRNA2 and shRNA4 were found to be most effective. A combination of both ShRNA2 and 4 was therefore used for all futher depletion experiments. Shown in Fig. 3.5 are representative cells transfected with ShRNA2 and ShRNA4 using Lipofectamine reagents. Seventy two hours after transfection, cells were fixed and stained for GM130. Quantification of the images indicated that up to 75% of the endogenous GM130 was depleted in cells expressing shRNA. (Fig. 3.5).

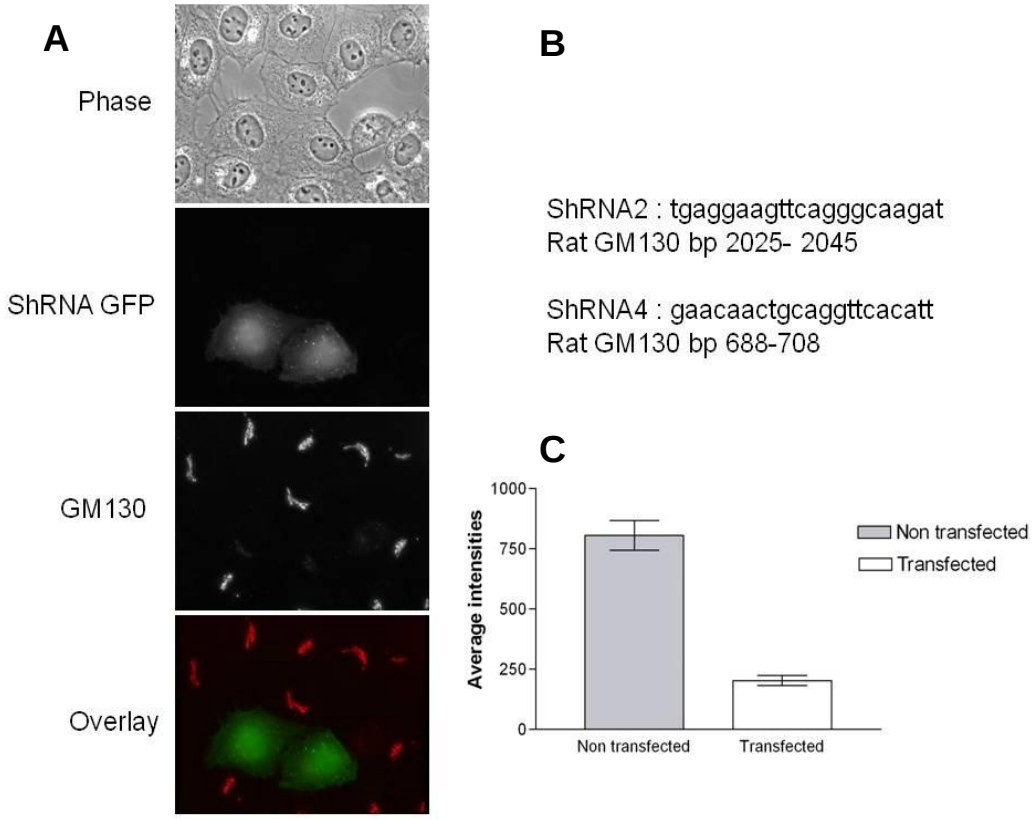


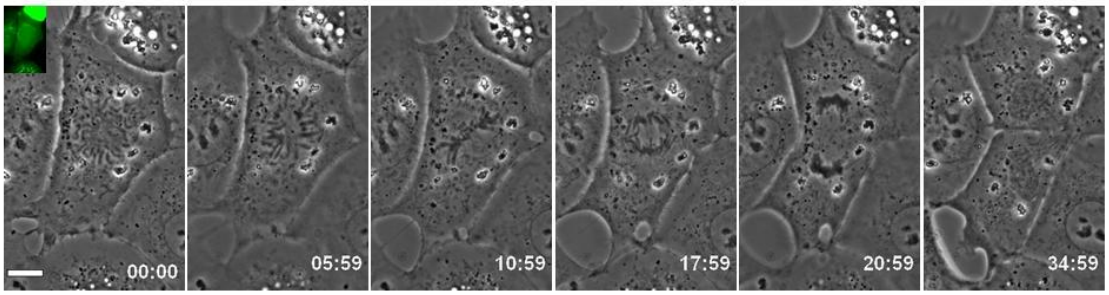
Figure 3.5**: ShRNA against rat GM130 depleted endogenous GM130 efficiently.** Cells were transfected with a mixture of two ShRNA against two different regions of rat GM130 (GFP acts as a marker for transfection) and were processed for immunofluorescence 72 hours after transfection. (A) Cells transfected with the ShRNA showed decreased fluorescence intensity upon GM130 staining when compared to the non transfected cells. (B) GM130 sequence based on which the ShRNAs were designed (C) Quantification of fluorescence intensity of GM130 staining in ShRNA transfected cells and controls. (n=2) Values represent mean ± SEM.

3.6 Depletion of GM130 does not affect mitotic progression

After confirming the depletion efficiency of the ShRNA, the mitotic progression of cells depleted of GM130 were followed by time lapse microscopy. Cells were transfected with the shRNA mixture (shRNA2 and

shRNA4) and incubated for 72 hours after which they were imaged. The GM130 depleted cells were identified by the GFP signal and their mitotic progression was monitored. It was found that depletion of endogenous GM130 did not lead to any defects in mitotic progression. The cells were able to segregate their chromosome normally and undergo cytokinesis in normal time duration as compared to control cells (36 minutes).

