ADHESION-DEPENDENT REGULATION OF GOLGI ORGANIZATION AND FUNCTION IN NORMAL VS CANCER CELLS

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I declare that this written submission represents my idea in my own words and where others' ideas have been included; I have adequately cited and referenced the original sources. I declare that I have acknowledged collaborative work and discussions wherever such work has been included. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that violation of the above will be cause for disciplinary action by the Institute and can also evoke penal action from the sources which have thus not been properly cited or from whom proper permission has not been taken when needed.

The work reported in this thesis is the original work done by me under the guidance of

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I certify that the thesis entitled, *"Adhesion-dependent regulation of Golgi organization and function in normal vs cancer cells"*, presented by Ms. Prachi B Joshi, represents her original work which was carried out by her at IISER, Pune, under my guidance and supervision, during the period from August 1st, 2017 to 15th January 2024. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other University or institution. I further certify that the above statements made by her regarding her thesis are correct to the best of my knowledge.

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CONTENTS

Essential abbreviations

ABD	Arf binding domain
Arf1	ADP-ribosylation factor 1
BFA	Brefeldin-A
BIG1	Brefeldin A-inhibited guanine nucleotide exchange factor 1
BIG2	Brefeldin A-inhibited guanine nucleotide exchange factor 2
CCLE	Cancer Cell Line Encyclopedia
DEGs	Differentially expressed genes
ER	Endoplasmic reticulum
FN	Fibronectin
GalTase	Beta-1,4-galactosyltransferase
GAP	GTPase-activating factor
GBF1	Golgi specific brefeldin A-resistance nucleotide exchange factor 1
GCA	Golgicide-A
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GGA3	Golgi localised γ-ear containing Arf-binding protein 3
GM130	Golgi matrix protein 130
ManII	Mannosidase II
ml	milliliter
MT	Microtubule
MTOC	Microtubule organizing center
RFP	Red fluorescent protein
WT-MEFs	Wild-type mouse embryonic fibroblasts
µl	microliter
µM	micromolar

ABSTRACT

Cancer cells are unique in overcoming the requirement for cell-matrix adhesion (anchorage dependence) to grow, proliferate and metastasize. Among the cellular pathways regulated by adhesion include its ability to control Golgi organization and function. In non-transformed cells, loss of adhesion causes dramatic disorganization of the Golgi accompanied by a change in cell surface glycosylation. Arf1 inhibitor-mediated disruption of the Golgi differentially affects the glycosylation signature of cells. If and how this adhesion-mediated regulation of Golgi is perturbed in cancers remains unclear. To address this, we screened multiple cancer cell lines for their Golgi organization when stable adherent and saw distinct changes. On loss of adhesion, some cancer cell lines interestingly maintained their intact Golgi organization, while others did not. This lead us to identify a pair of lung cancer cells, A549 and CaLu1, with distinct Golgi organizations. An extended screen of CCLE and other databases led us to shortlist differentially expressed genes (DEGs) between these lung cancer cell lines, which could potentially regulate their Golgi organization. Careful evaluation reveals the receptor tyrosine kinase AXL, detected to be localized in the Golgi, to mediate adhesion-independent Golgi organization in A549 cells. This work studies this regulation and evaluates how AXL-dependent Golgi organization could contribute to its oncogenic role in anchorage-independent lung adenocarcinoma cells.

Thesis Title: Adhesion-dependent regulation of Golgi organization and function in cancer cells

SYNOPSIS

INTRODUCTION

Cell-matrix adhesion is essential for most eukaryotic cells survival, growth, and proliferation (Reddig & Juliano, 2005). It regulates multiple cell signaling pathways, which control cellular functions, including cell growth, cell division, and cell migration (Berrier & Yamada, 2007), all deregulated in pathologies such as cancer (Maziveyi et al., 2017). Cancer cells are unique in their ability to overcome the requirement for cell-matrix adhesion, which promotes their anchorage-independent growth and metastasis (Janiszewska et al., 2020; Läubli & Borsig, 2019a; Pawar et al., 2016). The known alterations driving this anchorage independence in cancers are adhesion-independent growth signaling and changes in protein or cell surface glycosylation (Läubli & Borsig, 2019a; Pawar et al., 2016). In cancer cells, sustained activation of growth receptors or sustained trafficking of growth receptors to the plasma membrane without adhesion promotes adhesion-independent signaling (Pawar et al., 2016). Alterations in glycosylation, on the other hand, encourage anchorage independence by overcoming the loss of adhesion-mediated cell death (anoikis) and evasion of death ligands (Petrosyan et al., 2014; Piyush et al., 2017). Glycan alterations are mainly driven by changes in the expression or localization of glycosylation-associated proteins and by changes in Golgi organization, which is the central hub for glycosylation reactions (Bhat et al., 2017; Pinho & Reis, 2015). Cancer cells are indeed seen to carry an altered Golgi morphology, which could be driving the alterations in glycosylation cellular traffic, mechanisms for which are not well understood (Kellokumpu et al., 2002; Läubli & Borsig, 2019b; Petrosyan, 2015; Very et al., 2018)

The Golgi apparatus comprises multiple membranous sacs called cisternae, which stack up in a defined sequence (cis-, medial and trans-Golgi). In mammalian cells, lateral linking between the stacks forms a Golgi ribbon localized to the perinuclear region (Marsh & Howell, 2002; Nakamura et al., 2012; Rambourg & Clermont, 1997). Together with the ER-Golgi intermediate compartment (ERGIC) and the trans-Golgi network (TGN), the Golgi functions as a complex and dynamic system which is the site for post-translational modifications (PTMs) and trafficking events (Huang

& Wang, 2017; Ward et al., 2001). These Golgi functions are dependent on several Golgi-associated regulatory pathways, including the intact organization of the Golgi ribbon (Petrosyan, 2019; Stanley, 2011; X. Zhang & Wang, 2015).

While adhesion is known to regulate membrane trafficking (Pawar et al., 2016), its role in regulating Golgi, which is also involved in membrane traffic, was not known. Studies from the lab have recently shown that adhesion can regulate Golgi organization. Loss of adhesion-mediated Golgi disorganization was found to be mediated by the inactivation of the small GTPase ARF1 and was established to promote distinct changes in cell surface glycosylation. How changes in Golgi organization regulate cell surface glycosylation levels in non-adherent cells remains to be understood. This suggests the presence of a Cell adhesion – Arf1 – Golgi Organization – Glycosylation pathway in normal cells (Singh et al., 2018). If and how this adhesion-mediated regulation of Golgi is perturbed in cancers remains unclear. Such differential regulation of the Golgi in cancers could further impact their glycosylation signature and function.

This study, therefore, investigates the possible role and regulation of Golgi organization and subsequent Golgi function in normal and anchorage-independent cancer cells. To address this, we defined specific objectives in our study as follows -

Specific objectives of the thesis

1. Golgi organization in anchorage-independent cancers and the role of Arf1
2. Identifying regulator(s) of Golgi organization in lung cancer cell lines
3. Role of AXL in the regulation of Golgi organization and function in lung cancer cell lines
4. Loss of adhesion-dependent Golgi organization and it's effect on cell surface glycosylation.

1. Golgi organization in anchorage-independent cancers. Role of Arf1.

We performed a simple screen in adherent cancer cells for their Golgi organization phenotype, using the trans-Golgi marker GalTase-RFP. The Golgi organization was observed to be variable across the eight different cancer cell lines tested. A549 (lung cancer), MDAMB231 (breast cancer), T24, UMUC3, J82 (bladder cancer) and DLD1 (colorectal cancer) showed a visibly 'normal' intact Golgi organization. CaLu1 (lung cancer) and MCF7 (breast cancer) showed a disorganized Golgi phenotype in adherent cells. Knowing that both adhesion-dependent signaling and Golgi organization are often altered in cancers, we asked whether these cancers illustrate adhesion-mediated regulation of Golgi organization. To test this, we looked at the effect loss of adhesion has on Golgi organization using GalTase-RFP in lung cancer (A549 and CaLu1), breast cancer

(MCF7 and MDAMB231) and bladder cancer cells (T24, UMUC3). The disorganized Golgi phenotype in adherent CaLu1 and MCF7 cells was retained on loss of adhesion, while MDAMB231 cells showed loss of adhesion-mediated Golgi dispersal.

Interestingly, the intact Golgi A549, T24, and UMUC3 cells remained unchanged on the loss of adhesion, suggesting an adhesion-independent regulation of Golgi in these cells. This indicates that adhesion-dependent regulation of Golgi organization is variable across cancers. This supports the differential regulation of Golgi organization in these cells. Adhesion-dependent regulation in normal cells is mediated by Arf1 activation, which could be deregulated in these cancers. To test this, we looked at the effect of loss of adhesion has on active Arf1 levels, using GST-GGA3 pull-down, in cancers showing adhesion-independent regulation of Golgi (CaLu1, A549, T24 and UMUC3). Except for T24 cells, other cell lines tested showed a significant decrease in active Arf1 levels on loss of adhesion, similar to 'normal' cells (). Arf1 activation in T24 cells was maintained on loss of adhesion, which could support their intact Golgi organization. Arf1 inhibition using Brefeldin A (BFA; 10ug/µL) disrupted the Golgi as seen for the cis-medial (ManII-GFP) and trans-Golgi (GalTase-RFP) compartments, confirming the role adhesion-independent Arf1 activation has in keeping the Golgi intact in non-adherent T24 cells. BFA-mediated disruption of Golgi was observed in CaLu1, A549 and UMUC3, suggesting that the Golgi organization in these cells is sensitive to further loss in Arf1 activation. The possibility of basal active Arf1 levels being higher in these cancer cells was ruled out since expression and activation levels of Arf1 were tested and found to be comparable between CaLu1, A549, T24, and UMUC3. The same was also similar between CaLu1, A549 and normal lung epithelial cells – BEAS2B, where Golgi showed loss of adhesion-mediated disorganization. This suggests that the regulation of Arf1 by adhesion could also be differential across cancers. These results, however, do point towards the possible role of additional regulatory proteins in causing Golgi organization to be altered in cancers.

2. Identifying regulator(s) of Golgi organization in lung cancer cell lines

CaLu1 and A549 cells have different Golgi organization when stable adherent and non-adherent cells. These differences in Golgi organization could stem from the differential expression of Golgi regulators in these cells. To identify regulators of Golgi organization in these cells, we designed an *in-silico* study screening for genes with significantly different gene expressions (DEGs) between CaLu1 and A549 cells. Golgi-associated genes listed using literature referencing and the NCBI Gene Ontology tool were tested for differential mRNA expression based on data from the CCLE database. We identified 35 DEGs with a fold change of 5 or more, which were further

shortlisted using two scoring steps – i) a first score based on the known effect of the gene knockdown (KD) in regulation of Golgi organization and ii) a second score based on number of Golgi associated primary interactors, derived from protein interaction network which was obtained using STRING database. Combining these scores, 15 genes with a score of 3 and higher were selected as the top candidates of interest. These genes could be potential regulators of Golgi organization in CaLu1 and A549 cells and were hence considered for further evaluation.

3. Role of AXL in regulating Golgi organization and function in lung cancer cell lines

Of the top 15 candidate genes identified, 11 were overexpressed in CaLu1 relative to A549, and four were overexpressed in A549 relative to CaLu1. To begin with, we focused on the 11 genes overexpressed in CaLu1, confirming their differential mRNA expression using RT-PCR. The mRNA used for cDNA synthesis was tested for its quality, and the primers used were tested for their specificity and efficiency before RT-PCR experiments. From the genes showing differential mRNA expression between A549 and CaLu1, we arrived at the gene - AXL as our top candidate for further study. The choice of AXL was based on the highest combined score obtained for AXL, from scores for protein interaction network and the known effect of AXL KD on Golgi organization. The selection of AXL was further supported by the fact that AXL knockdown promotes an intact Golgi phenotype (Chia et al., 2012a), which agrees with its relatively lower expression in A549 cells with an intact Golgi. As a potential regulator of Golgi in CaLu1 and/or A549 cells, AXL was further evaluated for its protein expression. In line with mRNA expression data, AXL protein levels were also higher in CaLu1 than in A549. Immunostaining further revealed a distinct pool of AXL to localize at cis-Golgi (GM130) in both CaLu1 and A549 cells. These data suggested a possible role for AXL at the Golgi in these cells.

AXL is a receptor tyrosine kinase that regulates primary cell functions such as cell proliferation, migration and cell survival (Dagamajalu et al., 2021; Zhu et al., 2019). Though AXL has been extensively studied as an oncogene, very little is known about its possible role at the Golgi (Chia et al., 2012a; Zajac et al., 2020). Therefore, we aimed to test the role of AXL in regulating Golgi organization in both CaLu1 and A549 cells. Golgi organization is differentially altered in these cells; hence, we tested the effect of AXL inhibition in adherent CaLu1 cells (Golgi disorganized) and in non-adherent A549 cells (Golgi stays intact) to see if 'normal' Golgi organization under these conditions is restored. Treatment with 1μM R428, a selective inhibitor for AXL (Holland et al., 2010), for 12hrs, was found to inhibit Akt activation, which is a known readout of AXL inhibition (Holland et al., 2010) since phospho-AXL antibodies were poorly detected and are

actively being optimized in the lab. R428 mediated AXL inhibition did not affect the disorganized Golgi in adherent CaLu1 cells, tested with cis-medial Golgi marker (ManII-GFP).

Interestingly, R428 treatment promoted Golgi disorganization in non-adherent A549 cells, as confirmed with cis- (GM130), cis-medial (ManII-GFP), and trans-Golgi (GalTase-RFP) markers. We further found that AXL inhibition by R428 led to the loss of AXL from the Golgi, which could drive the Golgi disorganization observed in non-adherent A549 cells. Although the loss of adhesion seemed to affect AXL localization at the Golgi slightly, a pool of AXL was distinctly retained. This AXL localized to the Golgi in untreated control cells was significantly more than in non-adherent A549 cells treated with R428, as quantified using Pearson's coefficient. These data together suggest that inhibition of Golgi-associated AXL with R428 could be responsible for restoring adhesion-dependent Golgi organization in A549 cells. We identified the small GTPase Arf1 as a potential regulator working downstream to AXL from a group of different adhesion-dependent Golgi regulatory proteins, which were tested for a role in mediating the regulation of Golgi by AXL. R428-mediated AXL inhibition did not affect Arf1 activation (measured using GST-GGA3 pull-down assay) in non-adherent A549 cells, where active Arf1 levels inherently show a distinct drop in loss of adhesion. This inhibition, however, led to the loss of the active Arf1 retained at the Golgi (detected using ABD-GFP), which could potentially drive Golgi dispersal seen in these cells.

Interestingly, expressing constitutively active Arf1 (Q71L-ARF1) in these cells restored Golgi organization in the presence of AXL inhibition by R428. Active Arf1 pull-down fraction, when probed for AXL, also revealed the association of AXL possibly bound to active Arf1, which was unaffected by R428-mediated AXL inhibition. Our data suggests that a possible crosstalk between AXL and Arf1 could be responsible for the adhesion-independent regulation of Golgi organization in A549 cells. Our data further showed a modest but significant drop in cell surface binding of ConA (specific for mannose glycans) and WGA (specific for Sialic acid/N-GlcNAc glycans) lectins in non-adherent A549 cells treated with R248. This effect of AXL inhibition on cell surface glycosylation levels could be mediated by its impact on the Golgi in these cells, which remains to be tested. AXL inhibition also significantly affected the anchorage-independent growth of A549 cells, which could again be driven by its effect on Golgi dispersal in these cells. Our data thus identifies AXL as a potential regulator of Golgi organization and function in A549 cells. Its possible crosstalk with Arf1 in mediating this regulation and its role in the regulation of Golgi function and cancer cell function is worth investigating in the future.

4. Loss of adhesion-dependent Golgi organization. Effect on cell surface glycosylation.

Loss of adhesion-mediated Golgi disorganization was reported to affect cell surface glycosylation levels (Singh et al., 2018). Using a carefully defined range of increasing BFA concentrations (0.2 μM, 0.7μM, 1.8μM, 6μM and 17.8μM) in non-adherent WTMEFs, we obtained a consistent and reproducible gradient in Golgi organization (disorganization vs fragmentation). Along this gradient, non-adherent WTMEFs with a disorganized Golgi gradually showed an increase in the percentage of cells with complete Golgi fragmentation (and fallback of cis-Golgi into ER). The effect of BFA-mediated changes in Golgi organization on corresponding levels of cell surface glycan was tested using Flow cytometry analysis. Median intensities for cell surface binding of ConA (lectin specific to mannose glycans) showed a steep and consistent decrease.

In contrast, those for cell surface binding of WGA (lectin specific to Sialic acid/N-GlcNAc binding) showed little to no effect. These results were obtained using ConA and WGA tagged with two distinct fluorophores, making the data that much more comprehensive. Changes seen on 30 minutes of BFA treatment were interestingly retained for 90 minutes. This reveals differential regulation of glycan substrates by BFA-mediated changes in Golgi organization. WGA is known to be synthesized from ConA substrate in the Golgi (Sharma et al., 2014), making the regulation of their cell surface levels interpretable in the context of their relative processing in the Golgi. The Golgi organization could uniquely regulate this. We further tested if there were any differences in the endocytosis of these glycan substrates on treatment with BFA since this could contribute to their observed cell surface levels. BFA had no significant effect on endocytosis of these glycan substrates. A comprehensive quantitative evaluation of single-cell lectin binding data (Flow cytometry) and endocytosis data (confocal image analysis) was carried out, and the data was normalized to help derive a model for how Golgi processing could affect lectin levels at the cell surface (Done in collaboration with Dr Mukund Thattai and Aashish Satyajith from NCBS).

A mathematical model was derived from an existing understanding of the Golgi-mediated glycosylation and trafficking events in cells. Our experimental data, when looked at in the context of the model, was used to revise the same to better understand how the Golgi organization affects glycosylation. The revised model suggests that at lower BFA concentrations, depletion of influx from Golgi could affect the synthesis of ConA substrate, which seems to impact its cell surface levels directly. ConA substrate synthesis, while reduced, could accumulate to affect levels of WGA substrate in the trans-Golgi. At higher concentrations of BFA, Golgi fragmentation affects influx,

which depletes ConA substrate, thereby decreasing WGA substrate levels at the cell membrane. The proposed model provides a plausible explanation for differential processing and representation of cell surface glycans (ConA and WGA substrates) in response to changing Golgi organization. The interplay of recycling and new synthesis of glycans within a time frame of 90 minutes and the proposed impact of BFA on trans-Golgi are amongst the significant quantitative outcomes of our study. This relatively sparse model shows precisely and quantitatively how Golgi morphology impacts cell-surface glycan levels as a function of concentration and time.

5. Role of active Arf1 in regulating Golgi disorganization mediated by DNA damage

Recent studies reported DNA damage-mediated Golgi dispersal in cancer cells. This was shown to be mediated by DNA-PK phosphorylation, which in turn prevents GOLPH3 recruitment at the Golgi (Buschman et al., 2015a; Farber-Katz et al., 2014). We tested a role for Arf1 activation in DNA damage-mediated regulation of Golgi disorganization. A distinct increase in phosphorylated DNA-PK levels (S2056) seen with immunostaining, post 6h, 12h and 24hrs of induction confirmed the induction of DNA damage by UV radiation ($10J/m^2$) in adherent MCF10A (normal human breast epithelial) cells, used in the study. At these time points, Golgi dispersal observed using immunostaining for cis-Golgi marker (GM130) was seen to be increasingly more distinct, with mild enlargement at 6h, to complete disorganization at 24h. To test if DNA damage could mediate Golgi dispersal through its effect on Arf1 activation, we measured active Arf1 levels at the early (6h) and late (24h) time points post-induction, using GST-GGA3 pull-down assay. At 6h post UV induction, we observed a modest but significant drop in levels of active Arf1; at 24h, these levels recovered completely, with no significant difference relative to control. This could suggest a possible role for Arf1 activation downstream to DNA damage in mediating Golgi disorganization at an early time point post-induction. At later time points, however, recovery in levels of active Arf1 points towards a different mechanism at play, which could be responsible for sustaining the Golgi disorganization in these cells, even in the presence of active Arf1. Present data discusses preliminary results and more evaluation would be required to comment further on the above observations.

SUMMARY

Present work shows that adhesion-dependent regulation of the Golgi organization is variable in different cancers. Our in-silico study identified AXL as a novel regulator of the adhesion-independent Golgi organization in A549 cells. This role of AXL at the Golgi could possibly be mediated by a regulatory crosstalk between AXL and the small GTPase Arf1, which needs further

exploring. AXL inhibition by R428 also affected the cell surface glycosylation and anchorage-independent growth in these cells. This could be driven by the regulatory role of AXL at the Golgi and its effect on its organization in non-adherent A549 cells. Taken together, such an AXL-mediated Golgi regulation could also contribute to its oncogenic potential. The role AXL activation through its ligands like Gas6 might have in mediating the AXL-Golgi crosstalk is important to consider in the above context.

The known change in cell surface glycosylation as the Golgi organization changes is vital to understanding how cells (including cancer cells) use the Golgi to drive cell function. Using non-adherent WTMEFs as a unique Golgi organization tool has allowed us to disrupt Golgi organization with BFA concentration range and achieve distinct Golgi phenotypes that can then be evaluated for cell function. A profile for changes in cell surface glycosylation levels, along a gradient of changing Golgi organization, was arrived at, along with changes in glycan endocytosis. Using a comprehensive mathematical model, looking at Golgi trafficking, processing of glycans, their delivery to the plasma membrane and their endocytosis, our data was used to provide early mechanistic insights into how changes in Golgi organization could variably affect cell surface glycosylation. It is worth testing if this predicted understanding of Golgi structure-function dynamics holds in experimental outcomes.

REFERENCES

Berrier, A. L., & Yamada, K. M. (2007). Cell-matrix adhesion. *Journal of Cellular Physiology*, *213*(3), 565–573. https://doi.org/10.1002/jcp.21237

Bhat, G., Hothpet, V.-R., Lin, M.-F., & Cheng, P.-W. (2017). Shifted Golgi targeting of glycosyltransferases and α-mannosidase IA from giantin to GM130-GRASP65 results in formation of high mannose N-glycans in aggressive prostate cancer cells. *Biochimica et Biophysica Acta*, *1861*(11 Pt A), 2891–2901. https://doi.org/10.1016/j.bbagen.2017.08.006

Buschman, M. D., Xing, M., & Field, S. J. (2015). The GOLPH3 pathway regulates Golgi shape and function and is activated by DNA damage. *Frontiers in Neuroscience*, *9*, 362. https://doi.org/10.3389/fnins.2015.00362

Chia, J., Goh, G., Racine, V., Ng, S., Kumar, P., & Bard, F. (2012). RNAi screening reveals a large signaling network controlling the Golgi apparatus in human cells. *Molecular Systems Biology*, *8*, 629. https://doi.org/10.1038/msb.2012.59

Dagamajalu, S., Rex, D. A. B., Palollathil, A., Shetty, R., Bhat, G., Cheung, L. W. T., & Prasad, T. S. K. (2021). A pathway map of AXL receptor-mediated signaling network. *Journal of Cell Communication and Signaling*, *15*(1), 143. https://doi.org/10.1007/S12079-020-00580-5

Farber-Katz, S. E., Dippold, H. C., Buschman, M. D., Peterman, M. C., Xing, M., Noakes, C. J., Tat, J., Ng, M. M., Rahajeng, J., Cowan, D. M., Fuchs, G. J., Zhou, H., & Field, S. J. (2014). DNA damage triggers Golgi dispersal via DNA-PK and GOLPH3. *Cell*, *156*(3), 413–427. https://doi.org/10.1016/j.cell.2013.12.023

Holland, S. J., Pan, A., Franci, C., Hu, Y., Chang, B., Li, W., Duan, M., Torneros, A., Yu, J., Heckrodt, T. J., Zhang, J., Ding, P., Apatira, A., Chua, J., Brandt, R., Pine, P., Goff, D., Singh, R., Payan, D. G., & Hitoshi, Y. (2010). R428, a selective small molecule inhibitor of Axl kinase, blocks tumor spread and prolongs survival in models of metastatic breast cancer. *Cancer Research*, *70*(4), 1544–1554. https://doi.org/10.1158/0008-5472.CAN-09-2997/655769/P/R428-A-SELECTIVE-SMALL-MOLECULE-INHIBITOR-OF-AXL

Huang, S., & Wang, Y. (2017). Golgi structure formation, function, and post-translational modifications in mammalian cells. *F1000Research*, *6*, 2050. https://doi.org/10.12688/f1000research.11900.1

Janiszewska, M., Primi, M. C., & Izard, T. (2020). Cell adhesion in cancer: Beyond the migration of single cells. *The Journal of Biological Chemistry*, *295*(8), 2495–2505. https://doi.org/10.1074/jbc.REV119.007759

Kellokumpu, S., Sormunen, R., & Kellokumpu, I. (2002). Abnormal glycosylation and altered Golgi structure in colorectal cancer: dependence on intra-Golgi pH. *FEBS Letters*, *516*(1–3), 217–224. https://doi.org/10.1016/s0014-5793(02)02535-8

Läubli, H., & Borsig, L. (2019a). Altered cell adhesion and glycosylation promote cancer immune suppression and metastasis. *Frontiers in Immunology*, *10*, 2120. https://doi.org/10.3389/fimmu.2019.02120

Läubli, H., & Borsig, L. (2019b). Altered cell adhesion and glycosylation promote cancer immune suppression and metastasis. *Frontiers in Immunology*, *10*, 2120. https://doi.org/10.3389/fimmu.2019.02120

Marsh, B. J., & Howell, K. E. (2002). The mammalian Golgi–complex debates. *Nature Reviews. Molecular Cell Biology*, *3*(10), 789–795. https://doi.org/10.1038/nrm933

Maziveyi, M., Alahari, S. K., Maziveyi, M., & Alahari, S. K. (2017). Cell matrix adhesions in cancer: The proteins that form the glue. *Oncotarget*, *8*(29), 48471–48487. https://doi.org/10.18632/ONCOTARGET.17265

Nakamura, N., Wei, J.-H., & Seemann, J. (2012). Modular organization of the mammalian Golgi apparatus. *Current Opinion in Cell Biology*, *24*(4), 467–474. https://doi.org/10.1016/j.ceb.2012.05.009

Pawar, A., Meier, J. A., Dasgupta, A., Diwanji, N., Deshpande, N., Saxena, K., Buwa, N., Inchanalkar, S., Schwartz, M. A., & Balasubramanian, N. (2016). Ral-Arf6 crosstalk regulates Ral dependent exocyst trafficking and anchorage independent growth signalling. *Cellular Signalling*, *28*(9), 1225–1236. https://doi.org/10.1016/j.cellsig.2016.05.023

Petrosyan, A. (2015). Onco-Golgi: Is Fragmentation a Gate to Cancer Progression? *Biochemistry & Molecular Biology Journal*, *1*(1). https://doi.org/10.21767/2471-8084.100006

Petrosyan, A. (2019). Unlocking Golgi: Why Does Morphology Matter? *Biochemistry. Biokhimiia*, *84*(12), 1490. https://doi.org/10.1134/S0006297919120083

Petrosyan, A., Holzapfel, M. S., Muirhead, D. E., & Cheng, P.-W. (2014). Restoration of compact Golgi morphology in advanced prostate cancer enhances susceptibility to galectin-1-induced

apoptosis by modifying mucin O-glycan synthesis. *Molecular Cancer Research*, *12*(12), 1704–1716. https://doi.org/10.1158/1541-7786.MCR-14-0291-T

Pinho, S. S., & Reis, C. A. (2015). Glycosylation in cancer: mechanisms and clinical implications. *Nature Reviews. Cancer*, *15*(9), 540–555. https://doi.org/10.1038/nrc3982

Piyush, T., Rhodes, J. M., & Yu, L.-G. (2017). MUC1 O-glycosylation contributes to anoikis resistance in epithelial cancer cells. *Cell Death Discovery*, *3*, 17044. https://doi.org/10.1038/cddiscovery.2017.44

Rambourg, A., & Clermont, Y. (1997). Three-dimensional structure of the Golgi apparatus in mammalian cells. In E. G. Berger & J. Roth (Eds.), *The Golgi Apparatus* (pp. 37–61). Birkhäuser Basel. https://doi.org/10.1007/978-3-0348-8876-9_2

Reddig, P. J., & Juliano, R. L. (2005). Clinging to life: cell to matrix adhesion and cell survival. *Cancer Metastasis Reviews*, *24*(3), 425–439. https://doi.org/10.1007/s10555-005-5134-3

Sharma, V., Ichikawa, M., & Freeze, H. H. (2014). MANNOSE METABOLISM: MORE THAN MEETS THE EYE. *Biochemical and Biophysical Research Communications*, *453*(2), 220. https://doi.org/10.1016/J.BBRC.2014.06.021

Singh, V., Erady, C., & Balasubramanian, N. (2018). Cell-matrix adhesion controls Golgi organization and function through Arf1 activation in anchorage-dependent cells. *Journal of Cell Science*, *131*(16). https://doi.org/10.1242/jcs.215855

Stanley, P. (2011). Golgi glycosylation. *Cold Spring Harbor Perspectives in Biology*, *3*(4). https://doi.org/10.1101/cshperspect.a005199

Very, N., Lefebvre, T., & El Yazidi-Belkoura, I. (2018). Drug resistance related to aberrant glycosylation in colorectal cancer. *Oncotarget*, *9*(1), 1380–1402. https://doi.org/10.18632/oncotarget.22377

Ward, T. H., Polishchuk, R. S., Caplan, S., Hirschberg, K., & Lippincott-Schwartz, J. (2001). Maintenance of Golgi structure and function depends on the integrity of ER export. *The Journal of Cell Biology*, *155*(4), 557–570. https://doi.org/10.1083/jcb.200107045

Zajac, O., Leclere, R., Nicolas, A., Meseure, D., Marchiò, C., Vincent-Salomon, A., Roman-Roman, S., Schoumacher, M., & Dubois, T. (2020). AXL Controls Directed Migration of Mesenchymal Triple-Negative Breast Cancer Cells. *Cells*, *9*(1). https://doi.org/10.3390/CELLS9010247

Zhang, X., & Wang, Y. (2015). GRASPs in Golgi Structure and Function. *Frontiers in Cell and Developmental Biology*, *3*, 84. https://doi.org/10.3389/fcell.2015.00084

Zhu, C., Wei, Y., & Wei, X. (2019). AXL receptor tyrosine kinase as a promising anti-cancer approach: functions, molecular mechanisms and clinical applications. *Molecular Cancer 2019 18:1*, *18*(1), 1–22. https://doi.org/10.1186/S12943-019-1090-3

Chapter 1

Introduction and Review of Literature

1.1 The Golgi apparatus in mammalian cells

Introduction with historical background

The complex nature of the cellular structure and function has intrigued and overwhelmed the scientific community for ages. Understanding cell biology is limited by the techniques available for studying cells (Barbara Mullock & Paul Luzio, 2000). Therefore, discoveries in the field have always been time-tied with technological advancements. The invention of the light microscope was one of the first essential breakthroughs that led to the discovery of the cell by Robert Hooke in 1665. (Ribatti, 2018). However, better insights into the intracellular architecture were obtained only 200 years later when studies with improved versions of compound microscopes revealed the internal complexity of living cells. In the early 1800s, studies by Brown, Schleiden and Schwann suggested that all eukaryotic cells have a well-defined nucleus (Ribatti, 2018). Several other nanoscopic cell organelles, including the mitochondria, the lysosomes, chloroplasts, etc., were identified and described in the 19th century, aided by the development of chemical-based staining procedures. Discovery of the 'Golgi apparatus', or the 'Golgi complex', happened around the same time and was first reported by its namesake Camillo Golgi (Mazzarello et al., 2009).

In April 1898, Golgi observed a reticular structure in the cell body of neurons from a mouse cerebral cortex sample. This reticular network was not physically connected to the plasma membrane or the nucleus. Golgi referred to it as '*apparato reticulare interno*, ' which in Latin means internal reticular apparatus (Fig 1.1). Since this observation was based on staining experiments done with heavy metal impregnation, the scientific community believed it to be an experimental artefact (Jamieson, n.d.; Mazzarello et al., 2009). The invention of electron microscopy in the 1930s accelerated studies of intra-cellular organelles, which supported the claims regarding the existence of the Golgi complex to a certain extent (Barbara Mullock & Paul Luzio, 2000; Jamieson, n.d.). Finally, in the 1950s, Dalton and Felix published electron microscopy-based images of the Golgi apparatus, which, in addition to convincingly proving the Golgi apparatus's existence, also provided structural insights into the nature of the Golgi apparatus and its organization (Dalton & Felix, 1956.; FARQUHAR & PALADE, 1998). Over the years, many independent studies

have established that the Golgi apparatus is a highly conserved cell organelle in every eukaryotic cell type across species (Petrosyan, 2019).

a) b)

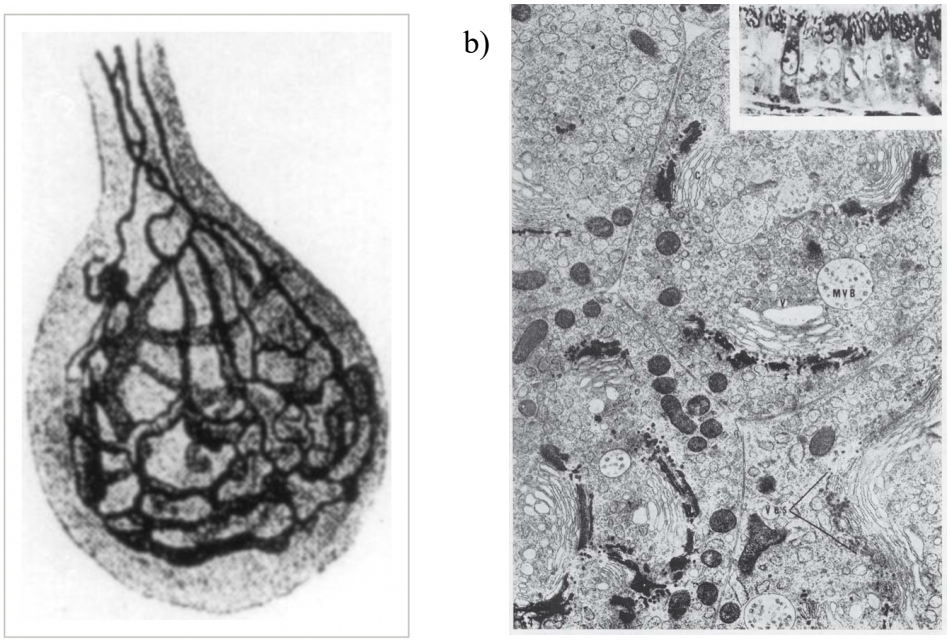


Figure 1.1 a) First published illustration of the Golgi apparatus as provided by Camillo Golgi in Journal of Microscopy, 1989 Source – Mazzarello et al., FEBS Letters, Volume: 583, 2009. b) Appearance of the Golgi in cells from the mouse epididymus after impregnation with OsO4. The cells photographed by light microscopy demonstrates the ribbon-like structure of the Golgi. C=Golgi cisternae, V - vacuole, MVB - multivesicular body, VES - vesicles. Source – Madame Curie Bioscience database, NCBI Bookshelf. Original image from Amer. J. Anat. 117, 145.

However, the Golgi apparatus's morphology differed in different organisms (Griffing, 1991; Klute et al., 2011; Kondylis & Rabouille, 2009; Papanikou & Glick, 2009). The Golgi morphology was also variable, depending on the type of cell and the physiological changes occurring in a cell for a particular organism (Kulkarni-Gosavi et al., 2019). Further, the role of the Golgi apparatus was established in the processing and trafficking of lipids and proteins in the cells. The Golgi apparatus is a highly complex system, its structure and function being intricately regulated by over 500 proteins in cells. Its role in disease is still largely unknown (Stanley, 2011). This study aims to understand the regulation of the Golgi in mammalian cells, with a special focus on anchorage-independent human cancer cells.

1.1.1 Golgi organization in physiology

The Golgi apparatus is a complex and dynamic assembly of numerous smaller structures and compartments that coordinate as part of the endomembrane system in all metazoan cell types. (Hua & Graham, n.d. 2000). The endomembrane system refers to the components of the secretory and endocytic pathways, including the Endoplasmic reticulum (ER), Golgi apparatus, endosomes, lysosomes, secretory and transport vesicles and the associated dynamics at the plasma membrane. This facilitates the synthesis and supply of biomolecules such as proteins and lipids and the recycling of components across the cell. The Golgi apparatus, which is the primary focus of this study, plays a central role in the functioning of the endomembrane system (Agliarulo & Parashuraman, 2022; Alberts et al., 2002).

1.1.1.1 Structure of the Golgi apparatus

Even though the Golgi structure is dynamic, it is functionally and spatially divided into cis-, medial- and trans-Golgi compartments composed of membranous sacs called cisternae. Besides the cisternal stacks, the Golgi apparatus includes the ER-Golgi intermediate compartment (ERGIC) and the trans-Golgi network (TGN) (Lowe, 2011). Each of the Golgi stacks is made up of 3 to 20 cisternae, and in mammalian cells, these stacks are often laterally linked to form a crescent moon-shaped structure, also referred to as the Golgi ribbon. This Golgi ribbon is located near the centrosome in cells. (Nakamura et al., 2012)

The Golgi stacks show a particular polarity in their organization, which is common in most eukaryotic cell types (Hua & Graham, n.d.2000). The cis-Golgi membranes face towards the ER, the trans-Golgi faces the plasma membrane side of the cell, while the medial Golgi is positioned between the cis- and the trans-Golgi compartments (Fig.1.2) (Lowe, 2011; Marsh & Howell, 2002)

Vesicles with cargo budding off the ER exit sites move through the ERGIC region along the microtubules and dock at the cis-Golgi membranes (Marra et al., 2007). The cis-, medial, and trans-Golgi compartments sequentially process the cargo, mainly consisting of proteins and lipids. They also release and receive transport vesicles to help shuttle specific cargo and recycle the endomembrane system's components. The trans-Golgi membranes

continue into a tubular and branched structure called the Trans-Golgi Network (TGN) (Glick & Nakano, 2009).

The TGN facilitates cargo trafficking from the Golgi to its appropriate destination sites in the cell or the plasma membrane for secretion. Therefore, the organization of the Golgi membranes complements the distinct functions of the different Golgi compartments, which extend into vesicular-tubular networks (Carlton et al., 2020; Glick & Nakano, 2009)

1.1.1.2 Structure-function relation within the Golgi apparatus

Though structurally similar, the cis-, medial- and trans-Golgi compartments differ in the proteins localized at these membranes, lipid composition, and the luminal environment. (Cluett et al., 1997; Day et al., 2013; Munro, 2005). The cis-Golgi membranes are rich in phospholipids, similar to the ER membranes. Towards the TGN end of the Golgi, the membrane composition reflects the composition of the plasma membrane and is rich in sphingolipids and cholesterols (Agliarulo & Parashuraman, 2022).

The Golgi membranes recruit three types of Coatomer complex proteins (COPI, COPII and COPIII or Clathrin) for the trafficking of transport vesicles, which are specific to the different Golgi cisternae. The trans-Golgi and TGN recruit and associate with the Clathrin protein-coated vesicles, while the cis- and medial-Golgi membranes primarily associate with the COPI and COPII vesicles. Through the vesicular system, the Golgi apparatus carries out anterograde and retrograde cargo trafficking in transit. This kind of bi-directional trafficking also helps to retain the localization of the proteins and enzymes native to the respective Golgi compartments (Glick & Nakano, 2009; Stalder & Gershlick, 2020a).

Specific localization of these enzymes and proteins at their respective Golgi compartments is crucial for carrying out accurate modifications on the cargo, which flows sequentially through the Golgi cisternae (Banfield, 2011; Gill et al., 2010; Huang & Wang, 2017). Taken together, the Golgi compartments' organisation is, therefore, closely linked to the proper functioning of the Golgi apparatus.

1.1.1.3 Different types of Golgi organization in physiology

The architecture of the Golgi apparatus is defined and yet transient. Depending on the physiological functions occurring in a cell, the Golgi structure assumes different morphological forms (Makhoul et al., 2019). Generally, mammalian cells have a Golgi ribbon located in the perinuclear region (Fig 1.3a). However, this architecture changes differently under other physiological circumstances. During embryo development and cellular differentiation, the intact ribbon-like Golgi architecture is lost, and Golgi components are dispersed in the cytoplasm around the perinuclear region (Lu et al., 2001; Zhong, 2011).

This morphology of the Golgi apparatus was often referred to as a disorganized Golgi structure (Fig 1.3b). The translational epithelial lining of the bladder in adult mammals also presents a disorganized Golgi architecture owing to the highly secretory nature of these cells (Kreft et al., 2022). During cell division, the Golgi is fragmented, where most of the Golgi membrane-associated proteins and enzymes fall back into the ER (Fig 1.3c) (Ayala et al., 2020). When the Golgi is in fragmented form, specific structural matrix proteins remain tethered to the microtubules, which is suggested to aid rapid Golgi biogenesis on completion of cell division. These retained structural components are known as Golgi remnants (Lowe, 2002; Ward et al., 2001). The Golgi fragmentation occurring during cell division serves as an essential cell cycle checkpoint, ensuring accuracy in cell division and equal distribution of Golgi components to the daughter cells (Ayala & Colanzi, 2022; Carlton et al., 2020).

Thus, Golgi organization is transiently disorganized or fragmented to promote cell function in normal physiology. These morphological changes are often irreversibly altered in disease. Since Golgi organization is closely connected to the proper functioning of the Golgi apparatus, it affects the cellular function in these cells (Liu et al., 2021; Makhoul et al., 2019).

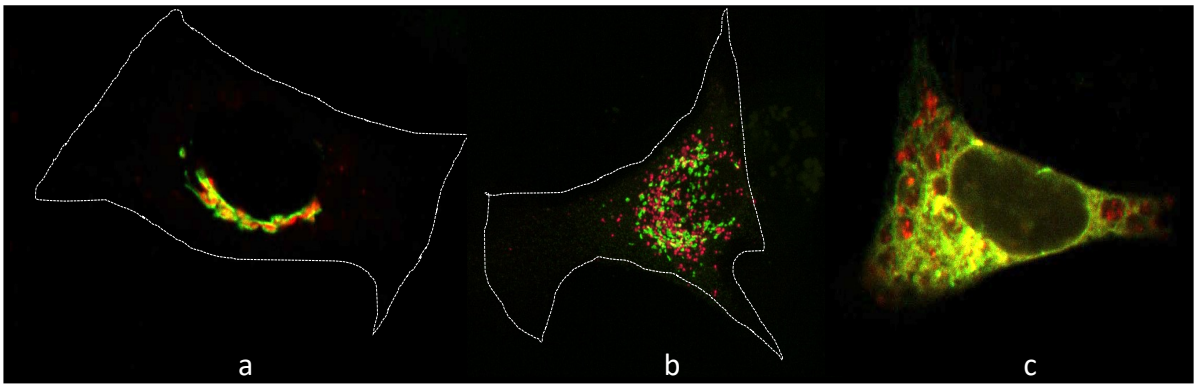


Figure 1.2 : Different types of Golgi organization Representative images depicting Golgi organization in stable adherent Mouse embryonic fibroblasts cells. a) Cell shows organized or intact Golgi ribbon in the perinuclear region. b) Cells shows numerous Golgi objects dispered in the near nuclear region, which is refered to as the disorganized Golgi phenotype. c) Cell shows Golgi haze through the cytoplasm, indicating the fall back of Golgi components into the ER. This morphology of the Golgi is refered to as fragmented Golgi phenotype. Cells are transfected with cis-Golgi marker MannosidaseII-GFP (in green) and trans-Golgi marker GalTase-RFP (in red). Images taken at Zeiss confocal microscope (LSM710).

1.1.2 Functions of the Golgi apparatus

The Golgi apparatus is a central player in the functioning of the endomembrane system, which in turn regulates the supply of proteins, lipids and specific polysaccharides. Despite its highly complex architecture, the general functions of the Golgi are very much conserved across the eukaryotic organisms (Klute et al., 2011; Mowbrey & Dacks, 2009). The Golgi apparatus is a major hub for glycosylation of lipids and post-translational modifications of proteins, thereby regulating the repertoire of proteins and lipids in cells (Glick & Nakano, 2009; Huang & Wang, 2017; C. Xu & Ng, 2015). Through trafficking and secretion of this processed cargo, the Golgi apparatus regulates cellular homeostasis, cell signaling, gene expression, and cell behaviour, concerning migration, differentiation, and cell survival, amongst other cellular functions (Cockcroft, 2021; Hurtado et al., 2011; Kulkarni-Gosavi et al., 2019; Luchsinger et al., 2018).

In addition, as discussed earlier, the dynamics of the Golgi organization are also crucial for the cell cycle process, where the Golgi morphology serves as a checkpoint through the events of cell division (Ayala & Colanzi, 2022). Furthermore, recent studies have revealed that the Golgi apparatus is a secondary site for microtubule nucleation in cells (Sanders & Kaverina, 2015). Considering the scope of the present study, I will now elaborate on the

extensively studied role of the Golgi apparatus in the processing and trafficking of proteins and lipids in cells.

1.1.2.1 Processing of proteins and lipids by the Golgi

Most proteins and lipids are synthesized at the ER, followed by their entry into the ER lumen, where lipids and proteins' primary processing occurs. The immature proteins and lipids are packaged into vesicles that bud off at ER exit sites and move towards the cis-Golgi membranes. At the Golgi membranes, lipids mainly undergo glycosylation, while proteins assume diverse post-translational modifications, including glycosylation, phosphorylation, sulfation and acetylation (Stanley, 2011).

Glycosylation constitutes a sequential cascade of enzymatic reactions, which label proteins, saccharides, and lipids with specific sugar moieties. The glycosylation process is highly complex, orchestrated by numerous glycosyltransferases and glycosidases. More than 250 such enzymes are native to the Golgi apparatus, essentially the hub of glycan modifications on proteins in lipids (Pothukuchi et al., 2019a; X. Zhang & Wang, 2016).

Glycan refers to all such carbohydrate moieties, including monosaccharides, oligosaccharides, and polysaccharides, which may also have one or more covalently linked phosphate and/or sulfate groups. In all, ten monosaccharides are involved in the generation of complex glycan structures on proteins and lipids – Galactose, Fucose, Xylose, Mannose, Glucose, Glucuronic acid, Iduronic acid, Sialic acid, N-acetyl Glucosamine (N-GlcNAc), N-acetylgalactosamine (N-GalNAc) (Lodish et al., 2000; Stanley, 2011). These glycan epitopes influence the interaction between the biomolecules and their microenvironment, contributing to their function. Therefore, the glycoproteome of a cell is responsible for cell adhesions, homeostasis, cell signaling, cell movement in the context of chemotactic migration, self-nonself recognition system, protein trafficking and secretion, and modulation of the cell microenvironment.

The ER and Golgi membranes harbour glycosylation enzymes, integral membrane proteins in these organelles, with their active sites facing the lumen. Each glycosylation enzyme is highly specific for the sugar being added and the particular substrate to which the addition is made. The complete glycosylation cascade includes glycosyltransferases and certain glycosidases specific to the membrane compartment where the target is glycosylated (Petrosyan et al., 2012; Witkos et al., 2019).

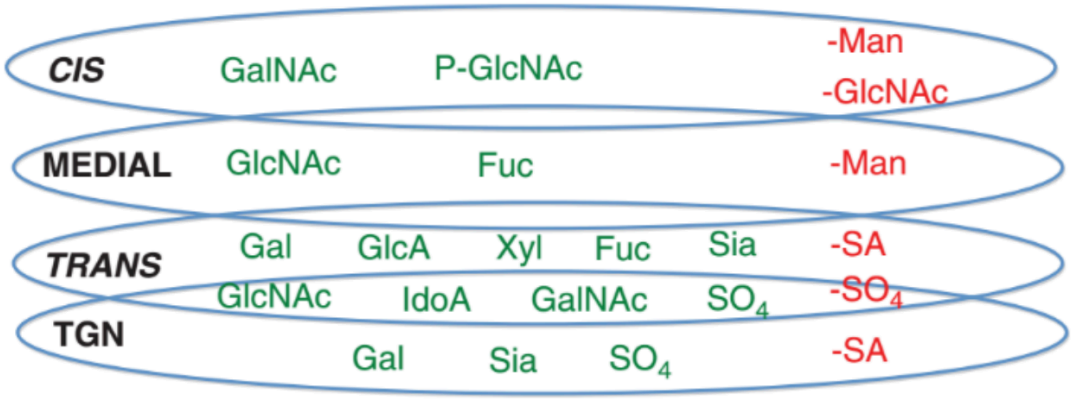


Figure 1.3 : Distribution of glycosylation reactions across Golgi cisternae – schematic showing the sequential order in which glycosylation reactions occur across the Golgi stacks. Golgi associated glycosylation begins at cis-Golgi, followed by medial-Golgi, trans-Golgi and trans-Golgi network. Glycan moieties in green indicate the residues which get added to substrates on protein, and in glycan moieties in red indicate the residues which get removed in respective compartments. *Image source – Stanley, Cold Spring Harb Perspect Biol, 2011*

N-glycosylation begins in the ER and then undergoes further modifications in the Golgi compartments, while O-glycosylation exclusively occurs across the Golgi compartments (Aebi, 2013; Gill et al., 2010; Stanley et al., 2015). Post preliminary modifications on the proteins in ER, they enter the cis-Golgi compartment. Whether these membranes are moved from cis to medial to trans- Golgi or whether the proteins are trafficked between compartments is still in debate. However, data supporting both possibilities exist, and the process could happen both ways simultaneously. The cis-medial- and trans-compartments are considerably different in the enzymes they carry as native residents (Colley et al., 2015; Stowell et al., 2015; X. Zhang & Wang, 2016).

Fig 1.3 represents the distribution of glycosylation reactions across Golgi compartments, including sugars or inorganic groups added *(in green)* or removed *(in red)*. The process commonly involves removing mannosidase groups in the *cis*- and *medial-Golgi*

compartments and adding N-GlcNAc residues. The *trans*-Golgi harbours the processing of glycan substrates to give terminal sialylation and/ sulphation and phosphorylation of substrate groups besides the addition of one or more of several sugar moieties (Galactose, Fucose, GalNAc, GlcNAc, and Xylose) (Stanley, 2011).

Specific cargo vesicles follow an unconventional processing route, independent of the reactions occurring on the Golgi membranes or lumen. Due to their size, these vesicles often carry macromolecular complexes and are believed to be processed outside the Golgi. The matrix proteins associated with the Golgi membranes, such as GRASPs, are shown to be involved in processing and trafficking through unconventional routes (Ahat et al., 2022; Gee et al., 2018; Rabouille & Linstedt, 2016)

1.1.2.2 Trafficking of vesicles through the Golgi

Proteins and lipids go through preliminary processing in the ER. They are then sent to the Golgi membranes, undergoing highly complex modifications to form mature and functional proteins and lipids. The cargo in process and the mediators of these modifications are trafficked to and from the Golgi. The traffic flow from ER to Golgi (anterograde) is balanced by the retrograde traffic, which recycles components back to Golgi and ER membranes (Chia et al., n.d.; Fisher & Ungar, 2016; Glick & Nakano, 2009).

Broadly, the trafficking at the Golgi can be divided into three categories – i) trafficking from ER to the Golgi, ii) trafficking within the Golgi compartments and back to the ER and iii) secretion of cargo from the trans-Golgi interface.

Trafficking from ER to the Golgi – Proteins and lipids synthesized at the ER are pinched off from ER exit sites in the form of vesicles and sent through ERGIC to the cis-Golgi membranes. The vesicles at the ERGIC region often fuse to form a continuous membrane structure or a tubular network, as discussed earlier. COPII proteins are explicitly recruited for packaging and trafficking of these anterograde flow of vesicles (Alberts et al., 2002; Ward et al., 2001).

Trafficking within the Golgi compartments and back towards ER – Cargo molecules docked at the cis-Golgi membrane travel sequentially through the Golgi cisternae, where

modifications such as glycosylation reactions occur. Post-modification process, specific cargo such as ER resident proteins are sent back to the ER via the retrograde flow of vesicles (Chia et al., n.d.; Gill et al., 2010; P. Sengupta et al., 2015). As for the anterograde trafficking, whether the cis-Golgi membranes themselves transgress from cis- to medial- to trans- Golgi or whether the proteins are trafficked between compartments is still in debate (Glick & Nakano, 2009).

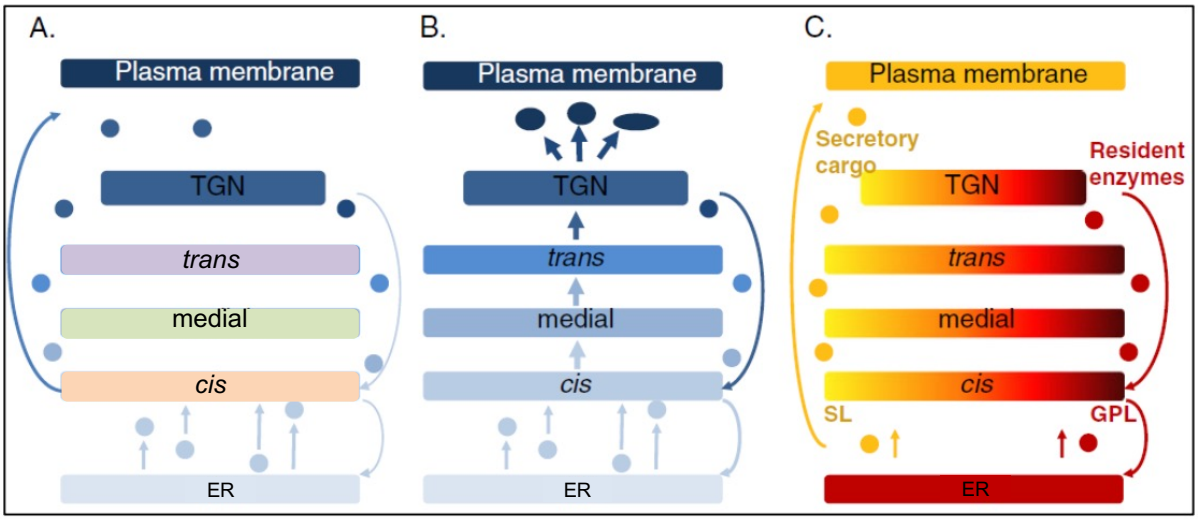


Figure 1.4 : Models for trafficking through the Golgi cisternae – a) Vesicular shuttle model – The Golgi stacks are static in space, and transport is facilitated by vesicles. b) Cisternal maturation model – The cis-Golgi itself mature into medial-, and trans-Golgi membranes through sequentialy processing of the cargo in it's lumen c) Rapid partitioning model – different functional domains exist in a unfied Golgi stack, which traffick cargo at different rates and variable modification reactions. *Image Source – Modified scheme of models reviewed by Glick and Nakuno in Ann Rev Cell Dev Biol, 2009*

Currently, three models explaining the system have been contemplated. **The cisternal maturation model** suggests that the cis-Golgi membranes evolve into medial-Golgi and then trans-Golgi compartments per the completion of the reactions occurring in the lumen (Fig1.4). Vesicles arising from the Golgi membranes are packaged with COPI proteins, which facilitate the retrograde flow for delivering proteins to the newly formed cis-Golgi from the trans-Golgi compartments, which have matured from the older cis-Golgi membranes. **The vesicular shuttle model** – suggests that the cis-, medial- and trans-Golgi membranes hold static positions in cytoplasmic space, and all the shuttling of cargo and native proteins happens through transport vesicles (Fig 1.4). This model, however, fails to explain the processing and trafficking of macromolecular complexes that are too large for

the space enclosed by transport vesicles. **Rapid portioning model** – According to this model, the differences in lipid composition of the Golgi membranes give rise to different functional domains in a unified Golgi structure. This was reported based on studies that suggested that some cargo moves slowly across the membranes while cisternal maturation rates are higher (Fig 1.4). The accepted theory is that cisternal maturation and vesicular shuttling exist simultaneously to facilitate variable cargo movement as required (Glick & Nakano, 2009; Luini, 2011).

Secretion of cargo from trans-Golgi membrane

Transport of the glycosylated proteins and lipids from the Golgi to sites within the cell or to the plasma membrane for secretion is achieved through the packaging of cargo in a target-specific assembly of transport vesicles (Spang et al., 2010). The exit sites for these vesicles are often at the Trans-Golgi and TGN, which recruit Clathrin proteins and adaptors complexes to package the cargo for secretion (Stalder & Gershlick, 2020b). Most proteins require the presence of a specific glycan motif to serve as a signal for engagement of the cargo protein with the correct transport vesicle . This ensures accurate targeting of the secretory vesicle to its destination (Fisher & Ungar, 2016). For example, lysosomal enzymes, after the glycosylation process, are labelled with Mannose-6-phosphate residues, which bind Mannose-6-phosphate receptors in the lysosomal membrane, facilitating their transport to lysosomes (Kudo et al., 2006; M. Xu et al., n.d.). Lack of correct signaling glycans often contributes to mis-sorting processed proteins, rendering them non-functional or promoting non-specific interactions. (Bhat et al., 2017; Linders et al., 2020; Petrosyan et al., 2014)

1.2 Regulation of the Golgi organization and Golgi function

1.2.1 Structural regulation of the Golgi apparatus

Many proteins and signaling pathways regulate the Golgi structure and function dynamics to ensure fidelity in the complex system. Different classes of regulatory systems are associated with the Golgi structure, mainly including the Golgi matrix proteins, Small GTPases, mineral ions, and cytoskeletal systems.

1.2.1.1 Golgi matrix proteins

Golgi matrix proteins include several structural proteins physically associated with the Golgi structure. These proteins are a tether between the Golgi cisterna to hold the stack together. The matrix proteins link the Golgi stacks laterally to form a Golgi ribbon. Different types of Golgi matrix proteins are often specific to the separate Golgi compartments and are integral to the maintenance of the Golgi structure (Xiang & Wang, 2011). Golgins and GRASPs' family of proteins are some of the most extensively studied groups of Golgi matrix proteins. The Golgin proteins have a coiled-coil domain, which imparts flexibility to the conformations taken up by the protein. This structural feature favours the tethering function of Golgins to extend over lengths, thereby facilitating the transient nature of the cisternal organization in a stack or the laterally aligned Golgi stacks. GM130, Golgin84, Giantin, Golgin160, etc., are some of the extensively studied Golgins (Smits et al., 2010; Witkos & Lowe, 2015; S. Yadav et al., 2012).

Other families of Golgi matrix proteins, known as the GRASPs (Golgi Reassembly and Stacking Proteins), are of two types – GRASP65 and GRASP55. Both the GRASPs are similar in structure and function but differ with respect to their binding partners at the Golgi and localization at the Golgi. GRASP65 binds to the Golgin GM130 and is native to the cis-Golgi, whereas GRASP55 binds to the Golgin-45 and localizes to the medial-Golgi compartments. In non-phosphorylated form, they maintain the tethering between the cisternae in a Golgi stack and between the Golgi stacks laterally (Ahat et al., 2022; Bekier et al., 2017; Rabouille & Linstedt, 2016; X. Zhang & Wang, 2015). Depletion of one or more of these proteins leads to altered Golgi structure and, hence, Golgi function (Fig 1.5). During Golgi fragmentation in physiology, some Golgi matrix proteins are speculated to behave as Golgi remnants, which help rapid biosynthesis of the Golgi to restore intact Golgi organization (Lowe, 2002; Ward et al., 2001). Such changes in Golgi morphology are orchestrated by cell signaling pathways, which mediate their effect on the Golgi by changing the activation or structural conformation of one or more Golgi matrix proteins.

Therefore, the Golgi matrix proteins often show physical binding or regulatory crosstalk with several other Golgi-regulating proteins and complexes (Cervigni et al., 2015; Puthenveedu et al., 2006; Sütterlin et al., 2005; Tang et al., 2016; Xiang et al., 2013a).

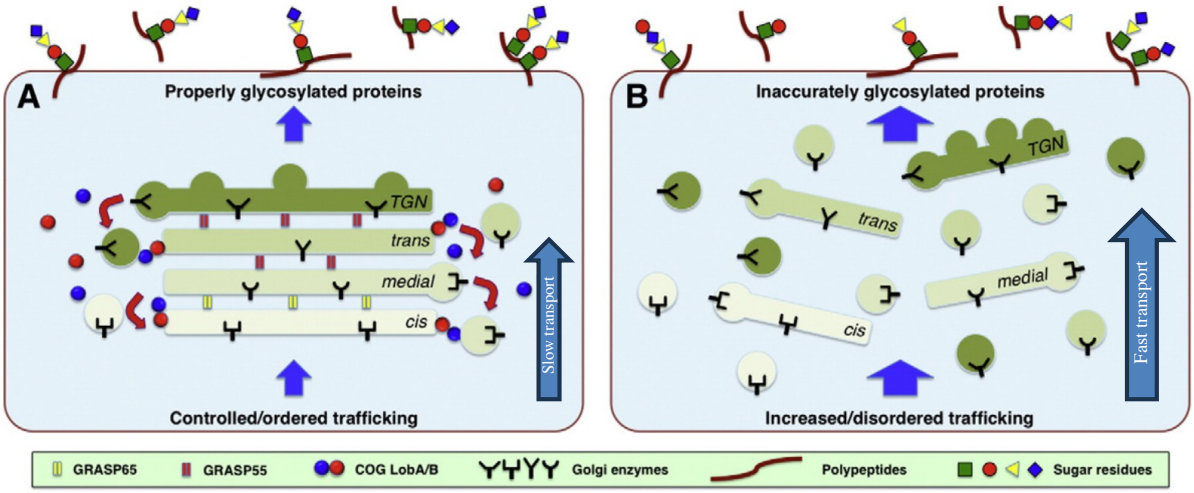


Figure 1.5 : Role of Golgi matrix proteins in regulation of Golgi organization and Golgi function – a) GRASPs holding together the Golgi cisternae to maintain an intact Golgi organization, and COG complexes regulate trafficking across the Golgi, which together promotes accurate glycosylation, rate of trafficking and sorting of cargo proteins. b) Depletion of GRASPs and COGs leads to Golgi disorganization which causes affects retention times of cargo in Golgi, glycosylation and hence sorting of proteins. *Image Source – Modified schematic adapted from Zhang et al., J Mol Biol 2016, Xiang et al., Front. Cell Dev. Biol, 2013*

1.2.1.2 Small GTPases

Small GTPases are proteins of around 21kD Mw, functioning as molecular switches in cells to regulate many transient cellular systems such as the Golgi complex. Amongst the extensively studied small GTPases, Ras, Rho, ARF and Rab GTPases are known to localize at the Golgi and play an essential role in regulating the structural and functional dynamics of the Golgi apparatus (Fig 1.6).

Arf GTPases

Arf GTPases are G proteins that play a significant role in regulating membrane traffic and organelle architecture. Like for several other GTPases, the function of Arf proteins is controlled by GEFs (Guanine nucleotide exchange factors) and GAPs (GTPase activation proteins) (Spang et al., 2010; Walton et al., 2020). In mammals, the ADP-ribosylation factor (Arf) family of GTPases is divided into three classes based on similarities in protein

structure. Class I comprises Arf 1 and Arf 3, class II comprises Arf 4 and 5, and class III is composed only of Arf6.

Certain Arf GTPases contribute to the structural integrity of the Golgi organization. For

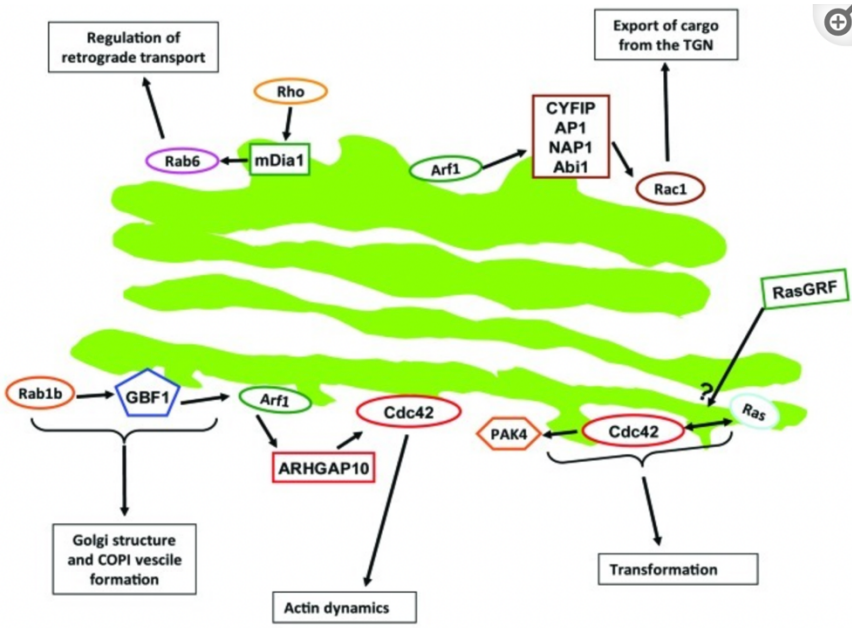


Figure 1.6 : Crosstalk of the small GTPases at the Golgi – Schematic shows the Golgi associated small GTPases (labelled in ellipses) and the proteins they interact with at the Golgi (labelled in polygons. Cellular functions regulated as a result of these interactions are also shown. *Image Source – Baschieri et al., Small GTPases, 2012*

example, active Arf1 associates with and recruits Dynein motor protein at the Golgi membrane (Ward et al., 2001)(S. Yadav et al., 2012). Specific inhibitors against Arf1 GEFs, BIG1/2 and GBF1 cause Golgi to fragment and affect Golgi function (Sciaky et al., 1997c; Ward et al., 2001). Besides, studies suggest that the members of the Arf family – Arf1, Arf3, Arf4 and Arf5 in combinations of two, differentially regulate the budding of COPI-coated vesicles as part of retrograde traffic (Volpicelli-Daley et al., 2005). A classical study by Pennauer and colleagues, where sequentially and combinational KO of the different Arf GTPases, shows distinct and redundant roles of other ARF1 GTPases in regulating Golgi structure and function (Pennauer et al., 2022). The study confirmed that Arf1 plays a significant role in vesicle formation at the Golgi and suggests that Arf4 alone is sufficient for cell viability.

The Arf family includes Sar1 and more than 20 Arf-like proteins (ARLs). Arls, like Arl2, regulate the assembly of microtubules at the centrosome and, therefore, contribute to the positioning of the Golgi in cells (Baschieri & Farhan, 2012). Arl3 localizes to the Golgi, and its knockdown leads to dramatic fragmentation of the Golgi apparatus (Zhou et al.,

2006). Therefore, the Arf family of GTPases are essential for vesicle and tubule formation and maintaining the Golgi apparatus's structural integrity.

Rho GTPases

Rho GTPases are a family of small G-proteins composed of over 20 members that function as critical regulators of the cytoskeleton, thereby modulating cell functions such as cell migration, neurite growth, vesicle trafficking and cytokinesis (Baschieri & Farhan, 2012). The three main groups within the Rho family are Rho, Rac and Cdc42. Rho GTPases, particularly Cdc42, have been shown to play an active role in the Golgi. Cdc42 was shown to localize to the Golgi and, by interaction with the γCOP subunit of the coatomer, regulates transport between the Golgi and the endoplasmic reticulum (Farhan & Hsu, 2016; Park et al., 2015).

Ras GTPases

Ras GTPase regulates many cellular processes like cell proliferation, differentiation, and apoptosis. In mammals, there are three genes for Ras GTPases – H-Ras, N-Ras and K-Ras. The Ras GTPases shuttle between the Golgi and the plasma membrane to regulate cell signaling in a spatio-temporal manner. Ras proteins get palmitoylated at the Golgi, which facilitates targeting of Ras to the inner leaflet of the plasma membrane. Depalmitoylation of the Ras proteins can occur anywhere in the cells, which again targets the Ras proteins to the Golgi membranes (Goodwin et al., 2005).

Besides, Ras GTPases regulate another important signaling pathway at the Golgi: the Raf-MEK-ERK mitogen-activated protein kinase cascade. RasGRP1 (GEF) activates Ras at Golgi, eliciting an ERK response in the downstream signaling (Chiu et al., 2002).

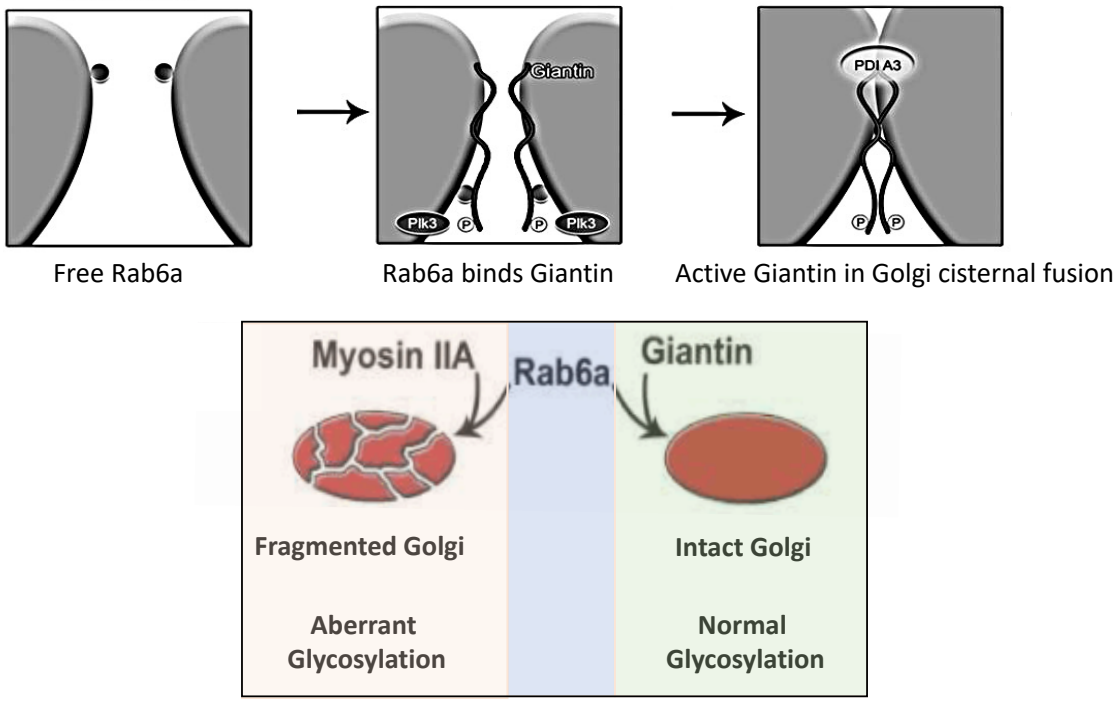


Figure 1.7 : Role of Rab6 GTPase at the Golgi – Schematic shows regulation of Golgi organization and function by GTPase Rab6. Binding of Rab6 to Giantin, promotes activation of Giantin and maintains intact Golgi organization. Actin associated motor protein, myosinIIA sequesters Rab6 preventing activation of Giantin and causing Golgi fragmentation and aberrant glycosylation of proteins. Image Source – A. Petryosan et al., Mol Cancer Res., 2014

Rab GTPases

Rab GTPases are the most prominent family of Ras-related GTPases. Certain Rab GTPases associate with the Golgi apparatus and regulate its organization and membrane trafficking events (Baschieri & Farhan, 2012). Specifically, the Rab GTPases Ypt1 (Rab1) and Ypt31/32 (Rab11) are critical for the dynamics of Golgi maturation cis-Golgi to trans-Golgi form (J. J. Kim et al., 2016). Petryosan and others (Petrosyan et al., 2014) demonstrated the role of Rab6 in maintaining the structural integrity of the Golgi apparatus. MyosinII motor protein binds to sequester Rab6 GTPase, which prevents Rab6 from binding and activating the Golgi matrix protein Giantin. In the presence of free Rab6, active Giantin tethers the Golgi cisternae to maintain an intact Golgi organization (Fig 1.7) (Petrosyan et al., 2014). Other Golgi-associated Rab GTPases include Rab1, Rabb2, Rab6, Rab18, Rab33B and Rab43 (Goud et al., 2018; Liu et al., 2021).

Small GTPases regulate a wide range of homeostatic processes such as cellular homeostasis, cytoskeletal organization, cell migration, and membrane trafficking, as well as in pathologic conditions such as cancer and neurodegeneration (Baschieri & Farhan, 2012; Manser, 2002; Thomas & Fromme, 2020). Golgi regulating small GTPases also often talks to major cell signaling pathways to mediate a required effect on Golgi's organization. Although important, it is difficult to understand the regulation of small GTPase signaling, given their transient nature of activation and localization, and on account of intricate crosstalk between different small GTPase families.

1.2.1.3 Cytoskeletal elements and Golgi structure

Most cytoskeletal elements, including the microtubules, actin, and motor proteins at the microtubule organizing centre (MTOC, i.e. centrosome), are closely associated with the Golgi membrane dynamics. Depolymerization of **microtubules**, using inhibitory agents such as nocodazole, causes unlinking of the Golgi ribbon and resulting Golgi mini stacks disperse throughout the cytoplasm (Burkhardt, n.d.; Haase & Rabouille, 2015). The centrosome-derived microtubules also regulate the positioning of Golgi in cells in a steady state and during events such as cell migration, when the Golgi positions itself at the leading edge of the cells (Hurtado et al., 2011; Mascanzoni et al., 2022a). Several studies have reported that Golgi often acts as a second **MTOC** in cells. Unlike centrosomal microtubules, the Golgi-derived microtubules have hyperacetylation and detyrosination, imparting more stability to these microtubules (Sanders & Kaverina, 2015). GM130, along with AKAP450, promotes the nucleation of microtubules at cis-Golgi (Egea et al., 2015; Rios, 2014). Interactions with multiple Golgi structural proteins further stabilize the microtubules (see Fig 1.8). Depletion of one or more of these Golgins or GRASPs affects their interactions with microtubules and motor proteins and, as a consequence, causes alterations to the Golgi structure (Marra et al., 2007; Puthenveedu et al., 2006; Smits et al., 2010).

The **Actin** filaments also play an essential role in maintaining the integrity of the Golgi organization and its positioning in the cell (Egea et al., 2006). Actin also contributes to maintaining Golgi structure regulation of secretory and endocytic pathways in cell and vesicle biogenesis (Chakrabarti et al., 2021; Porat-Shliom et al., 2013).

Apart from microtubules and Actin, a range of cytoskeletal **motor proteins** associated with Golgi are integral to its maintenance (Fig 1.8). This includes actin-associated motor proteins such as myosin-II, V and VI), and microtubule-associated motor proteins, including kinesin1, 2, and 3, plus-ended and cytoplasmic dynein-1 minus-ended motor proteins. Depending on their directionality, the microtubule-associated motor proteins regulate the traffic of transport vesicles to and from the Golgi (Brownhill et al., 2009; Burkhardt, n.d.1998). Inhibition of Dynein with Ciliobrevin is known to promote Golgi disorganization (Jaarsma & Hoogenraad, 2015a).

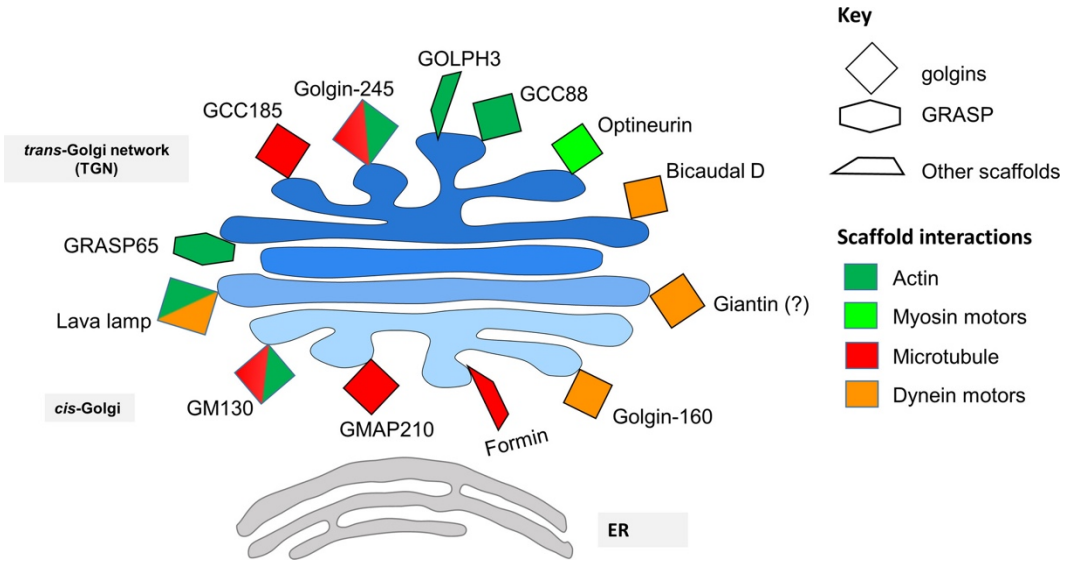


Figure 1.8 : Regulation of Golgi organization and function by Cytoskeletal elements – Schematic shows protein complexes associated with actin filaments, myosin motors, dynein and microtubules which together regulate Golgi organization and Golgi function. Known Golgi associated matrix proteins, specific to respective cytoskeletal scaffolds and to Golgi cisternae are also highlighted in polygons as given in the legend. *Image Source – Kulkarni-Gosavi et al., FEBS Letters, 2019*

1.2.2 Cell processes and pathways that regulate Golgi organization and function

Cells during migration, cell division and apoptosis modulate the Golgi organization through different cell signaling pathways. The proteins from these cell signaling pathways transiently regulate the Golgi organization during particular cellular functions, and their roles in the context of the entire cell are not exclusive to the Golgi dynamics. A comprehensive study by Chia et al. screened the phosphotome and kinome in HeLa cells,

revealing over 188 candidates that have a role in the regulation of Golgi organization and Golgi function with respect to cell surface glycosylation (Chia et al., 2012b). Such proteins include Integrins, Aurora Kinases, SRC kinases, AMPK, etc. For all such proteins, regulation of the Golgi organization is indirect. One or more Golgi-associated direct regulatory proteins play intermediate roles in mediating the effect on the Golgi complex. Mechanistic details on important cellular events such as cell division, apoptosis and DNA damage are discussed below.

1.2.2.1 Cell Division

The Golgi ribbon observed in a cell at a steady state results from a dynamic equilibrium between the formation and disassembly of membranes and tubular networks at the Golgi. This equilibrium is particularly relevant during the cell division cycle, where Golgi fragmentation is required for equal distribution of Golgi components in the daughter cells.

As the figure describes, the Golgi ribbon undergoes unlinking in the G2 phase, forming isolated cisternal stacks dispersed in the cytoplasm. However, these isolated stacks maintain their cisternal polarity (Shima et al., 1997) and can perform certain cargo processing and trafficking functions effectively (Wei & Seemann, 2010). The unlinking of Golgi stacks occurs in the G2 phase and is integral for cells to proceed into the Mitotic phase, often referred to as the 'Golgi checkpoint' in the cell cycle (Sü et al., 2002). Through prophase and metaphase, Golgi stacks further vesiculate, and most of the Golgi components fall back into the ER, forming a 'Golgi haze'.

Towards late Anaphase, the Golgi apparatus reforms into stacks around the spindles, where the ribbon forming factors are assembled to aid the restoration of Golgi ribbon structure in daughter cells (Colanzi & Corda, 2007). Mechanisms behind the regulation of the Golgi dynamics during the cell cycle are not entirely understood. However, I will discuss certain insights available into the complex process, divided into three steps – unlinking the Golgi ribbon, disassembly of Golgi stacks into vesicles and clusters, and reassembling the Golgi components into Golgi ribbon post mitosis (Fig 1.9).

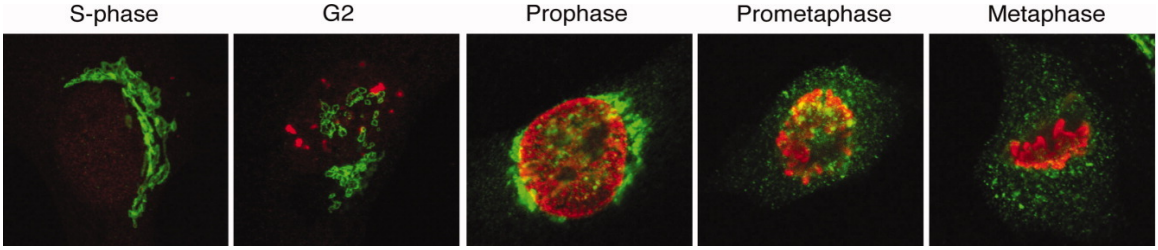


Figure 1.9 : Golgi membranes through the cell cycle – Images show changes in Golgi organization with Giantin marker, through the cell cycle in Hela cells. Nucleus is labelled with anti-phosphohistone H1 and H3 to mark different phases of cell cycle. *Image Source – Adapted from Colanzi et al., The EMBO Journal 2007 and Corda et al., IUBMB Life, 2012*

Unlinking of Golgi stacks –

Specifically, during G2, the kinase PKD activates the RAF1/MEK1/ERK1 signaling cascade, which phosphorylates GRASP55 (Colanzi et al., 2003; Feinstein & Linstedt, 2007), while JNK2 and PLK1 are known to phosphorylate GRASP65 (Cervigni et al., 2015; D. Sengupta & Linstedt, 2010). These phosphorylations coincide with the blocking of tethering events, which results in Golgi unlinking (Rabouille & Linstedt, 2016).

Crossing of the 'Golgi mitosis checkpoint' promotes activation of the Golgi localized pool of SRC kinases (Weller et al., 2010) and of AMPK kinases (Lee et al., 2015; Mao et al., 2013), which in turn activates Aurora Kinase A. Active Aurora Kinase A is recruited at the centrosome, which triggers entry into mitosis and centrosome maturation (Barretta et al., 2016; Kimura et al., 2018).

Golgi disassembly in Mitosis –

At the onset of mitosis, CDK1 phosphorylates several Golgi matrix proteins, including various Golgins, GRASPs and adaptor proteins aiding membrane fusion. Also, AMPK has been suggested to phosphorylate ARF1 GEF GBF1, leading to loss of active ARF1 at the Golgi and hence Golgi fragmentation (Mao et al., 2013). This promotes extensive vesiculation and disassembly of the Golgi components (Fig 1.9). Extensive fragmentation of the Golgi apparatus is essential for mitotic progression, as blocking the disassembly of stacks affects spindle formation, thereby arresting cells in metaphase (Guizzunti & Seemann, 2016).

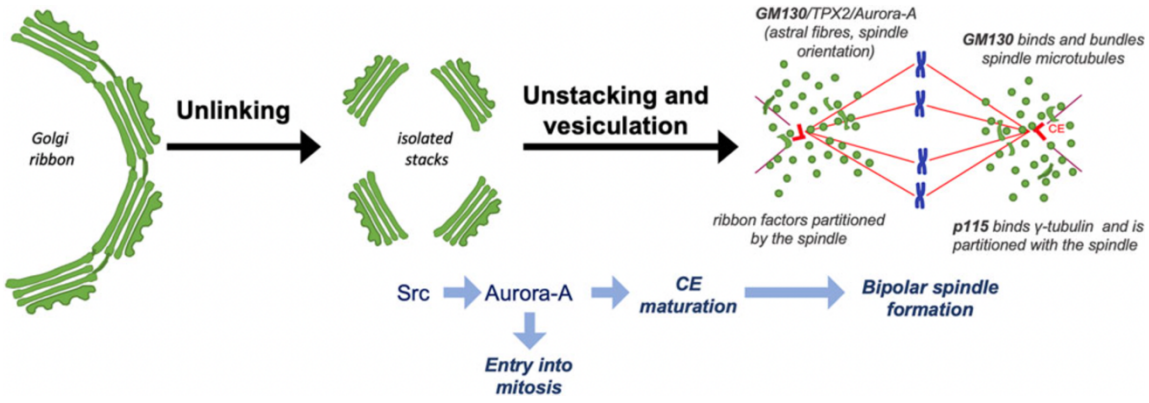


Figure 1.10 : Regulation of mitotic Golgi disassembly – Schematic describes unlinking of Golgi stacks in G2 phase, which activates Src/Aurora-A signaling axis. This in-turn drives centrosome maturation and entry into mitosis. With the initiation of mitosis, the Golgi complex further undergoes extensive fragmentation, where numerous Golgi vesciles and clusters are dispersed in the cytoplasm. Golgi associated proteins, including GM130 and p115 engage with the microtubules to form a bipolar, symmetric and correctly oriented spindle, which ensures equal distribution of Golgi components to the daughter cells. Image Source – Ayala and Colanzi, Front. Curr. Dev. Biol, 2022

Re-assembly of Golgi membranes –

During metaphase, Golgi matrix proteins such as GM130, p115, and GRASP65 were observed to be present at each of the two spindle poles. GM130 was shown to bind and stabilize the microtubule spindles, while GRASP65 and p115 are suggested to aid in the proper segregation of chromosomes in the daughter cells. The pool of Golgi matrix proteins at the spindle poles, along with a separate pool of Golgi enzymes and associated proteins distributed in the cytoplasm of each daughter cell, helps rapid restoration of the Golgi membranes post-mitosis (Radulescu et al., 2011; Sütterlin et al., 2005).

1.2.2.2 Apoptosis and DNA damage

In physiology, cells occasionally undergo different stress conditions, including pH alterations, abnormal redox flux, pathogen infiltration and DNA damage. Although stressed cells often present an abnormal Golgi morphology, the mechanisms behind the observation are still largely unknown (Ignashkova et al., 2017; Machamer, 2015a). When a cell proceeds towards apoptosis, the death ligands, for example, Fas protein, bind their corresponding receptor to initiate a cascade of programmed cell death (Waring & Müllbacher, 1999). Fragmentation of Golgi is found to be an early event in the apoptotic cascade and is mediated by the cleavage of several Golgi-associated proteins by protein

caspases. Golgi fragmentation precedes the release of cytochrome C from the mitochondria. Cytoskeletal elements are also implicated in apoptotic Golgi fragmentation (Mukherjee et al., 2007).

A separate study in breast cancer cells suggested that DNA damage caused by UV radiations causes dramatic dispersal of the Golgi membranes throughout the cytoplasm. In response to DNA damage, DNA-PK phosphorylates GOLPH3, increasing interaction with MYO18A, which applies a tensile force to the Golgi. Interference with the Golgi DNA damage response by depletion of DNA-PK, GOLPH3, or MYO18A was shown to reduce survival after DNA damage. In reverse, the overexpression of GOLPH3 was found to impart drug resistance towards DNA-damaging agents (Anandi et al., 2017; Farber-Katz et al., 2014). Besides DNA damage, an increase in oxidative stress and autophagy pathways also alters Golgi structure and function as an event in the cascade or as an apoptotic precursor.

1.2.3 Role of cell-matrix Adhesion in the Regulation of Golgi Organization and Function

Cell-matrix adhesion, mediated by integrin signaling, is integral to several critical cellular functions, including cell proliferation, migration, differentiation, survival etc. One of the primary functions of the Golgi apparatus is the recycling of membrane raft microdomains, which carry major cell signaling receptors, including growth factor receptors (Berrier & Yamada, 2007; Pawar et al., 2016).

In non-transformed mammalian cells, it was observed that these membrane raft domains were internalized through endocytic pathways on the loss of adhesion to substrate. On re-adhesion to the matrix, integrin-dependent activation of RalA drives activation of Arf6, which traffics back the membrane raft domains to the plasma membrane. In this way, cell-matrix adhesion regulates membrane trafficking through the endocytic pathways (Pawar et al., 2016). The Golgi apparatus is also a significant participant in the endocytosis process, where endocytic vesicles fuse with the trans-Golgi compartments and transiently exit back to the plasma membrane. Therefore, the Golgi apparatus and adhesion signaling seems to function in tandem as a part of the membrane recycling process (Stalder & Gershlick, 2020a).

1.2.2.3 Cell-matrix adhesion regulates Golgi organization and function

A recent lab study by Vibha et al. addressed the possibility of a crosstalk between cell-matrix adhesion and the Golgi complex. Interestingly, we found that on the loss of cell-matrix adhesion, the Golgi undergoes dramatic disorganization into the cytoplasm in mouse fibroblast cells (Fig 1.11) (Singh et al., 2018), human fibroblasts (BJ cells) and human mammary epithelial cells (MCF10A) (Unpublished data). The disorganization of Golgi components into several objects was limited to the cytoplasm, with no fall back into the ER. This was observed for cis-Golgi marker GM130, cis-medial-Golgi marker – MannosidaseII-GFP and trans-Golgi marker – GalTase-RFP.

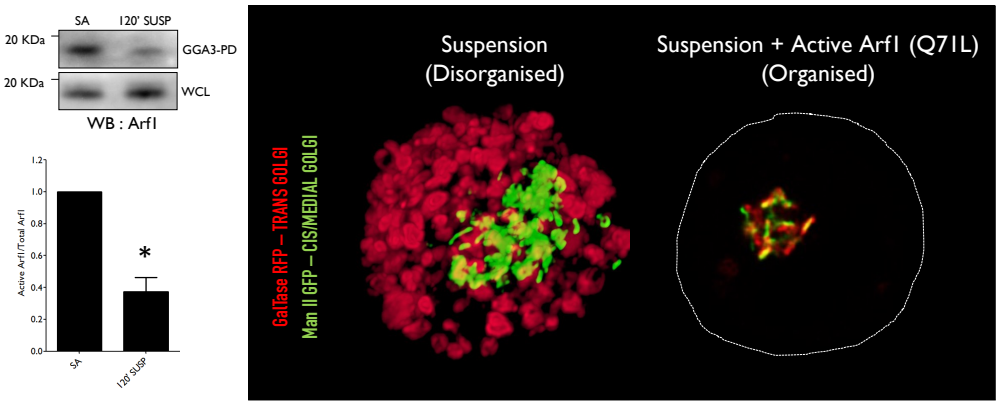


Figure 1.11 : Adhesion-dependent regulation of Golgi organization is mediated by active ARF1 – Loss of cell matrix adhesion causes active Arf1 levels to drop significantly. This leads to Golgi disorganization in Mouse embryonic fibroblasts, as shown with cis-Golgi marker MannosidaseII-GFP and trans-Golgi marker GalTase-RFP. Expressing constitutively active ARF1 (Q71L) maintains intact Golgi organization in non-adherent cells. *Image Source – Adapted from Singh et al., JCS, 2018*

This change in Golgi organization in non-adherent cells was also found to affect Golgi function with respect to an increase in cell surface glycosylation levels in MEFs, as observed with cell surface glycan-binding lectins – ConA (binds Mannose), WGA (binds Glucose and N-Acetylglucosamine), PNA (binds Galactose and N-AcetylGalactosamine) and UEA (binds Fucose). How the change in Golgi organization affects Golgi function and what the change in Golgi organization and function means to the cells is still not clearly understood.