Figure 3.6: **Depletion of GM130 did not affect mitotic progression**. Cells were transfected with a mixture of 2 ShRNAs against rat GM130 and



incubated for 72 hours after which they were imaged. Cells that were transfected with GM130 ShRNA showed no significant defects or delays in mitotic progression (n=6). Shown in the inset is the GFP to indicate transfected cell. Scale bar represents 5 μm. Time elapsed is shown.

3.7 GM130 S25A over expression did not affect mitotic progression or Golgi vesiculation

While depletion of GM130 did not affect mitotic progression, over expression of the phosphorylation deficient mutant (S25A) was hypothesised to induce a block in Golgi vesiculation and hence mitotic progression. Over expression experiments were carried out with the S25A mutant by co transfecting an excess of the S25A mutant along with the shRNA mixture. After 72 hours of incubation, cells were imaged to

visualize the effect of GM130 S25A over expression. In cells over expressing the S25A mutant, the Golgi apparatus regained the ribbon architecture, indicating that the S25A mutant was indeed able to rescue the interphase morphology of the Golgi. However, over expression of the S25A mutant did not affect mitotic Golgi vesiculation or mitotic progression. The Golgi underwent fragmentation in G2 phase and subsequently underwent vesiculation as the cells progressed through mitosis (Fig. 3.7). The cells proceeded through mitosis with kinetics similar to control cells (Fig. 3.7, time 55.10) suggesting that the over expression did not affect mitotic progression.