1.2.2.4 Role of Arf1 activation in adhesion-mediated regulation of Golgi complex

Arf1 activation was found to drop significantly on loss of adhesion compared to stably adherent and early re-adherent cells, as measured by active Arf1 pull-down assay in MEFs. On the expression of constitutively active Arf1 (Q71L), the Golgi organization stayed intact in suspension, which suggested that the presence of active Arf1 reverses the loss of adhesion-mediated Golgi dispersal (Fig 1.11) (Singh et al., 2018).

Expression of active Arf1 also prevented the increase in cell surface lectin binding, which is observed when Golgi is disorganized in non-adherent cells. Further, the loss of active Arf1 in non-adherent MEFs was found to affect the recruitment of Dynein at the Golgi, which we suggest could be the reason for the dispersal of the Golgi membranes. In line with this hypothesis, the Dynein fraction that binds to active Arf1 was present in the active Arf1 pull-down fraction in adherent cells but was absent in the pull-down fraction from non-adherent cells (Singh et al., 2018; S. Yadav et al., 2012). Taken together, we saw that loss of cell-matrix adhesion causes Golgi disorganization and increases cell surface glycosylation. This regulation is mediated by adhesion-dependent activation of ARF1

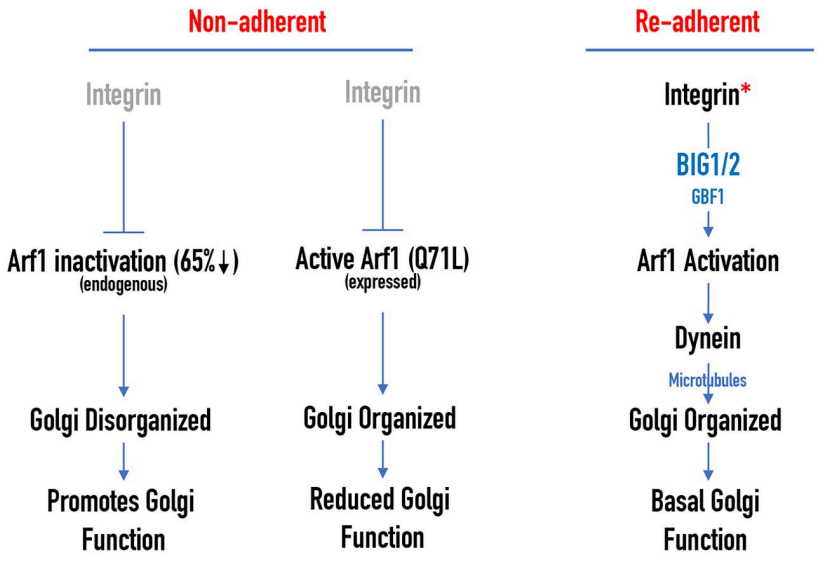


Figure 1.12 : Adhesion regulates Golgi organization and function – In absence of integrin mediated adhesion, activation of Arf1 is diminished, leading to Golgi disorganization and subsequent increase in cell surface glycosylation. On re-adhesion to matrix, integrin mediated signaling is activated promoting Arf1 activation, which recruits Dynein at the Golgi. Dynein in turn engages the Golgi membranes with microtubules, maintaining intact Golgi organization and hence Golgi function. *Image Source – Schematic adapted from Singh et al., JCS 2018*

GTPase, which binds Dynein in its active form, thereby engaging the Golgi membranes with microtubules (Fig 1.12).

In summary, it is worth noting that different cellular pathways regulate the Golgi dynamics to facilitate cellular functions. Any small alteration in the positioning or function of one or more of these regulatory components can lead to an alteration in the integrity of Golgi membranes and, hence, Golgi function, the pathological consequences of which will be discussed in the next section.

1.3 Role and Regulation of Golgi Apparatus in Diseases and Disorders

1.3.1 Golgi organization in diseases/disorders/cancers

The Golgi apparatus's structure and function are maintained under spatio-temporal regulation by several Golgi regulating proteins and signaling complexes. Changes in the Golgi structure and function can result from normal physiological processes occurring in a cell, like cell division or can be induced by using biochemical (e.g. BFA, GCA, etc.) or DNA damaging agents (e.g. UV radiation, cisplatin) as discussed earlier. Depending upon the cause of perturbation at the Golgi, the effect on Golgi organization or function is variable (Gonatas et al., 2006). Induced perturbations, spontaneous mutation events or infectious agents targeting one or more of the Golgi regulating proteins or regulatory pathways affect the integrity of Golgi structure and function. This may lead to a range of pathological conditions, including – neurodegenerative diseases, cancer, infectious diseases and cardiovascular diseases (Haase & Rabouille, 2015; Jaarsma & Hoogenraad, 2015b; Machamer, 2015b).

1.3.1.1 Neurodegenerative diseases

Golgi fragmentation has been observed in several **neurodegenerative diseases**, including Amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Spinocerebral ataxia type 2, corticobasal degeneration and Parkinson's disease (Gonatas et al., 2006). In literature, Golgi fragmentation often refers to disassembling the intact Golgi ribbon into multiple Golgi objects dispersed in the cytoplasm. Increasing evidence suggests that Golgi

fragmentation is a relatively early event in the cascade of progressive neurodegeneration diseases and often precedes the onset of clinical and pathological symptoms. The mechanisms behind the relevance of Golgi alterations in neurodegenerative diseases are recently being explored (Ángel Martínez-Menárguez et al., 2019).

Gonatas et al. studied the role of mutations in the gene Superoxide dismutase (SOD1) in transgenic mice having familial ALS. They showed mice with G93A mutations in SOD1 cause Golgi fragmentation months before the onset of paralytic symptoms (Gonatas et al., 2006). Further, a cellular mimic model of Parkinson's disease, Golgi fragmentation, was shown to precede the formation of inclusion bodies and its dependency on Rab and SNARE proteins was established by Rendon and colleagues (Rendón et al., 2013). Another study of progressive motor neuropathy in mice suggests that Golgi fragmentation observed in diseased cells could be regulated by a crosstalk between Arf1 and a cis-Golgi localized tubulin binding cofactor E (TBCE) gene. In these mice, a mutation in TBCE affects Golgi-derived microtubules, further affecting the integrity of the Golgi structure. Besides, alterations in VAP proteins (Vesicle-Associated Membrane Protein (VAMP)-Associated Protein) have been shown to affect the Golgi structure and function in multiple forms of ALS (Haase & Rabouille, 2015). In HeLa cells, depletion of VAP-B caused extensive fragmentation of the Golgi (Peretti et al., 2008), which could be on account of the role of VAP proteins in membrane trafficking, ER/cytoskeleton interactions, the unfolded protein response (Lev et al., 2008) and axonal transport of mitochondria (Bor et al., n.d.2012). Taken together, mutations or alterations in Golgi-associated proteins, membrane trafficking pathways or the microtubules affect Golgi integrity in neurodegenerative diseases.

However, an axonal injury, which can potentiate neuronal disruption, did not affect the integrity of the Golgi structure. A few other studies reported that the transection of sciatic or facial nerves in rats did not cause the Golgi to fragment (Bellouze et al., n.d.; Fujita et al., 2011). Further, non-apoptotic cell death was observed in astrocytes expressing Tau protein, the accumulation of which contributed to Golgi fragmentation in these cells (Gonatas et al., 2006). Conforming to these observations, a comprehensive review by Martinez-Menarguez also suggests that Golgi fragmentation could be a driver of neurodegenerative diseases, though more research is needed (Ángel Martínez-Menárguez et al., 2019).

1.3.1.2 Cancer

Apart from neurodegenerative disease, alterations in Golgi organization are rampant across several types of cancers. Cancer cells inherently show a disorganized Golgi structure, which is not surprising and has been reported frequently since the 1950s (Fig 1.13) (Petrosyan, 2015). However, understanding the mechanisms behind altered Golgi in cancers is unknown. Only recently has research in the field of onco-Golgi accelerated and revealed the diverse and complex nature of dysregulated Golgi in cancers. Aberrant Golgi dynamics help cells override their regulatory mechanisms and alter the tumor microenvironment and immune landscape. These behaviours improve cell resilience and enhance cancer cells invasive and metastatic potential (Stowell et al., 2015).

Golgi-associated gene mutations are ubiquitous in most cancers and are responsible for altering Golgi structure and function to become pro-metastatic (Bajaj et al., 2022). Many of these mutations are associated with fragmentation of the Golgi ribbon, for example, defects in the conserved oligomeric Golgi complex (COG) in congenital disorders of glycosylation (Miller & Ungar, 2012). Rab GTPases, such as Rab6a in prostate cancer (Petrosyan et al., 2014) and Rab25 in Breast and ovarian cancer (Yin et al., 2012), have been linked to promoting Golgi fragmentation.

Golgi fragmentation has also been reported in several other cancer types, including breast, prostate, colon, ovarian and lung cancer (Fig 1.13) (Bhat et al., 2017; Kellokumpu et al., 2002). Fragmentation or any alteration in the Golgi organization itself has been suggested to be a gateway into cancer progression (Petrosyan, 2015). This is often attributed to the effect Golgi disruption could have on different parts of Golgi function. Mathematical modelling by Mani and Thattai suggests that when Golgi membranes are disorganized into numerous Golgi objects, the surface is available for pinching off vesicles, increasing significantly (Mani & Thattai, 2016). Therefore, the retention time of cargo within the Golgi lumen decreases, which affects both the rate of trafficking in the cell and accurate modifications occurring in the Golgi lumen. Altered modifications to the cargo can further affect the sorting signal on these molecules, thereby also affecting the transport of vesicles to appropriate target sites in a cell (Xiang et al., 2013b; X. Zhang & Wang, 2016).

Besides, Golgi disorganization affects the specific localization of glycosylation enzymes through the different Golgi compartments (Petrosyan et al., 2014). The mislocalization of proteins and enzymes native to the Golgi changes the accessibility of target proteins to their glycosylating enzymes, thereby altering the glycan signatures on cargo. This was shown in prostate cancer cell lines DU145 (which has a disorganized Golgi) in comparison with LnCAP C33 (which has an organized Golgi) (Bhat et al., 2017).

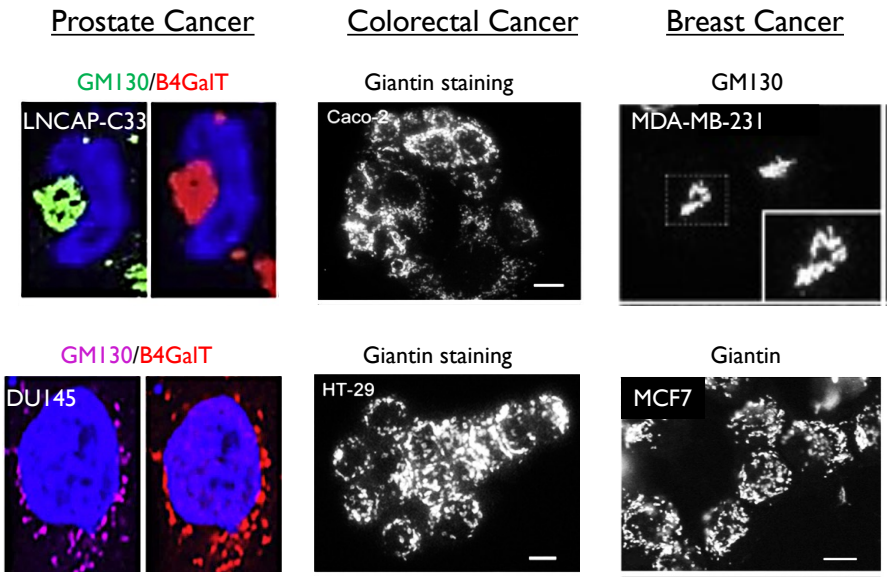


Figure 1.13 : Representative images showing variability in Golgi organization across different types of cancers. LNCAP-C33 and MDAMB231 show organized Golgi architecture. Golgi in DU145, Caco-3, HT-29 and MCF7 is observed to be inherently disorganized. *Image Source – Data adapted from Bhat et al., BBA Subjects, 2017 and Kellokumpu et al., FEBS Letters, 2002*

Differences in the Golgi organization between LnCAP C33 and DU145 cell lines could reflect the tumor aggressiveness in both these cell lines since the latter is highly aggressive compared to the former (Bhat et al., 2017). However, this correlation has not been established or understood in the field since instances suggest otherwise. MDAMB231, which is highly aggressive with respect to invasive behaviours, presents an organized Golgi morphology, whereas MCF7, which is mildly aggressive, have a disorganized Golgi morphology (Kellokumpu et al., 2002).

Knowing that aberrant Golgi morphology is highly implicated in cancers, few studies have attempted to target the Golgi organisation as a therapeutic intervention. It was seen that in

lung cancer cells, Golgi fragmentation induced by treating cells with Arf1 GEF inhibitor mCOP-A promoted sensitivity of the cancer cells to certain receptor tyrosine kinase inhibitors such as Gefitinib and Erlotinib (targeting EGFR on the cell surface) (Ohashi et al., 2018). However, the cause-effect relation of Golgi disruption in cancer is not clearly understood, and mechanisms behind the role of altered Golgi organization in cancers are largely unknown.

1.3.2 Membrane trafficking altered in diseases/disorders/cancers

Membrane trafficking pathways are integral for normal cell functioning, growth, and accommodation in its physical and chemical environment. This encourages proper intra- and inter-cell communication to promote coordination and function in complex multicellular organisms. The fidelity of trafficking pathways depends on two key aspects – accurate targeting of vesicles in traffic and rate of trafficking (Hirschberg et al., 1998a; Linders et al., 2020). Both these aspects can vary across cell types to sustain cells in different environments and produce variable functions in cells as required.

For example, the membrane flux in some cells is low, while in some cells, it is higher, but in specialized cells and tissue types, it can be enormous. Pancreatic acinar cells synthesize and secrete several enzymes, from which 'amylase' is a digestive enzyme, produced at a rate of approximately 0.5% of cellular protein mass per hour (Allfrey et al., 1953). In the case of Schwann cells, the rate of trafficking to the membrane must correlate with the several thousand-fold expansions of the cell surface, which occurs during myelination (Yarwood et al., 2020). Therefore, small alterations in transport machinery can affect cell function and contribute to disease manifestations.

Parkinson's disease (PD) is a well-studied neurodegenerative disease which shows several defects in endocytic traffic, including increased uptake of toxic alpha-synuclein aggregates (Abeliovich & Gitler, 2016). Additionally, deficiency in VPS26 and VPS35, two subunits of the retromer complex for endosomal recycling, have been observed in the case of Alzheimer's Disease (AD), which is one of the many different alterations reported in AD (Small et al., 2005; Yarwood et al., 2020).

Defective COPI-dependent recycling from the Golgi apparatus to the ER is associated with two multi-systemic genetic disorders affecting the liver. The COPI accessory protein

SCYL1 mutation causes low γ-glutamyl-transferase cholestasis, acute liver failure, and neurodegeneration (CALFAN) syndrome. The CALFAN syndrome further manifests into hepatocyte death and liver failure. This may also cause ataxia resulting from cerebellar neurodegeneration (Lenz et al., 2018; Schmidt et al., 2015).

Even in cancer, alterations in membrane trafficking are intimately linked with tumor progression. Changes in the expression levels or degree of phosphorylation of endocytic trafficking machinery can correlate with cancer prognosis. Oncogenic mutations in RAB35, although rare, have been shown to cause its constitutive activation and promiscuous growth factor signaling from endosomal compartments, contributing to poor prognosis (Wheeler et al., 2015).

Mutation of the ER-to-Golgi trafficking protein LMAN1 in colorectal cancers leads to reduced secretion of the LMAN1 partner protein α-1-antitrypsin, an angiogenesis inhibitor, thereby contributing to tumor blood supply and growth (Roeckel et al., 2009) (Roeckel et al., Cancer Res. 2009).

Since the Golgi complex is at the hub of trafficking events in a cell, changes in Golgi organization also affect the rate of trafficking through the Golgi. As discussed earlier, Golgi disorganization can increase the rates of trafficking and missorting of cargo (Fig. 1.6). Mechanisms behind how altered Golgi morphology could affect membrane trafficking events in disease are not well known, especially in the context of tumor progression.

1.3.3 Glycosylation changes associated with diseases/disorders/cancers

Glycans influence the metabolic flux within a cell by regulating the function and localization of biomolecules, cellular signaling, endocytosis, and trafficking, as discussed earlier. Defects in glycan processing thus can cause various anomalies with mild to severe pathological consequences (Makhoul et al., 2019; Ohtsubo & Marth, 2006). Commonly known defects caused by mutations in glycosylation enzymes, such as glycosidases, include storage-associated disorders - Tay Sach's, Gaucher's and Sandhoff's syndromes. 'I' cell disease is the earliest known **storage disorder** attributed to defects in glycan synthesis (M. Xu et al., n.d.). It arises from mutations in Golgi localized enzyme, GlcNAc phosphotransferase alpha/beta subunit precursor, which leads to the absence of lysosomal sorting signal – Mannose-6-phosphate on hydrolases native to the lysosome.

Altered glycosylation can also promote **autoimmune response**. The mammalian immune system can distinguish between self and non-self-glycan antigens and thereby defends the body specifically against foreign pathogens. However, defective glycan processing can lead to newer glycan structures which the system recognizes as non-self, thereby inflicting an autoimmune response. Co-clustering of T cell receptors is observed in the case of mutated or deficient beta-1,6-N-acetylglucosaminyltransferase V (Mgat5), causing a reduction in the threshold requirement of these receptors on the surface. This increased signaling leads to autoimmune diseases bypassing immune activation thresholds (Demetriou et al., 2001).

Many **Congenital diseases** of glycosylation (CDG) have been well characterized and are known to cause severe abnormalities in growth, metabolism, and development. CDGs can arise due to mutations in glucosidases, glycosyltransferase or associated proteins integral to their function (Hennet & Cabalzar, 2015; Linders et al., 2020). Some CDGs caused by a deficiency of carriers of nucleotide sugar residues across organelle membranes can be rescued by alterations in dietary intake. The study of congenital diseases of glycosylation has been difficult since most of the mutations have been proven to be lethal (Aebi & Hennet, 2001; Varki, 2017).

Besides, redundancy in molecular functions and integration of multiple metabolic and signaling pathways particularly favour **cancer** cells to escape in-built surveillance, regulatory systems, and therapeutic strategies. The diverse glycoproteome of a cell provides yet another cell function outcome that can be regulated and exploited by cancer cells (Fig 1.14). An increasing number of studies show that the glycoproteome of cancer cells is very often altered to varying extents. An abnormal increase in terminal sialylation motifs, Sialyl-Lewisx, Sialyl-Lewisa, Tn, STn and TF antigens are the prominently detected phenotypes in most epithelial cancers (Fig. 1.15) (Reticker-Flynn & Bhatia, 2015).

Another study showed that the *O*-linked glycosylation on the extracellular domain of integral membrane protein MUC1 imparts anoikis resistance to cancer cells. The bulky nature of glycans on this protein probably plays a significant role in restricting access of the cell surface apoptotic signal receptor molecules such as E-Cads, Integrins, and Fas receptors to the ‘death-inducing’ ligands, thereby conferring resistance to anoikis (Piyush et al., 2017).

Almost all glycosylation reactions happen through the Golgi compartments. Therefore, any subtle changes to the Golgi architecture reflect on alteration in glycosylation signatures generated on proteins, lipids and the cell surface glycome. Glycosylation enzymes at the Golgi are often bound to Golgi matrix proteins, emphasizing the importance of Golgi organization to glycosylation. Defective Giantin in androgen-independent prostate cancer cells promoted a disorganized Golgi architecture. It also resulted in a shift of Golgi targeting of glycosyltransferases and alpha-mannosidases IA from their usual binding partner – Giantin to GM130-GRASP65 complex. Consequently, trans-Golgi enzymes and

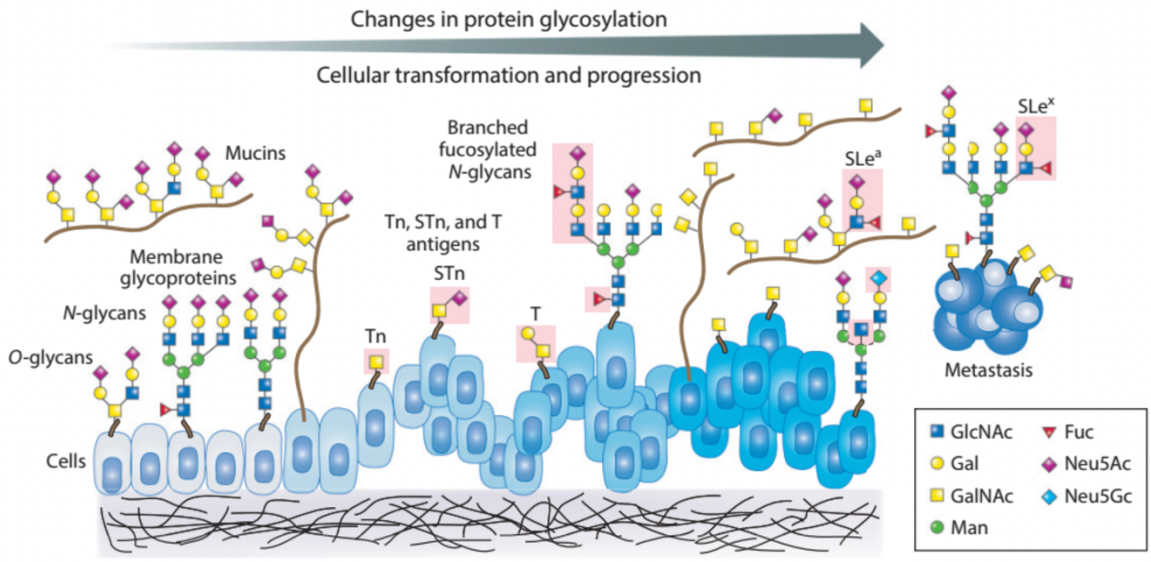


Figure 1.14 : Schematic showing glycosylation in cellular transformation – T, STn and Tn antigens are shown among several other glycan modifications occurring in epithelial cells as they undergo transformation and promote disease progression. *Image Source: Stowell et. al. Annu. Rev. Pathol. Mech. Dis. 2015*

cell surface glycoproteins were observed to have a higher content of mannose N-glycans, which are absent in cells with functional Giantin and organized Golgi architecture (Bhat et al., 2017).

Altered Golgi morphology and subsequent alterations in Golgi function are advantageous to cancer cells in multiple ways, which promotes malignant behaviours, survival of tumor cells in an unfavourable environment and also imparts resilience in cancers. Understanding the mechanisms behind changes in the Golgi complex in promoting cancers will be vital in developing and improving therapeutic strategies against tumor progression.

1.3 Hypothesis and Objectives of the thesis

As evidenced from literature studies, the varying Golgi organisation is often of much consequence in diseases, including cancer. In cancers, the Golgi organisation is highly variable, with some showing distinct disruption of Golgi organisation and others having intact 'normal' Golgi. These variable cancer cell Golgi phenotypes continue to support their transformation. How changes in Golgi organisation impact cancer cell function remains poorly understood. Knowing that cell-matrix adhesion can also regulate Golgi organisation in normal cells, the impact this has in anchorage-independent cancers remains to be tested. The effect this could have on the role of the Golgi in cancers may add significantly to how we think about this regulation. While there are multiple ways to regulate Golgi organisation (and function), cancer cells could also hold clues to revealing novel candidates that might be of consequence in 'normal' Golgi organisation and function. It is these questions that the first aim of my thesis will address.

The **first part** of my thesis is dedicated to understanding and testing the variable organisation of the Golgi apparatus across cancer cell types when adherent and on loss of adhesion. In focussing on lung cancer cell lines A549 and CaLu1, with distinctly differing Golgi phenotypes, I use *in silico* analysis to identify differentially expressed Golgi-associated genes that could regulate Golgi organisation. Ranking these genes first led me to test AXL, a receptor tyrosine kinase. Localising on the Golgi, I find AXL to be a novel mediator of Golgi organization and function. This regulation is seen in non-adherent A549 cells, where Golgi organization is adhesion-independent. I further investigate how AXL regulates Golgi organisation in A549 cells, its regulatory crosstalk with Arf1 and its effect on Golgi-dependent cell surface glycosylation. How this and other cancers might use AXL-dependent regulation of the Golgi to drive cancer cell transformation and migration will be of much significance.

In the **second part** of my thesis, I investigate quantitative changes in cell surface glycosylation levels as a function of changing Golgi organization. I created a range of Golgi fragmentation phenotypes in non-adherent mouse fibroblasts using a concentration gradient of Brefeldin A, which targets Arf GEFs to regulate ARF1 activation. Using an optimised combination of lectins and data modelling, I help reveal how Golgi organisation affects differential cell surface glycosylation, which could affect cell function. The

implications this could have in how Golgi organisation changes affect cancer cell function will be particularly interesting.

Specific objectives of the thesis

1. Golgi organization in anchorage-independent cancers and the role of Arf1
2. Identifying regulator(s) of Golgi organization in lung cancer cell lines
3. Role of AXL in the regulation of Golgi organization and function in lung cancer cell lines
4. Loss of adhesion-dependent Golgi organization and its effect on cell surface glycosylation.

Chapter 2

Materials and Methods

2.1 MATERIALS

2.1.1 Reagents

Accutase (Cat. No. #A6964), Fibronectin (Cat. No. #F2006) and Triton-x 100 (Cat. No. #T8787) were purchased from Sigma. Lipofectamine 2000 was purchased from Invitrogen (Cat. No. # 11668019). Fluorophore conjugated lectin probes were purchased from Invitrogen Molecular probes - ConA-Alexa488 (Cat. No. #C11252), ConA-Alexa647 (Cat. No. # C21421), WGA-Alexa488 (Cat. No. #W11261), WGA-Alexa647 (Cat. No. #W32466), PNA-Alexa488 (Cat. No. #L21409), PNA-Alexa647 (Cat. No. # L32460). UEA-FITC lectin was purchased from Sigma (Cat. No. # L9006). DMSO was purchased from Sigma (Cat. No. # D2438). Trypsin Inhibitor was purchased from Roche (Cat. No. #10109886001). Inhibitors Brefeldin A (Cat. No. #B7651) and Golgicide A (Cat. No. #G0923) were purchased from Sigma. Ciliobrevin (Cat. No. #250401) was purchased from Calbiochem. Bemcentinib (R428) was purchased from Cayman USA (Cat. No. #21523). Immobilon Western Chemiluminescence substrate was purchased from Millipore (Cat. No. #WBKLS0500). Trizol was purchased from Ambion (Cat. No. # 15596018). Fluoramount-G was purchased from Southern Biotech (Cat. No. #0100-01). cDNA synthesis kit (Takara Cat. No. #6210A) was obtained from Dr. Nishad Matange IISER Pune. SYBR green mix was purchased from Takara (Cat. No. #RR820A). BCA protein estimation kit (Cat. No. #23225) was purchased from Pierce.

2.1.2 Antibodies

pAKT S473 Cat. No. #4060S, AKT Cat. No. # 9272S, pAMPK T172 Cat. No. #2535, AMPK Cat. No. #2352, AXL Clone C89E7 Cat. No. #8661S, pAURK T288A/B/C (Cat. No. #2914S) and EGFR (Cat. No. #2232S) were purchased from Cell Signaling. GAPDH Cat. No. #9545 was purchased from Sigma. AXL (Cat. No. #AF154 SP), pAXL (Cat. No. #AF2228 SP), and pEGFR Y1173 (Cat. No. #AF1095) were purchased from R&D technologies. Beta Tubulin (Cat. No. #E7) was purchased from DSHB. GM130 Clone 35, Cat. No. # 612008, AURKA (Cat. No. #610939), and RALA (Cat. No. #610221) were purchased from BD Transduction. pRALA S194 (Milipore Cat. No. #07-2119), , Vimentin (Cat. No. #ab92547), E-Cadherin (Cat. No. #ab1416) N-Cadherin (Cat. No. #ab76057) and yH2AX (Cat. No. #ab26350) were obtained from Dr. Kundan Sengupta IISER Pune. LC3

A/B (Cat. No. #4108S) was obtained from Dr. Madhulika Dixit (IITM). ARF1 Clone 1D9, Abcam, Cat. No. # ab2806 (1:500). βeta-tubulin Clone E7, Developmental Studies Hybridoma Bank, Cat. No. #AB_2315513 (1:1000). Phalloidin-488 was purchased from Invitrogen (Cat. No. #A12379)

Secondary Fluorescent conjugated antibodies (Alexa488 and Alexa647) were purchased from Invitrogen Molecular Probes and used at a dilution of 1:1000. HRP-conjugated secondary antibodies were purchased from Jackson Immunoresearch and used at a dilution of 1:5000

2.1.3 Plasmids and Oligos

GalTase-RFP and Manosidase-GFP constructs were obtained from Dr. Jennifer Lippincott Schwartz. ABD-RFP and WTARF1-GFP constructs were obtained from Dr. Satyajit Mayor, National Centre for Biological Sciences, Bangalore, India. GFP-tagged Arf1-Q71L construct was made in the lab by Vibha S. Briefly, site-directed mutagenesis was performed with GFP-Arf1-WT as a template and using the following primers: (forward) 5'-GACGTGGGTGGCCTGGACAAGATCCGG-3' and (reverse) 5'-CCGGATCTTGTCCAGGCCACCCACGTC-3'

Primers used for RT-PCR experiments were designed using a Primer design tool from Integrated DNA Technologies and were ordered from Eurofins. Table 1 provides a listing of sequences for primers used in the study. AXL siRNAs were purchased from Bioserve oligos (sense strand #1 - 5'GAAAGAAGGAGACCCGUUA3' sense strand #2 – 5'CCAAGAAGAUCUACAAUGG3') AXL sgRNA primers were designed using CHOPCHOP and CCTOP tools and were purchased from Bioserve oligos (Table 2). All primer sequences were checked for target specificity using the NCBI nucleotide BLAST tool.

TABLE 2.1 : Primer sequences used for RTPCR

SR NO	GENE NAME	PRIMER SEQUENCE
1	MECOM FP	AGGCATCTTCCAGTTTACCC
2	MECOM RP	GTTCCCAATCATGTCAGGTG
3	TXK FP	CAGTGGTATGTGGCTGAAAG
4	TXK RP	CTGGTAAACAACTGCCCATC

5	EPHB1 FP	CAGGCAAGAGGGAAATCTAC
6	EPHB1 RP	CTCCAGGCGAATGATGTTAG
7	PAK3 FP	GTGGCTCTCTGACTGATGTG
8	PAK3 RP	TGGATCACCTGGTTTGAGTG
9	PAG1 FP	ACAACGTGACTCCCAAGTAG
10	PAG1 RP	TCGCACATCCTATCACATCG
11	TICAM2 FP	CTACCAGTACCGGGATCTGCACAC
12	TICAM2 RP	AGGAGGCTTGACTTACTTGCATGCTC
13	PDE4DIP FP	GCTGCTGATATGGAGTCTCT
14	PDE4DIP RP	AGTAAGACTTCCTGGGTCAG
15	SPTBN5 FP	GTTGGGAGAGAGCTGTTGAG
16	SPTBN5 RP	CTCAGTCATCTTGCGGTTCA
17	MST1 FP	TCCAGAGCTGCGGCATCAAA
18	MST1 RP	CTGGTGCGGGTGCTGAAGAT
19	HCK FP	GAAGACGATGAAGCCAGGGA
20	HCK RP	ACTCCGTGATGATGTAGATGGG
21	AXL FP	GTCTAGCTGACCGTGTCTAC
22	AXL RP	CCTGGCGCAGATAGTCATAA

2.2 METHODS COMMONLY USED THROUGHOUT THE STUDY

2.2.1 Cell culture and transfections

Mouse Embryonic Fibroblast (MEF) cells were obtained from Dr. Richard Anderson's lab (University of Texas Health Sciences Centre, Dallas, TX). CaLu1, T24, UMUC3 and MDAMB231 cell lines were obtained from ECACC. A549, DLD1 and J82 cell lines were obtained from ATCC. The MCF7 cell line was obtained from Prof. Gupta at ACTREC, Navi Mumbai, India. DLD1 cells were cultured in Gibco RPMI medium from Thermo Fisher Scientific with 10% Penstrep and 5% FBS added. Normal human lung epithelial cell line BEAS2B was a generous gift from Dr. Shantanu Chowdhary, IGIB Delhi. BEAS2B cells were cultured using Gibco LHC9 medium from Thermo Fisher Scientific (Cat No. #12680013). All other cell lines were cultured using Gibco DMEM from Thermo Fisher Scientific, with the addition of 10% Penstrep and 5% FBS. 0.05% trypsin was used to detach cells, and an excess of culturing medium was used to neutralize the action of trypsin. For BEAS2B cells, a trypsin inhibitor (Roche) was used to neutralize trypsin.

For transfection studies, cells were seeded in 6cm dishes to attain a confluency of 60% and allowed to attach and spread for 5 hours. Using Gibco OptiMEM medium and transfection agent Lipofectamine 2000 from Thermo Fisher Scientific, the transfection mix was prepared and kept at room temperature for 30 minutes before adding to the cells seeded in

6cm dishes. Media in transfected dishes was changed 12 hours post-transfection, and cells were used for experiments 36 hours post-transfection.

2.2.2 Suspension assay experiments

Cell lines maintained in their respective media were grown up to 75% confluency in 10cm or 6cm dishes, as required. WTMEF cells were serum-deprived using low serum DMEM (0.2% FBS) for 14.5 hours before detaching cells for suspension assay. 0.05% Trypsin or Accutase (for lectin labelling experiments) used to detach cells was washed out using excess media. Trypsin inhibitor (Roche) was used in the case of BEAS2B cells treated with Trypsin. From collected cells, one aliquot was kept aside and processed for the 5-minute (just detached) timepoint if required. For the suspension assay, cells were gently mixed with culture medium containing 1% methylcellulose and incubated for a specified duration at 37^0C with 5% CO_2. Post-suspension, methyl cellulose was washed out using culture medium, and 4^0C conditions were maintained throughout to harvest cells from suspension. Collected cells were distributed to suspension and re-adhesion time points as required. For re-adhesion time points, cells post-suspension were seeded on 22x22mm coverslips or 6cm dishes pre-coated with 2mg/ml or 10mg/ml Fibronectin, respectively. For inhibition experiments, respective inhibitors were added to the suspension mix, media washes and re-adhesion timepoints. Each timepoint was then processed for lectin labelling or fixed with PFA for IFA/imaging or kept at -80^0C for preparing cell lysates and for active ARF1 pulldown experiments.

2.2.3 Lectin labelling and Flow Cytometry analysis

Cells harvested from suspension were given a PBS wash to remove residual media, then added to a lectin labelling mix containing fluorophore-conjugated lectins diluted in 1XPBS at required lectin concentrations. In experiments with dual labelling, cell sample was incubated with two lectins having different glycan substrates and different fluorophore conjugates, simultaneously in the same reaction mix. The labelling reaction was kept in ice under dark conditions for 15 minutes, followed by two washes with cold 1xPBS. PFA (3.5%) was used to fix the lectin-labelled cell samples, which were then resuspended in cold 1xPBS (350µl) for Flow cytometry analysis. Samples were run on BD Celesta Flow Cytometer. A morphologically uniform cell population presented by the forward and side scatter (FSC and SSC) plot was selected using polygon gating for data acquisition. A maximum of 15000 events were recorded in the gated area for every sample. Data obtained from BD Celesta was further analysed using FlowJo software to get single-cell data for each sample. For optimizing concentrations of lectin to be used, a range of concentration

was tested for each lectin using Flow Cytometry (as shown in Table 2.1). The lectin concentration giving intensity measurements in the range of 5000 – 10000 arbitrary units was selected and used in all lectin labelling experiments for that particular lectin.

Table 2.2 : Range of lectins concentration used to identify optimal concentration for labelling

Concentration range used for Lectin standardization in Lung cancer cells		
Sr no.	**Lectin**	**Concentration**
1	ConA green	25ng/µl
		50ng/µl
		100ng/µl
2	HPA green	25ng/µl
		50ng/µl
		100ng/µl
5	WGAgreen	0.5ng/µl
		1ng/µl
		2ng/µl

Concentration range used for Lectin standardization in WTMEFs		
Sr no.	**Lectin**	**Concentration**
1	ConA green	25ng/µl
		50ng/µl
		100ng/µl
2	ConA red	2.5ng/µl
		5ng/µl
		10ng/µl
5	WGA green	0.125ng/µl
		0.25ng/µl
		0.5ng/µl
6	WGA red	0.125ng/µl
		0.25ng/µl
		0.5ng/µl

2.2.4 Immunofluorescence assay (IFA)

For IFA, cells fixed with PFA were incubated in a Permeabilization buffer for 15 minutes at room temperature. Permeabilization buffer was made with Triton X 100 (0.05%) diluted in 5% BSA, which was made in 1x PBS. Post permeabilization, two washes were given with 1X PBS followed by blocking with 5% BSA at room temperature for 30min. Two washes were given after blocking. Samples were incubated with Primary antibody overnight at 4^0C. Three washes were given post-incubation with primary antibody followed by a 1-hour incubation with secondary fluorescent antibody at room temperature. Samples were given three washes and then mounted on slides using Fluoramount-G. Slides were maintained at room temperature under dark conditions to dry properly, then moved to 4^0C until confocal imaging.

2.2.5 Inhibitor studies

Cells grown to 75% confluency were treated with AXL inhibitor R428 at 1μM concentration for 12 hours before using cells for suspension assay, preparing lysates or preparing slides for confocal imaging. For BFA and GCA experiments, cells were held in suspension for 30 minutes, or 90 minutes with indicated concentrations of BFA or GCA. Nanoparticle-MLN8237 and Compound C are inhibitors of AURKA and AMPK, respectively. NpMLN (0.04μM) was added to 50% confluent cells for 24hrs and 48hrs at indicated concentrations. Compound C was used at 20μM concentration for 2.5 hours before using cells for experiments. In R428, BFA, GCA and Compound C experiments, DMSO was used as a control. During the experiment, respective inhibitors were added in every step of processing cells to prevent reversal of inhibition. For Confocal and Flow cytometry experiments, samples were fixed immediately at required timepoints. For western blotting procedure with or without Arf1 activity assay, experimental timepoints were frozen at -80^{0}C until completion of experiments, so as to proceed with lysis of all due timepoints simultaneously.

2.2.6 Testing endocytosis of cell surface-bound lectins

WTMEFs serum-deprived for 14.5hrs at 70% confluency were detached using Accutase. Cells were held in suspension for 30 minutes at 37^{0}C and 5% CO_2, with the indicated concentrations of BFA. Post-suspension cells were harvested, followed by dual labelling with lectins - ConA (Alexa488) and WGA (Alexa647) for 15 minutes at 4^{0}C under dark conditions. Unbound lectins were washed off by giving two washes with 1X PBS. Labelled cells were further distributed to three-time points - 0min endocytosis timepoint was fixed with PFA immediately, 15min and 30min samples were kept in 500μl low serum media (with BFA) at 37^{0}C to allow endocytosis of surface lectins for respective timepoints followed by fixing with PFA. Throughout the process, 4^{0}C conditions were maintained unless otherwise specified. All samples were mounted on slides using Fluoramount-G. Slides were maintained at room temperature under dark conditions to dry properly, then moved to 4^{0}C until confocal imaging

2.2.7 Arf1 activity assay

Activity assay was performed simultaneously for all time points/samples in an experiment in order to ensure that the same amount of beads was used across the samples that were to be compared. Samples for pulldown assay were processed immediately at respective timepoints or frozen at -80^{0}C, before performing activity assay; for experiments looking at active ARF1 in MCF10A, untreated or UV radiation-treated adherent cells were a gift from the lab of Dr. Mayurika Lahiri, IISER Pune. Briefly, stably adherent cells were exposed to UV radiation for 1 second, causing 10 Joule/m^2 DNA damage. Samples were kept for 6 hours, 12 hours and 24 hours, respectively, before freezing cells at -80^{0}C, followed by ARF1 activity assay for all time points together.

Active ARF1 pulldown was performed using Sepharose A beads bound to fusion protein GST-GGA3. Samples were lysed with 500µl activity assay buffer containing Tris-HCl (HiMedia), $MgCl_2$ (HiMedia), TritonX 100 (), NaCl (HiMedia), Protease Inhibitor Cocktail (PIC) (1x), Sodium Fluoride (NaF; Sigma), and Sodium Orthovanadate (NaOr; Sigma). 60ug of GST-GGA3 was given a quick wash with a complete buffer. 400µl of lysate was added to 60ug of GGA3 beads and incubated at 4^{0}C for 35min at 9rpm. The remaining 100µl of lysate was mixed with Laemmli buffer; this was used as the whole cell lysate fraction. Post incubation with GGA3 beads; the samples were given three washes with activity assay buffer. After the last wash, the maximum supernatant was removed, and beads were mixed with Laemmli buffer. Samples were boiled at 95^{0}C and given a short spin before loading gels or kept at -20^{0}C for long-term storage. Western blotting results were analysed using ImageJ software.
To determine the percentage activity of Arf1, the following calculation was used:

$$\text{Percentage activity} = \frac{\text{Pulldown Band Intensity X 100}}{\text{Corresponding WCL Band Intensity X Dilution factor}}$$

The dilution factor was calculated as the ratio of the amount of total cell lysate used for the pulldown (400 µl) and the amount of this lysate resolved by SDS PAGE in the whole cell lysate (WCL) lane (24 µl WCL + 6 µl 5X Lamelli buffer). The dilution factor was hence 400 ÷24 = 16.66. This ratio was kept constant in all experiments.

2.2.8 Protein estimation with Pierce BCA

Samples were lysed in RIPA buffer with freshly added protease inhibitors and kept in ice for 30 minutes for lysis. Lysates were then spun down at 14000rpm;4^0C for 15min. Supernatent was collected in a fresh tube as the lysate. 10µl of samples were set aside for BCA, and the rest was lysed in Laemmli buffer and boiled at 95^0C followed by a short spin. Lysates were stored at -20^0C until gel run and western. Protein estimation was done as follows : in a 96 well plate, unknown samples diluted to 1:5 times with RIPA buffer were loaded in triplicates along with freshly prepared standards for BSA diluted in RIPA buffer (range – 0mg/ml to 2mg/ml). Working reagent from the Pierce BCA kit was prepared by mixing Reagent A and Reagent B in a ratio of 50:1. 200µl of working reagent was added to each well containing 10µl of sample or BSA standard. The plate was incubated at 37^0C for 30 minutes and then scanned at 562nm to determine absorbance using a plate reader (Varioscan/PerkinElmer Ensight). Absorbance values for BSA concentrations were plotted to obtain a standard curve, using which concentration for the unknown sample was determined.

2.2.9 SDS-PAGE and Western Blotting

20ug of protein was loaded in gels for SDS-PAGE. Protein samples run on gels were transferred to PVDF membrane using a methanol buffer or sodium bicarbonate buffer. Post transfer, the PVDF membrane was blocked in 5% skimmed milk (made in 1xTBS-Tween20) at room temperature for 1hr at 10rpm. The membrane was washed with 1xTBST; then, individual blots were incubated in respective Primary antibody solutions overnight at 4^0C. Blots were then washed three times with 1xTBST, followed by incubation at RT with respective HRP-conjugated secondary antibody solutions made in 2.5% skimmed milk. Post-secondary antibody incubation blots were given three washes with 1xTBST and then developed with direct or diluted Immobilon Substrate using LAS4000 Chemiluminescent Imager. Densitometric analysis was done using ImageJ FIJI software.

2.2.10 Confocal imaging and Image analysis

Slides were imaged at ZEISS confocal (LSM710) using a 63X oil immersion objective. For profiling data, cells observed at confocal were categorized manually and counted in to two groups. A group of cells with organized Golgi and group of cells with disorganized

Golgi architecture for Golgi profiling data. For ABD-RFP profiling data the two groups were - cells showing localization of ABD-RFP at the Golgi vs cells not showing localization of ABD-RFP at the Golgi. A minimum of 100 and a maximum of 200 cells were counted and categorized according to their phenotype to arrive at a percent population of cells in either category. For lectin endocytosis experiments, cross-sectional images were acquired at the equatorial plane of cells. Images were captured with an average of 4 and zoomed at 5 for suspended cells and zoomed at 2 for stable adherent cells. The scan speed applied was 5 for cross-sectional images and 7 when obtaining z-stacks. Leica DM6 was used for imaging samples from active ARF1 localization experiments using an ABD-RFP marker.

2.2.11 Image Analysis and quantitation

For all imaging experiments, ImageJ FIJI was used to process images with scale bars until otherwise specified. Huygens software was used to perform deconvolution of LSM files. Deconvoluted images were used to generate Maximum Intensity Projection (MIP) images and 3D Surface Rendered (SR) images of the Golgi organization in cells using the Visualization tool in Huygens. Colocalization analysis for data showing AXL localization at the Golgi was performed using the 'Colocalization Analyzer Advanced' tool from Huygens software. Costes' method was used to set a threshold for each image, and the GM130 (Alexa488) channel was manually thresholded at 80 and analysed by the software to obtain Pearson's coefficient value.

2.2.12 Gene expression analysis for regulators of Golgi organization (In-silico study)

A comprehensive listing of genes associated with the Golgi apparatus was done using the NCBI Gene Ontology (GO) tool. A search with the terms 'Golgi organization human' yielded 131 human genes associated with the term Golgi organization. Gene expression data (RPKM) was obtained from the CCLE database using the UCSC Xena browser for cell lines A549 and CaLu1. Fold change between gene expression values of the cell lines was calculated, and a difference of $\geq$5 was considered significant to select differentially expressed genes (DEGs). STRING-DB version 11.0 was used to create a primary protein interaction network for each DEG. No restriction was applied over the number of interactors displayed in the network to cover all possible primary interactions. Interactions based on experimental evidence with a confidence score of $\geq$0.4 were considered in the

study. Each of the primary interactors obtained was evaluated for any known role in the regulation of Golgi organization or Golgi function.

Scoring of genes - Knowing the Golgi organization phenotype in stably adherent A549 and CaLu1 cells, the primary list of selected genes was scored based on available data in the literature for the effect of gene knockdown on Golgi organization. Accordingly, genes agreeing with the Golgi phenotype on KD were scored as 2, those that have an effect as shown in literature but not agreeing with the observed phenotype were scored as 1, and the genes having no available data to suggest a direct effect on Golgi organization were scored as 0. A second score was given based on the number of primary interactors obtained from STRING analysis that have a direct role in Golgi's organization or function. The two scores were added for each gene, and the DEGs were further shortlisted to 14 genes, which had a combined score of 3 or higher. These 14 genes were validated further using RT-PCR.

2.2.13 RNA isolation and cDNA synthesis

For preparing RNA samples, $8x10^5$ cells were seeded in 6 well plates for BEAS2B, A549 and CaLu1 cell lines. Post 14hrs of seeding, the samples were lysed in Trizol (Ambion), followed by RNA isolation using the PCI method (Phenol, chloroform and Isopropyl alcohol). Post ethanol washes, the RNA were allowed to dry for 15 minutes at 37^0C and resuspended in 50µl of Nuclease Free Water (NFW). RNA concentration and purity were checked using NanoDrop 2000. 1ug of RNA was mixed with Gel loading buffer and run on 10% Agarose gels to check the quality of RNA samples. Stock of

RNA samples were stored at -80^0C. Using the PrimeScript cDNA synthesis kit (Takara), cDNA samples were prepared from at least five different sets of RNA (1ug) for BEAS2B, A549 and CaLu1 cell lines each. Details of the PCR cycle run were as follows - 25^0C for 2min, 25^0C for 15 seconds, 37^0C for 25min, 85^0C for 1min 30seconds. cDNA samples were stored at -20^0C and used RTPCR runs for primer evaluation or comparative gene expression analysis.

2.2.14 Real-Time PCR

Primers purchased for the 15 selected DEGs were tested for primer efficiency by running RT-PCR for a range of cDNA dilutions. Primer efficiency was determined by three criteria

– single peak for melt curve, Ct value for the highest concentration of cDNA, and slope value for a range of cDNA concentrations. Post RT-PCR, the product was run on Agarose gels to confirm if the product ran at the expected band size. All RT-PCR experiments for comparative gene expression analysis were performed using 1:1 dilution of cDNA. SYBR green mix (Takara) was used for the detection and quantification of gene expression. Actin was used as the standard control. 5µl reaction mix was loaded in triplicates for each sample. The plate was given a quick spin before running the RTPCR cycle, the protocol for which was as follows: 95^0C for 3 minutes (once per sample). Plate reading was recorded after the two steps of 95^0C ; 10 seconds – 60^0C ; 25 seconds; this cycle was repeated 40 times per sample. Recorded data was analysed for Delta Ct and Fold change. The relative gene expression between BEAS2B, A549, and CaLu1 cell lines was determined to identify differentially expressed genes.

2.2.15 Statistics

All the statistical analysis was done using Graphpad Prism analysis software. Statistical analysis for western blotting using absolute data (not normalized to a condition or a control) was done using the two-tailed unpaired Mann-Whitney t-test. For western blotting data using data normalized to control or a certain experimental condition, the two-tailed single sample Wilcoxon t-test was used. Statistical analysis for Golgi profiling data, comparing the percentage of cells with a specific Golgi phenotype under different experimental conditions, was done using one-way ANOVA, multiple comparison's test, with Tukey's method for error correction. The Pearson's coefficient analysis data for localization of AXL at the Golgi was tested for statistical significance using on-way ANOVA, multiple comparison's test with Tukey's method for error correction.

Chapter 3

Golgi organization in anchorage-independent cancers on loss of adhesion. Role of Arf1

3.1 RATIONALE

In most eukaryotic cells, the Golgi apparatus consists of multi-membrane stacks in the perinuclear region. These stacks link laterally to form a ribbon-like architecture in normal mammalian cells. The Golgi structure is functionally and spatially divided into cis-, medial- and trans-Golgi compartments, each composed of membranous sacs called cisternae (Lowe, 2011; Rambourg & Clermont, 1997). The organization of the Golgi is integral to its proper functioning, including the processing and trafficking of lipids and proteins (Glick & Nakano, 2009; Stanley, 2011). Therefore, the maintenance of Golgi organization is intricately regulated by several proteins and cell signaling pathways (Bekier et al., 2017; Rabouille & Linstedt, 2016; Witkos & Lowe, 2015; X. Zhang & Wang, 2015). Cell-matrix adhesion was shown to regulate Golgi organization and function in normal cells (Singh et al., 2018). Loss of adhesion causes dramatic disorganization of the Golgi membranes, accompanied by increased cell surface glycosylation. This adhesion-dependent control was mediated by regulation of the activation of GTPase ARF1, which causes loss of Arf1 and hence Dynein from the Golgi in non-adherent WTMEFs (Singh et al., 2018). In anchorage-independent cancer cells, which often show altered Golgi morphology (Kellokumpu et al., 2002; Petrosyan, 2015), adhesion-mediated signaling is also deregulated, thereby promoting anchorage independence (Maziveyi et al., 2017; Pawar et al., 2016). We asked if and how adhesion-mediated regulation of the Golgi could be altered in cancers. Knowing that the Adhesion-dependent regulation of Arf1 mediates the regulation of Golgi in normal cells, we further tested the role of Arf1. This aim evaluates the adhesion-dependent regulation of the Golgi in cancers and the possible role Arf1 could have in mediating the same.

3.2 Adhesion-independent regulation of Golgi organization in cancers

Anchorage-independent cancer cells are often seen to have an altered Golgi morphology as well as alterations in adhesion-mediated signaling, which is known to regulate Golgi organization (Singh et al., 2018). The possible regulation of the Golgi in cancers, particularly downstream of adhesion, remains to be evaluated.

3.2.1 Golgi organization in adherent cancer cells

The Golgi apparatus assembles as a compact structure in the perinuclear region of 'normal' – non-transformed cells, such as the mouse embryonic fibroblasts (Lowe, 2011; Singh et al., 2018). Recent studies from the lab suggest a role for cell-matrix adhesion in regulating Golgi organization. On loss of cell-matrix adhesion, the Golgi apparatus showed dramatic disorganization with Golgi objects dispersed through the cell cytoplasm (Fig 3.1). This disorganization of the Golgi is mediated along the microtubule network and, interestingly,

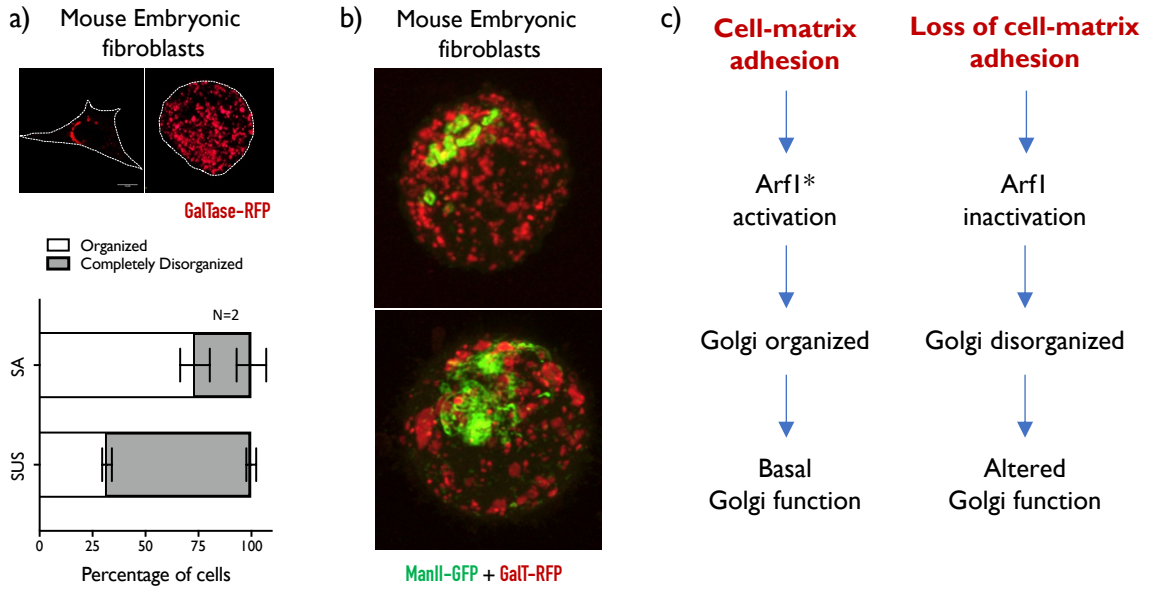


Figure 3.1 : Adhesion regulates Golgi organization in non-transformed cells – a) Golgi organization in adherent vs suspended mouse embryonic fibroblast cells. Z-stack images with maximum intensity projection and percentage profile of cells with the trans-Golgi marker GalTase-RFP. B) Representative images showing organization of cis-medial Golgi relative to the trans-Golgi. c) Schematic showing adhesion mediated regulation of Golgi organization in non-transformed cells, mediated by active Arf1, has effect on Golgi function

was variable across Golgi compartments (Singh et al., 2018). The trans-Golgi (labelled with GalTase-RFP) was found to disorganize to a more significant extent compared to the cis-Golgi (labelled with ManII-GFP) (Fig 3.1.b). This suggests that loss-of-adhesion mediated change in cell signaling could differentially regulate Golgi compartment organization in 'normal' cells. These studies in 'normal' mouse fibroblasts were done in low serum conditions, ensuring adhesion-dependent signaling was the primary mediator.

In cancer cells, however, the requirement for adhesion-dependent signaling is bypassed as cells transform and become anchorage-independent (Guadamillas et al., 2011; Pawar et al., 2016). Hence, we wanted to test if adhesion-dependent regulation of the Golgi organization is altered in anchorage-independent cancer cells. Therefore, we performed a screen for Golgi organization across multiple cancer cell lines expressing the trans-Golgi marker GalTase-RFP. Since on loss of adhesion, the trans-Golgi is extensively dispersed in mouse embryonic fibroblast cells (Singh et al., 2018), the trans-Golgi marker GalTase-RFP is a robust marker for detecting changes in Golgi organization in cancer cells.

GalTase-RFP labelled trans-Golgi organization was observed to vary across cancer cell lines tested in this screen. Adherent lung cancer CaLu1 and breast cancer MCF7 cells showed a predominantly disorganized Golgi phenotype. Golgi objects were observed to be dispersed in the cytoplasm, with a distinct perinuclear localization. However, lung cancer A549, breast cancer MDAMB231, bladder cancer T24, UMUC3 and J82 and the colorectal cancer DLD1 cells showed a predominantly organized Golgi phenotype (Fig 3.2).

A systematic and focused approach to determining Golgi organization phenotypes in cancer cells could be very revealing. Our simple screen above already shows the variable nature of Golgi organization in these cells. For cancer cells with a seemingly 'normal' (intact) Golgi architecture when adherent, it is also worth asking if and how the regulation of Golgi organization and function is different on loss of adhesion.

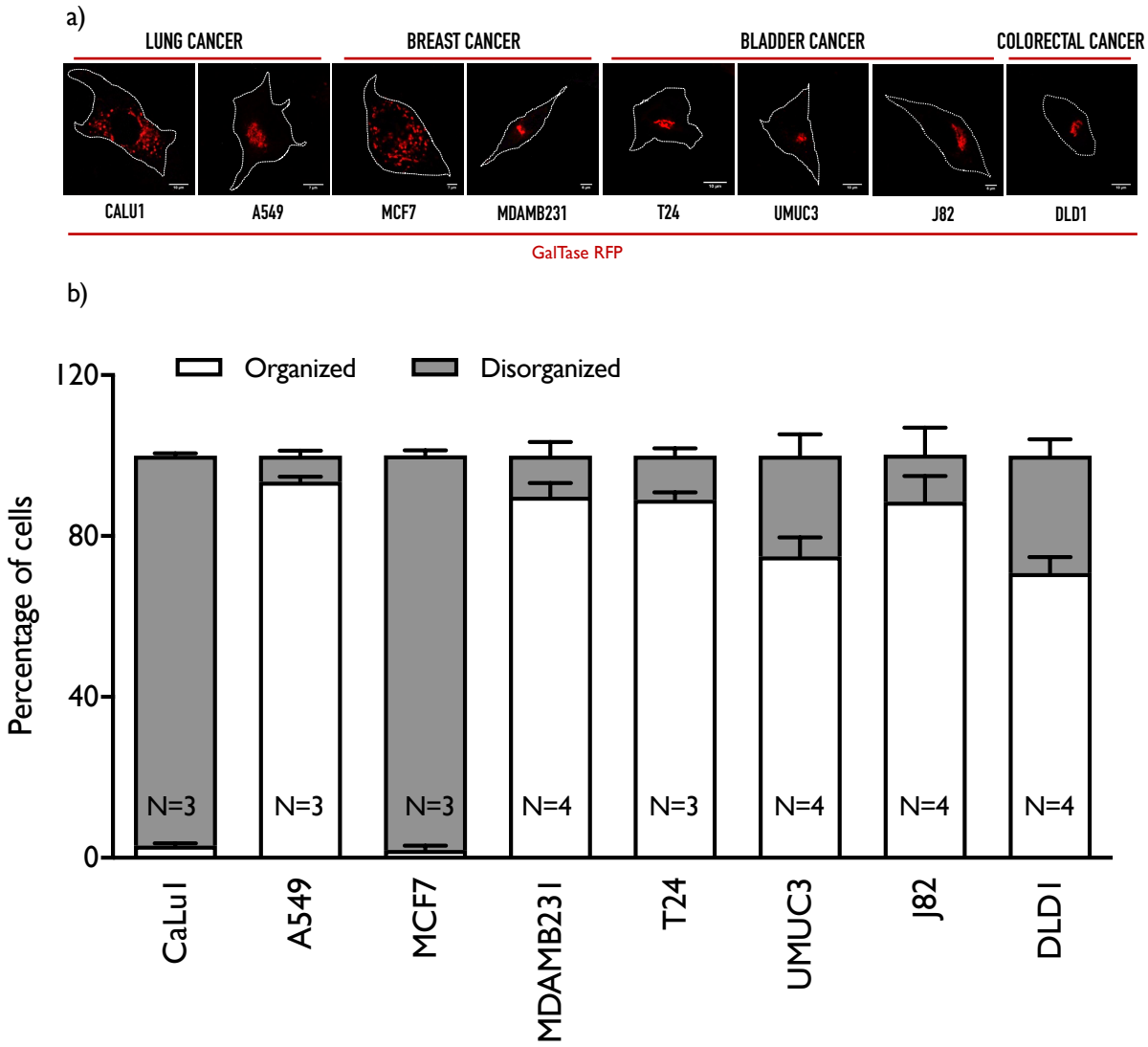


Figure 3.2 : Golgi organization across different cancer cell lines – a) Cross-sectional image of the Golgi organization phenotype representing majority of population in indicated cancer cell lines, evaluated using GalTase-RFP in stable adherent cells b) Graphs show distribution profile for percentage of cells (n≥200 cells) showing organized or completely disorganized Golgi phenotypes.

3.2.2 Testing adhesion-dependent regulation of Golgi organization in cancer cells

We wanted to ask if, in anchorage-independent cancer cells, adhesion-mediated regulation of Golgi organization exists. To test this, a pair of cancer cell lines, each from lung (A549, CaLu1), breast (MCF7, MDAMB231), and bladder (T24, UMUC3) cancer, were chosen to look at their Golgi organization when stable adherent and on loss of adhesion. The choice of cell lines was in part influenced by the distinct difference in Golgi organization between them when adherent, despite their common tissue origin.

Using the trans- Golgi specific marker, GalTase-RFP, these cancer cells were detached and held in suspension (in the presence of serum). CaLu1 and MCF7 cells with a disorganized Golgi retained their Golgi phenotype on loss of adhesion (Fig 3.3).

In A549, T24 and UMUC3 cells, the intact Golgi organization was observed to stay intact on loss of adhesion (Fig 3.3). This is distinctly different from the loss of adhesion-mediated dispersal of the Golgi reported in 'normal' cells. In MDAMB231 cells, however, the intact Golgi (when stable adherent) was found to be dispersed on loss of adhesion (Fig 3.3), effectively mimicking the 'normal' Golgi phenotype (Singh et al., 2018). The variable adhesion-dependent regulation of the Golgi in these cancer cells further raises the question of what regulatory pathway downstream to cell-matrix adhesion could control Golgi organization in cancers.

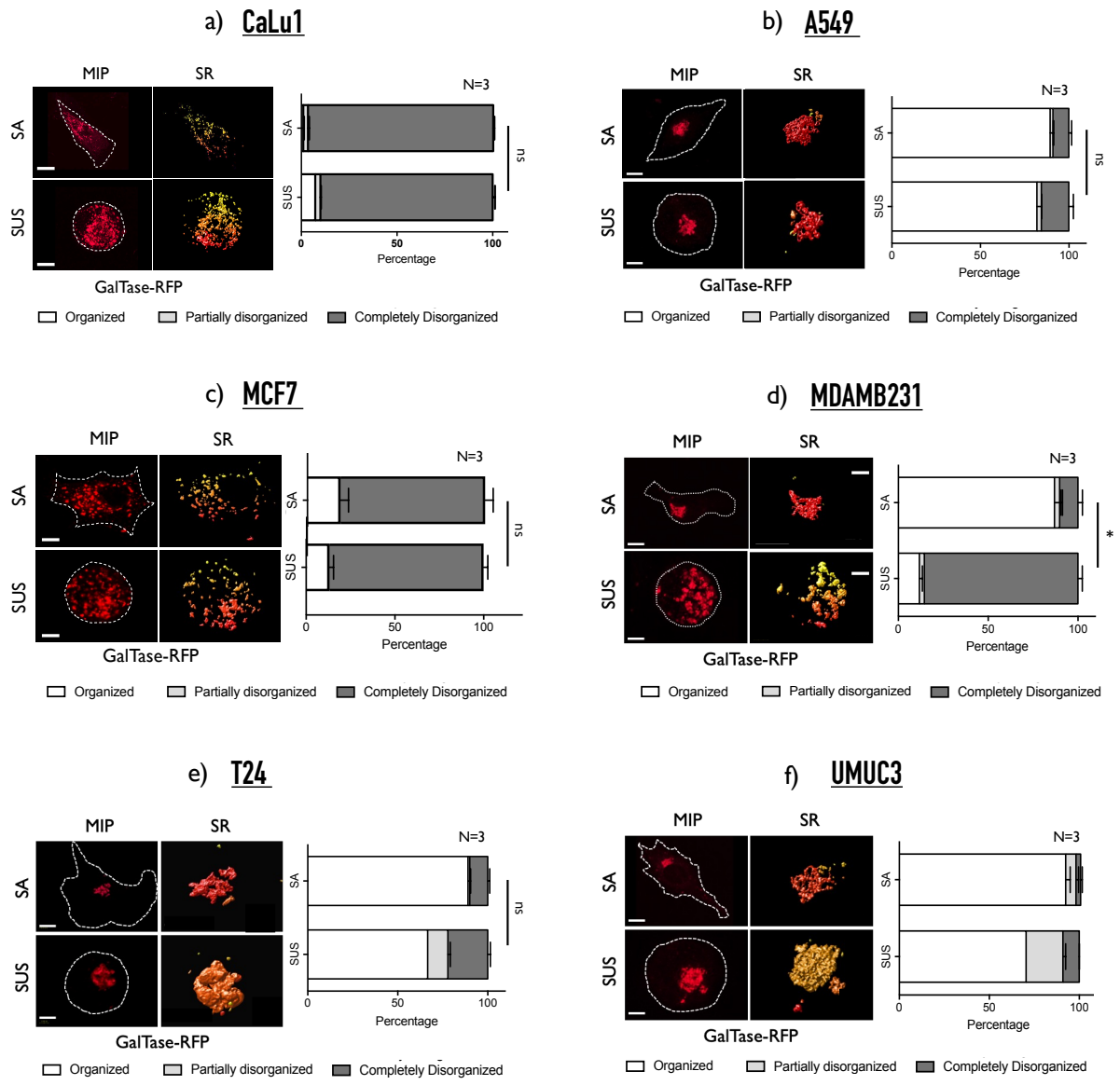


Figure 3.3 : Adhesion-dependent regulation of Golgi organization in cancer cells – a) - f) Golgi organization in stable adherent vs non-adherent cancer cells is shown using GalTase-RFP. Representative images show deconvoluted Z-stacks with maximum intensity projection (MIP) along with a zoomed image of the Golgi with surface rendering (SR). Graphs show distribution profile for percentage of cells ($n \geq 200$ cells) showing organized, partially disorganized or completely disorganized Golgi phenotypes. Statistical analysis done using one-way ANOVA.

3.3 Role of Arf1 in adhesion-independent Golgi organization in cancers

3.3.1 Expression and activation of Arf1 and its regulation by cell-matrix adhesion in cancer cells

Lung and Bladder cancer cells do not show loss of adhesion-mediated effect on Golgi organization. In 'normal' cells, adhesion-dependent regulation of Golgi is mediated by Arf1 activation, which could be altered in these cancer cells, making the Golgi adhesion-independent.

The known regulators of anchorage independence and cancer progression include the small GTPase Arf1 (Ward et al., 2001). Arf1 is a Golgi-associated GTPase, which is found to be overexpressed in several cancer types, including breast cancer, prostate cancer, lung cancer (Casalou et al., 2016a; Xie et al., 2016) etc. Arf1 expression strongly correlates with decreased overall survival and poor prognosis in cancer patients (Xie et al., 2016). Deregulation in Arf1 activity and function has also been shown to promote drug resistance in breast cancer. Inhibition of Arf1 by specific agents such as mCOPA or BrefeldinA was established to promote sensitivity to chemotherapeutic agents such as ActinomysinD and Vinblastine (Luchsinger et al., 2018).

Recent studies from the lab have revealed a role for active Arf1 in mediating the adhesion-dependent regulation of Golgi organization in mouse embryonic fibroblasts (Singh et al., 2018). Both adhesion-dependent signaling (Pawar et al., 2016) and Arf1 activity (Casalou et al., 2016a) are known to be altered in different cancers. In line with this, Golgi organization was observed to be adhesion-independent in the lung (A549 and CaLu1) and bladder cancer (T24 and UMUC3) cells (Fig 3.3). Therefore, we wanted to test the possible role Arf1 could have in regulating the Golgi organization in these cancer cells. In WTMEFs, active ARF1 levels dropped significantly on the loss of adhesion, while total ARF1 levels remained unaffected. This decrease in active ARF1 led to the dramatic dispersal of Golgi organization in WTMEFs. Restoring the activation of Arf1 (by expressing constitutively active Arf1) in non-adherent WTMEFs restored Golgi organization (Singh et al., 2018). A similar role for constitutively active Arf1 can be envisaged in cancer cells to drive anchorage-independent Golgi regulation.

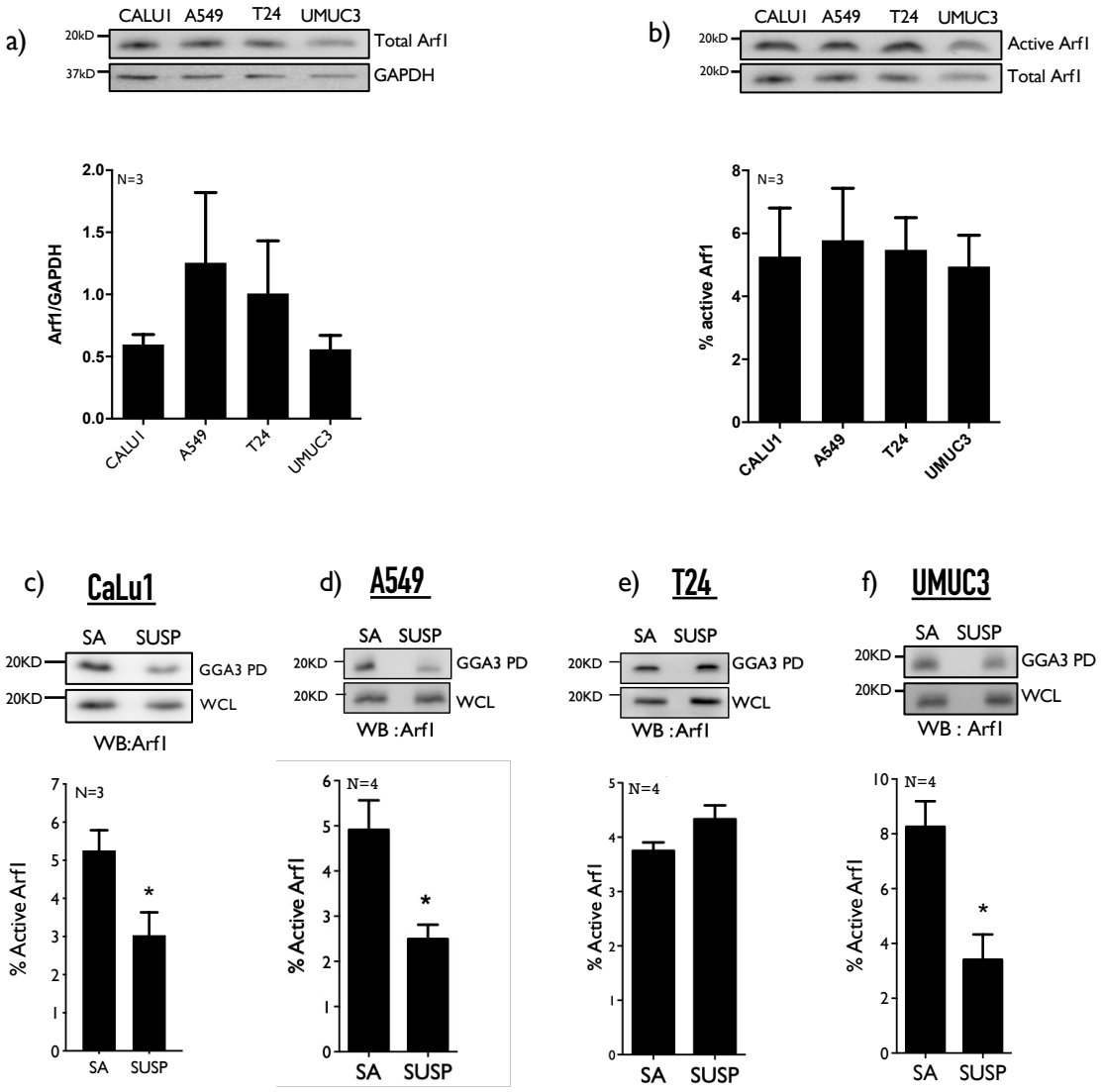


Figure 3.4 : Arf1 activation and its adhesion mediated regulation by cell-matrix adhesion in cancers – a) Total Arf1 expression levels compared between cancer cells when cells are stably adherent. b) Basal levels of active Arf1, (measured using GST-GGA3 pull-down followed by western blotting) compared between cancer cells when cells are stably adherent c) – f) Effect of loss of adhesion on active Arf1 levels tested in different cancer cell lines. Statistical analysis used is unpaired, Mann Whitney t test.

To test for such a role, we compared the basal levels of expression and activation for Arf1 between A549, Calu1, T24 and UMUC3 when stably adherent. The total Arf1 expression is comparable between these cell lines (Fig 3.4a). Active Arf1 levels were measured using a GST-GGA3 pull-down assay and showed Arf1 activation to be similar between these cancer cell lines (Fig 3.4b). However, the regulation of Arf1 activation on loss of adhesion could still be distinctly altered in cancer cells. Hence, we compared active Arf1 levels in stable adherent vs non-adherent cancer cells. CaLu1, A549, and UMUC3 showed a significant drop (50%) in the activation of Arf1 on loss of adhesion (Fig 3.4). Surprisingly,

this loss in active Arf1 levels did not affect the intact Golgi organization in A549 and UMUC3 cells. In CaLu1 cells, the disorganized Golgi, in adherent cells, did not further fragment on loss of adhesion. This suggested the regulation of Golgi in CaLu1, A549, and UMUC3 could be independent of Arf1. If the organization of the Golgi in these cells is sensitive to Arf1, regulation remains to be confirmed. T24 bladder cancer cells, however, retained Arf1 activation on loss of adhesion (Fig 3.4e), which could support their organized Golgi phenotype.

3.3.2 Role of Arf1 in Regulating Golgi organization in non-adherent Bladder Cancer Cells

In bladder cancer T24 and UMUC3 cells, Arf1 activation does not drop in T24 but does in UMUC3 cells on loss of adhesion. Golgi organization, however, stays intact in both on loss of adhesion. It thus raises the question of whether the Golgi in these cells is sensitive to active Arf1-mediated regulation. To test this, we used an inhibitor of Arf1 activation, Brefeldin A (BFA), which targets the Arf1 GEFs (Guanine nucleotide exchange factors), GBF1 and BIG1/2, causing inactivation of Arf1 in cells (Sciaky et al., 1997a; Ward et al., 2001).

Treating suspended T24 and UMUC3 cells with BFA caused a significant drop in active Arf1 levels (Fig 3.5). Fragmentation of the cis-medial Golgi (ManII-GFP), known to be affected by BFA (B.R. et al., 2023), is observed in both cell types, confirming their sensitivity to Arf1 (Fig 3.5). The trans-Golgi is affected more prominently in UMUC3 cells than in T24 cells. They suggest the Golgi in T24 and UMUC3 cells are sensitive to active Arf1 levels.

The lack of drop in active Arf1 levels in T24 cells could hence be responsible for their organized Golgi phenotype on loss of adhesion. In UMUC3 cells, the drop in active Arf1 levels not seen to affect Golgi organization could suggest additional regulatory changes downstream of Arf1 in these cells.

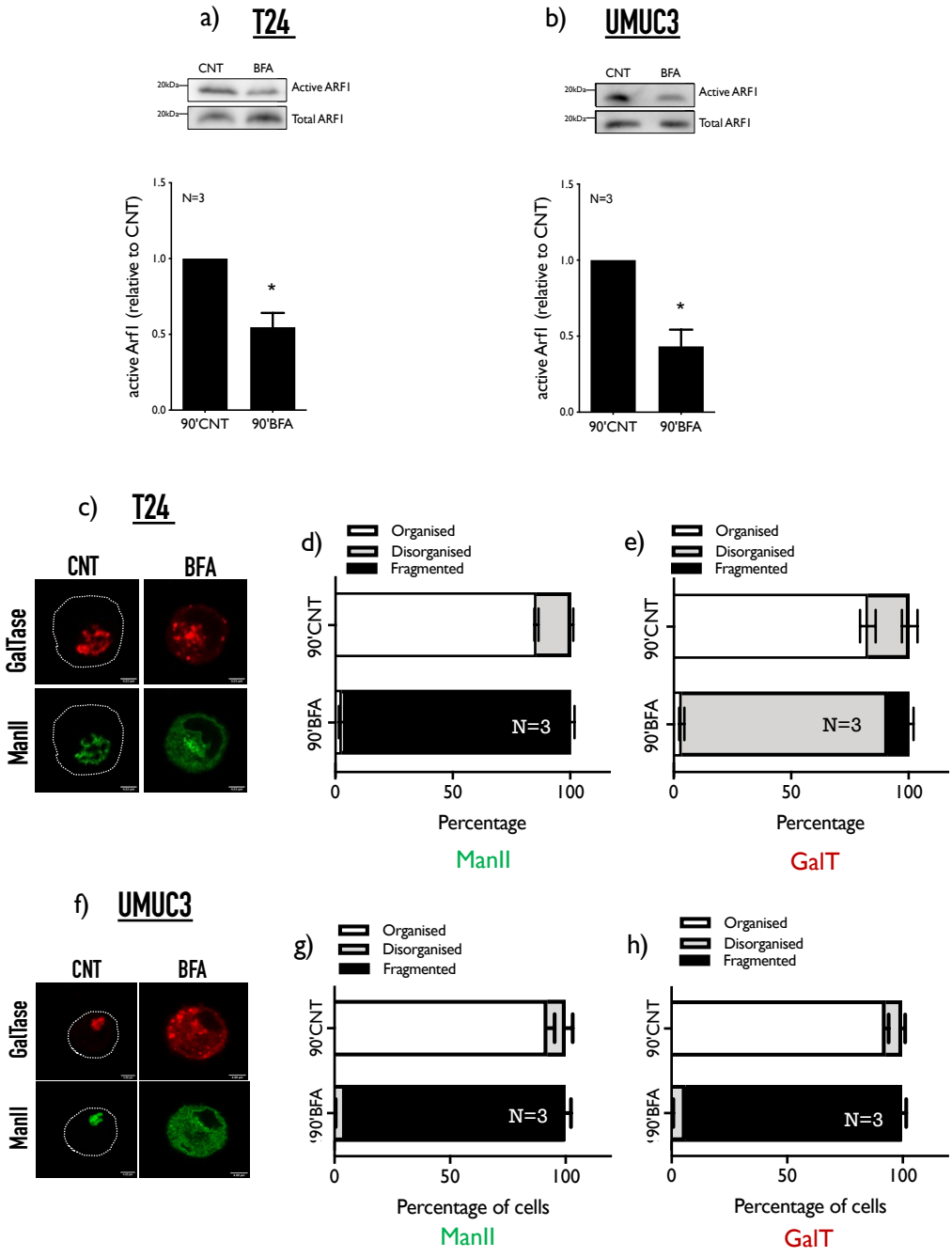


Figure 3.5 : Role of Arf1 in regulation of Golgi organization in bladder cancer cell lines – Inhibition of Arf1 activation shown in non-adherent T24 (a) and UMUC3 cells (b) using inhibitors BFA (10ug/ml), (measured by using GST-GGA3 pull-down assay followed by western blotting). Effect of this inhibition on Golgi organization is shown with cross-sectional images of non-adherent T24 (c) and UMUC3 (f) cells expressing GalTase-RFP and ManII-GFP. Graphs show distribution profile for percentage of cells ($n \geq 200$) with organized, disorganized, and fragmented Golgi phenotypes in presence and absence of BFA in T24 (d-e) and UMUC3 (g-h) cells.

3.3.3 Role of Arf1 in regulating Golgi organization in non-adherent lung cancer cells

In lung cancer CaLu1 and A549 cells, Arf1 activation drops in both cell types on loss of adhesion. Golgi organization, however, does not change on loss of adhesion in both CaLu1 and A549 cells. It thus raises the question of whether the Golgi in these cells is sensitive to active Arf1-mediated regulation. To test this, we used BFA to look at the effect of Arf1 inhibition on the Golgi organization in these cells and the loss of adhesion.

Treating non-adherent CaLu1 and A549 cells with BFA caused a significant drop in active Arf1 levels (Fig). Fragmentation of the cis-medial Golgi (ManII-GFP), known to be affected by BFA (B.R. et al., 2023), is observed in both cell types, confirming their sensitivity to Arf1 (Fig 3.6). The trans-Golgi is affected more prominently in A549 cells than CaLu1 cells. Together, they suggest the Golgi in CaLu1 and A549 cells to be sensitive to active Arf1 levels. However, the drop in active Arf1 levels on their loss of adhesion, not affecting Golgi organization in both cell types, could mean i) active Arf1 levels need to drop further than seen on loss of adhesion to support Golgi dispersal ii) additional regulatory changes downstream of Arf1 might be supported by such a drop in its activity to promote Golgi dispersal.

In summary, these observations have led us to consider which cancer cell lines from our screen might be best suited for an in-depth evaluation for regulation of the Golgi organization. A pairing of two or more cancer cell lines of similar origin with differential Golgi organization will ideally be suited for such a study. As tested above, lung cancer cell lines A549 and CaLu1 could be such a pairing. These two Ras-dependent (K-Ras) cells lines support anchorage-independent signaling and growth. Their Golgi organization is distinct in adherent and non-adherent conditions and sensitive to conventional regulators like Arf1. Very little is known about how Golgi's organization is regulated in these cell types, making them suitable for a comprehensive evaluation. Our studies going forward have hence focused on A549 and CaLu1.

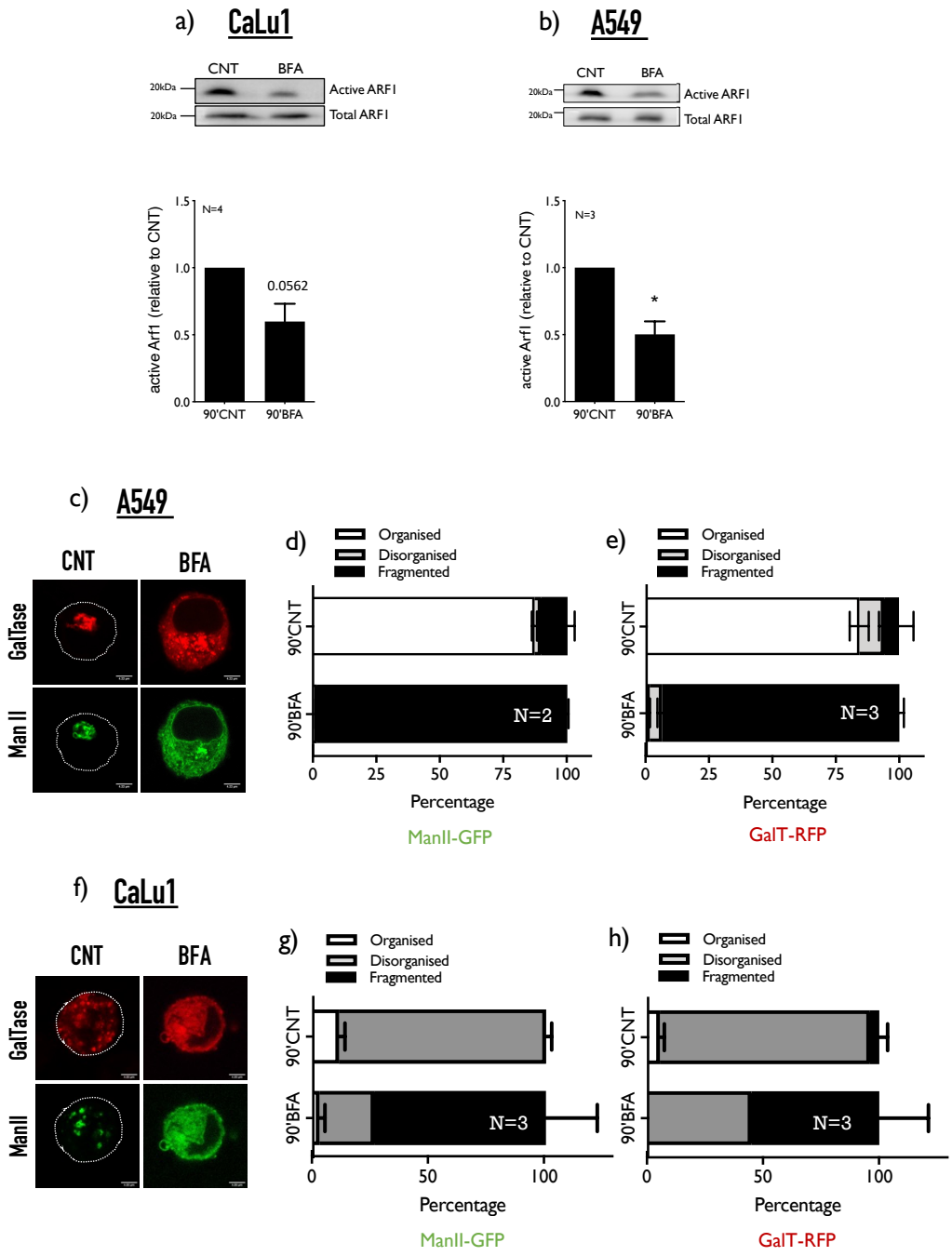


Figure 3.6 : Role of Arf1 in regulation of Golgi organization in lung cancer cell lines – Inhibition of Arf1 activation shown in non-adherent A549 (a) and CaLu1 cells (b) using inhibitors BFA (10ug/ml), (measured by using GST-GGA3 pull-down assay followed by western blotting). Effect of this inhibition on Golgi organization is shown with cross-sectional images of non-adherent A549 (c) and CaLu1 (f) cells expressing GalTase-RFP and ManII-GFP. Graphs show distribution profile for percentage of cells ($n \geq 200$) with organized, disorganized, and fragmented Golgi phenotypes in presence and absence of BFA in A549 (d-e) and CaLu1 (g-h) cells.

3.4 Golgi organization and function in normal lung epithelial vs lung cancer cells

3.4.1 Adhesion-dependent regulation of Golgi organization in normal vs cancer cells.

Having selected lung cancer A549 and CaLu1 cells for our studies, we must evaluate their regulation of Golgi organization in the context of how normal lung epithelial cells behave. In non-transformed 'normal' cells, Golgi organization is regulated by cell-matrix adhesion (Singh et al., 2018). We used a non-transformed, human lung epithelial cell line, BEAS2B. We evaluated its Golgi organization relative to lung cancer cells using cis-medial Golgi marker ManII-GFP and trans-Golgi marker GalTase-RFP.

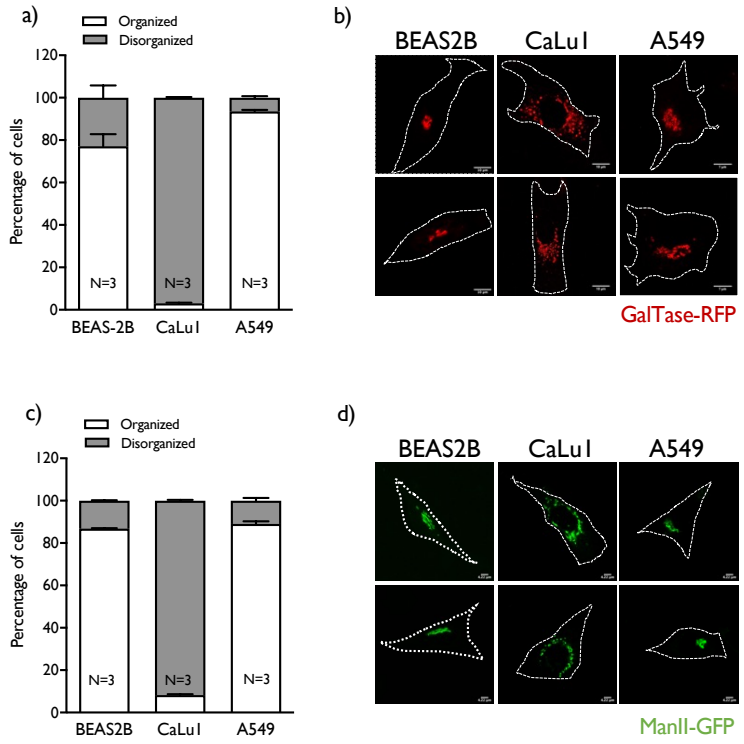


Figure 3.7 : Golgi organization in adherent lung epithelial cells; normal vs cancer – Golgi organization in stable adherent BEAS2B, A549 and CaLu1 cells, transfected with GalTase-RFP (a)-(b) or ManII-GFP (c)-(d). (a) and (c) show distribution profiles for percentage of cells with organized or disorganized Golgi in indicated cell types. Representative cross-sectional images of the predominant Golgi organization phenotype observed in BEAS2B, A549 and CaLu1 cells are shown for GalTase-RFP (b) and ManII-GFP (d) expressing

The Golgi organization in BEAS2B cells was intact for both cis-medial and trans-Golgi markers in adherent cells (Fig 3.7). This was comparable to A549 cells with a similar distribution profile (organized vs disorganized). In both normal and cancer cells, the presence of a small but consistent population of cells with a Golgi phenotype distinctly

different from most cells is worth noting. CaLu1 cells have a distinctly disorganized Golgi phenotype, as reported earlier.