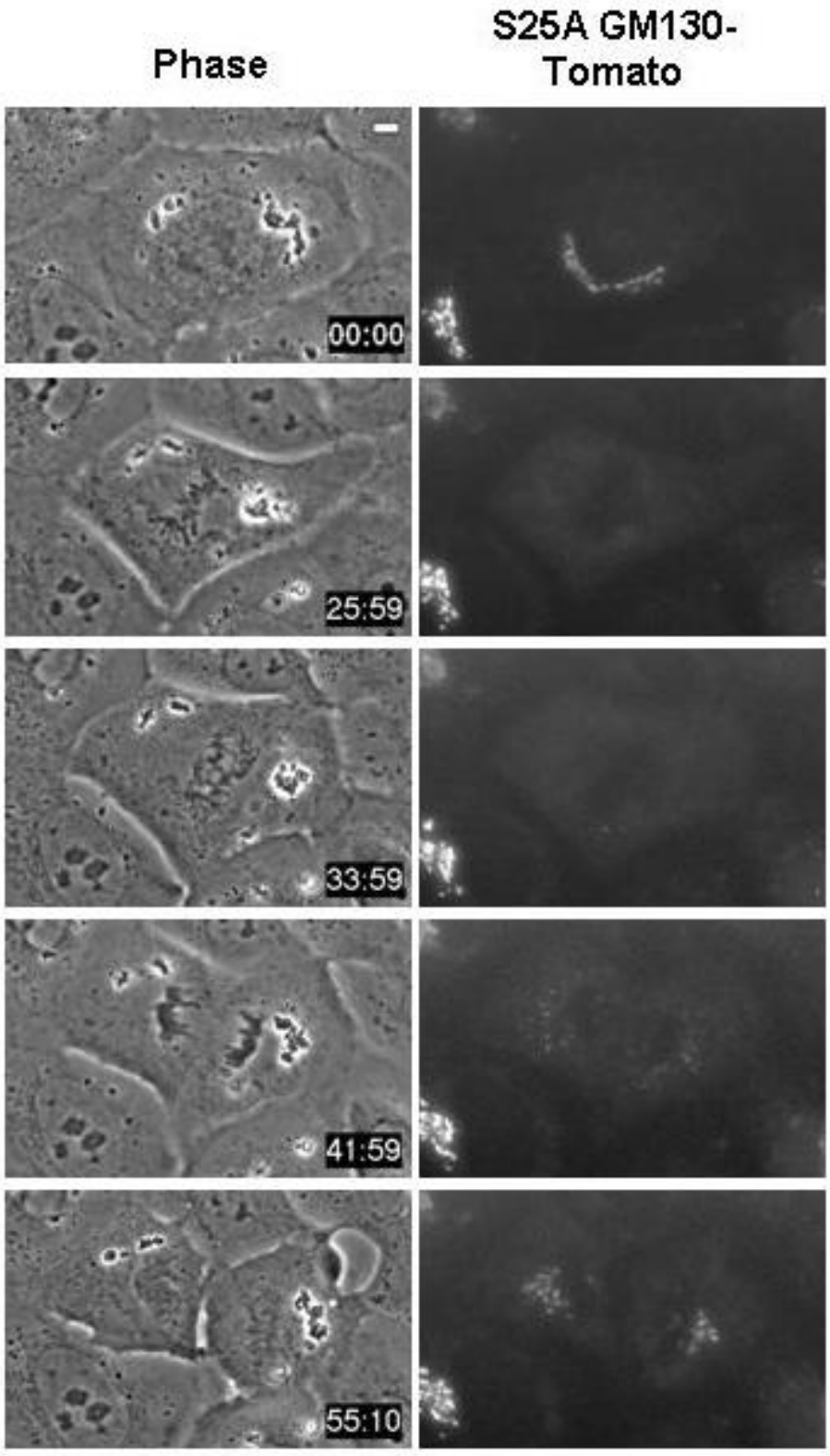


Figure 3.7**: Over expression of S25A GM130-tomato in cells depleted of Gm130 does not affect Golgi vesiculation.** Cells were co transfected with ShRNA and Tomato-GM130 S25A and were incubated for 72 hours before being subjected to imaging. The cells neither showed any visible defects in mitotic progression nor was Golgi dynamics in these cells affected. (n=3) Scale bar represents 5 μm. Time elapsed is shown.