We further extended this study to ask how the loss of adhesion affects the cis-medial (ManII-GFP) and trans- (GalTase-RFP) Golgi organization in BEAS2B cells relative to CaLu1 and A549 cells. The intact Golgi in BEAS2B cells was observed to disorganize on the loss of adhesion for both cis-medial and trans-Golgi markers (Fig 3.9). Unlike BEAS2B cells, the Golgi organization does not change on loss of adhesion in CaLu1 and A549 cells. Additionally, differences in the relative organization of the cis-medial vs trans-Golgi between BEAS2B, CaLu1 and A549 cells are worth noting (Fig 3.8). In non-adherent BEAS2B cells, trans-Golgi dispersed to a much larger extent than the cis-medial Golgi. This is comparable to their differential disorganization in WTMEF cells (B.R. et al., 2023; Singh et al., 2018). However, in non-adherent CaLu1 cells, the cis-medial and trans-Golgi compartments are disorganized comparably with little overlap (Fig 3.8). In non-adherent A549 cells, the Golgi, in staying organized, keeps its cis-medial and trans-Golgi compartments together. Spatial reorganization of Golgi compartments could affect how the Golgi functions in cells.

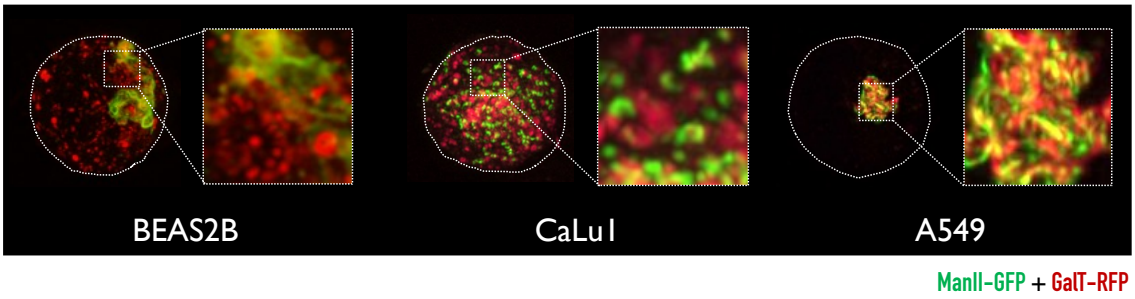


Figure 3.8 : Spatial organization of the Golgi compartments on loss of adhesion in normal vs cancer cells –Deconvoluted Z-stacks with MIP images showing relative organization of cis-medial (ManII-GFP) vs trans-(GalTase-RFP) Golgi compartments compared between non-adherent BEAS2B, A549 and CaLu1 cells. Boxed images depict zoomed in selections from the MIP images.

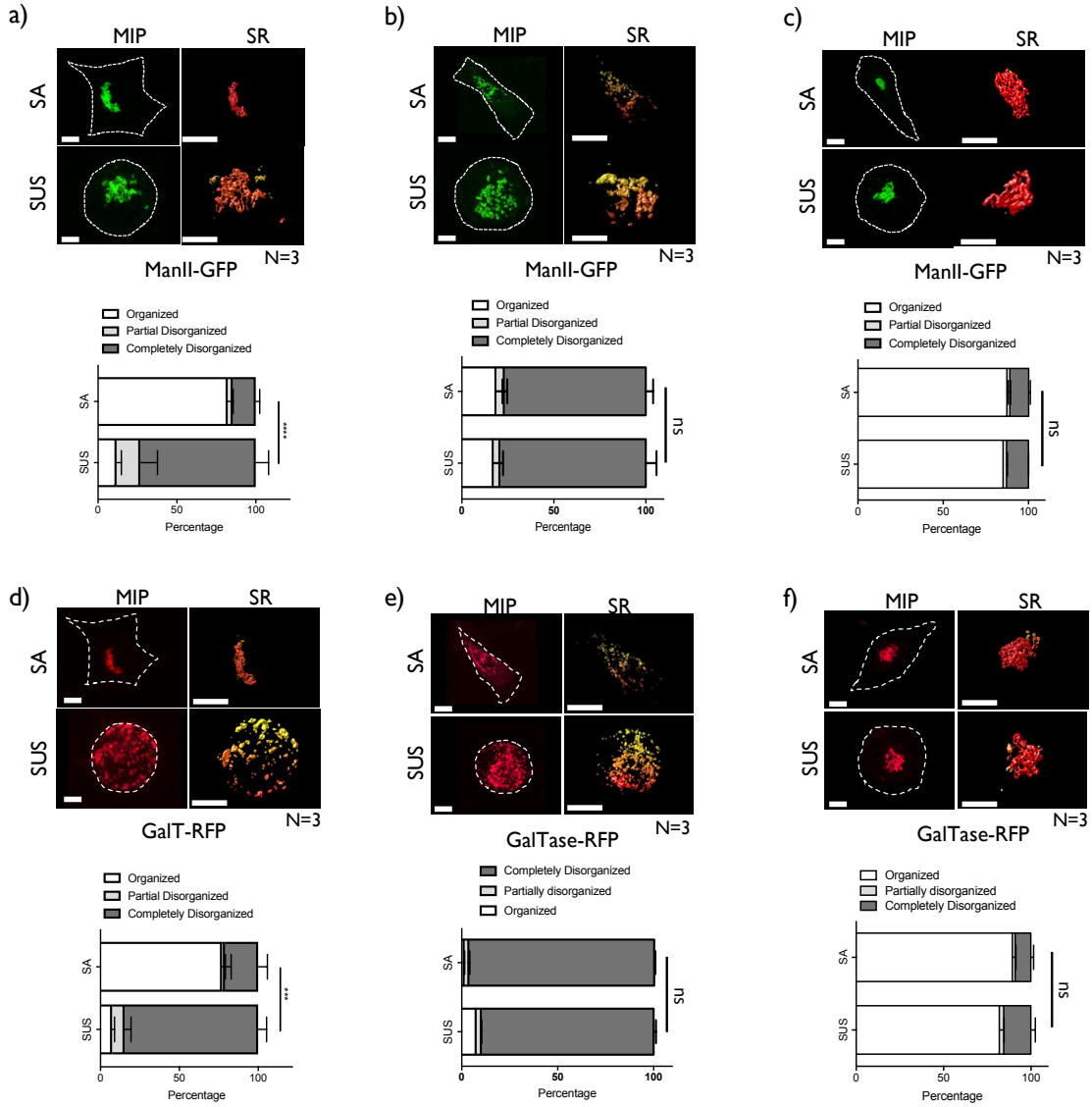


Figure 3.9 : Adhesion-dependent regulation of Golgi organization in normal vs cancer cells – Golgi organization in adherent vs non-adherent cells - BEAS2B (a and d), CaLu1 (b and e) and A549 (c and f) is shown. Cells transfected with GalTase-RFP (a, b, c) or ManII-GFP (d, e, f) were held in suspension for 120min and then fixed for suspension and stable adherent timepoints. Representative images given are deconvoluted Z-Stacks with Maximum intensity projection (MIP) along with a zoomed image of the Golgi with surface rendering. Graphs give distribution profile for percentage of cells showing organized and disorganized Golgi phenotype. Statistical analysis is done using one way ANOVA.

3.4.2 Role of Arf1 in the regulation of Golgi organization in normal vs cancer cells

In 'normal' WTMEFs, loss of adhesion-mediated drop in active Arf1 causes Golgi disorganization (Singh et al., 2018). BEAS2B cells show similar disorganization of the Golgi on loss of adhesion. This is distinctly different from CaLu1 and A549 cells that keep their disorganized and organized Golgi phenotype, respectively, on loss of adhesion. Interestingly, Arf1 activation drops on loss of adhesion in both these cells but does not affect their Golgi organization. Arf1 expression and/or activation in BEAS2B vs CaLu1 vs A549 could contribute to their differential regulation of the Golgi. To test this, we looked at Arf1 expression levels across BEAS2B, CaLu1 and A549 cells when stably adherent and found them to be comparable (Fig 3.10). We also measured the basal activation levels of Arf1 in these cells using GST-GGA3 pull-down assay. These active Arf1 levels were similar between these cell lines (Fig 3.10). This suggests basal Arf1 expression and activation are unlikely mediators of the differential Golgi organization in BEAS2B, CaLu1 and A549 cells. A comparable drop in Arf1 activation is also seen in CaLu1 and A549 cells on loss of adhesion. Thus, the anchorage-independent Golgi phenotype in CaLu1 and A549 cells could be driven by regulators in addition to or independently of Arf1.

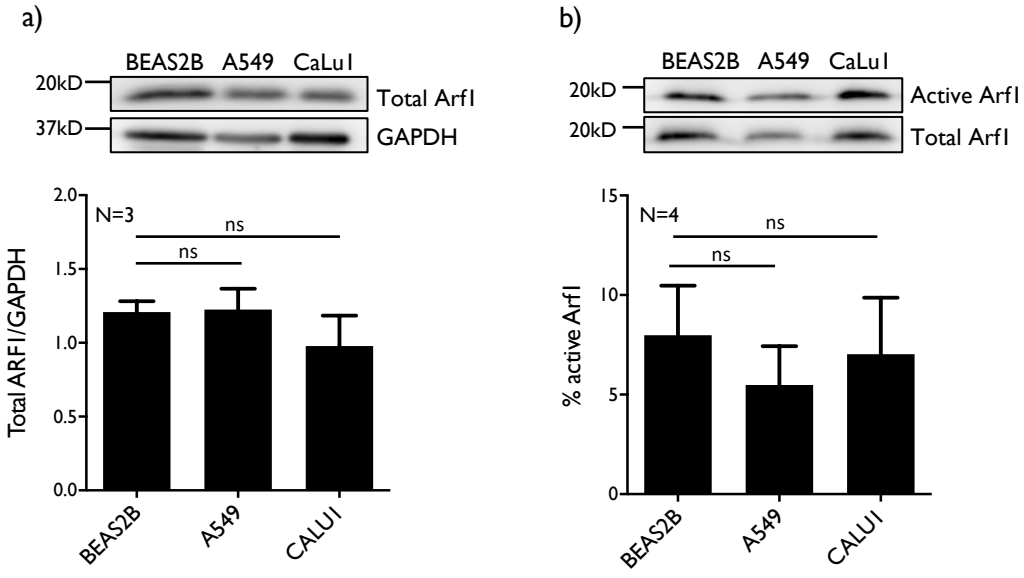


Figure 3.10 : Expression and activation levels of Arf1 compared between in normal vs cancer cells when stably adheren – a) Total Arf1 expression compared between BEAS2B, A549 and CaLu1 cells in stable adherent cells using protein estimated cell lysates, with equal protein (20ug) loading. b) Basal Arf1 activation compared between BEAS2B, A549 and CaLu1 cells (measured using GST-GGA3 pull-down assay followed by western blotting). Statistical analysis done using unpaired Man-Whitney test .

3.4.3 Cell surface glycosylation in normal vs cancer cells on loss of adhesion

The functional significance of the distinct differences in Golgi organization on the loss of adhesion in BEAS2B, CaLu1 and A549 cells is essential to assess. They could, in turn, also help ascertain the role Golgi organization plays in these cells. Their cell surface glycosylation signature is a necessary and rapid Golgi-mediated change in cells (Stanley, 2011). Glycosylation changes are also influenced by the organization status of the Golgi and implicated in cancer progression (X. Zhang & Wang, 2016). Cell surface glycosylation was measured by Flow cytometry analysis using fluorescently tagged lectins (ConA, PNA, and WGA) that have specific binding affinities towards a set of cell surface glycans (Table 3.1). Labelling cells with optimal lectin probes required careful standardization and is vital for comparative evaluation (Fig 3.11). Comparing surface lectin binding in non-adherent cells will allow for the quantitative assessment of changes therein. Normal vs cancer cells held in suspension for increasing times and labelled for lectins could provide an accurate quantitative evaluation of Golgi function in cells. In BEAS2B cells, the cell surface binding of ConA, PNA, and WGA in suspension after 60 and 120 minutes was comparable across time (Fig 3.12). However, A549 (organized Golgi) and CaLu1 (disorganized Golgi) cells showed significantly decreased cell surface binding of lectins ConA and WGA at 60 minutes and 120 minutes, respectively. PNA levels dropped substantially in CaLu1 cells at 60min and 120min. No such change was observed in A549 cells (Fig 3.12). This study shows the lectin profile (and hence Golgi function) for non-adherent lung cancer cells to be distinctly different from BEAS2B cells. A549 and CaLu1, with differences in the spatial organization of Golgi compartments, also show distinct changes in their lectin binding profiles. Glycosylation signatures at the cell surface are reflective of Golgi processing and in part dependent on Golgi organization and the time cargo spends in distinct Golgi compartments (Mani et al. 2016). The distinct difference in Golgi organization on loss of adhesion in these cells (Fig 3.8), could reflect in the lectin profile changes observed over time (Fig 3.12). It is striking to note that in both CaLu1 and A549 the lectin profile for ConA and WGA show a comparable change despite distinctly different Golgi organizations. Change in surface PNA levels with time does differ distinctly in these cells. Together they do suggest that different cancer cell types could interpret changes in Golgi organization very uniquely. The impact organized vs disorganized Golgi have on cell function (in this case cell surface glycosylation) could indeed be subtle and cancer specific.

Table 3.1 : List of lectins used in the study and their respective glycan binding specificities

LECTIN	BINDING SPECIFICITY
ConA	Mannose
HPA/PNA	Both bind Galactose and N-Acetyl Galactosamine (N-GalNAc)
WGA	Sialic acid and N-Acetyl Glucosamine (N-GlcNac)

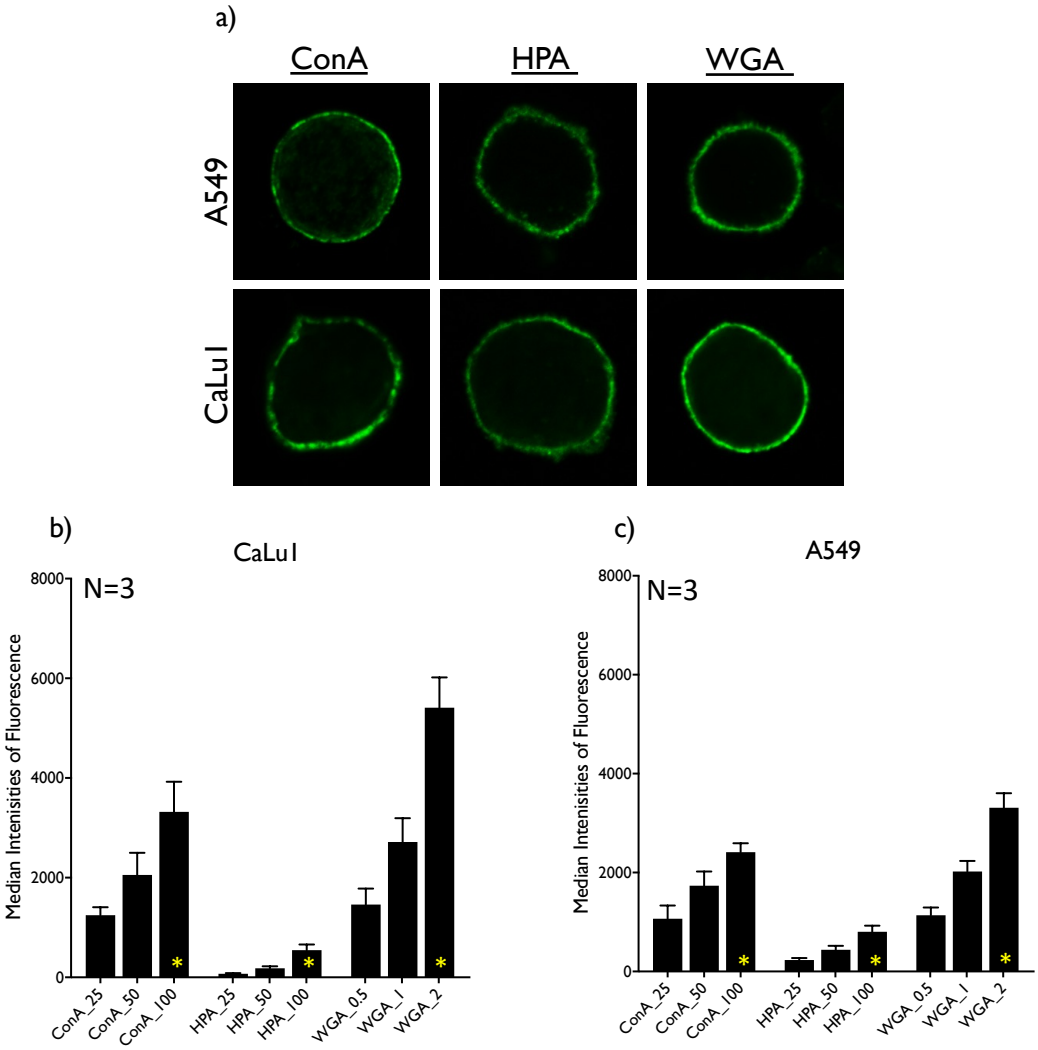


Figure 3.11 : Optimization of lectin concentration for labelling in non-adherent lung cancer cells – Cells detached with Accutase and labelled with fluorescently conjugated (Alexa488) ConA, HPA and WGA lectins (15min in ice) were fixed and imaged were analyzed by Flow cytometry and also imaged at confocal . a) Cross-sectional images shot at the equatorial plane of cell (highest perimeter), show cell surface localization of lectin signal in CaLu1 and A549 cells indicated surface labelling of glycans. Standardizing of lectin concentrations in CaLu1 (b) and A549 (c) cells was done with a range of different concentrations of lectins (indicated on the x-axis; concentrations in ng/µl) by Flow cytometry. Optimal lectin concentration selected for experiments is marked with the asterix symbol. Median intensities of fluorescence for the lectins ConA, WGA and HPA are plotted against their corresponding suspension timepoints. Statistical analysis was done using one sample t test normalized to respective 5min suspension timepoints.

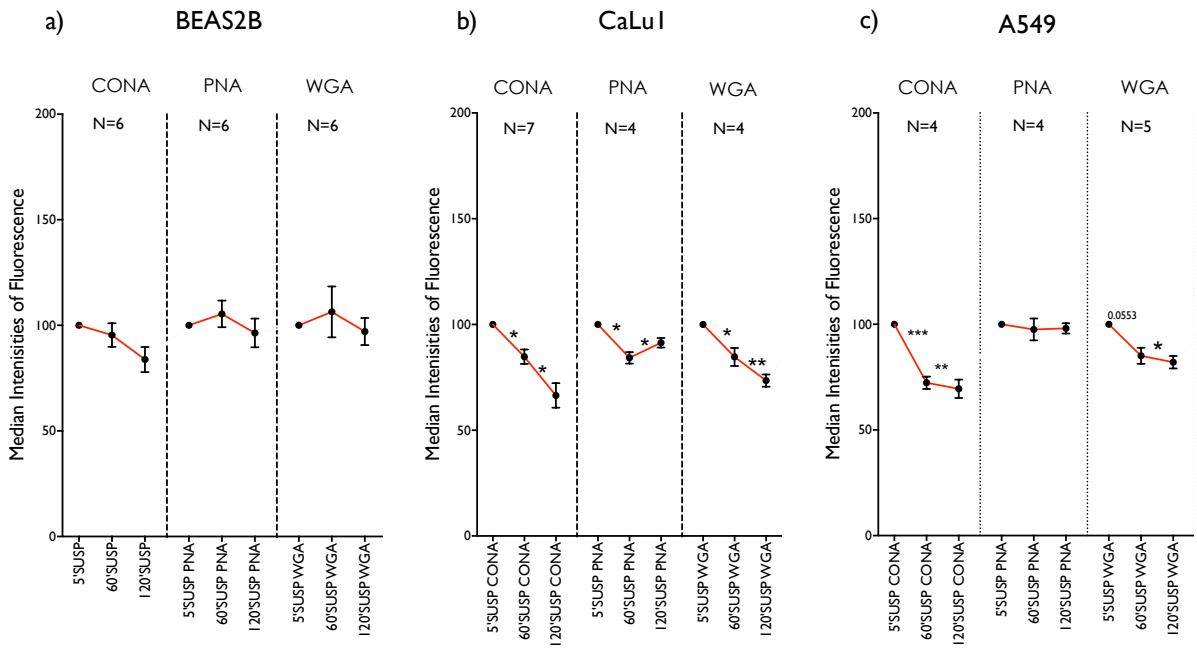
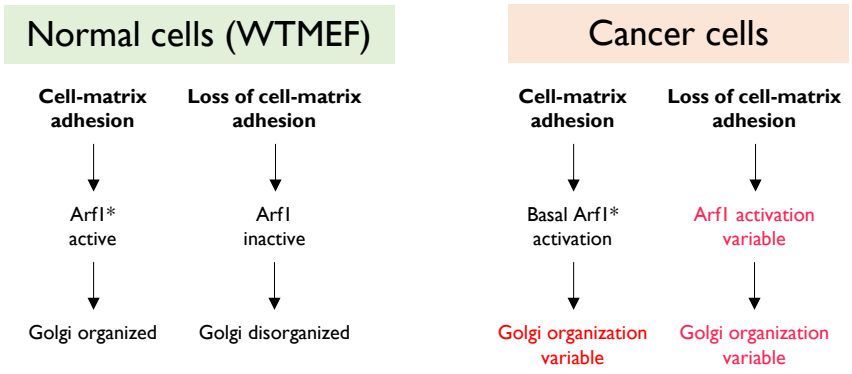


Figure 3.12 : Differential effects of loss of adhesion on cell surface glycosylation in normal vs cancer cells – Median intensities of fluorescence obtained for cell surface binding of indicated lectins using Flow Cytometry, are plotted in above graphs. Data shows cell surface binding levels of glycans (proportional to their respective lectin binding intensities) for non-adherent BEAS2B (a), CaLu1 (b) and A549 (c) at 60min and 120min timepoints each normalized to their respective 5min (basal) timepoint. Statistical analysis was done using one sample Wilcoxon t test normalized to respective 5min (basal) timepoints.

In Summary

Cellular functions	CaLu1	A549	MDAMB231	MCF7	T24	UMUC3
Golgi organization in adherent cells	Disorganized	Organized	Organized	Disorganized	Organized	Organized
Golgi organization in non-adherent cells	Disorganized	Organized	Disorganized	Disorganized	Organized	Organized
Arf1 activation in non-adherent cells	Drops significantly	Drops significantly	Drops significantly	Drops significantly	Does not drop	Drops significantly
Effect of BFA on Golgi in non-adherent cells	Golgi dispersal	Golgi dispersal	Not tested	Not tested	Golgi dispersal	Golgi dispersal



Chapter 4

Identifying regulator(s) of Golgi organization in lung cancer cells

4.1 RATIONALE

Changes in the expression or activation of Golgi regulatory proteins could mediate differential Golgi organization in cancers. Differential expression of genes is integral to the changes that drive cancer cell transformation (Garnis et al., 2004). This could also cause changes in Golgi organization phenotypes, further affecting cancer cell function. Alterations in Golgi's organization and its regulation by adhesion were detected and described in Chapter 3. This led us to evaluate further the differential regulation of the Golgi lung cancer cell lines, CaLu1 and A549. To do this, we designed an in-silico study to identify differentially expressed genes between CaLu1 and A549. This aimed to identify potential regulators of the altered Golgi organization in these cells. The study employed several different online tools, including Cancer Cell Line Encyclopedia (CCLE) (Barretina et al., 2012), UCSC Xena browser (Goldman et al., 2020), NCBI Gene Ontology (Harris et al., 2004) and STRING database (Szklarczyk et al., 2015). We began with a literature survey and the NCBI Gene Ontology search tool to obtain a comprehensive list of Golgi-linked genes. The differential expression of these genes (DEGs) was evaluated using the CCLE database, and genes were further shortlisted by their association/regulation of the Golgi. The final list of candidate genes was ranked and evaluated as novel regulators of Golgi organization in cancers.

4.2 *In silico* analysis for differentially expressed genes (DEGs). A549 vs CaLu1

The Golgi organization in stable adherent CaLu1 and A549 cells is distinctly different. Differences in gene expression of Golgi structure and regulatory proteins can drive this. Our *in-silico* analysis uses a bottom-up approach, screening for differentially expressed Golgi organization-associated genes using the NCBI Gene Ontology database (Fig 4.1). This was combined with known regulators of Golgi structure and function obtained from literature referencing. A combined listing of 390 genes was arrived at, and their differential mRNA expression in the CCLE database was compared between CaLu1 and A549 cells. This revealed 35 differentially expressed genes (DEGs) showing a fold change difference of 5 or more between CaLu1 and A549. A scoring method was now applied to these 35 DEGs (Fig4.2).

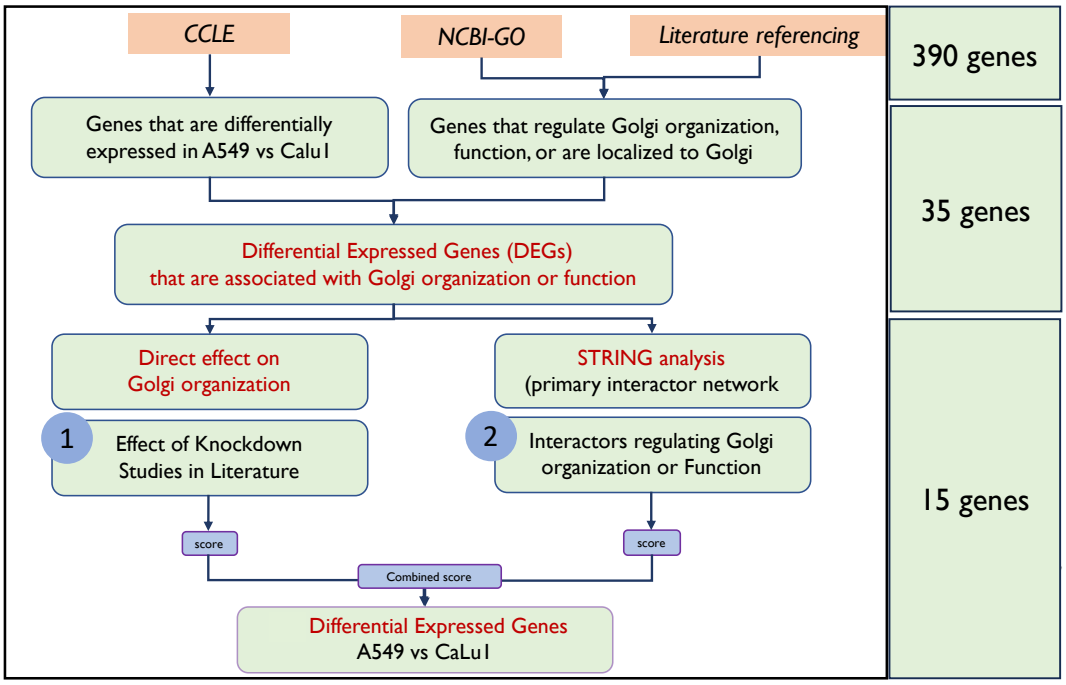


Figure 4.1 – Schematic representation showing the sequence of steps followed in the *in silico* study. Golgi associated genes (390) obtained from NCBI GO and literature referencing were screened for their differential gene expression (fold change ≥ 5) between A549 and CaLu1 using mRNA expression data from CCLE database. 35 DEGs were identified were further shortlisted using a score based on their known effect on Golgi organization and a second score based on number of primary interactors known to be associated with the Golgi. Primary interaction network was derived using STRING analysis. Top 15 DEGs having a combined score of 3 or more were considered to be potential candidate regulators of the Golgi in these cells, and were hence selected for further evaluation.

The first score given was based on the effect knockdown of a gene may have on Golgi organization in cells from literature. The second score was based on the number of primary interactors involved in regulating Golgi structure or function, identified using STRING database protein interaction networks (see Fig 4.2). The sum of these two scores gave a combined score for each gene. Setting a cut-off of 3 or more for this combined score, we ranked the top 15 genes as candidate regulators of differential Golgi organization between CaLu1 and A549 cells (Table 4.1).

`

DEGs	CaLu1> A549	Effect of gene KD on Golgi organization		Golgi Associated interactors	Combined Score
Candidate A - AXL	**5.943321**	**Condensed**	**2**	**4**	**6**
Candidate B	**583.4711**	**Condensed**	**2**	**3**	**5**
Candidate C	**142.9857**	**Condensed**	**2**	**1**	**3**
Candidate D	50.63102	Disorganized	1	9	10
Candidate E	19.6512	Disorganized	1	5	6
Candidate F	9.375613	Disorganized	1	3	4
Candidate G	6.017117	Disorganized	1	3	4
Candidate H	77.38672	Disorganized	1	2	3
Candidate I	15.59441	Unknown	0	5	5
Candidate J	8.744106	Unknown	0	3	3
Candidate K	7.605945	Unknown	0	3	3
DEGs	**A549 > CaLu1**	**Effect of gene KD on Golgi organization**		**Golgi Associated interactors**	**Combined Score**
Candidate L	**13.4463075**	**Disorganized**	**2**	**8**	**10**
Candidate M	**9.94107248**	**Disorganized**	**2**	**3**	**5**
Candidate N	9.76517947	Condensed	1	9	10
Candidate O	37.2033589	Unknown	0	5	5

Table 4.1 – Final shortlist of top 15 DEGs ranked according to i) Score for effect of gene knockdown on Golgi organization ii) Combined score. 11 candidate genes are overexpressed in CaLu relative to A549 and 4 candidate genes are overexpressed in A549 relative to CaLu1

	Candidate A - AXL		Candidate L	
STEP1 mRNA expression from CCLE	Cell Line	mRNA expression from CCLE (RPKM)	Cell Line	mRNA expression from CCLE (RPKM)
	CaLu1	300	CaLu1	0.2803
	A549	50.5	A549	3.77
	Fold Change >5 CaLu1/A549	**5.941**	Fold Change >5 A549/CaLu1	**13.446**

STEP2

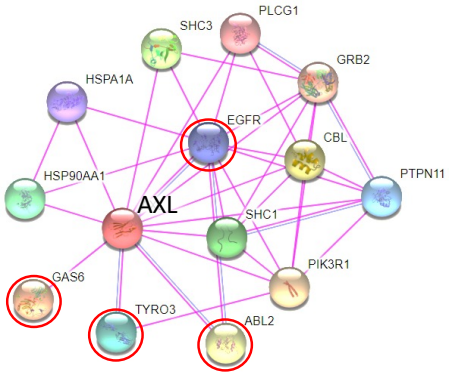
Scoring based on effect on Golgi

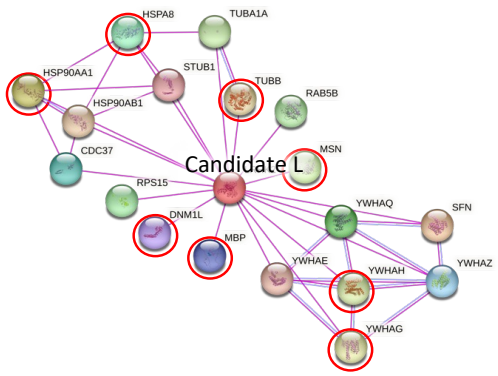
Effect of gene KD on Golgi organization known	Gene expression and Golgi organization agrees with our data in A549 and CaLu1	Score given to the Gene
YES	YES	2
YES	NO	1
NO	NA	0

Effect of AXL KD on Golgi	Gene expression and Golgi organization AGREES with data in A549 and CaLu1	**Score given to AXL**	Effect of Gene-L KD on Golgi	Gene expression and Golgi organization AGREES with data in A549 and CaLu1	**Score given to AXL**
Golgi is intact	AXL is CaLu1>A549 Golgi in A549 is intact	**2**	Golgi is intact	Gene-L is A549>CaLu1 Golgi in Calu1 is disorganized	**2**

STEP3

Scoring based on STRING analysis





4 of the primary interactors are known to affect Golgi organization and function
Score given - 4

8 of the primary interactors are known to affect Golgi organization and function
Score given - 8

STEP4

Combine score from STEP 2 and STEP 3

Scoring based on effect of gene KD on Golgi – 2

Primary interactor score – 4

Combine score – 6

Scoring based on effect of gene KD on Golgi – 2

Primary interactor score – 8

Combine score – 10

Figure 4.2 : In-silico analysis explained in detail for the two top candidate genes, candidate A (CaLu1>A549) and candidate L (A549>CaLu1).

4.3 AXL as the potential regulator of Golgi organization in lung cancer cells

The final shortlist of 15 candidate genes obtained had 11 genes overexpressed in CaLu1 relative to A549, while four genes were overexpressed in A549 relative to CaLu1. Both could be potential mediators of their different Golgi organizations. To begin with, we have focused on the 11 genes overexpressed in CaLu1. They were evaluated for their differential mRNA expression using RT-PCR. For RT-PCR experiments, the quality of isolated RNA was tested before using the same for cDNA synthesis (Fig 4.3a). The primers used for RT-PCR were tested for their gene specificity and efficiency (Fig 4.3b-d). These were then used to compare mRNA expression between CaLu1 and A549. We also looked at the expression of these genes in 'normal' BEAS2B cells by RT-PCR to understand possible changes observed between normal and cancer cells.

The mRNA expression for 6 of the 11 genes was significantly downregulated in A549 relative to BEAS2B (Fig 4.4). Since both these cells have intact Golgi when stably adherent but differ in their Golgi organization on loss of adhesion, this differential expression of genes could support anchorage-independent regulation of the Golgi in A549 cells. On the other hand, comparable mRNA expression profile for 9 out of 11 genes in BEAS2B (intact Golgi) and CaLu1 (disorganized Golgi) cells (Fig 4.4) suggests the likely presence of additional genes that could drive these differential phenotypes. The screen might be limited in identifying all genes mediating variable Golgi organization in cancer cells.

We narrowed down AXL as our top candidate for further study from the genes with confirmed significantly different mRNA expression between A549 and CaLu1 cells. (Fig 4.4). The knockdown of AXL, shown to promote an condensed Golgi phenotype (Chia et al., 2012a), which agreed with its downregulation in A549 cells (having an intact Golgi) reinforced the choice of AXL. Going ahead, our studies have hence evaluated AXL as a potential candidate regulator of Golgi organization in lung cancer.

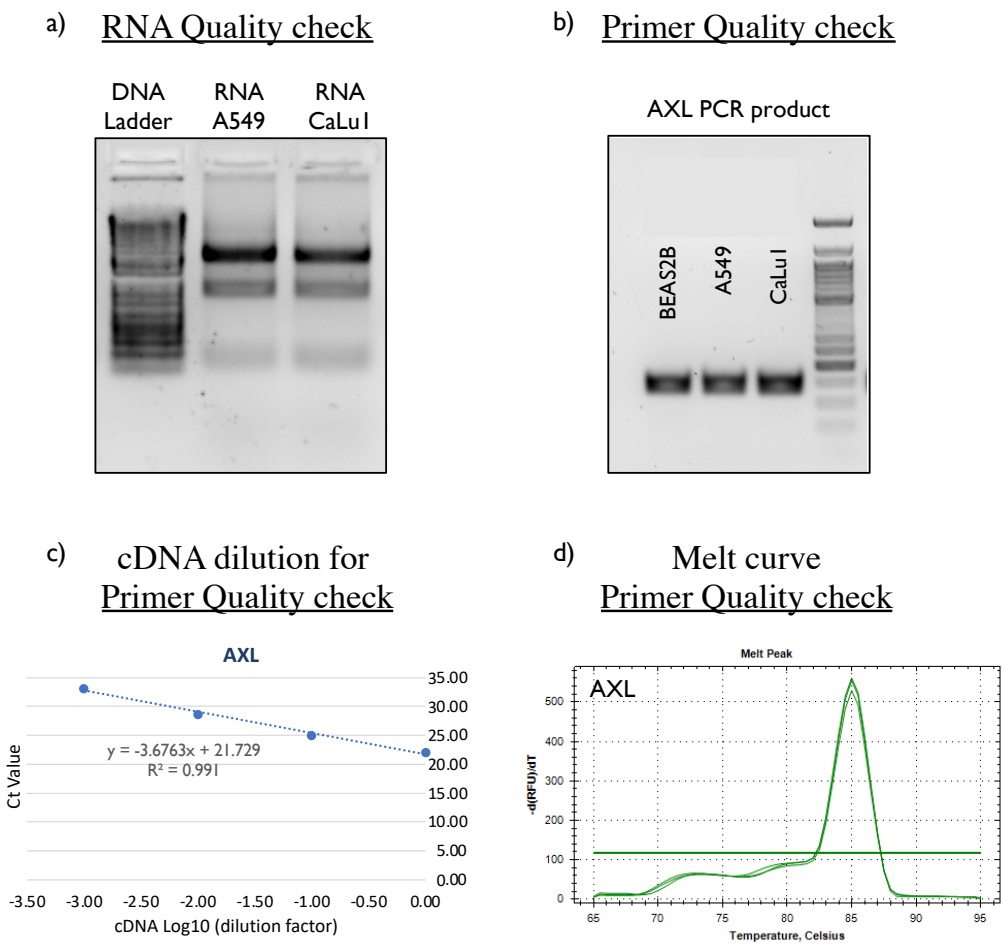


Figure 4.3 : Testing RNA quality and primer efficiency – a) 2ug of RNA run on 0.8% agarose gels shown for A549 and CaLu1 samples, next to 1kbp DNA ladder. b) RTPCR product run for AXL amplicon from BEAS2B, A549 and CaLu1 samples, next to 100 bp DNA ladder c) Slope for AXL primer efficiency obtained from RTPCR performed with cDNA dilutions for CaLu1 sample. Slope of around 2.8 to 3.5 was considered to be appropriate for given primer. d) Melt curve peak for RTPCR run using AXL primer in cDNA sample from CaLu1 cells.

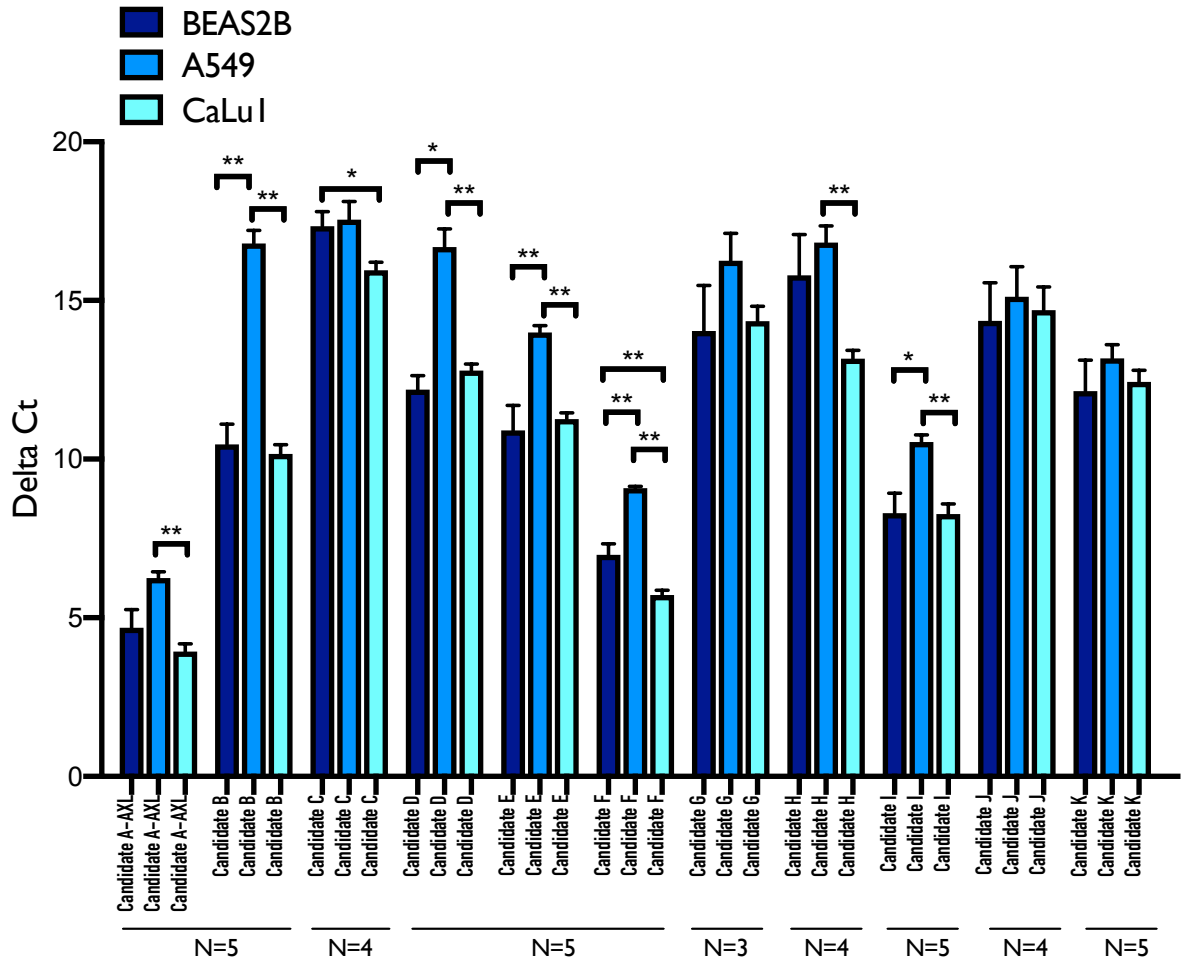


Figure 4.4 : Comparative mRNA expression analysis in normal vs lung cancer cells - Delta Ct values obtained from RTPCR analysis are plotted for each gene, for the cells lines in order - (L-R) BEAS2B A549 and CaLu1. Statistical analysis was done using Unpaired Mann-Whitney t-test.

Chapter 5

Role of AXL in the regulation of Golgi organization and function in lung cancer cells

Introduction to AXL

5.1 Role of AXL in Golgi and Cancer

5.1.1 AXL as a receptor tyrosine kinase

AXL was discovered as an oncogene in a screen for genes involved in the progression of chronic myeloid leukaemia (CML) to blast. The name AXL is derived from the Greek word '*anexelekto',* which means uncontrolled, alluding to its role in cancer progression. AXL, also known as UFO, ARK, Tyro7, or JTK11, is a member of the TAM family of receptor tyrosine kinases (RTKs) (Wium, Ajayi-smith, et al., 2021a). Structurally, the AXL receptor comprises an extracellular ligand binding domain, a transmembrane region, and an

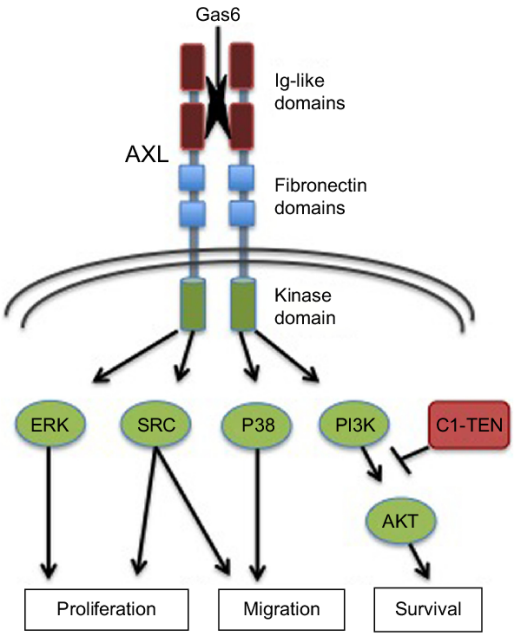


Figure 5.1 : AXL receptor regulation of cell signaling – AXL is a transmembrane receptor tyrosine kinase. Schematic shows a AXL heterodimer bound to it's ligand Gas6, leads to activation of it's intracellular kinase domains. AXL receptor mediated cell signaling regulates major cell signaling pathways including ERK, SRC, P38, PI3K and AKT. *Image Source – Okimoto et al., Lung Cancer Targets and therapy, 2015*

intracellular domain. Like other TAM family members, the extracellular domain has two immunoglobulin (Ig)-like domains and two fibronectin III domains involved in ligand binding. The intracellular domain is the tyrosine kinase domain and has important auto-phosphorylation sites (Fig 5.1). The tyrosine kinase domain reflects the oncogenic capacity and can be activated with or without extracellular stimulation (Auyez et al., 2021a).

AXL receptor binds to different ligands, some of which are shared amongst the TAM family. Gas6, protein S, Tubby, Tubby-like protein (TULP-1), and Galectin-3 are examples of some ligands that bind one or more of the TAM family receptors. Gas6 can attach to all three members of the TAM family, whereas protein S binds only to MER and TYRO3. The affinity of Gas6 is 3–10 times higher for AXL than that for the other two family members (Scaltriti et al., 2016; Zhu et al., 2019).

Upon binding of the ligand, the AXL receptor undergoes homo-dimerization and subsequent trans-autophosphorylation within the intracellular kinase domain, thus recruiting adaptor molecules and effector proteins containing Src homology 2 (SH2) or other phosphotyrosine-binding domains (PTBs) and activating downstream signaling pathways. Six phosphorylation sites have been found in AXL, Tyr698, Tyr702, Tyr703, Tyr779, Tyr821 and Tyr866. Three N-terminal tyrosine residues, 779, 821 and 866, are related to auto-phosphorylation and AXL activation. At the same time, the other three C-terminal sites are relatively conserved among the TAM receptors and indispensable for the complete functions of the kinase (Majumder et al., 2022; Zhu et al., 2019).

AXL receptors can function as monomers, binding to monomeric receptors on other cells to aid cell-cell adhesion and signaling. It can carry out intracellular signaling as a homodimer, or cluster of receptors, and form heterodimers with other protein receptors such as EGFR (Scaltriti et al., 2016).

AXL is found to be expressed in specific embryonic tissues and is suggested to be involved in mesenchymal and neuronal development. In adult tissue, its expression is limited chiefly to smooth muscle cells and tissue cells primed to respond to injuries, such as alveolar macrophages, Langerhans cells of the skin and splenic dendritic cells (Gjerdrum et al., 2010; Wium, Ajayi-smith, et al., 2021b).

AXL plays a significant role in multiple cellular processes, including cell growth, proliferation, cell survival, cell adhesion, and apoptosis. Given this, the involvement of AXL in cancer progression is not unexpected. It has been associated with different high-grade cancers and correlated with poor prognosis. Furthermore, overexpression of AXL has been observed in highly invasive cancers, unlike less invasive cancers, indicating an

association with migration and invasiveness of cancer cells (Auyez et al., 2021b; Zajac et al., n.d.).

5.1.2 AXL as an oncogene; role in tumor progression

Overexpression of Gas6/AXL has been shown in several human malignancies, including breast cancer (Holland et al., 2010; Zajac et al., 2020), acute myeloid leukaemia (C. C. Hong et al., 2008), non-small cell lung cancer (NSCLC) (Iida et al., 2017; Z. Zhang et al., 2012), ovarian cancer (Kanlikilicer et al., 2017), glioblastoma (Onken et al., 2016), neuroblastoma (Debruyne et al., 2016), etc. This altered expression is associated with disease progression and shortened overall survival (OS)

AXL was shown to be a driving force in the spread of tumors in both *in vivo* and *in vitro* studies (Ye et al., 2010; Zhu et al., 2019) (Ye et al., 2010). AXL activity is vital for cell migration phenotypes, including increased GTP-binding proteins Rho and Rac (Koorstra et al., n.d.) and filopodia formation (Lay et al., 2007). The overexpression of AXL in cells with low metastatic colonization potential leads to increased migratory and invasive abilities. Also, AXL expression has been shown to mediate Yes-associated protein (YAP)-dependent oncogenic functions that potentiate migration and invasion in hepatocellular carcinoma (HCC) (M. Z. Xu et al., 2011).

Besides, AXL expression is also known to drive EMT and enable cells to maintain a mesenchymal phenotype. In human breast cancer epithelial cells, exogenous expression of SLUG and SNAIL into MCF10A cells led to an increase in AXL expression, which induced the loss of epithelial morphology and the gain of mesenchymal markers (Gjerdrum et al., 2010).

AXL is also shown to have a role in angiogenesis, however there are contradictory arguments regarding whether AXL activation promotes or suppresses angiogenesis (Zhu et al., 2019). The autocrine and paracrine signaling derived from AXL is suggested to enhance endothelial cells' activity and proliferation and regulate integrin signaling, cell migration and cell survival through AKT signaling (Graham et al., 2014). Knockdown of the *AXL* or *Gas6* gene will impair endothelial tube formation and functional circulation (Linger et al., 2010; Pinato et al., 2015). However, Gallicchio et al. implied an anti-

angiogenic role for AXL by showing that the Gas6/AXL axis might antagonize VEGFR2-dependent angiogenesis (Gallicchio et al., 2005)

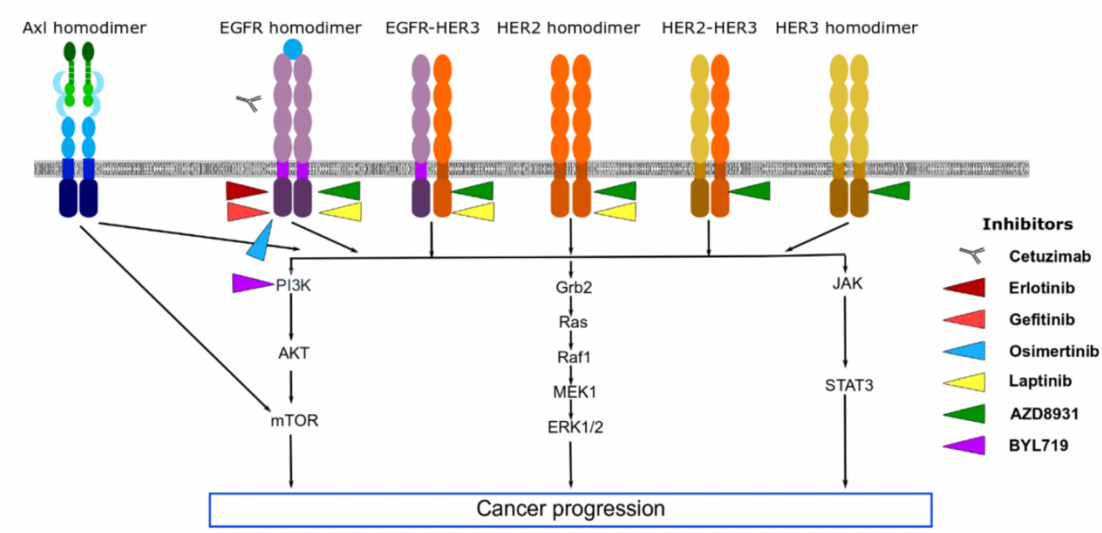


Figure 5.2 : AXL expression promotes resistance in cancers against EGFR inhibitors – Cancer cells treated with HER2 (EGFR) and HER3 inhibitors often show acquired resistance, mediated by overexpression of AXL. AXL signaling sustains the downstream pathways of the inhibited HER2 nad HER3 receptors, thereby promoting drug resistance. *Image Source – Wium et al., Cancers, 2021.*

The role of AXL in promoting **drug resistance in cancers** has also been extensively researched. AXL expression associated with drug resistance has been reported in prostate, breast, ovarian, colorectal and lung cancers (Auyez et al., 2021a). In most instances, therapy against targets independent of AXL led to increased AXL expression, which sustained cell signaling and cell functions even when major cell signaling pathways such as ERK, BRAF, EGFR, and VEGFR stay inhibited (Fig 5.2). The suppression of AXL, knockdown or inhibition, was however, shown to effectively overcome such chemoresistance to specific drugs in an otherwise resistant cell line (C. C. Hong et al., 2008; Taniguchi et al., 2019; Wang et al., 2019; Z. Zhang et al., 2012).

These underlying mechanisms might involve crosstalk between AXL and other RTK family members. Since AXL can form heterodimers with other RTKs, such as EGFR, MET and PDGF, the effects of specific RTK inhibitors could be rendered ineffective (Zhu et al., 2019). Further, AXL was found to sustain the signaling downstream to EGFR or AXL-EGFR heterodimer. For instance, the hetero-interaction between AXL and HER2 receptor maintained the downstream signaling by PI3K/AKT and ERK, thereby promoting

Lapatinib resistance in HER2-positive breast cancer. AXL is, therefore, a significant driver in cancer, promoting invasiveness, cell survival, drug resistance, etc and is a promising target in cancer therapy.

5.1.3 Role in Golgi organization and function of normal and cancer cells

Though it regulates multiple cellular functions that are also common to the Golgi complex's functioning, few studies investigate crosstalk between AXL and the Golgi membrane dynamics. A comprehensive survey by Chia et al. looked at the entire repertoire of kinases and phosphatases in Hela cells for identifying novel regulators of the Golgi structure. By performing knockdowns for all the genes in the screen, the authors arrived at over 188 genes, for which knockdown of the gene directly affected the Golgi organization. Alterations to the Golgi structure were categorized into three types – condensed Golgi (compact Golgi), disorganized Golgi (Golgi stacks or vesiculated clusters dispersed in the cytoplasm) and fragmented Golgi (fall back of Golgi components to ER; Golgi haze)
Further, the function of the Golgi regulating genes was studied in mediating cell surface

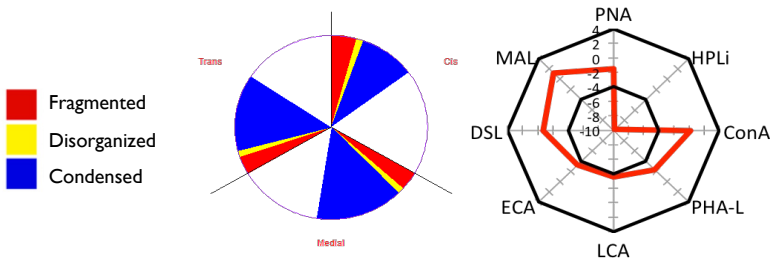


Figure 5.3 : Knock-down of AXL regulation Golgi architecture and cell surface glycosylation : Knock-down of AXL in Hela cells led to condensed Golgi architecture as indicated in pie graph, as detected with Golgi markers – HPL (cis-Golgi), ManII (medial-Golgi), and TGN46 (trans-Golgi). AXL knock-down also led to decrease in glycan binding to HPLi lectin as shown in radial graph. Image Source – Adapted from Chia et al., Molecular Systems Biology, 2012

glycosylation and rates of cellular trafficking. AXL was one of the genes identified to regulate Golgi structure and function, at which point, its knockdown in Hela cells caused the Golgi to have a condensed morphology. This change in the Golgi organization was also reflected in the cell surface glycosylation levels for galactose glycans, as binding of HPA lectin on the cell surface decreased significantly in AXL knockdown cells (Fig5.3) (Chia et al., 2012a). A second study in breast cancer cells suggests that expression and activation

of AXL plays a role in Golgi alteration-mediated cancer progression. The study first showed that expression of AXL is higher in mesenchymal triple-negative breast cancer (Hs578t, MDAMB231) cells than in luminal breast epithelial cancer cells (MCF7) (Zajac et al., 2020). AXL also displayed a polarized localization at the Golgi apparatus in mesenchymal TNBCs Hs578t (Fig 5.4). The inhibition of AXL with the inhibitor R428 affected the Golgi localization of AXL, as seen with cis-Golgi marker GM130. In

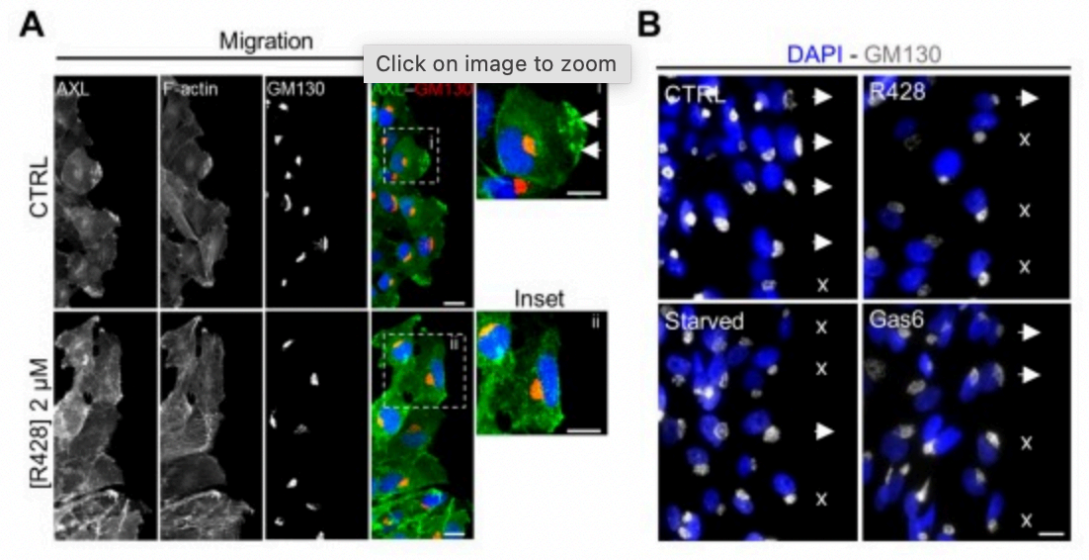


Figure 5.4 : AXL regulates polarize position of Golgi apparatus duing directed cell migration – Representative images of breast cancer cells - Hs578t treated with DMSO (CNT) or 2µM R428 (AXL inhibitor). A) Cells stained with F-actin, AXL (green) and GM130 (red). B) Cells treated with DMSO or R428 (upper panel), or starved and then stimulated with Gas6, showing DAPI (blue) and GM130 staining. *Image Source – Zajac et al., Cells 2020.*

migratory cells, inhibition with R428 displaced AXL and F-actin from the leading edge of the cell to the lateral area between the front and rear of the cells, as well as affected the direction of cell migration (Fig 5.4)(Zajac et al., 2020). Besides, as discussed earlier, both AXL and altered Golgi morphology are somewhat implicated in promoting resistance to RTK inhibitors and chemotherapeutic agents in several aggressive cancers, including NSCLC and Breast cancer (Luchsinger et al., 2018; Ohashi et al., 2018; Ramkumar et al., 2021; Z. Zhang et al., 2012). Investigating the role of AXL in mediating cancer progression through its regulatory function at the Golgi complex is a potential field of research. Insights into the AXL-Golgi crosstalk could help resolve the challenges in existing therapeutic strategies and help design better treatment regimes in the context of cancer management.

5.2 RATIONALE

The receptor tyrosine kinase – AXL, mainly localizes to the cell membrane. On binding its ligand Gas6, AXL forms homodimers or heterodimers with other kinase receptors. These dimerization events promote phosphorylation of the AXL intracellular domains, activating multiple downstream signaling cascades and further regulating cell proliferation, migration, and survival (Zhu et al., 2019).

AXL, a known oncogene, has been extensively studied and established as a driver of cancer progression. It is often upregulated in aggressive cancer cells and promotes resistance to RTK inhibitors and chemotherapeutic drugs (Auyez et al., 2021a). Sustained AXL activation is correlated with the invasiveness of cancer cells and, in turn, poor survival, making it an excellent therapeutic target for several cancers (Wium, Ajayi-Smith, et al., 2021). Many therapeutic interventions targeting AXL are actively being tested in clinical trials with promising outcomes (Wium, Ajayi-Smith, et al., 2021; Zhu et al., 2019). Mechanisms by which AXL promotes cancer progression remain to be fully understood. Studying the localization of AXL in cell could have valuable insights for understanding its role.

Our *in silico* analysis suggested AXL could be a potential regulator of Golgi organization that is differentially expressed in lung cancer cells CaLu1 and A549. Expression of AXL is significantly more in CaLu1 (disorganized Golgi) than A549 (organized Golgi), which could be driving their differential Golgi organization. Though evidence suggests that targeting AXL suppresses tumor progression across cancer cells (Wium, Ajayi-Smith, et al., 2021), very little is known about if and how AXL could have a role at the Golgi (Chia et al., 2012a; Zajac et al. 2020). We aim to test its role in regulating Golgi organization, which could provide mechanistic insights into the AXL-Golgi-glycosylation-cancer pathway.

5.3 Role of AXL in regulation of Golgi organization in lung cancer cells

Our *in silico* analysis suggests AXL could be a potential regulator of Golgi organization that is differentially expressed in lung cancer cells CaLu1 and A549. When considered in the context of studies that suggest AXL could have a Golgi localization, this makes testing its role in lung cancer particularly interesting.

5.3.1 AXL expression and localization in adherent lung cancer cells

AXL, a receptor tyrosine kinase, is implicated in cancer cell progression (Zhu et al., 2019). Its localization at the Golgi is tentative at best, with very little known about its possible role in regulating Golgi organization or function (Chia et al., 2012a; Zajac et al., 2020). The lung cancer cell lines A549 and CaLu1 could be an exciting pairing for evaluating the same. As a first step to testing the role of AXL in these cells, we compared its mRNA and protein expression between CaLu1 and A549 cells. In line with the CCLE data, mRNA and protein expression were higher in CaLu1 cells than in A549 cells (Fig 5.5). We further tested if by immunostaining AXL localizes at the Golgi apparatus in these cells.

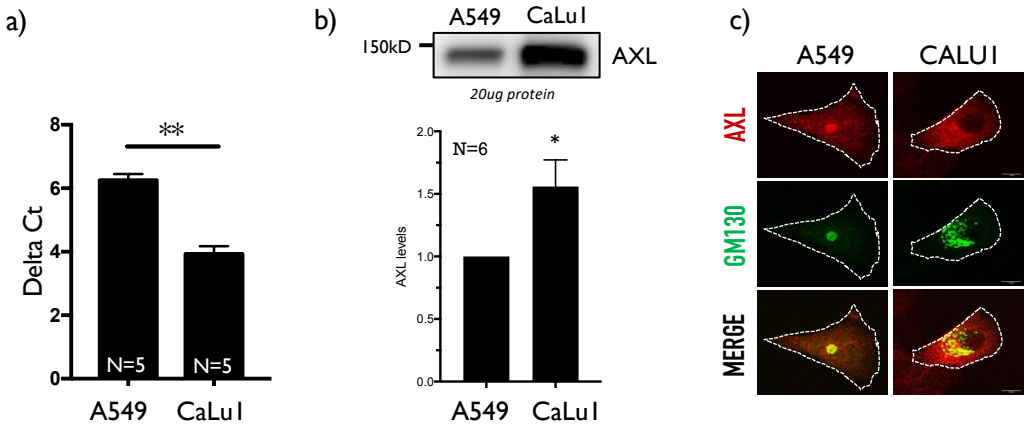


Figure 5.5 : AXL expression and localization in A549 vs CaLu1 cells – a) mRNA expression shown by delta Ct values in A549 vs CaLu1 cells. b) Protein expression as observed with western blot data compared between A549 and CaLu1 using 20ug of protein estimated cell lysates. c) AXL localization seen with GM130 at the Golgi in A549 vs CaLu1 cells. Statistical analysis was done using unpaired Mann-Whitney t-test for RTPCR data and single sample Wilcoxon t test for western blotting data.

Interestingly, in adherent A549 cells, where the Golgi is intact, a distinct pool of the cellular AXL is colocalized well with the cis-Golgi marker (GM130) (Fig 5.5). In CaLu1 cells, the dispersed cis-Golgi (GM130) also overlapped with AXL (Fig 5.5). Together, they strongly suggest a role for AXL at the Golgi in lung cancer cells.

5.3.2 Effect of AXL inhibition on Golgi organization in A549 and CALU1 cells

In 'normal' BEAS2B cells, the Golgi is organized when adherent and disorganizes on loss of adhesion. In CaLu1 cells, with a 2-fold increase in AXL levels (relative to BEAS2B), the Golgi is disorganized when adherent. In A549 cells with a 2-fold decrease in AXL levels (relative to BEAS2B), the Golgi stays organized on loss of adhesion. Hence, we asked if targeting AXL in adherent CaLu1 and non-adherent A549 cells can restore their Golgi organization to 'normal' as observed in BEAS2B cells (Fig 5.6).

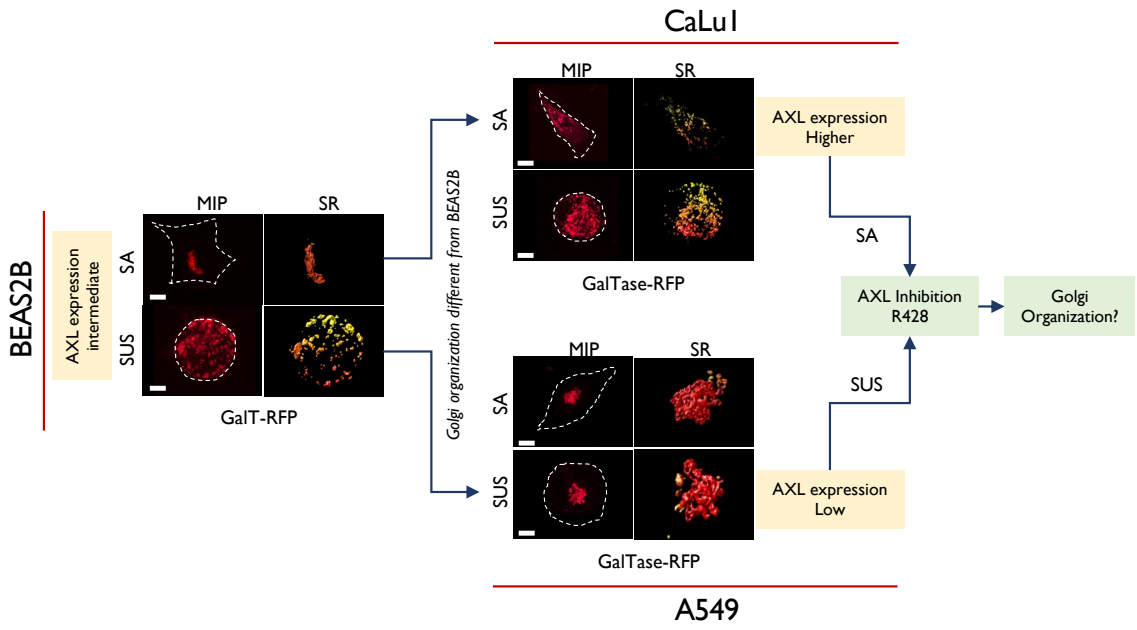


Figure 5.6 – Schematic representing differential Golgi organization between normal vs cancer cells

We used a selective ATP competitive inhibitor of AXL – R428 (Bemcentinib) to test this. R428 blocks AXL activation by preventing phosphorylation of AXL at its tyrosine residues (Y702, Y779, Y821). AXL activation also promotes AKT activation, affecting its phosphorylation at the Ser473 residue. AKT inactivation is used as a readout of AXL

inhibition by R428 (Holland et al., 2010). The optimal concentration of R428 that specifically targets AXL with minimal/no non-specific inhibition could vary between studies. A range of AXL concentrations between 1μM and 5μM has been used across studies (Chen et al., 2018; Holland et al., 2010). Preliminary studies in CaLu1 cells with a range of R428 concentrations (1μM, 2μM, 5μM, 10μM) caused cells to be sick at 5uM and 10μM concentrations in 6 hours. This led us to focus on the lower 1μM and 2μM concentrations for our standardizations. CaLu1 cells treated with R428 at both these concentrations showed significant and comparable inhibition of Akt activation. This led us to choose 1μM R428 for all further investigations. Evaluation of AXL inhibition by detecting a change in its phosphorylation (Y702, Y779, Y821) was tested with poor antibody detection outcomes. We are in the process of trying multiple phospho-AXL antibodies to improve these outcomes.

We first tested if R428 (1μM) mediated AXL inhibition can restore Golgi organization in adherent CaLu1 cells. Interestingly, Golgi organization remained unaffected upon AXL inhibition for both incubation times tested (6H and 12H). A significant drop in Akt activation on R428 treatment confirmed the AXL inhibition (Fig 5.7). We then tested the effect AXL inhibition could have on Golgi organization in non-adherent A549 cells. R428 treatment of these cells causes the Golgi to disorganize distinctly. This is seen for the cis-(GM130), cis-medial (ManII-GFP), and trans-Golgi (GalTase-RFP) (Fig 5.8). This behavior is similar to the disorganization of the Golgi observed in 'normal' BEAS2B cells on loss of adhesion. R428 treatment causes significant inhibition of Akt in A549 cells, supporting AXL inhibition. The effect R428 treatment has on Akt activation was more pronounced in non-adherent A549 cells. This could reflect a more prominent regulation (and hence inhibition) of Akt by AXL or a more pronounced inhibition by R428 of AXL itself. Together, this AXL inhibition can restore adhesion-dependent regulation of Golgi organization in A549 cells.

We further tested if and how R428 treatment affects AXL localization at the Golgi in adherent vs non-adherent A549 cells. AXL colocalization at the Golgi was lost comparably in both adherent and non-adherent cells. This is reflected in its colocalization with cis-Golgi (GM130). On loss of adhesion, AXL localization at the Golgi is seen to reduce compared to its overlap with the Golgi in stable adherent A549 cells (Fig 5.9). This suggests a possible regulation of AXL by adhesion at the Golgi. The AXL retained at the

Golgi in non-adherent A549 cells (intact Golgi) seems sufficient to keep the Golgi intact (Fig 5.9). Its inhibition further by R428 treatment and resultant loss from the Golgi is required for Golgi disorganization. The localization of AXL at the Golgi in A549 cells could hence be responsible for its organization being retained on loss of adhesion, unlike BEAS2B cells. The possible role adhesion-dependent Golgi-associated proteins, like AMPK, AURKA and Arf1, could have in mediating the role of AXL in the Golgi is also worth exploring.

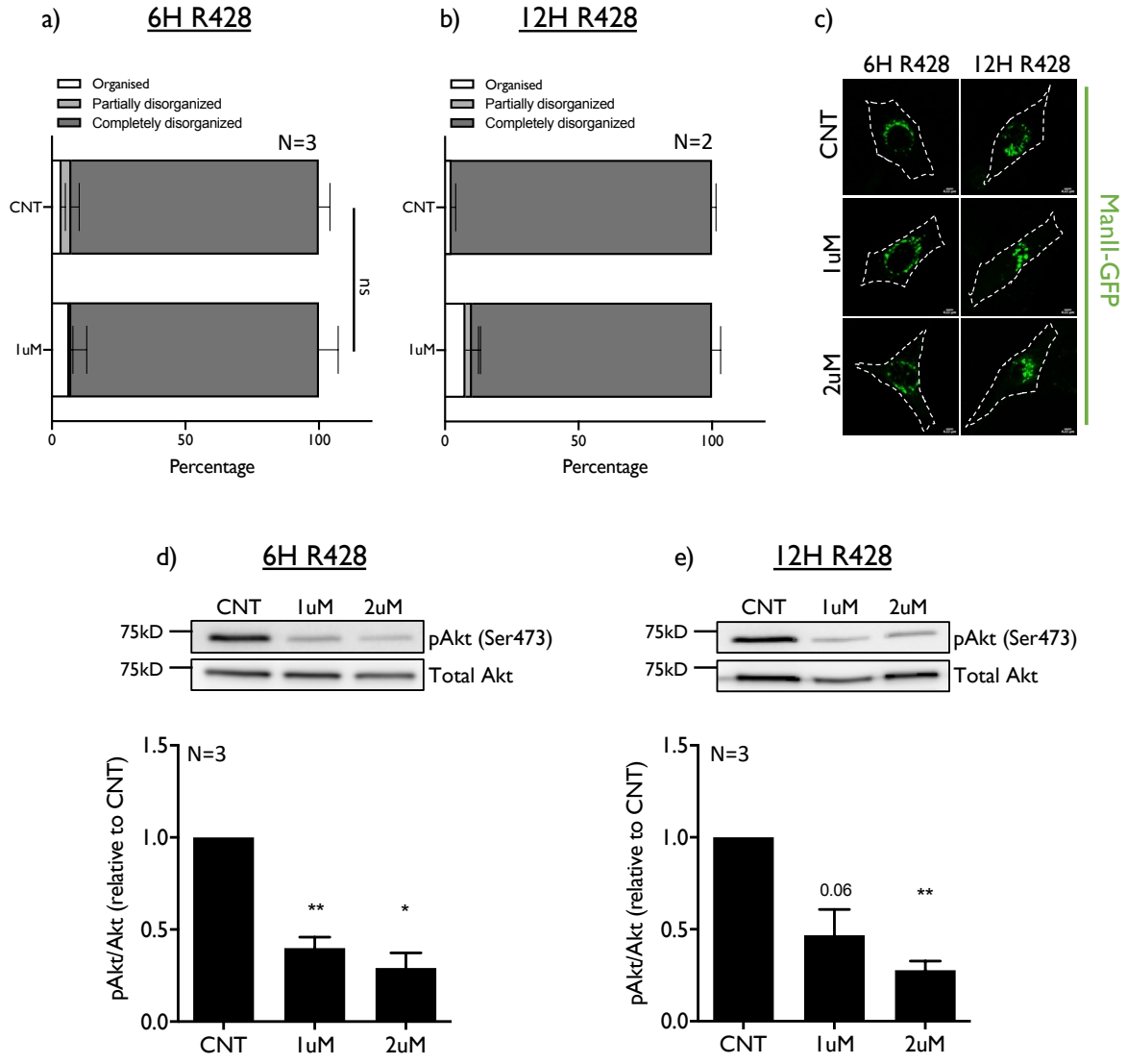


Figure 5.7 : Effect of AXL inhibition on Golgi organization in CaLu1 cells – Distribution profile for percentage of cells showing organized an disorganized Golgi in adherent CaLu1 cells, (expressing cis-medial Golgi marker ManII-GFP) treated with DMSO (CNT), 1µM R428 or 2 µM R428 for 6H (a) and 12H (b). c) Representative cross-sectional images for Golgi organization in adherent CaLu1 cells with AXL inhibition done for 6hours and 12hours. (d) and (e) Effect of R428 treatment mediated AXL inhibition on Akt activation using 6hours and 12hours of incubation times with R428. Statistical analysis was done using one way ANOVA for the distribution profiles and single sample Wilcoxon t test for western blotting results.

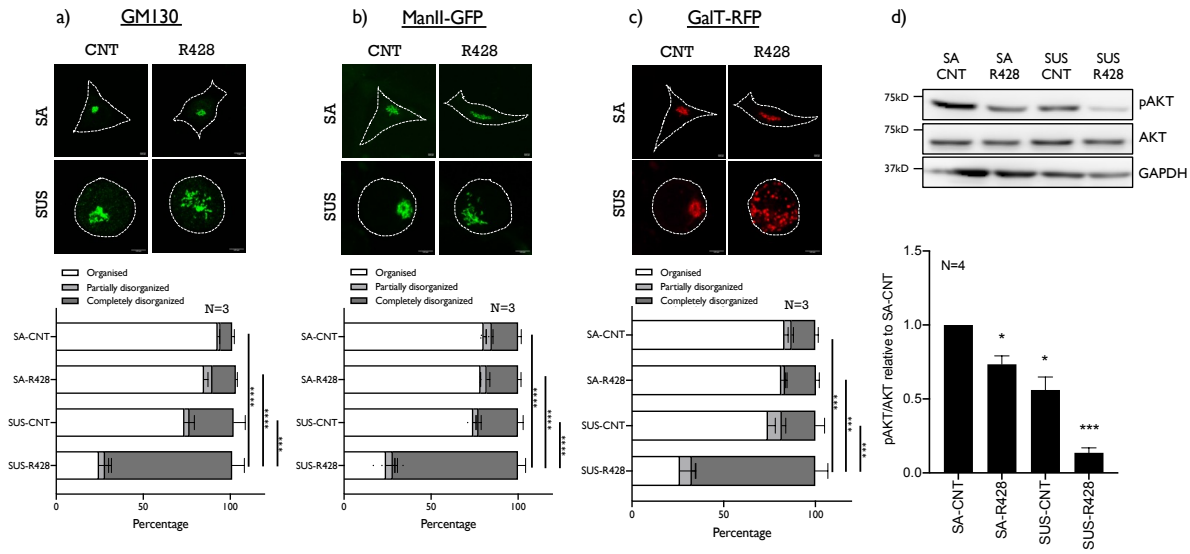


Figure 5.8 : Effect of AXL inhibition on Golgi organization in A549 cells – Effect of AXL inhibition (1µM R428 treated for 12hours before suspension) on Golgi organization in adherent vs non-adherent A549 cells, shown as distribution profiles for percentage of cells having organized, partially disorganized or disorganized Golgi, using Golgi markers (a) GM130, (b) ManII-GFP and (c) GalTase-RFP. Representative images shown are deconvoluted z-stacks showing Maximum intensity projection (MIP) images. (d) Effect of R428 treatment mediated AXL inhibition on Akt activation in adherent and non-adherent A549 cells treated with 1µM of R428 for 12hours, before suspension assay. CNT represent untreated samples. Statistical analysis was done using one way ANOVA for the distribution profiles and single sample Wilcoxon t test for western blotting results.

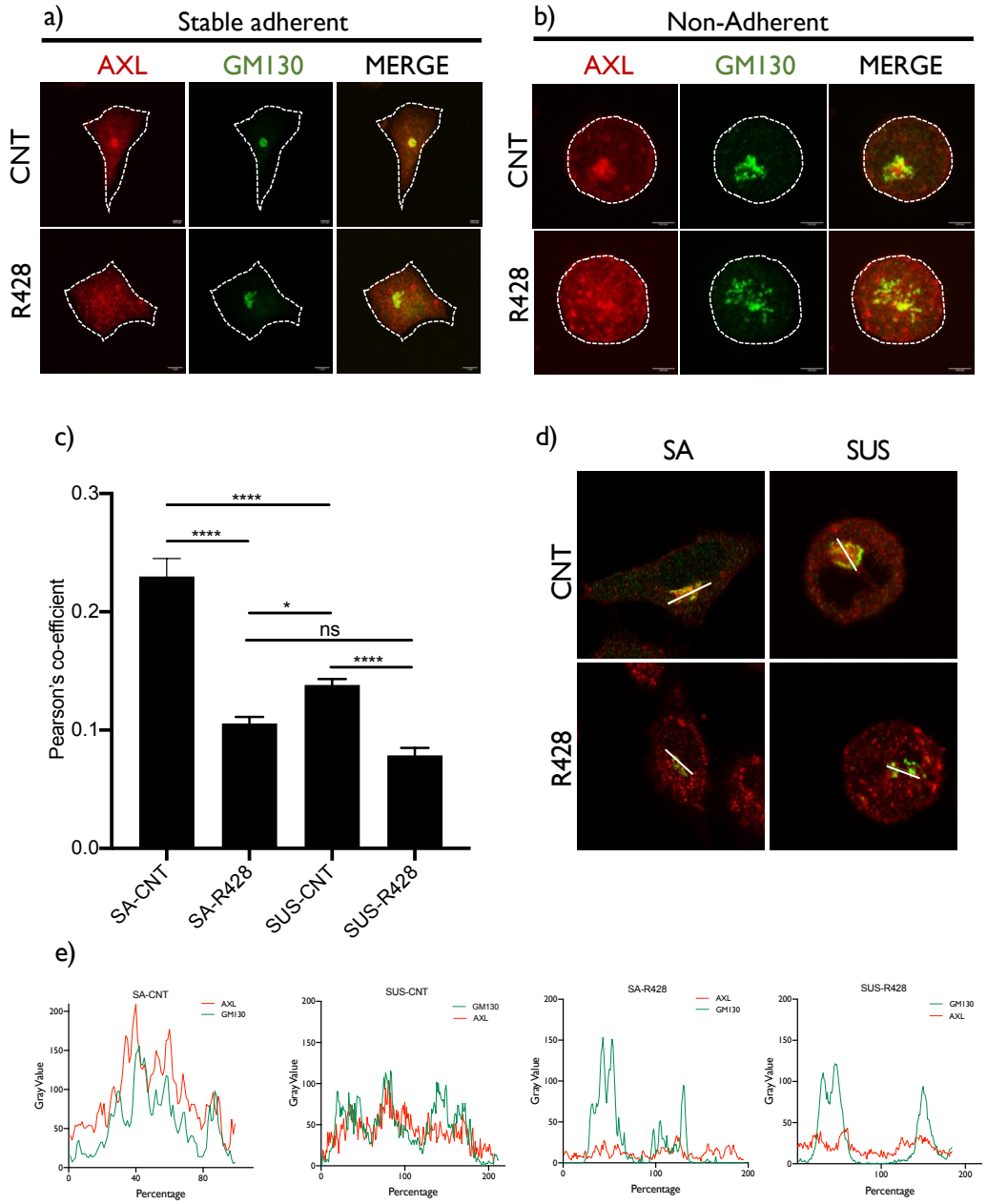


Figure 5.9 : Effect of AXL inhibition on localization of AXL at the Golgi – Deconvoluted z-stacks given as Maximum intensity projection (MIP) images showing AXL localization in adherent (a) vs non-adherent (b) A549 cells, in presence and absence of AXL inhibition (1µM R428). c) Pearson's corelation coefficient derived for colocalization of AXL and GM130 in the above deconvoluted z-stack images compared between untreated (CNT-DMSO) and treated (1µM R428) A549 cells in adherent and non-adherent conditions d) Cross sectional images for AXL localization with Golgi marker GM130, used for line plot analysis given in e). Statistical analysis was done using one way ANOVA.

5.3.3 How does AXL regulate adhesion-dependent Golgi organization in A549 cells?

R428 treatment and the resulting loss of AXL localization at the Golgi could regulate the disorganization of the Golgi in non-adherent A549 cells. This further supports the role of AXL in keeping the Golgi intact in untreated non-adherent A549 cells. Considering that 'normal' BEAS2B cells disorganize their Golgi on loss of adhesion, this A549 Golgi phenotype could reflect their anchorage-independent phenotype. Cancer cells also sustain the activation of multiple adhesion-dependent signaling pathways to drive their oncogenic transformation (Pawar et al., 2016). A possible role for such an adhesion-independent Golgi-associated signaling pathway in supporting an AXL-dependent Golgi organization is hence worth evaluating. Activation of AMPK (Sundararaman et al., 2016), AURKA (INCHANALKAR, 2014 unpublished data), and Arf1 (Singh et al., 2018) are all known to be regulated by adhesion. AMPK (Lee et al., 2015; Mao et al., 2013), AURKA (Kimura et al., 2018), and Arf1 (Donaldson et al., 2005) are all also known to regulate Golgi organization in cancers. Through these regulators, R428 treatment-mediated inhibition of AXL could control Golgi organization in A549 cells.

We first evaluated the effect R428 treatment has on AMPK and AURKA activation in non-adherent A549 cells. Previous studies have seen AXL targeting to affect AMPK activation but not AURKA (J. Hong et al., 2022). Interestingly, the R428 treatment caused a significant decrease in AMPK and AURKA activation in non-adherent A549 cells (Fig 5.10). A drop in AMPK activation is reported to promote Golgi organization (Miyamoto et al., 2008), contrary to the disorganization seen in R428 treated non-adherent A549 cells. These data rule out AMPK as a possible collaborator for AXL. A drop in AURKA activation is reported to cause Golgi disorganization (Kimura et al., 2018), as seen in R428 treated non-adherent A549 cells. A role for AURKA downstream of AXL is new and does need further exploration. The possible role AURKA could have in the AXL-dependent Golgi organization can be explored in our studies. We tested whether AURKA inhibition independent of R428 can affect Golgi organization in non-adherent A549 cells. Nanoparticle loaded with MLN8237, a known specific AURKA inhibitor (unpublished reagent developed by Kajal Singh in collaboration with Prof. Jaykannan at IISER Pune), is seen to inhibit AURKA activation across a concentration range dramatically. The lowest 0.04μM concentration seen to inhibit AURKA when tested comprehensively did not affect

Golgi organization in non-adherent A549 cells (Fig 5.10). This suggests that AURKA is not involved in the AXL mediated Golgi organization of A549 cells.

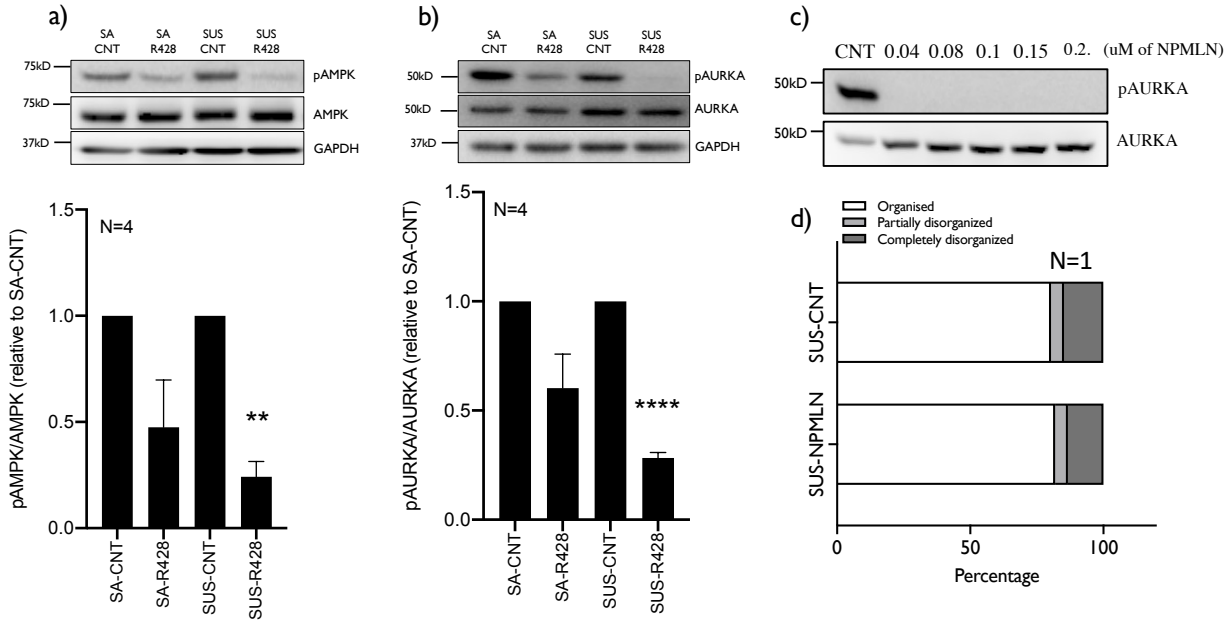


Figure 5.10 : Testing role of AMPK and AURKA signaling downstream of AXL in regulation of Golgi organization in A549 cells – a) Effect of AXL inhibition on AMPK activation in adherent and non-adherent A549 cells. b) Effect of AXL inhibition on AURKA activation in adherent and non-adherent A549 cells. c) AURKA inhibition with a range of indicated NPMLN concentrations, showing significant inhibition of AURKA activation in adherent A549 cells. d) Distribution profile for percentage of cells with organized, partially disorganized or disorganized Golgi showing effect of AURKA inhibition by NPMLN at 0.04µM concentration on Golgi organization in non-adherent A549 cells. Statistical analysis was done using single sample Wilcoxon t test.

Arf1 is well established as a vital regulator of Golgi organization and function (Singh et al., 2018; Ward et al., 2001). This, we know, depends on its activation status, which is regulated by adhesion and deregulated in cancers. Activation of Arf1 in A549 cells is seen to be adhesion-dependent and drops on loss of adhesion. However, this is insufficient to cause disorganization of the Golgi unless there is a simultaneous inhibition of AXL (by R428). This, combined with the fact that AXL inhibition (by R428) does not affect the Golgi in stable adherent A549 cells (with active Arf1), suggests a possible crosstalk between Arf1 and AXL could be required to sustain Golgi organization in non-adherent A549 cells. Inhibition of AXL could use this same crosstalk to cause Golgi disorganization. To test this, we first evaluated the effect R428 treatment has on Arf1 activation in non-adherent A549 cells and observed that Arf1 activation remains unaffected (Fig 5.11). R428 could still affect Arf1 localization at the Golgi in non-adherent A549 cells. Using the ABD-

GFP construct to localize active Arf1 in A549 cells, we detected a loss of ABD-GFP localization from the Golgi on the R428 treatment (Fig 5.11). This suggests a change in active Arf1 localization at the Golgi while net active Arf1 levels remain the same. This loss of active Arf1 may be responsible for the Golgi disorganization on R428-mediated AXL inhibition. Could AXL have a role in regulating active Arf1 localization at the Golgi in non-adherent A549? This is now worth addressing.

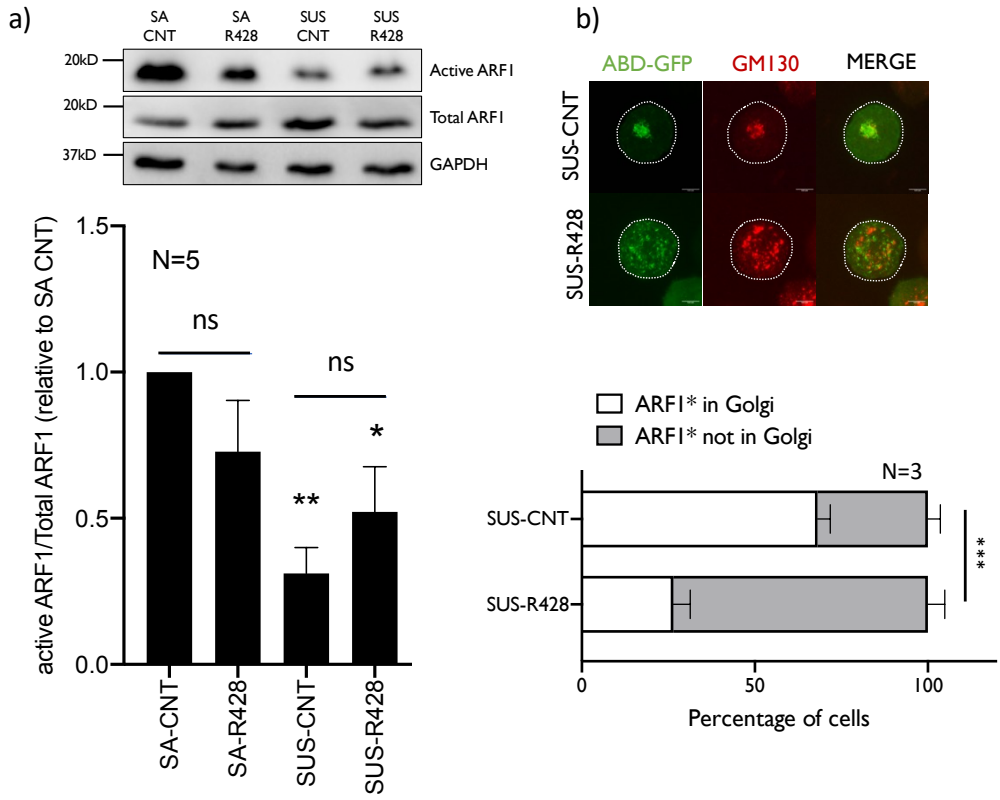


Figure 5.11 : Testing role of AXL in regulation of activation and localization of ARF1 in A549 cells - a) Effect of AXL inhibition (1µM R428; 12H before suspension) on active ARF1 levels, in adherent and non-adherent A549 cells (measured using GST-GGA3 pull-down assay followed by western) b) Effect of AXL inhibition (1µM R428; 12 H before suspension) on localization of active ARF1, using ABDGFP construct and immunostaining with GM130 in non-adherent A549 cells. c) Distribution profile showing percentage of cells with or without localization of ABDGFP at the Golgi in non-adherent A549 cells in presence or absence of AXL inhibition. Statistical analysis for distribution profiles was done using one way ANOVA, and for western blotting data single sample Wilcoxon t test was used.