4. Discussion

4.1 Golgi dynamics during mitosis is dependent on Cdk 1

The Golgi breakdown during mitosis has been postulated to be a very important step that controls cell cycle progression. While the initial fragmentation step was shown to offer cells a checkpoint on which to decide cell cycle progression, the reason for the mitotic Golgi vesiculation has been the source of an intense debate for quite some time (Colanzi, Suetterlin et al. 2003). Though the mitotic Golgi haze has been reported in many different mammalian cell types, the nature of the haze depended on the Golgi marker used for visualization. In this study we used a Golgi resident enzyme, and found that the haze appeared uniform with very few bright spots representing mitotic Golgi clusters. However when we used a Golgi matrix protein, a far greater number of Golgi spots were observed. This possibly reflects the different fates of the Golgi constituents wherein the Golgi enzymes enter the ER and the Golgi matrix components remain independent of the ER (Seemann, Pypaert et al. 2002; Pecot and Malhotra 2004).

The molecular mechanism behind the mitotic Golgi vesiculation has remained elusive. It was previously proposed that Cdk 1 acting through its Golgi localized target GM130 played a role in Golgi vesiculation (Lowe, Rabouille et al. 1998). The Cdk 1 inhibitor Purvalanol A was shown to inhibit Cdk 1 levels very effectively in cells (Villerbu, Gaben et al. 2002; Hu, Coughlin et al. 2008). Based on imaging at different concentrations, we decided to use 15μM of the drug to inhibit Cdk 1

activity. This was because purvalanol A was known to inhibit Cdk 1 and Cdk 2 and hence it could cause a G1 and a G2 arrest in addition to its inhibitory activities during mitosis. However at concentrations of 15 uM, the drug seemed to permit cells to enter mitosis while perturbing normal mitotic activity.

In cells treated with purvalanol A, at prophase, the Golgi was observed to be composed of individual blobs, suggesting that Cdk 1 phosphorylation might not be involved in the initial fragmentation process and that this process is mediated by other kinases as described before (Acharya, Mallabiabarrena et al. 1998), (Preisinger, KoÌˆrner et al. 2005). While the experiments with purvalanol A demonstrated the requirement of Cdk1 for mitotic Golgi vesiculation and mitotic progression, it is known that Cdk 1 controls a number of targets in the cell (Nigg 2001) and hence the block in mitotic Golgi vesiculation may or may not be a specific effect of Cdk 1 inhibition. Moreover, the efficiency of the drug seemed to wear off with time, with cells imaged immediately after purvalanol A treatment showing very strong effect compared to the cells which were imaged subsequently. For the above mentioned reasons, a more specific means to perturb mitotic Golgi vesiculation was probed.

4.2 Perturbing GM130 does not modulate mitotic Golgi vesiculation

The Golgi localized target of Cdk 1 during mitosis was then the obvious choice for testing our hypothesis that blocking Cdk1 based phosphorylation events could inhibit Golgi vesiculation and hence mitotic progression. The Golgi matrix protein GM130 was known to be

mitotically phosphorylated by Cdk 1 through biochemical experiments (Nakamura, Lowe et al. 1997; Lowe, Rabouille et al. 1998; Lowe, Gonatas et al. 2000). However, microscopic observation of the GM130 dynamics with and without Cdk1 based phosphorylation was not performed in living cells. In this study, two different variants of GM130 fused to GFP, the full length version and the other being a phosphorylation deficient mutant (S25A, that was incapable of being mitotically regulated), were used. Over expression of the GFP- full length GM130 or the phosphorylation deficient mutant showed normal Golgi dynamics and mitotic progression with the cells capable of forming the characteristic Golgi haze and undergoing cytokinesis in time durations comparable to control cells.

In the above mentioned over expression experiments, we could not exclude the possibility that the endogenous GM130 was able to carry out its function of promoting Golgi vesiculation even in the presence of the excess of the S25A mutant. This necessitated the removal of the endogenous GM130 from the cells. There had been many reports wherein depletion of GM130 was performed by using RNA interference (Puthenveedu, Bachert et al. 2006; Kodani and SuÌˆtterlin 2008; Rivero, Cardenas et al. 2009) . The experiments provided equivocal views on the effect of the depletion on the cells. While some reports suggested that depletion of GM130 affected secretion only mildly, another report suggested that depletion of GM130 in HeLa cells affected cell cycle progression severely. In our experiments, depletion of GM130 in NRK cells was achieved by using ShRNAs. However depletion of GM130 did