To evaluate this, we first aimed to confirm their functional crosstalk in Golgi organization. Constitutively active Arf1 (Q71L-ARF1 GFP), expressed in A549 cells, was seen to restore Golgi organization lost on R428 treatment (Fig 5.12). WT-Arf1, however, was unable to

do this, suggesting Arf1 activation to be vital in mediating this functional crosstalk. This also means that loss of both AXL and active Arf1 from the Golgi could be needed to cause Golgi disorganization. The association between AXL and Arf1 protein in these cells further supports the above regulation. Considering active Arf1 was required for the above regulation, we used GGA3-GST to pull down active Arf1 and ask if it could bind AXL. GGA3 pull-down fractions were seen to contain Arf1 (active) and AXL. R428 treatment caused a small, non-significant increase in active Arf1 levels and a comparable small change in AXL binding (Fig 5.12). Together, this suggests the presence of Arf1-AXL association (direct/indirect) largely unaffected by R428 treatment. Does this mean that on R428 treatment, AXL bound to Arf1 is lost from the Golgi and supports Golgi disorganization? It is worth exploring if and how this AXL-Arf1 crosstalk affects Golgi and/or cancer cell function.

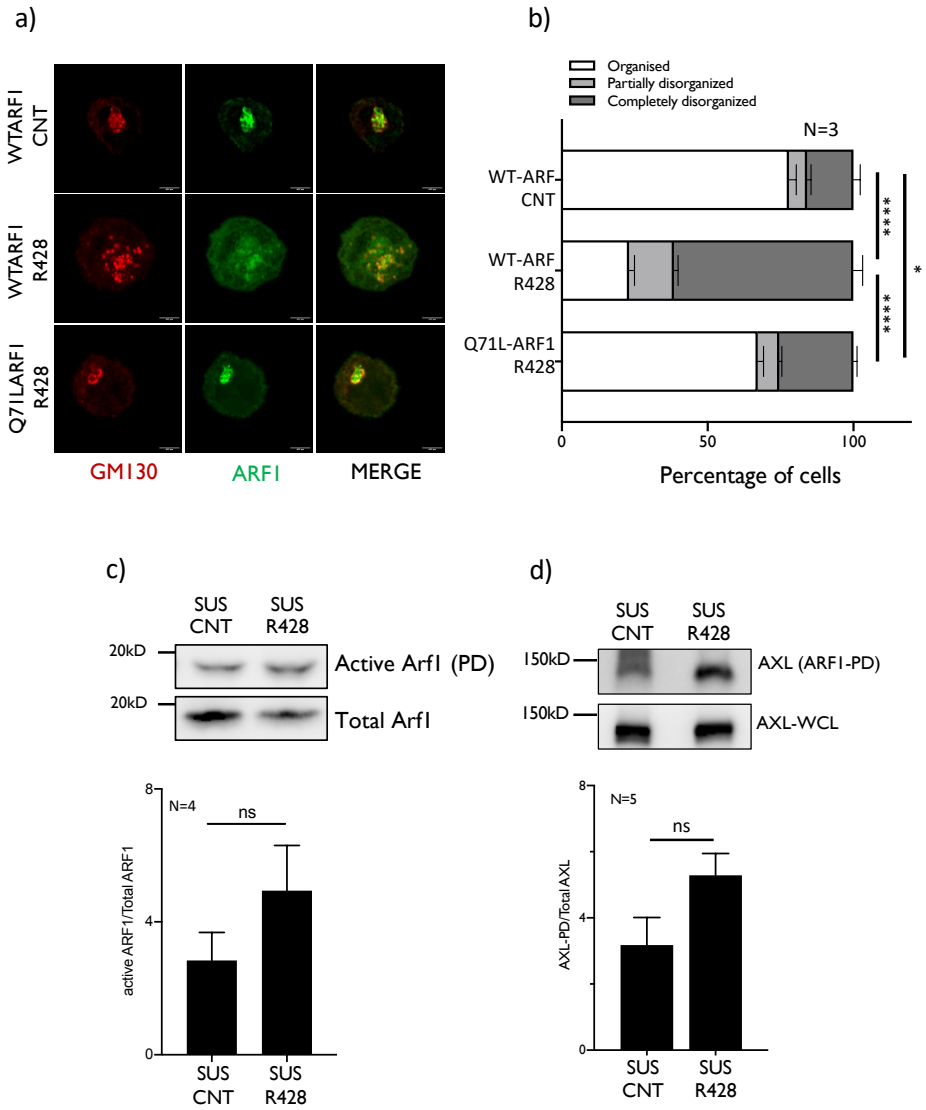


Figure 5.12 : Role of active ARF1 in AXL mediated regulation of Golgi organization in non-adherent A549 cells – a) Effect of expressing constitutively active ARF1 (Q71L-GFP) or WT-ARF1, on Golgi organization shown with GM130 immunostaining, in presence or absence of AXL inhibition (1µM R428; 12H before suspension) in non-adherent A549 cells. b) Distribution profile for percentage of (WT-ARF1/Q71L-ARF1 expressing) cells showing organized, partially disorganized, or completely disorganized Golgi in presence and absence of AXL inhibition in non-adherent A549 cells c) Effect of AXL inhibition on active Arf1 levels (c), and on association of AXL with activeArf1 shown by detection of AXL in fraction of GST-GGA3 pull-down of active Arf1 in non-adherent A549 cells in presence or absence of AXL inhibition by R428. Statistical analysis for distribution profile done using one way ANOVA, and for western blotting data using unpaired Man Whitney test.

5.4 Role of AXL in the regulation of Golgi function and cancer cell function

Golgi organization directly affects its functions in cells (Huang & Wang, 2017). Loss of adhesion in WTMEFs and the resulting disorganization of the Golgi are reported to regulate the cell surface glycosylation (Singh et al., 2018). In cancer cells, changes in their cell surface glycome are known to promote oncogenic behavior such as increased invasion (Arriagada et al., 2018; Bassagañas et al., 2014), evading immune response (Demetriou et al., 2001; Läubli & Borsig, 2019a), and apoptosis (Batisse et al., 2004; Valenzuela et al., 2007) and anoikis resistance (Piyush et al., 2017). Knowing that R428-mediated AXL inhibition causes Golgi disorganization in non-adherent A549, we evaluated the effect this could have on Golgi function in these cells. We used fluorescent lectin binding (ConA, PNA, WGA) to measure cell surface glycosylation levels to test this. In R428 treated cells, the cell surface binding of ConA and WGA lectins showed a modest but significant decrease relative to untreated non-adherent A549 cells (Fig 5.13). The cell surface binding of PNA lectin was observed to remain unaffected in these cells (Fig 5.9). This suggests that R428 treatment-mediated Golgi disorganization affects Golgi function, reflecting uniquely on different glycan substrates on the cell surface. These changes can further affect cancer cell function, which is worth exploring.

One such direct role for AXL could impact anchorage-independent growth in A549 cells. To test this, we compared the anchorage-independent growth in A549 cells with (disorganized) and without (intact Golgi) R428 and found AXL inhibition to suppress anchorage-independent growth significantly (Fig 5.14). This is reported in earlier studies (Kanlikilicer et al., 2017; Zhu et al., 2019) and could be in part mediated by AXL-dependent regulation of Akt, MAPK, ERK and Src signaling (Dagamajalu et al., 2021; Scaltriti et al., 2016). The relative contribution an AXL-dependent Golgi organization could make in supporting anchorage independence needs further validation.

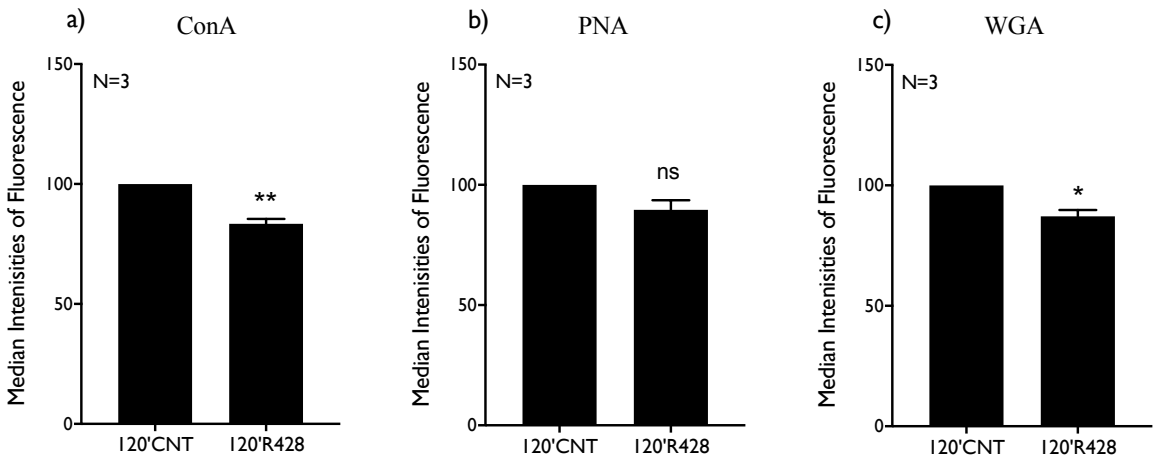


Figure 5.13 : Effect of AXL inhibition mediated Golgi disorganization on Golgi function – Median fluorescent intensities plotted for cell surface binding levels of lectins ConA (a), PNA (b) and WGA (c), obtained by Flow cytometry analysis, in presence and absence of AXL inhibition (1µM R428) in non-adherent A549 cells. Statistical analysis done using single sample Wilcoxon t test.

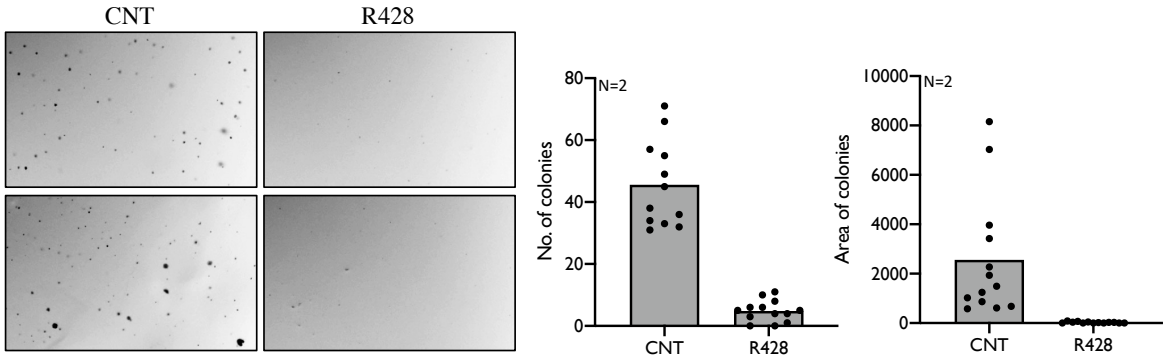
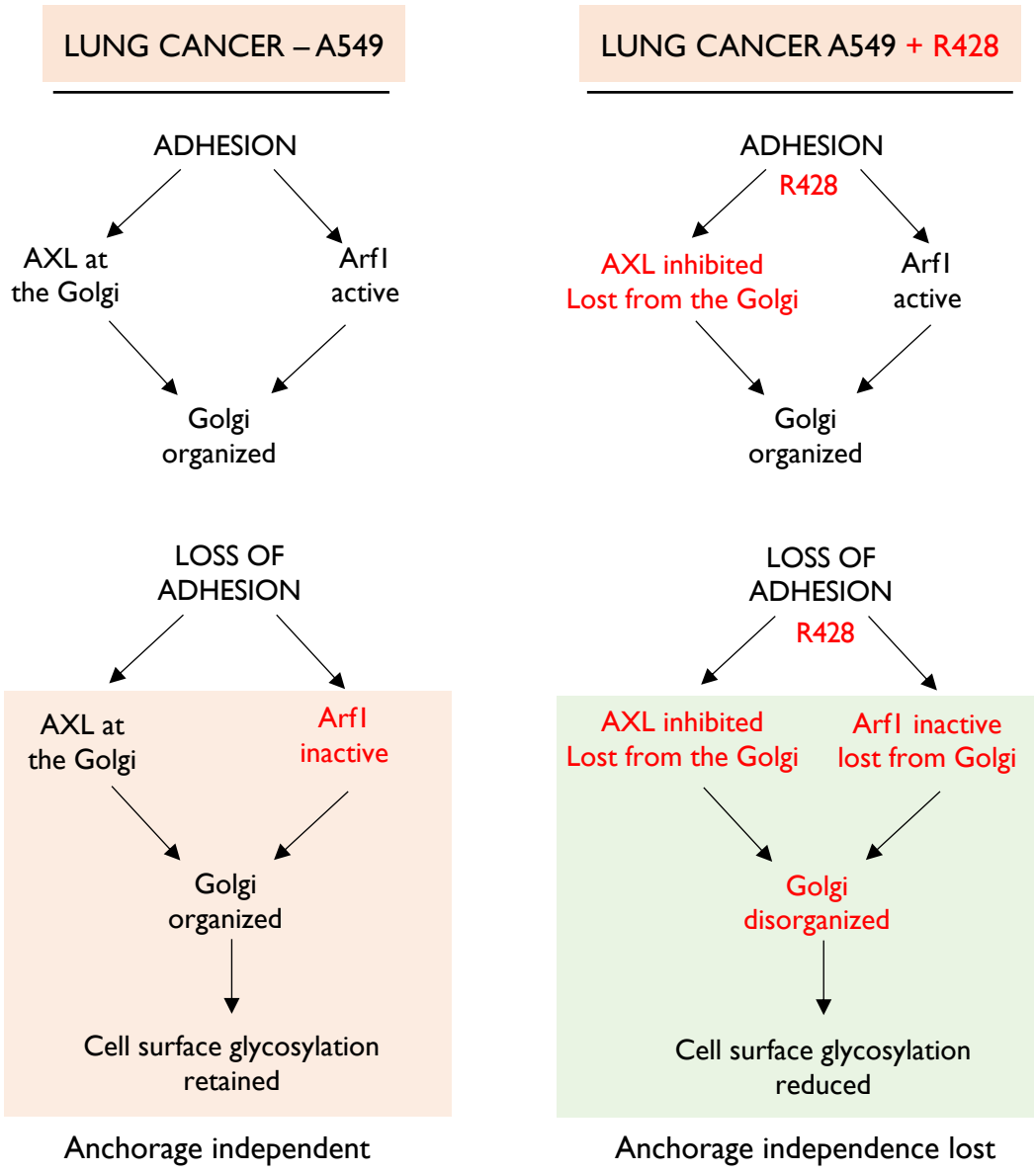


Figure 5.14 : Effect of AXL inhibition on anchorage-independent growth in A549 cells – a) Cells maintained in 0.3% soft agar gels for 14 days in presence or absence of AXL inhibition, showing growth of colonies. B) Total number of colonies plotted from 3 frames each in control vs R428 treated cells in soft agar wells, seeded in duplicates across 2 independent experiments. c) Average area of colonies obtained per frame plotted for cells in control vs R428 treated soft agar wells, seeded in duplicates.

In Summary


LUNG CANCER – A549
ADHESION
AXL at the Golgi
Arf1 active
Golgi organized
LOSS OF ADHESION
AXL at the Golgi
Arf1 inactive
Golgi organized
Cell surface glycosylation retained
Anchorage independent
LUNG CANCER A549 + R428
ADHESION
R428
AXL inhibited Lost from the Golgi
Arf1 active
Golgi organized
LOSS OF ADHESION
R428
AXL inhibited Lost from the Golgi
Arf1 inactive lost from Golgi
Golgi disorganized
Cell surface glycosylation reduced
Anchorage independence lost

Chapter 6

Quantitative evaluation of changes in Golgi organization mediated cell surface glycosylation in WTMEFs.

6.1 Rationale

Golgi-mediated glycosylation is orchestrated by over 250 proteins, most of which are native to the Golgi membranes (Petrosyan, 2015; Stowell et al., 2015). Organized Golgi architecture allows for appropriate localization of these proteins across different Golgi compartments (Petrosyan et al., 2012; C. Xu & Ng, 2015). This thereby regulates the fidelity of glycosylation and trafficking from the Golgi. Depending on the cause of perturbation, alterations in the Golgi organization and, subsequently Golgi function, are known to be variable (B.R. et al., 2023; Ward et al., 2001). How the changing Golgi organization variably affects Golgi function remains to be fully understood.

On loss of adhesion, rapid and reproducible changes in Golgi organization are known to affect cell surface glycosylation patterns (B.R. et al., 2023). Non-adherent cells also provide a unique regulatory and organizational standpoint of the Golgi that could allow us to evaluate its role in ways that haven't been done before. In this study, we asked if titrating BFA concentrations can create a reproducible gradient of Golgi disorganization to complete fragmentation. This gradient, if reproducible, can be used to measure corresponding changes in cell surface glycosylation as a read-out of Golgi function. The possible effect changes in Golgi organization have on endocytosis of cell surface glycans was also tested. Mathematical assimilation of the above data further helped generate a model for how changes in Golgi organization could differentially affect cell surface glycosylation. This study was done in collaboration with – Dr Mukund Thattai and Aashish S from NCBS Bangalore.

6.2 Standardizing BFA concentrations to get a gradient of Golgi organization in non-adherent WTMEFs

The Golgi organization in cells can assume a wide array of phenotypes, ranging from moderate disorganization to complete fragmentation with distinct effects on Golgi function (B.R. et al., 2023; Chia et al., 2012a; Makhoul et al., 2019). Understanding complex regulatory networks driving Golgi function outcomes is now being carefully explored. In previous studies, we reported the loss of adhesion to support Golgi disorganization that was distinctly different from known Golgi fragmentation that caused its fall back into the ER (Singh et al., 2018). Both outcomes depended on Arf1 activation status and differentially affected cell surface glycosylation signatures. If achieved, a careful evaluation of incremental changes in Golgi's organization (from disorganized to fragmented) could provide insights into how Golgi's organization kinetics could affect glycosylation. To achieve this, we used Brefeldin A (BFA), known to target Arf1 GEFs (GBF1 and BIG1/2), inhibiting Arf1 to regulate Golgi organization (Ward et al., 2001).

Increasing concentrations of BFA were tested at two different incubation times (30min and 90min) in non-adherent WTMEFs to evaluate how they impact the fragmentation of the disorganized Golgi. A reproducible gradient of Golgi fragmentation was characterized using the cis-medial Golgi marker (ManII-GFP), which is susceptible to Arf1 inhibition-mediated fragmentation and falls back to the ER. Evaluation of these Golgi phenotypes reveals that the BFA gradient causes a concentration-dependent increase in Golgi fragmentation (Fig 6.1). Interestingly, BFA treatment at said concentration range comparably disrupted Golgi organization on 30-minute and 90-minute treatments (Fig 6.1). This could, in part, reflect changes in how susceptible Arf1 at the Golgi is to inhibition by BFA in non-adherent cells.

The resulting range of varying Golgi phenotypes observed could be helpful in mapping corresponding changes in levels of cell surface glycans. This further led us to ask whether this comparable Golgi organization between 30 minutes and 90 minutes also generates similar outcomes at the level of cell surface glycosylation.

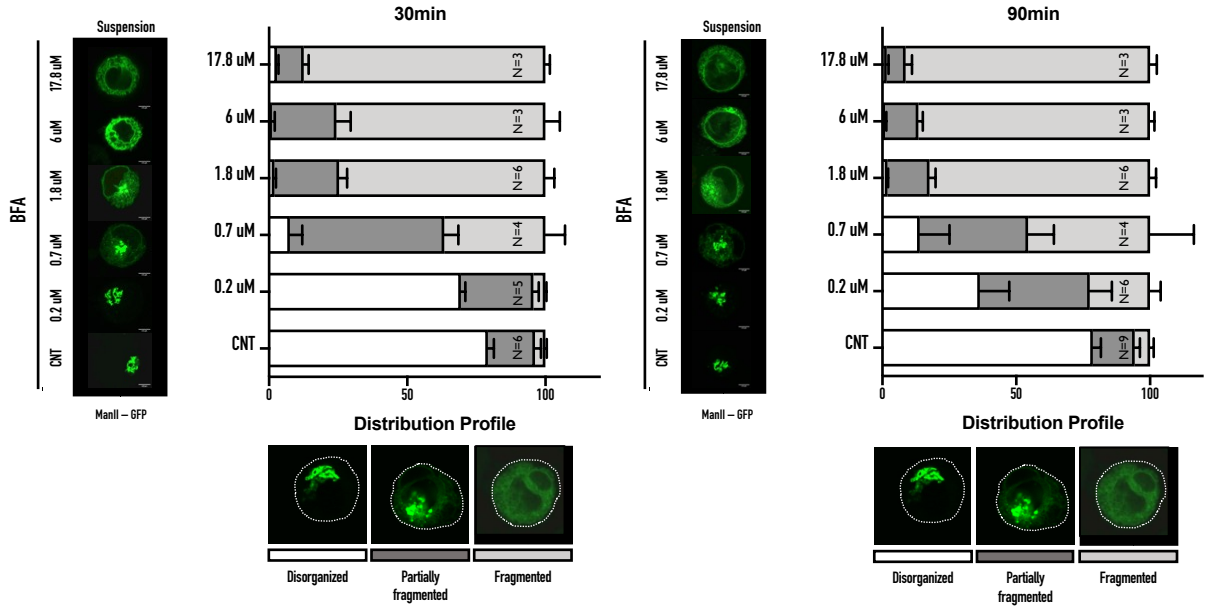
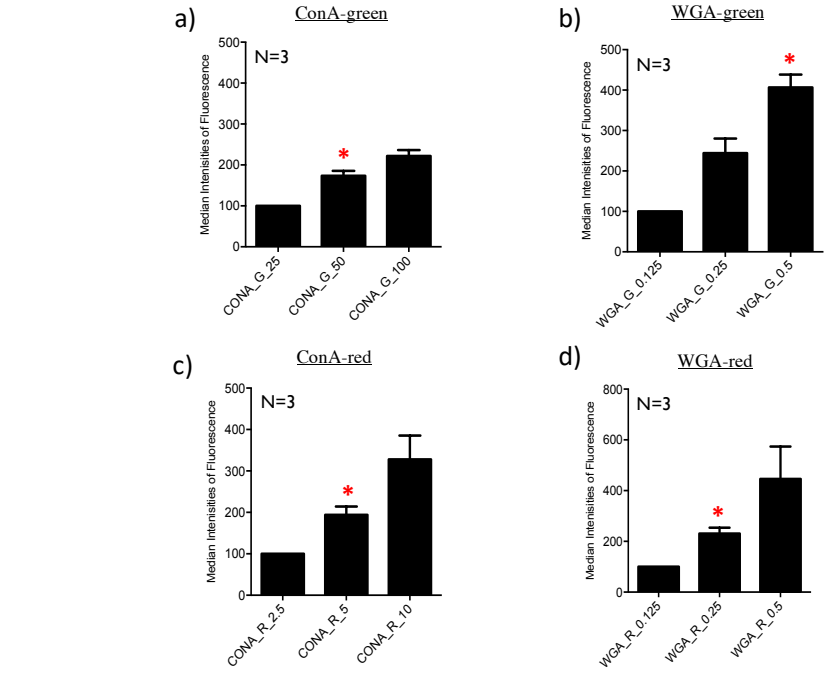


Figure 6.1 : Titrating a range of BFA concentrations to obtain a gradient of Golgi organization phenotypes – a) Cross-sectional images of non-adherent MEFs, representing the Golgi organization phenotype in majority of cells, for 30min of incubation with indicated BFA concentrations.. b) Golgi organization profile in non-adherent MEFs for 30min of incubation with indicated BFA concentrations. c) Cross-sectional images of non-adherent MEFs, representing the Golgi organization phenotype in majority of cells, for 90min of incubation with indicated BFA concentrations. d) Golgi organization profile in non-adherent MEFs for 90min of incubation with indicated BFA concentrations.

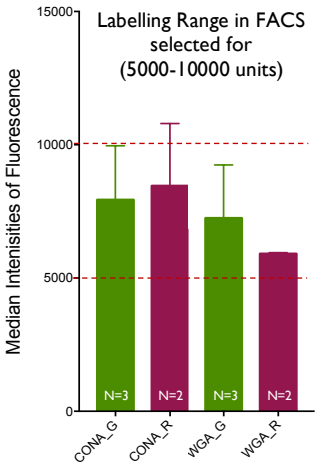
6.3 Optimizing concentrations of lectins used and dual-labelling in WTMEFs

Cell surface glycans were measured by Flow cytometry analysis using lectin binding assays. Lectins ConA and WGA used in the study were obtained in two different fluorescently tagged versions each, with both fluorophores having no spectral overlap (Alexa488 – green and Alexa647 – red). This allowed us to evaluate the levels of lectin binding by simultaneously labelling cells with both ConA and WGA lectins in the same population of cells. We first identified the optimal concentration for each lectin using a concentration range for labelling. A near-proportional increase in the median intensity of fluorescence for both lectins, as detected by Flow cytometry, allowed us to identify the optimal concentration to be used (Fig 6.2). Using these selected lectin concentrations, we asked if dual labelling might interfere with the detection of individual lectins. We compared the lectin binding levels from single and double lectin labelling to test this and found no significant change for ConA or WGA (Fig 6.3). This confirmed that dual labelling does not affect outcomes of individual lectin binding, allowing us to probe for two lectins in the same cells safely. A combination of ConA green + WGA red or ConA red + WGA green was hence possible to use.

Using non-adherent cells for these studies meant such labelling and analysis of individual cells could be done for a large population using Flow cytometry. Dual labelling data of individual cells further enhanced the outcomes of such a study.



e)



f)

Lectin	Binding specificity	Concentration (used in labeling reaction)
ConA Green	Mannose	50ng/ul
ConA Red		5ng/ul
WGA Green	N-acetylglucosamine/ Sialic acid	0.5ng/ul
WGA Red		0.25ng/ul

Figure 6.2 : Standardizations of lectin concentrations for labelling in MEFs – a) – f) Non-adherent MEFs detached and labelled with indicated lectins conjugated with Alexa488 OR Alexa647. Median fluorescent intensities obtained from flow cytometry were normalized to lowest concentration of respective lectin label, are plotted. Concentrations on x-axis are given in ng/µl . Asterix mark indicates selected lectin concentration. g) Absolute values for median intensities obtained for selected lectin concentrations are plotted to show intensity range across lectins used in the study. h) Table showing optimized lectin concentrations for each lectin, as used in the study.

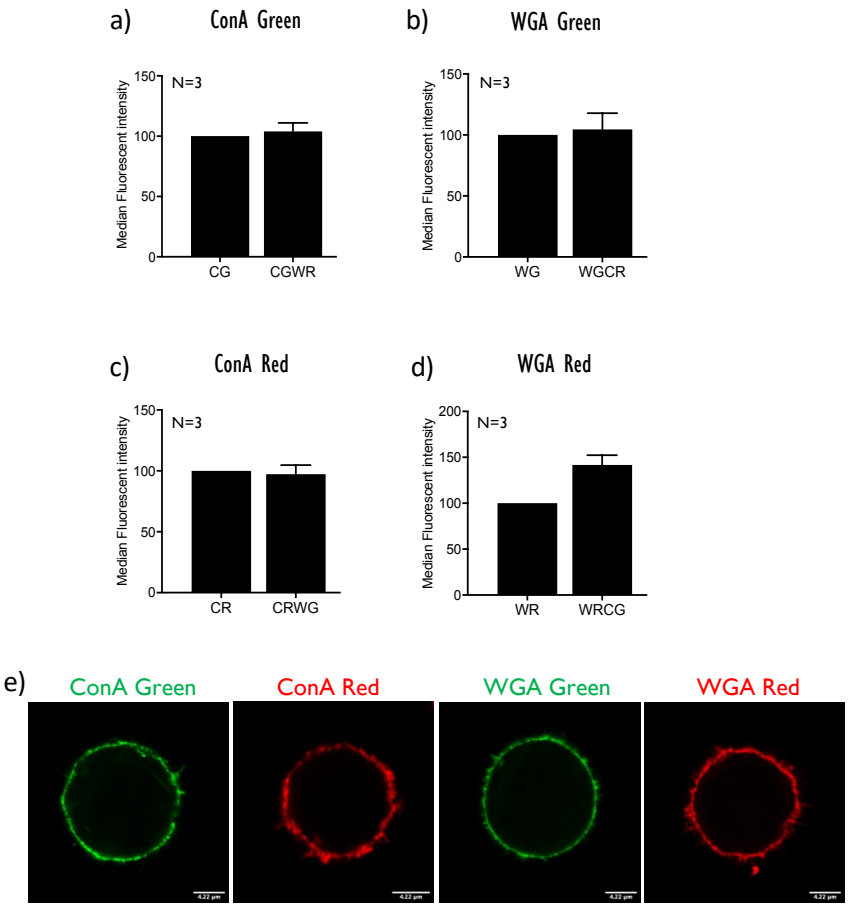


Figure 6.3 : Testing dual labelling and localization of lectin signal in MEFs – a) – d) Non-adherent MEFs detached and labelled with indicated lectins in combinations of two, were used to obtain data for median fluorescent intensity of a lectin in the pair. This value is normalized to median fluorescent intensity for single labelling of respective lectin and plotted in the graphs. Inset is key provided for identifying respective lectin signals labelled in pairs. e) Confocal imaging data shows cell surface localization of lectin signals in non-adherent MEFs. Red lectins have Alexa647 fluorophore conjugate and Green lectins have Alexa488 fluorophore conjugate.

6.4 Effect of BFA-mediated changes in the Golgi organization on cell surface glycosylation

A gradient of Golgi phenotypes, changing from predominantly disorganized to entirely fragmented, was obtained using increasing BFA concentrations at 30 vs 90-minute incubations. This change in Golgi was evaluated for corresponding changes in cell surface glycan levels using dual lectin labelling (ConA green – WGA red; ConA red – WGA green). When tested, we observed a steep and consistent decrease in cell surface ConA binding (ConA green and ConA red) on increasing BFA (0.2µM to 17.8µM) mediated Golgi fragmentation (Fig 6.4). Cell surface WGA binding, on the other hand, showed no effect at lower BFA concentrations. At the highest (6µM and 17.8µM) BFA concentration,

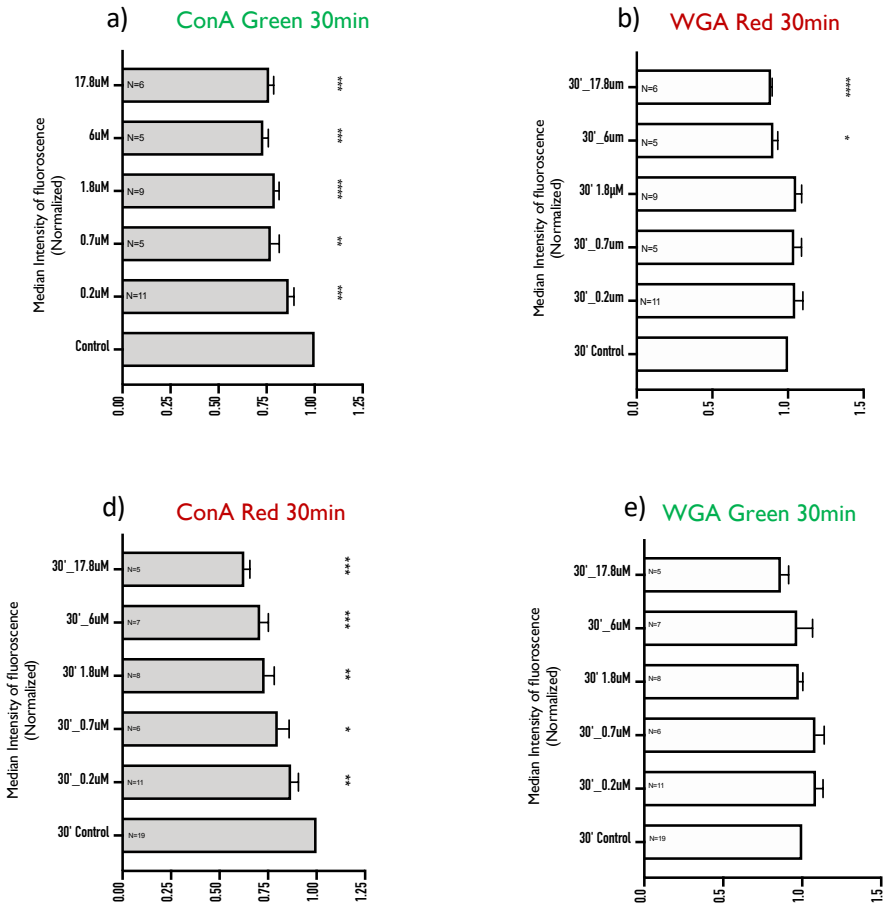


Figure 6.4 : Effect of 30min incubation with increasing BFA concentrations on cell surface glycans – MEFs held in suspension for 30min with indicated concentrations of BFA were labeled simultaneously with pair of lectins ConA-green (a) and WGA-red (b) or ConA-red (c) and WGA-green (d). Median intensities of fluorescence obtained from Flow Cytometry, were normalized to median intensity values of non-BFA treated control cells and plotted in the graphs above. Statistical analysis was done using single sample Wilcoxon t test.

WGA red only led to a small but significant drop that was not seen with WGA green (Fig 6.4).

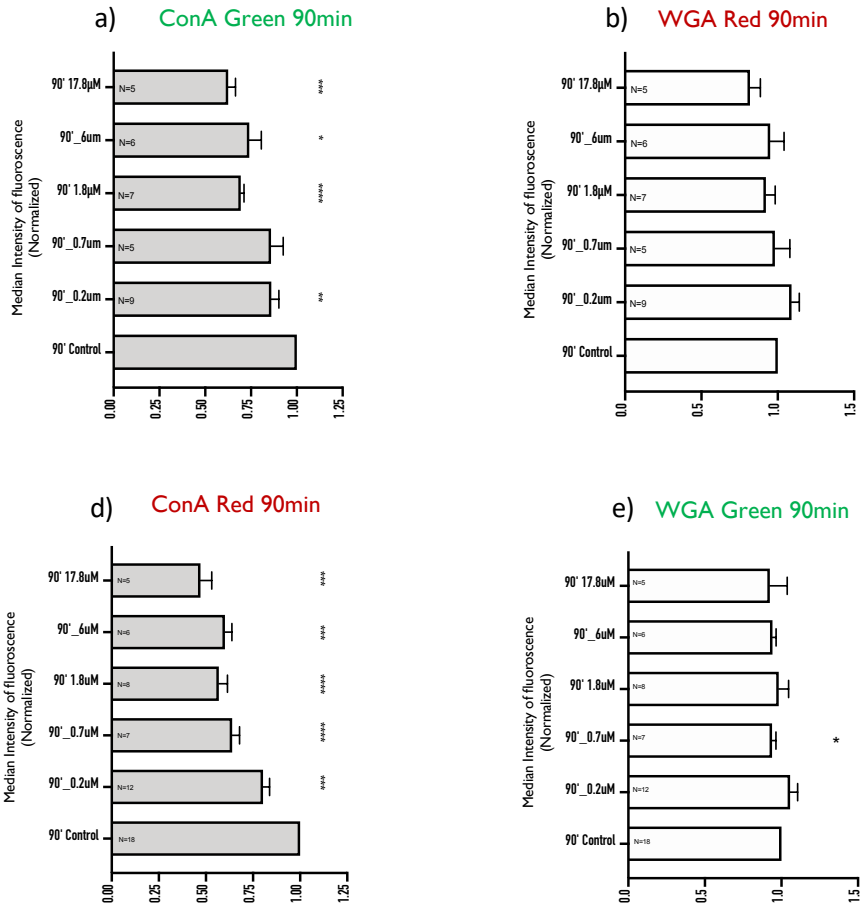


Figure 6.5 : Effect of 90min incubation with increasing BFA concentrations on cell surface glycans – MEFs held in suspension for 90min with indicated concentrations of BFA were labeled simultaneously with pair of lectins ConA-green (a) and WGA-red (b) or ConA-red (c) and WGA-green (d). Median intensities of fluorescence obtained from Flow Cytometry, were normalized to median intensity values of non-BFA treated control cells and plotted in the graphs above. Statistical analysis was done using single sample Wilcoxon t test.

This comparative evaluation with red and green fluorophore-labelled lectin pairs essentially gave comparable results at 30 and 90 minutes, confirming the fluorescent tag and labelling protocols' negligible contribution to lectin binding outcomes (Fig 6.4 and Fig 6.5). The consistency of this data is at the core of all further evaluations to be made. The consistency in Golgi fragmentation phenotypes along the range of BFA concentrations further supports this. The differences observed in cell surface binding of ConA and WGA lectins could be a consolidated outcome of changes in Golgi-mediated processing, trafficking and possibly endocytosis of lectins from the plasma membrane.

6.5 Testing the effect of BFA-mediated Golgi fragmentation on the endocytosis of cell surface glycans

BFA-mediated changes in Golgi organization and the resulting differential effects on cell surface glycans could be further regulated by endocytosis in WTMEFs. Earlier studies from the lab showed that loss of adhesion could trigger differential endocytosis from the plasma membrane (del Pozo et al., 2005; Pawar et al., 2016), making this regulation essential to evaluate for cell surface glycans. Differences in endocytic rates could contribute to net observed changes in cell surface lectin binding levels in response to the BFA concentration gradient. To test this, we chose two concentrations of BFA, representing distinctly different Golgi organization phenotypes. A 30-minute treatment with 0.1μM BFA causes the Golgi to be distinctly disorganized, while 1.8μM caused complete fragmentation of the Golgi. Cell surface lectin labelled control and BFA-treated live cells were i) fixed immediately as 0min pre-endocytic control and ii) incubated at 37^0C for 15min or 30min to allow for lectin endocytosis. Cells were fixed after the required time and showed the exclusive membrane localized lectin (at 0min) to be distinctly endocytosed into the cells after 15 and 30min of incubation. BFA treatment, however, did not affect the endocytosis of ConA or WGA in these cells when observed visually (Fig 6.6). A quantitative evaluation of the above data could provide valuable insights into how differences in the behaviour of ConA vs WGA glycan substrates could impact net cell surface level changes on Golgi disruption by BFA.

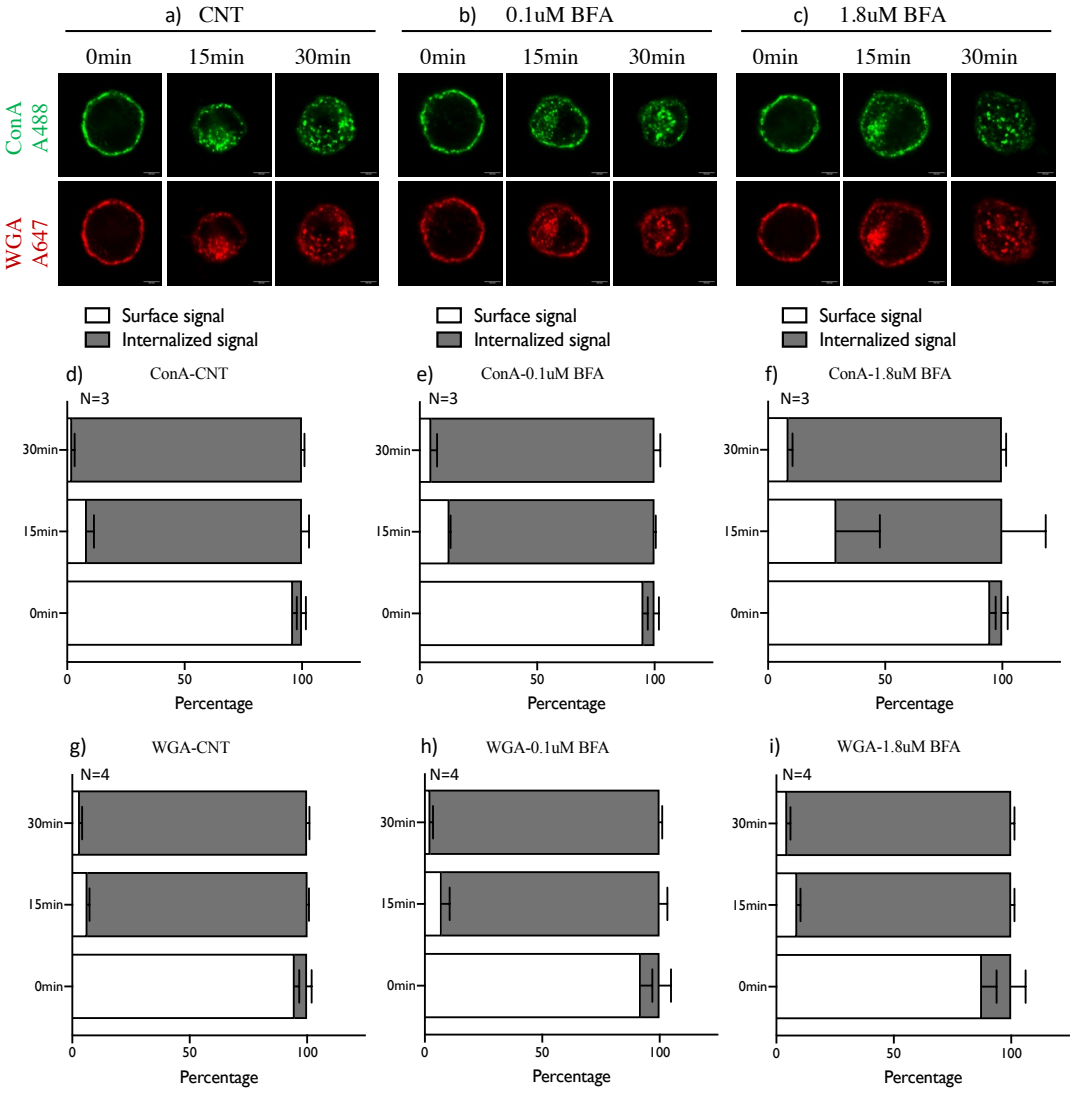


Figure 6.6 : Effect of BFA mediated changes in Golgi organization on endocytosis of cell surface bound lectin – MEFs detached with Accutase and held in suspension for 30min with DMSO-CNT (a), 0.1µM BFA (b) or 1.8µM BFA (c) were dualy labeled with ConA-Alexa488 and WGA-Alexa647 lectins. Cross-sectional images are shown to represent the localization of lectin signal in cell as seen in majority of cells for a given sample (a-c). Profiling of cells showing percentage of cells with lectin signal on the cell surface vs percentage of cells showing lectin signal predominantly in the cell interior for ConA-Alexa488 (d-f) and WGA-Alexa647 (g-i)

6.6 Mathematical analysis of cell surface glycan levels and glycan endocytosis in response to changing Golgi organization.

Increasing Golgi fragmentation on BFA treatment and its corresponding effects on cell surface glycosylation in non-adherent cells provides a unique reproducible system for in-depth analysis of Golgi organization-function correlation. Using two different lectins labelled with two different fluorophores on 10000 or more cells with distinct Golgi disruption, analyzed by Flow cytometry, gives us a comprehensive single-cell data profile for changes in lectin binding, which can then be equated to changing Golgi organization in the population. The complex nature of this data, considered in the context of the endocytic regulation of bound lectins, mandates the need for careful mathematical analysis to arrive at a possible model to explain this phenomenon. This was possible by collaborating with Dr Mukund Thattai and Aashish Satyajith from NCBS Bangalore.