not have any effect on mitotic progression or Golgi vesiculation although the interphase Golgi architecture was affected. This was in confirmation with previous reports that suggested that depletion of GM130 affected the ribbon architecture as GM130 could have a role in both cisternae stacking and integration of individual Golgi stacks into a ribbon (Puthenveedu, Bachert et al. 2006). The fact that depletion of GM130 did not have any effect on Golgi fragmentation can also be explained in the following scheme. In interphase cells, in the absence of GM130, the COP I vesicles are unable to dock and fuse with the Golgi however the continued activity of the anterograde transport (mediated by the COPII vesicles) and the ability of the COPI vesicles to fuse directly with the ER suggest that Golgi mediated transport is only slightly affected. During mitosis, the absence of GM130 resembled the mitotic phosphorylation state of GM130 and hence Golgi vesiculation proceeds as usual.

Following the depletion of GM130, over expression experiments were performed in cells depleted of GM130 with TdTomato-S25A GM130. The rationale behind the rescue experiments was that depletion of GM130 somehow resembles the mitotic phosphorylation state and hence had no effect on mitotic Golgi vesiculation. Over expression of the full length GM130 in cells depleted of endogenous GM130 would bring the Golgi architecture back into equilibrium. Due to the presence of the S25 residue, it can still be regulated mitotically. However, over expression of the S25A construct in cells depleted of GM130 can have drastically different effects for the cell. While the cell might regain the interphase Golgi architecture, the absence of the S25 residue will prevent mitotic

phosphorylation and hence will cause a block in mitotic Golgi vesiculation (as illustrated in figure 4.1).

However, over expression of the S25A GM130 protein in cells depleted of endogenous GM130 has no effect on mitotic Golgi vesiculation or mitotic progression.

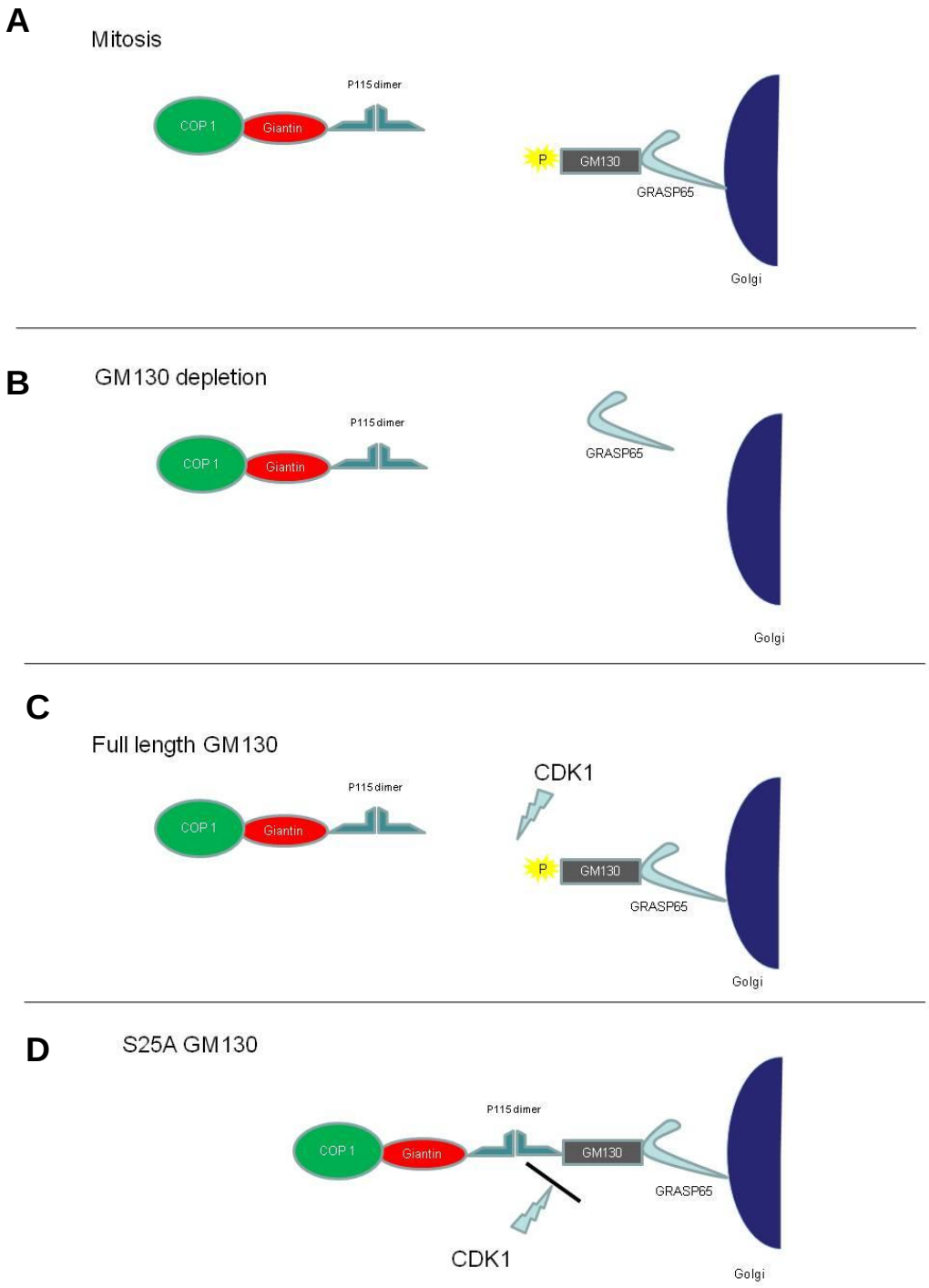


Figure 4.1 **Hypothesis for GM130 mediated Golgi vesiculation.** (A) During mitosis, phosphorylation of GM130 at S25 precludes p115 binding and hence prevents COPI vesicle tethering and could lead to Golgi vesiculation. (B) Depletion of GM130 could remove the scaffolding ability thereby inhibiting COPI vesicle tethering. However the continued anterograde transport maintains Golgi structure substantially. (C) Over expression of FL GM130 in the depletion background restores the mitotic regulatability of GM130 and Golgi structure, however, (D) over

expression of the S25A mutant in the depletion background, though restoring the interphase Golgi structure, could prevent mitotic phosphorylation and will allow COPI vesicle fusion even during mitosis thereby preventing mitotic Golgi vesiculation.

While the possibility of over expressing ShRNA resistant S25A-GM130 in cells depleted of GM130 will lend further support, the preliminary results from the current set of experiments suggest the following possibilities.

Firstly, it is possible that the Cdk 1 mediated phosphorylation of GM130 at the S25 residue may not have any role in mediating Golgi vesiculation. This suggests that the phosphorylation might serve some other purpose during mitosis. It has been shown previously that p115-GM130 interaction is essential for transport through the Golgi (Seemann, Jokitalo et al. 2000). It is possible that the mitotic phosphorylation of GM130 is merely to ensure that transport processes are suspended for the duration of mitosis so as to ensure proper partition of the Golgi. It has also been shown previously that GM130 binds to the t-SNARE component Syntaxin 5 and the small GTPase Rab1 in a p115 regulated manner. It is thus likely that mitotic phosphorylation of GM130 might serve to regulate transport during the process of mitosis and have no specific role in Golgi vesiculation.

A second possibility is that the level of depletion of GM130 that was observed (approx. 75%) was not sufficient to prevent the protein from carrying out its proposed mitotic function of vesiculating the Golgi. This may not be the case because at a comparable level of depletion, the

Golgi structure in terms of the ribbon architecture was found to be disrupted (Puthenveedu, Bachert et al. 2006). A previous report has suggested that up to 90% depletion of GM130 in HeLa cells prevented GRASP65 localization at the Golgi. The same study suggested that depletion of GM130 by up to 90% induced a G phase cell cycle block (Kodani and Sulˆtterlin 2008). In our experiments, NRK cells depleted of GM130 did not have any problems in entering and exiting mitosis. It is possible that the effect observed by the previous report was specific for HeLa cells.

Finally, it is possible that the cell relies on more than just one pathway to achieve mitotic Golgi vesiculation. This is a very attractive possibility since it suggests that mitotic phosphorylation of GM130 at S25 is neither necessary nor sufficient to induce Golgi vesiculation and implies that there are parallel pathways that might contribute to mitotic Golgi vesiculation. While it is very difficult to speculate on the identity of the proteins involved in these parallel pathways, one thing that can be inferred is that the proteins in the pathways are phosphorylated by Cdk 1. Purvalanol A treatment completely abrogated Golgi vesiculation and hence Cdk 1 is involved in Golgi vesiculation. An attractive model is that Cdk1 might mediate multiple phosphorylation events on Golgi localized targets during early prophase. This ensures that the Golgi undergoes vesiculation even if one component or pathway is inactivated. Some of the possible candidates in the pathway are the Golgi matrix proteins such as GRASP55 and GRASP65. GRASP65 was previously shown to have a major role in the first fragmentation event. It is also known to form

a complex with GM130 and the complex helps to maintain the Golgi ribbon architecture (Puthenveedu, Bachert et al. 2006). When GM130 was depleted, GRASP65 localization was also affected (Kodani and SuÌˆtterlin 2008). Thus it is an interesting possibility that Cdk 1 phosphorylates GRASP65 (Separately from the Plk1 based phosphorylation that mediates the initial fragmentation) to help in mitotic Golgi vesiculation. This model of multiple Cdk 1 targets in the Golgi underscores the importance that the cell gives to dispersing the Golgi apparatus not just from its pericentriolar locale but also from having a definite localization during mitosis. This reinforces the idea that the mitotic Golgi vesiculation might serve to accurately segregate the Golgi apparatus to both daughter cells. With regard to its role in regulating mitosis, further work in specifically perturbing Cdk 1 targets in the Golgi is necessary to categorically suggest a role for the Golgi structure in directly regulating mitotic progression in mammalian cells.

5 Conclusion

This study has focussed on understanding the role of Golgi vesiculation in mitotic progression in mammalian cells and more specifically the role of the Golgi matrix protein GM130 in regulating Golgi vesiculation. While previous studies have relied on *in vitro* evidence, this study has demonstrated *in vivo* that Cdk 1 based phosphorylation is essential for regulating mitotic Golgi vesiculation and mitotic progression in mammalian cells.

Previous *in vitro* studies had shown that the Golgi matrix protein GM130 is the target of Cdk1 activity and that mitotic phosphorylation by Cdk 1 regulates GM130 mediated Golgi breakdown. In our study we have shown that over expression of either full length or a phosphorylation deficient mutant of GM130 was incapable of inducing a block in Golgi vesiculation. Subsequent ShRNA experiments and rescue experiments showed that perturbing GM130 did not affect Golgi vesiculation or mitosis lending support to the idea that that Cdk 1 based phosphorylation of GM130 might not be the sole pathway that is responsible for Golgi breakdown during mitosis. Future work has to focus on identifying the Golgi localized targets of Cdk 1 that might act parallel to GM130 in inducing mitotic Golgi vesiculation.

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Appendix

Reagents used

0.05%Trypsin

0.25% trypsin (GIBCO)	10ml
Hank's balanced salt solution (HBBS, GIBCO)	40ml

STE (Per L)

NaCl	150 mM
Tris·HCl (pH 8.0)	44 mM
Tris-Base	6 mM
EDTA (pH 8.0)	1 mM

PBS (per L)

NaCl	80g
$Na_2HPO_4.7H_2O$	22g
KCl	2g
KH_2PO_4	2g