Single-cell Flow cytometry data from these experiments was put through a series of systematic normalizations to rule out contributions from non-specific parameters. These included cell size variations, red vs green fluorescence detection and day-to-day variations in experimental handling. This data showed a bivariate normal distribution after normalizations, which is ideal for further analysis. This normalized data was used to calculate and plot the median intensities obtained for ConA and WGA binding and showed their behavior to be qualitatively different across BFA concentrations. The endocytic rates of ConA and WGA glycan substrates were defined as the fraction of pixels in the cell boundary (taken to be a rim of the cell obtained using Cellpose (Stringer et al., 2020), as the 15% of the total area of the cell, starting from boundary and going inwards, plus a region of 5% area of the cell going outwards) that have intensity of lectin higher than median intensity of the region inside the rim. This was used to derive the endocytic index for ConA and WGA, suggesting a slightly higher endocytic rate for ConA relative to WGA. However, this difference in endocytic rates was seen to be independent of BFA treatment, thereby ruling out any contribution of changes in the Golgi organization.

Based on our understanding from the literature to date, the effect increasing BFA concentration mediated Golgi fragmentation has on the processing and trafficking of glycans in cells was modelled as described in the schematic (Fig 6.7). Our data for how

increasing BFA concentrations impact the Golgi fragmentation kinetics and possibly the endocytosis of cell surface glycans could now be incorporated (or fitted) into this model. The results from lectin binding studies were then used to adjust the model's parameters to fit the experimental data. Modelling the fallback of Golgi to the ER (fragmentation) on the addition of BFA, we show that Golgi's organization is influenced by both the concentration of BFA and the time elapsed after adding BFA. These changes in Golgi affect both the chemical modifications and trafficking rates of glycans. An interplay between both the changes and trafficking rates could regulate the cell surface levels of X (ConA glycan substrate) and Y (WGA glycan substrate) (Fig 6.7). (ConA binding sites on proteins with polymeric glycosylation (with or without branching) are referred to as ConA glycan substrate)

In fitting the model to our lectin binding and endocytosis of bound lectin data along with the increasing Golgi fragmentation observed, we arrived at a model which provides valuable preliminary insights into differential processing and trafficking of glycans by the Golgi. The proposed model is based on the following assumptions.

1) The flux of the glycoprotein substrate through the trans-Golgi is proportional to the volume of the cis-Golgi.
2) The time taken for a substrate to go from cis-Golgi to the plasma membrane is shorter than the time it takes for the volume of cis-Golgi to change (fragment). This difference in time is significant at lower BFA concentrations. From the literature, we see that the Golgi residence times in HeLa cells is <14 mins (Sun et al., 2021), and transport time in COS-7 cells from the Golgi to the plasma membrane is 3.8 mins (Hirschberg et al., 1998b). Our data shows that the time scale of volume change is 12.77 minutes for 1.8μM BFA and even shorter for higher BFA concentrations. So this assumption is, in effect, feasible up to 1.8μM BFA concentration.
3) The enzymes inside the Golgi are not saturated (Hirschberg et al., 1998b). This lets us employ first-order kinetics for the reactions happening in the Golgi.
4) We assume that there is a rate-limiting step between the formation of Y (WGA-binding substrate) from X (ConA-binding substrate). Since these rates cannot be directly measured, we leave open the possibility that β and γ (see schematic) are subject to change with BFA concentration and instead model the ratio of both the rates for which we assume the

simplest possible (linear) dependence on BFA concentration. A constant ratio of β and γ did not work (i.e. glycan levels being independent of BFA does not work)

5) From our data, we see that the rate constant of endocytosis for ConA and WGA substrates is not dependent on BFA concentration for the time scale of the experiment. This is not true for longer times (Prydz et al., 1992).
6) The time from when a glycoprotein gets endocytosed to the time it recycles is longer than the time of the experiment (90 minutes) (Montealegre & Van Endert, 2019).

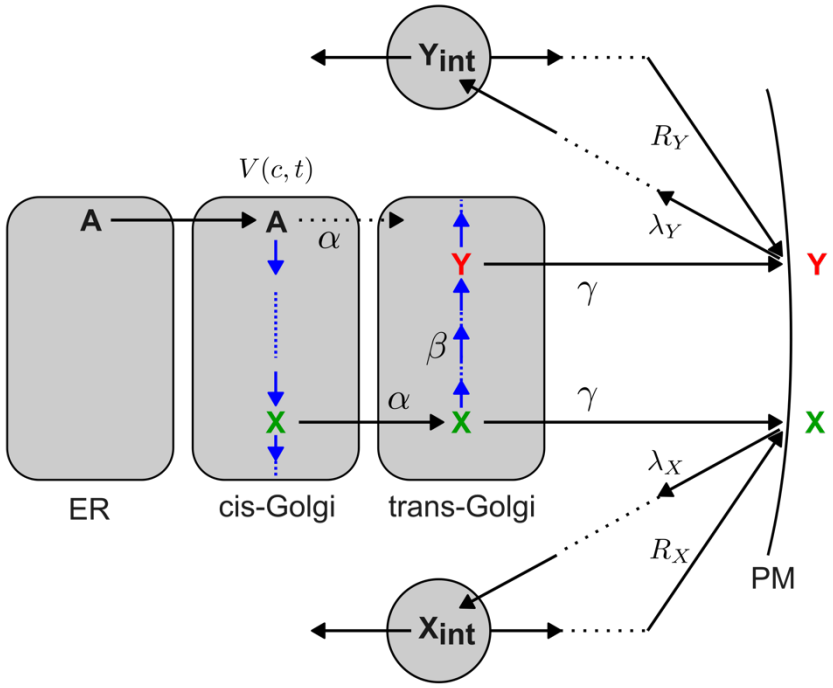


Figure 6.7 : Schematic of the proposed model – Given schematic representation of a cell, shows (glycoconjugate) input from ER moving through the cis-Golgi to trans-Golgi followed by trafficking to the cell membrane. *V(c,t)* denotes the effect of increasing BFA concentration on Golgi organization and hence the influx of the ConA substrate (denoted α). X denotes ConA glycan substrate (mannose glycans) and Y denotes WGA glycan substrate (sialic acid/N-GlcNAc glycans). β represents the reaction rate from X to Y, while γ represents the trafficking rates of glycans from trans-Golgi to the cell membrane. X_{INT} and Y_{INT} represent internalized pools of surface glycans X and Y with endocytic rates λ_X and λ_Y respectively. From the endocytic compartments these glycans (X and Y) are shown to recycle back to plasma membrane (PM) with trafficking rates R_X and R_Y respectively.

The above assumptions made to fit the model with experimental data revealed mechanistic insights into how changes in Golgi could differentially affect cell surface glycan levels. The proposed model (Fig 6.7) shows the processing of glycosylated cargo (A), modified to X at the cis-Golgi to form Con-A glycan substrate (Stanley, 2011), which then moves to the trans-Golgi. From trans-Golgi, X acts as an intermediate to produce Y (WGA substrate) (Sharma et al., 2014; Stanley, 2011), along with its trafficking to the cell membrane. We

propose that at lower BFA concentrations, depletion of influx into the Golgi could subsequently affect the availability of ConA substrate in the Golgi and on the plasma membrane. Our model suggests that lower concentrations of BFA *(c)* will lead to an increase in β relative to (β and mediating X to Y and X to cell membrane traffic, respectively), which further increases the production of Y (WGA substrate) in trans-Golgi and hence its levels at the cell membrane as is observed in our data. At higher concentrations of BFA *(c)*, Golgi fragmentation affects influx *V(c,t)*, which depletes X, thereby decreasing levels of both X and Y on the cell membrane.

These sequential events in response to varying BFA, revealed by the model, could explain the known/observed differential effects of increasing BFA concentrations on the cell surface glycans. Additionally, our data suggested that endocytic rates of cell surface bound ConA (λ_X) are higher than that of WGA (λ_Y), and BFA does not affect these rates. Fitting the model to our data, we can now propose that endocytosis of respective glycans could affect their cell surface levels independently of changes in the Golgi. This model provides early insights into the complex nature of glycosylation reactions occurring within the Golgi and how alterations to the Golgi organization can differentially affect cell surface levels of different glycan substrates.

(Additional chapters)

Chapter 7

Role of active Arf1 in regulating Golgi disorganization mediated by DNA damage

7.1 Rationale

Cancer cell progression is influenced by its sensitivity to DNA damage (Jeggo et al., 2016) and its ability to support anchorage-independent growth (Guadamillas et al., 2011; Pawar et al., 2016). While integrins act as the primary regulators of cell-matrix adhesion to drive anchorage-dependent signaling, the responsiveness of cells to DNA damage is mediated by multiple pathways. DNA damage and adhesion signaling regulate different cellular pathways as well as the dynamics of cell organelles (Berrier & Yamada, 2007; Jeggo et al., 2016). The Golgi apparatus is one such vital cell organelle that is shown to be affected by the loss of adhesion-mediated signaling as well as DNA damage (Buschman et al., 2015a; Singh et al., 2018).

DNA damage is also seen to rapidly fragment the Golgi. This was shown to be mediated by DNA-PK-dependent phosphorylation of the Golgi structural protein GOLPH3, which leads to Golgi dispersal (Farber-Katz et al., 2014). A study suggests that GOLPH3 and Arf1 could associated with each other in regulating Golgi (Iyer et al., 2018). This means a possible role for ARF1 in DNA damage-mediated dispersal of the Golgi organization. In this study, we aimed to evaluate a potential role for the small GTPase ARF1 in mediating the effect of DNA damage on Golgi organization.
This part of the study was done with Dr. Mayurika Lahiri and Abhijit K.

7.2 Effect of DNA damage on Golgi organization in adherent MCF10A cells

DNA damage-induced activation of DNA-dependent protein kinase (DNA-PK) prevents the recruitment of GOLPH3 at the Golgi, which promotes Golgi dispersal in normal and cancer cells (Farber-Katz et al., 2014). Using 'normal' MCF10A cells, we first confirmed the effect of DNA damage on Golgi organization in adherent cells using the cis-Golgi marker GM130. A distinct increase in DNA-PK activation confirmed the induction of DNA damage by UV treatment (Fig 7.1). The intact Golgi organization was observed in control; untreated MCF10A cells were found to be completely disorganized across 6, 12 and 24hrs post-UV treatment of cells. The extent to which Golgi disorganized in these cells varied across different time points post UV induction, from mild enlargement of the Golgi 6 hours post UV induction to complete Golgi disorganization observed at 24 hours (Fig 7.1). This observation was also supported by data for the area of the Golgi apparatus, which showed a significant increase between control vs UV-treated cells after 6, 12, and 24 hours post-UV induction. We also show a visible enlargement of nuclei in the UV-treated cells, which distinctly increased from 6 to 24 hours post-UV induction (Fig 7.1). This effect was also reported in earlier studies, wherein DNA damage induction by carcinogen MNU (n-methyl n-nitrosourea) caused a significant increase in the nuclei volume of MCF10A cells (Anandi et al., 2017). This data confirmed the impact of UV induction-mediated DNA damage in causing Golgi disorganization in adherent MCF10A cells. Knowing this effect on Golgi is regulated by the DNA-PK – GOLPH3 – Golgi axis and that GOLPH3 could associate with the small GTPase Arf1 (Iyer et al., 2018), we further tested a possible role for Arf1 activation in DNA damage mediated Golgi dispersal.

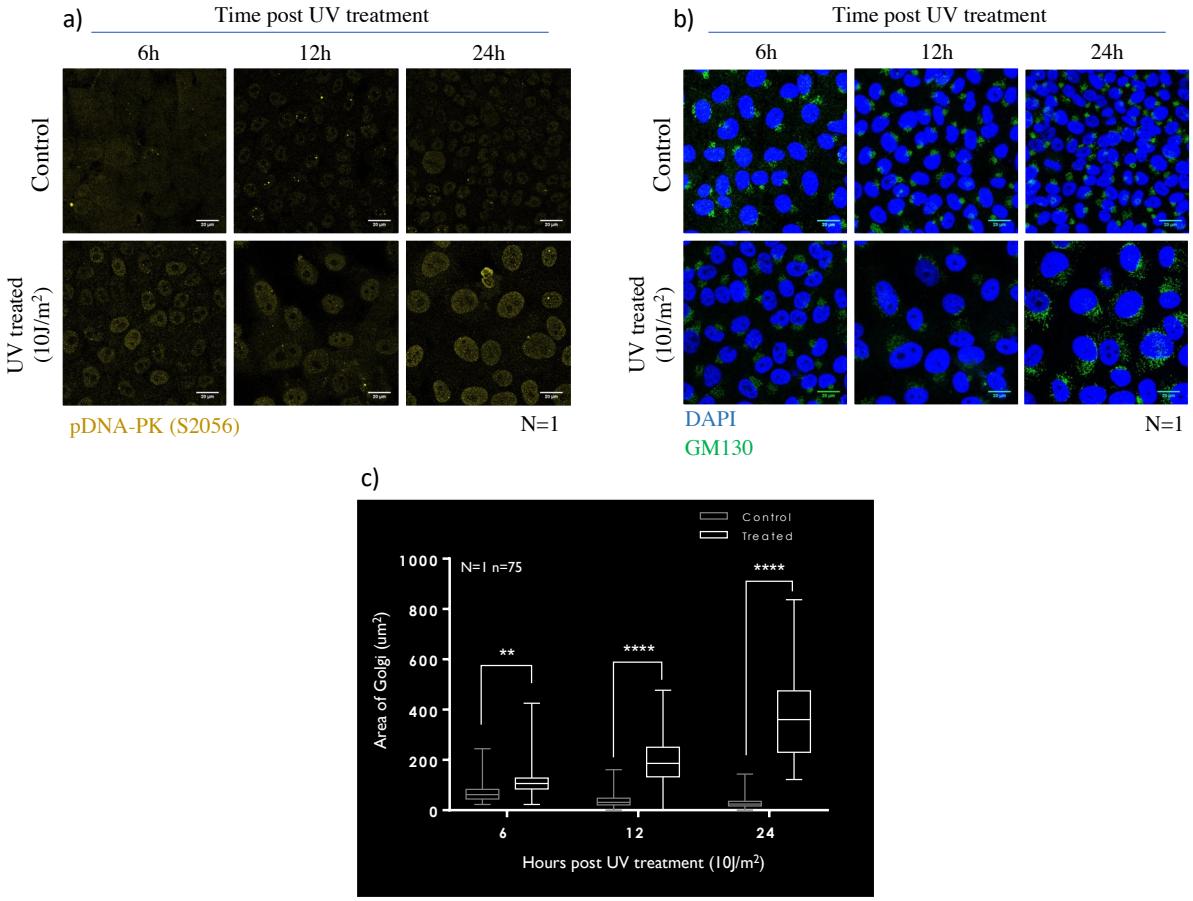


Figure 7.1: Effect of UV treatment mediated DNA damage on Golgi organization in adherent MCF10A cells – Normal human breast epithelial cells MCF10A were treated with UV radiation (10J/m^2), post 16hours of seeding. Cells were fixed post 6hours, 12hours and 24hours of UV induction and processed for IFA. a) Representative cross-sectional images of cells showing effect of DNA damage on activation of DNA-PK (S2056), post 6hrs, 12hrs and 24hrs of UV induction. B) Representative cross-sectional images showing effect of DNA damage on Golgi organization with cis-Golgi marker GM130, post 6hrs, 12hrs and 24hrs of UV induction. Nuclear staining with DAPI shows effect of DNA damage on nuclear morphology at indicated timepoints. c) Area occupied by Golgi objects in control vs UV treated cells at indicated timepoints post UV induction. Statistical analysis Kruskal-Wallis and Dunn's Multiple comparisons test. ** $p<0.01$, **** $p<0.0001$

7.3 Role of Active ARF1 in DNA damage mediated Golgi dispersal

To evaluate the possible involvement of active Arf1 in DNA damage-mediated Golgi disorganization, we tested the effect of UV-induced DNA damage on active Arf1 levels. We confirmed the induction of DNA damage by UV treatment by looking at p53 (phosphorylation at Ser15) activation in treated cells, a known DNA damage marker (Lewis et al., 2002). A significant increase in p53 activation levels at early (6h) and late (24h) time points post-UV treatment further confirmed the induction of DNA damage (Fig 7.2). The effect of DNA damage on active Arf1 was measured using GST-GGA3 pull-down assay in adherent MCF10A cells at these time points post-UV induction. We observed a modest but significant drop in levels of active ARF1 at 6 hours post-UV induction (Fig 7.2). This drop in active Arf1 could be responsible for the Golgi disorganization and significant increase in the Golgi area observed under these conditions. However, after 24 hours of UV induction, active ARF1 levels were comparable to those in untreated control cells (Fig 7.2). This further suggests that the initial drop in active ARF1 at 6 hours post UV treatment could be required to initiate the Golgi disorganization event, while at later time points, an Arf1 independent mechanism could be taking over to sustain the observed disorganized Golgi phenotype over 24hrs post DNA damage. The complete Golgi disorganization observed at 24hrs could also be maintained by regulatory proteins other than Arf1 as a part of DNA damage response signaling. Whether localization of active Arf1 could be lost upon DNA damage induction and could drive the observed Golgi dispersal over more extended hours, post-UV induction remains to be tested. Additionally, other DNA damage agents independent of UV treatment could have a differential effect on active ARF1 and be worth exploring. The above results discuss preliminary observations and require further evaluation to comment on the possible role of Arf1 in the DNA damage response pathway.

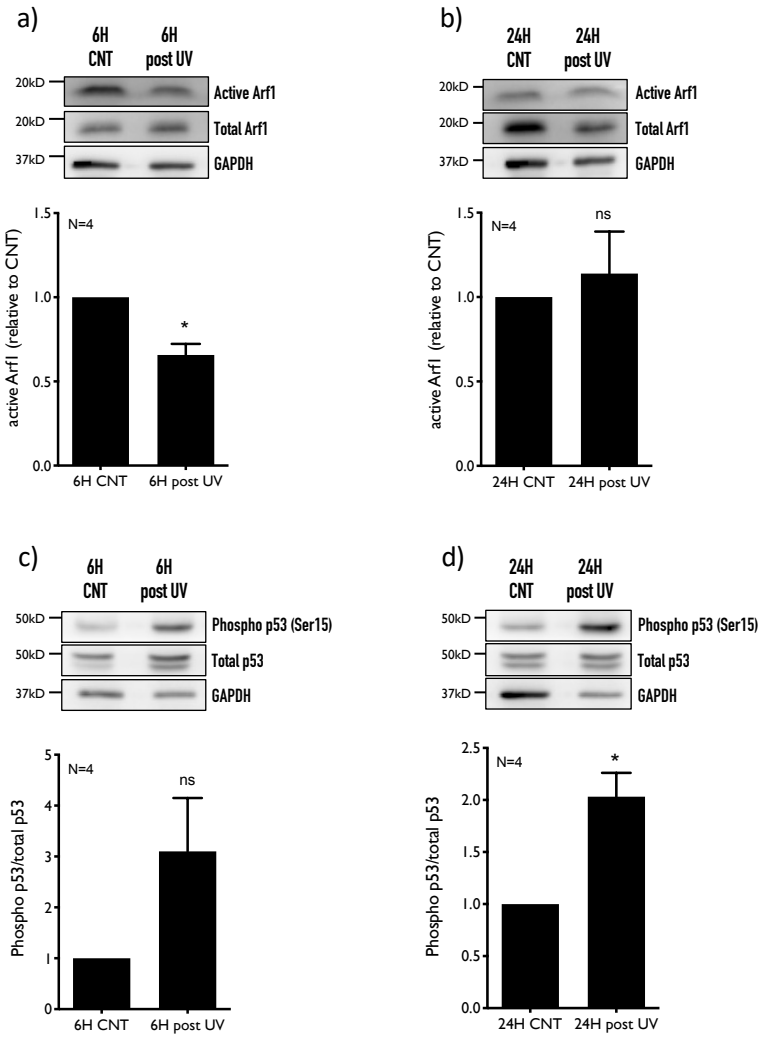


Figure 7.2: Effect of UV treatment mediated DNA damage on active ARF1 levels in adherent MCF10A cells – Normal human breast epithelial cells MCF10A were treated with UV radiation (10J/m^2), post 16hours of seeding. Cells were processed for active ARF1 pull-down 6hours and 24hours post UV induction. Active ARF1 levels 6hours (a) and 24hours (b) post UV treatment in adherent MCF10A cells. Effect of UV treatment on levels of DNA damage marker phosphorylated p53 post 6hrs (c) and 24hrs (d) of UV induction in adherent MCF10A cells. Statistical analysis was done using single sample Wilcoxon t test.

Chapter 8

Discussions

Discussion

The work presented in this thesis investigates the role and regulation of adhesion-dependent Golgi organization and function in normal vs cancer cells. It mainly focuses on how this regulation is perturbed in certain anchorage-independent cancers. We identify AXL as a novel regulator of the altered Golgi organization in cancer and provide some mechanistic insights into its regulatory role at the Golgi. Later, the thesis evaluates the changes in cell surface glycosylation levels as a function of gradual changes in the Golgi organization. We also propose a model explaining how alterations in the Golgi organization could variably affect different cell surface glycans.

8.1 Adhesion-dependent regulation of Golgi organization is variable in cancers

Variability in Golgi organization in adherent cancer cells is well documented in the literature (Kellokumpu et al., 2002; Petrosyan, 2015; Petrosyan et al., 2014), and this was confirmed by our data in several cancer cell lines as tested. We also show that adhesion-dependent regulation of Golgi organization, seen in normal cells (Singh et al., 2018), is variably altered in different cancers. In 'normal' cells, loss of adhesion-mediated Golgi disorganization affects cell surface glycosylation (Singh et al., 2018). In cancer cells, the effect of its altered Golgi organization on cancer cell function has only recently been explored (Petrosyan, 2015). The adhesion-independent regulation of the Golgi organization in cancers, shown in our study, is a novel change mediated by a physiological loss of adhesion, which could have implications for cancer progression. Studies have shown changes in Golgi organization to alter the cell surface glycosylation levels and protein glycosylation signatures (Bajaj et al., 2022; Bhat et al., 2017; Petrosyan et al., 2014) in cancers. Such changes in glycosylation are known to promote resistance to anoikis (Piyush et al., 2017), regulate growth factor signaling (Ferreira et al., 2018), invasiveness (Arriagada et al., 2018; Huanna et al., 2015), angiogenesis (Imamaki et al., 2018), evasion of immune response (Demetriou et al., 2001), regulate gene expression (Křivohlavá et al., 2018; Tajadura-Ortega et al., 2019), promote drug resistance (Lopez Sambrooks et al., 2018; Very et al., 2018), and cell migration (Arriagada et al., 2018; Janik et al., 2010; Petrosyan, 2015). Golgi organization being a major driver of glycosylation changes, its altered phenotype on loss of adhesion, as shown in our study, could have significant implications in anchorage-independent cancers, which remains to be thoroughly assessed.

In adherent cancer cells, both disorganized and intact Golgi phenotypes could be deemed as 'altered' since they both could be regulated in non-canonical ways (as seen in our study) and actively support cancer progression (Petrosyan, 2015). Luo et al. showed that maintaining Golgi organization supported tumor progression in nasopharyngeal carcinoma (Luo et al., 2021). On the contrary, Petryosan and group reported the disorganized Golgi in androgen refractory prostate cancer to support its more aggressive nature relative to non-androgen refractory cells having intact Golgi organization (Petrosyan et al., 2014; Ward et al., 2001). A study by Kellokumpu et al., as well as results from our study, showed that MDAMB231 cells have an intact Golgi, which is a known aggressive cancer cell line, relative to the mildly aggressive MCF7 cell line (Kellokumpu et al., 2002), which has a disorganized Golgi. Further, while Golgi compaction is suggested to support EMT (Tan et al., 2017), cancer EMT, often considered as a hybrid population (E/M), presents a heterogenous mix of both organized and disorganized Golgi (Bui et al., 2021). In our study, A549, known to be a hybrid population for EMT (N. Kim et al., 2023), predominantly shows an intact Golgi.

On the other hand, the mesenchymal cells, CaLu1, were observed to predominantly show a disorganized Golgi, unlike the intact Golgi observed in mesenchymal MDAMB231 cells. However, the disorganized Golgi in CaLu1 is similar to the Golgi in MCF7 cells, which is epithelial. Taken together, whether a conserved paradigm exists for either intact Golgi or disorganized Golgi, which is more frequently associated with aggressive cancers or certain cancer cell functions, remains unclear.

The altered Golgi organization phenotypes, shown in our data, also lead us to ask what implications restoration of Golgi organization in these cancers could have on the cells. Targeting the Golgi as a tool for cancer therapy has recently been explored as a promising approach (Khine & Sakurai, 2023; Ohashi et al., 2018), and restoration of altered Golgi in cancers could help our understanding of how, why and when Golgi targeting could be of relevance in cancer. The suggestion that anchorage-dependent Golgi could be de-regulated in cancers and could have implications for anchorage independence could also reflect how cancer cells respond to a matrix of varying stiffness. Changes in matrix organization and stiffness and their contribution to the oncogenic potential of cancers are well documented (Ishihara & Haga, 2022; Jiang et al., 2022). Studies also suggest Golgi apparatus's physical

and functional coupling to extracellular matrix stiffness (Martino et al., 2018; Mascanzoni et al., 2022b; Romani et al., 2019). Could the adhesion-dependent regulation of the Golgi affect its organization in response to changing matrix stiffness, and if this regulation is altered in cancers to impact their oncogenic and metastatic potential, it is worth exploring.

Our results discuss the Golgi organization in a cell line based on the predominant phenotype observed in a population of cells. In contrast, a small percentage of the population was constantly observed to present a different phenotype. Such heterogeneity in cancers supports their resilience and metastasis (Cluett et al., 1997; Pally et al., 2021). Could this heterogeneity observed in the Golgi organization promote cancer cell function? It is worth asking. Detection and physical separation of heterogeneous cell populations is rather challenging. Achieving this could, however, provide valuable insights into how heterogenous groups of the same cell population differ with respect to their regulation and functions.

8.2 Role of ARF1 activation in mediating altered Golgi organization in cancers

The Golgi-associated small GTPase Arf1 is required to maintain the Golgi organization, mediated by its activation at the Golgi membranes by specific GEFs (Donaldson et al., 2005; Ward et al., 2001). On loss of adhesion, a significant decrease in active Arf1 levels leads to Golgi disorganization (Singh et al., 2018). This adhesion-dependent regulation of Arf1 activation was found to be altered in T24 cells (bladder cancer), unlike most other cancers tested in our study. Overexpression of Arf1 has been reported in multiple cancers, including breast, prostate, gastric, hepatocellular, osteosarcoma and colorectal cancers (Casalou et al., 2016a; Haines et al., 2015; Xie et al., 2016). Its aberrant expression or activation is correlated with poor prognosis in cancers (Casalou et al., 2020) and is known to promote cancer cell functions such as migration (Lewis-Saravalli et al., 2013), drug resistance (Haines et al., 2015; Luchsinger et al., 2018), and invasiveness (Lewis-Saravalli et al., 2013; Schlienger et al., 2014). Our data suggests novel and variable roles for Arf1 in mediating adhesion-independent regulation of the Golgi in cancers, which could have implications for the above cancer cell functions.

In T24 cells, sustained Arf1 activation on loss of adhesion was seen to support an intact Golgi organization. In other cancer cells, A549, UMUC3, and CaLu1, loss of adhesion-mediated Arf1 depletion had no effect on Golgi's organization. This could suggest active Arf1 to be a bystander in maintaining Golgi in these non-adherent cancer cells. Further depletion of Arf1, however, led to Golgi disruption, meaning that all cancer cells tested in our study are sensitive to loss of active Arf1, as seen in published studies as well (Sciaky et al., 1997b; Ward et al., 2001), though the sensitivity seems to kick in at varying basal levels of active Arf1 for different cells, as shown in our study. Further, in A549 cells, we identified a possible crosstalk between Arf1 and AXL, which regulates the adhesion-independent Golgi organization in these cells. Taken together, how this differential regulation of Arf1, in different non-adherent cells, affects Golgi and hence cancer cell functions remain to be tested.

Additionally, it is worth exploring if sustained active Arf1 on loss of adhesion can support anchorage independence in cancers. Such alterations in Arf1 activation can, in turn, be driven by the altered role of their GEFs and GAPs effectors. In line with this, several cancers indeed show abnormal expression levels of different GEFs and GAPs, which affect the activation of Arf1 and, hence, cancer progression (Casalou et al., 2016b, 2020; Walton et al., 2020).

These roles for Arf1 could be mediated by its regulation of Golgi organization or even beyond the Golgi, such as through its role in post-Golgi trafficking events (Adarska et al., 2021; Donaldson et al., 2005). Altered regulation of Arf1 and other Arf family (Ar3, Arf4, Arf5) proteins could have concerted effects on Golgi and cancer cell functions. Arf3 is mainly associated with the TGN and endosomal compartments, while Arf4 and Arf5 are known to be associated with the ERGIC, cis-medial, and TGN compartments (Adarska et al., 2021; Manolea et al., 2010). All four Arfs are involved in post-Golgi trafficking events and are differentially implicated in cancer cell progression (Adarska et al., 2021). The sequence similarities between the Arf family present difficulties in studying specific roles of different Arfs family proteins. Systematic gene editing approaches have provided some early insights into redundant vs distinct roles of different Arfs (Pennauer et al., 2022). Whether differential adhesion-dependent regulation of Golgi-associated Arfs affects the Golgi organization and functional outcomes, and if these outcomes are altered in cancers is worth exploring.

Additionally, Arf1 is known to associate with other Golgi-associated proteins such as GMAP210, Golgin160 and AXL (Adarska et al., 2021; Casalou et al., 2020; Haines et al., 2015; S. Yadav et al., 2012). Whether regulation of onco-Golgi and other cancer cell functions, mediated by altered regulation of Arf1, involves a role for these Arf1-associated players in non-adherent cancer cells remains to be tested.

8.3 AXL regulates adhesion-independent Golgi organization in A549 cells

The screening for differentially expressed genes in cancer cell lines with differential Golgi organization and the selection of lung cancer cells to evaluate the same is well justified by the identification of AXL as a regulator of Golgi organization. Using multiple parameters to rank and shortlist genes could hence be a simple and practical approach to identifying candidates, as done here. Identifying AXL also suggests the other candidate genes identified could be essential. Further studies in the lab focus on other candidates in the screen and test their possible crosstalk with AXL.

AXL was first discovered as an oncogene in CML patients (Zhu et al., 2019) and has now been extensively studied for its various roles in cancer progression, including cell proliferation, gene expression, cell survival, drug resistance, cell migration and cell adhesion (Auyez et al., 2021b; Duan et al., 2019a; C. C. Hong et al., 2008; J. Li et al., 2012; Scaltriti et al., 2016; Zajac et al., n.d.). Our study identified a novel role of AXL in regulating adhesion-independent Golgi organization in lung cancer cells, A549. To date, only two studies suggest a direct role for AXL at Golgi. In breast cancer cells, Hs578t, AXL was tentatively shown to colocalize with cis-Golgi marker (GM130) and regulate Golgi positioning and polarity to promote cell migration (Zajac et al., 2020). In a separate study, Chia et al. conducted a kinome and phosphotome RNAi screen for regulators of Golgi organization in HeLa cells, which showed AXL KD to promote a condensed Golgi phenotype (Chia et al., 2012a). This effect of AXL targeting, being different from our study where AXL inhibition led to Golgi disorganization in non-adherent A549 cells, could stem from the differences in cells used to test the same (HeLa vs A549) and the possible role adhesion could have in mediating his. Chia et al. further showed that AXL KD causes a

decrease in cell surface glycans, GalNAc and GlucNAc, as is additionally seen in the R428 treatment of non-adherent A549 cells. A549 cells show a reduction in GlcNAc, Sialic acid and Mannose levels but not in GalNAc. This difference could reflect the differential Golgi organization between HeLa and A549 cells upon AXL targeting as well as the adherent vs non-adherent conditions under which these cells were tested. The processing of glycans is known to occur sequentially in defined Golgi compartments (Stanley, 2011). The differential effect AXL targeting has on the Golgi organization in A549 and HeLa cells could also differentially affect the spatial organization of cis- vs trans-Golgi compartments. This could further contribute to differences in the regulation of cell surface glycans, which is worth evaluating.

Together, these data suggest the need to evaluate the effect of AXL targeting on adhesion-dependent Golgi organization and function in different cancer cells. Further investigations in 'normal', non-transformed cells will also provide more insights into the conserved role of AXL at the Golgi in adherent and non-adherent conditions. Our future studies will be focusing on both.

Another study in breast cancer cells showed immunoprecipitation of Golgi-associated GTPase Arf1 to contain AXL (Haines et al., 2015), which indirectly suggests that AXL could be associated with Golgi organization or function. In line with this, results from our study indicate that AXL binds active Arf1 and restores adhesion-dependent Golgi organization in A549 cells, which crosstalk of AXL with Arf1 could mediate. This adhesion-dependent regulation of Golgi by AXL raises the possibility of adhesion regulating AXL expression and/or activation, which has not been tested so far. We observed a partial but significant loss in AXL localization at the Golgi in non-adherent A549 cells. This suggests a novel role for adhesion signaling in regulating AXL localization, which could be more pronounced in 'normal' cells and is worth exploring. Adhesion-dependent regulation of AXL, if confirmed, could further have far-reaching implications for the role of AXL in cells and its regulation of downstream signaling and function.

Additionally, the roles of AXL discussed above in regulating Golgi organization in cancer need to be looked at in context of its regulation by the Gas6 ligand. This ligand holds a high affinity for AXL relative to other TAM family receptors (Zhai et al., 2023). In our

study, whether the Gas6-mediated activation of AXL is involved in the AXL-mediated regulation of the Golgi organization in A549 cells remains to be tested. These studies are now in progress. Further, if this role for Gas6 is differential on the loss of adhesion, it is worth exploring how it could support AXL's adhesion-dependent regulation of Golgi. Most studies implicating roles for AXL in cancer progression invoke the Gas6-mediated activation of AXL (Chen et al., 2018; Duan et al., 2019b; Holland et al., 2010). However, this activation could be dispensable in some cancers that override the requirement for Gas6. For instance, AXL but not Gas6 knockdown rescued the AXL overexpression-driven drug resistance to EGFR TKI inhibitors in NSCLC (Z. Zhang et al., 2012). Is Gas6 activation AXL localized at the Golgi and what that could mean to its localization and role at the Golgi needs careful examination.

Among AXL's various oncogenic roles, its impact on promoting drug resistance is well established. Studies suggest AXL targeting overcomes drug resistance against chemotherapeutic agents and RTK inhibitors across different cancers such as NSCLC, breast cancer, pancreatic cancer, acute myeloid leukaemia, ovarian cancer and glioblastoma (Auyez et al., 2021a; Debruyne et al., 2016; C. C. Hong et al., 2008; Okura et al., 2020; Scaltriti et al., 2016; Z. Zhang et al., 2012; Zhu et al., 2019). Interestingly, while targeting the Golgi disruption in NSCLC tumors was also shown to promote resistance to EGFR-specific RTK inhibitors (Ohashi et al., 2018), Arf1 inhibition was established to promote resistance to Chemotherapy (Haines et al., 2015; Luchsinger et al., 2018; Onken et al., 2016), and RTK inhibitors, in breast cancer cells. We see that AXL, Arf1 and Golgi are all independently shown to promote drug resistance in cancers. However, there is a gap in our understanding of whether these players work together to support drug resistance in cancers. Our data presents a novel AXL-Arf1-Golgi organization regulatory axis, a role for which drug resistance is worth investigating. Similarly, AXL (Bi et al., 2017; Onken et al., 2016; Zajac et al., 2020), Arf1 (Casalou et al., 2016b; Lewis-Saravalli et al., 2013), and Golgi organization (Isaji et al., 2014; Millarte & Farhan, 2012; Zajac et al., 2020) are shown to enhance cancer cell migration. A concerted role for AXL, Arf1, and subsequently Golgi's organization in promoting cancer cell migration is worth testing.

In our study, AXL inhibition decreased cell surface levels of Mannose, Sialic acid and N-GlcNAc glycans. Upregulation of cell surface glycosylation is suggested to support anoikis

resistance (Piyush et al., 2017) and anchorage-independence in cancers (Reticker-Flynn & Bhatia, 2015; Stowell et al., 2015). We do see that AXL inhibition suppresses anchorage independence in A549 cells. This suggests that AXL-mediated regulation of Golgi organization and glycosylation could support anchorage independence in cancers that remain to be tested. The lectins used in the study bind glycan moieties that can be classified as N-glycans or O-glcyans (ConA- N-glycans, WGA – N- and O-glycan, PNA – O-glycans. It is unclear if AXL-mediated regulation of Golgi organization and resulting effect on glycosylation could be specific to either N- or O-glycans or if it can be a general effect.

Taken together, having arrived at AXL as a novel regulator of the Golgi organization, which we show localizes to the Golgi, several questions arise which remain to be explored. One primary question is how AXL localizes at the Golgi. Do endocytic pathways drive this localization? Does its possible binding to Arf1 solely mediate it, or are other Golgi-associated regulators involved? Further, it must be evaluated if AXL localization at the Golgi requires it to be in its active (phosphorylated) form. If and how R428 mediated inhibition of AXL pool at the cell membrane has a role in the regulation of Golgi organization or cell surface glycosylation and whether this inhibition overrides the action of Gas6 stimulation in non-adherent cells also remains to be addressed.

8.4 Gradual changes in Golgi organization differentially affect cell surface levels of ConA and WGA glycan substrates

Golgi apparatus can assume numerous organizational phenotypes from mild disorganization to complete fragmentation (Bajaj et al., 2022; Marsh & Howell, 2002; Petrosyan, 2019), which affects its glycosylation and trafficking functions in differential and complex ways (B.R. et al., 2023; Petrosyan et al., 2012; Pothukuchi et al., 2019b; Stanley, 2011). Non-adherent cells with a distinct loss of adhesion-mediated Golgi disorganization provide a unique model system, which helps characterize intact, disorganized, or fragmented Golgi organization phenotypes using BFA/GCA treatments. Tuning Arf1 activation with BFA, GCA treatment has a distinct and reproducible impact on the Golgi organization. Earlier studies using this paradigm have determined how Arf1 activation kinetics are relevant in regulating Golgi organization and function (B.R. et al., 2023), which has been largely unknown. Our study used an increasing range of BFA concentrations to obtain a gradient of Golgi phenotypes from disorganization to complete

fragmentation. This reproducible and fine-tuned gradient in Golgi phenotypes differentially affected the levels of cell surface glycans (detected using ConA and WGA lectins). These changes were broadly comparable (30 minutes vs 90 minutes of treatment). It is important to note that the ConA glycan substrate is processed predominantly in the cis-Golgi, while WGA glycan substrates are primarily processed in the trans-Golgi (Stanley, 2011).

Interestingly, our previous study reported that BFA caused complete fragmentation of cis-medial Golgi but not trans-Golgi, which maintains its disorganized phenotype in non-adherent cells on BFA treatment (B.R. et al., 2023). Could the varying response of ConA vs WGA surface binding to BFA stem from the differential response of their respective modification sites in Golgi? It is worth evaluating. Further, the endocytic rates of these surface-bound lectins were found to remain unaffected by BFA-mediated changes in the Golgi, suggesting the cell surface glycan levels to essentially be an effect of Golgi organization and independent of their endocytic regulation. Though BFA does not affect endocytosis in our study at the concentrations tested (0.1μM and 1.8μM), it may, however, increase endocytosis at higher concentrations, as shown in human fibroblasts (Damke et al., 1991) and MDCK (Prydz et al., 1992) cells.

Additionally, the contribution of Golgi to plasma membrane trafficking in mediating these differential effects on cell surface binding of lectins remains to be tested. BFA-mediated Arf1 inactivation prevents COP1 coat protein-mediated vesicle formation, which affects exocytosis (Sciaky et al., 1997c; Strous et al., 1993; Ward et al., 2001). Cellular trafficking is also known to be regulated by the Golgi organization, mechanisms for which are poorly understood (Stalder & Gershlick, 2020a; Yarwood et al., 2020). Studies have reported the Golgi disorganization-mediated increase in surface area available for vesicle budding in Golgi membranes, to support increased rates of trafficking to the cell membrane (Bekier et al., 2017; X. Zhang & Wang, 2015). A mathematical study by Mani et al. also supports this increase in trafficking rates. It suggests its effect on the retention times of cargo in the Golgi compartments, which further affects glycan processing (Mani & Thattai, 2016). However, complete Golgi fragmentation and fall back into ER on BFA treatment, could differently affect trafficking rates, suppressing Golgi function altogether. The effect of varying BFA concentrations on Golgi to cell membrane trafficking, localization and availability of glycosylation enzymes, and retention times of substrate glycans in Golgi

compartments, are all worth evaluating to obtain a deeper understanding of how Golgi dynamics regulate Golgi function.

Evaluation of such complex systems can benefit from mathematical data modelling to obtain hidden insights into their regulatory paradigms. Few such studies jointly measure Golgi morphology and function (glycosylation) in the analytical sense. Dr. Mukund and Aashish generated a mathematical model based on existing literature depicting glycan processing and trafficking in the cell through the Golgi apparatus. Our results from BFA-mediated fragmentation changes of the Golgi and the quantitative evaluation for endocytosis of cell surface-bound ConA vs WGA lectins were incorporated into the model to fill information gaps. The high throughput and comprehensive nature of single-cell data obtained with Flow cytometry experiments complemented the consistency in results obtained for both fluorophores with the same lectin. Following a series of systematic normalizations, this data for cell surface levels of ConA and WGA glycan substrates further helped bring the model closer to what happens in cells. As discussed earlier, ConA and WGA glycan substrates are processed in different compartments (Stanley, 2011), and ConA glycan substrate is also a forming intermediate for WGA (Sharma et al., 2014). This association also has significance in how changes in cell surface ConA vs WGA lectin levels are interpreted. Fitting the model to this understanding and the above data, we propose that at lower BFA concentrations, depletion of influx from Golgi, could affect synthesis of ConA substrate, which seems to directly reflect on its cell surface levels. Additionally, the synthesis rate of WGA glycan substrates could be higher at these low BFA concentrations, thereby explaining the initial lack of effect on its cell surface levels. The model thereby suggests a plausible theory for how differential regulation of Golgi could affect glycan processing and trafficking.

Therefore, we provide preliminary insights into the Golgi form and function interplay. The proposed model is straightforward in terms of unknown parameters, as most of the derivation is based on cell-biological measurements. The robustness of the fits with small number of parameters gives confidence in the assumptions underlying the model. The model also precisely and quantitatively shows how Golgi morphology impacts cell-surface glycan levels as a function of concentration and time. We believe this is the first time such a quantitative agreement has been obtained between cell biology and biochemistry of the Golgi. Understanding the contribution of recycling vs. new

biosynthesis of glycans for experiments up to 90 minutes is one of the critical quantitative outcomes of fitting our data. Additionally, BFA is shown to have an impact not just at the cis-Golgi but also directly at the trans-Golgi. We suggest this could be due to an increase in the residence time of glycans at the trans-Golgi, which is worth probing in the future.

Several such mathematical models have been generated using high throughput data, which are valuable in extracting information on complex biological systems. A similar study in by Fisher et al, build a computational model for glycan biosynthesis to understand how differential glycan profiles are generated in context of altered cell states, including intra-Golgi trafficking defects and cellular differentiation (Fisher et al., 2019). Another study proposes a mathematical model based on the chemical synthesis of glycans that quantitatively assessed a tradeoff between the number and specificities of glycosylation enzymes and the number of cisternae required to arrive at a cell surface glycosylation distribution of a defined complexity and accuracy (A. Yadav et al., 2022). The simple nature of the generated model could be vital in furthering our complex understanding of these processes. Such models are also suggested to help better understand the glycosylation changes in diseased states, identifying disease biomarkers and contributing to glycoengineering systems.

8.5 Role of ARF1 in DNA damage-mediated Golgi dispersal

DNA damage-mediated Golgi disorganization was recently reported in cancer cells. This was shown to be mediated by DNA damage-mediated activation of DNA-PK, which phosphorylates GOLPH3, thereby causing Golgi disorganization (Buschman et al., 2015b; Farber-Katz et al., 2014). Though GOLPH3 could be associated with the GTPase Arf1 (Iyer et al., 2018), whether Arf1 activation is involved in DNA-PK – GOLPH3 – Golgi disorganization axis, has not been tested so far. We present preliminary observations in adherent MCF10A cells for the effect of DNA damage on active Arf1 levels. At the early stages of DNA damage induction, we show that active Arf1 levels drop distinctly, suggesting a role in the early response pathway on UV induction. Recovery of active Arf1 at later time points, in the presence of Golgi disorganization, suggests a different regulatory path to sustain the observed Golgi disorganization. Whether this recovered pool of active Arf1 localizes to the Golgi remains to be tested. Many chemotherapeutic drugs used in

cancer therapy aim to induce DNA damage in cancer cells (Jeggo et al., 2016; Ramkumar et al., 2021; Torgovnick & Schumacher, 2015). While the effect of DNA damage on nuclear functions has been extensively studied, its impact on cytoplasmic activities is not well known (Farber-Katz et al., 2014). Understanding the mechanisms of DNA Damage response in cancer cells is particularly important given the use of DNA-damaging agents in therapy and the development of resistance against these agents in cancers (L. Y. Li et al., 2021). GOLPH3, which mediates DNA damage-induced Golgi disorganization, is shown to be overexpressed across several cancers (Hu et al., 2013; Scott et al., 2009). This overexpression is suggested to be strongly corelated with poor prognosis and resistance in cancer cells to DNA damaging agents (Farber-Katz et al., 2014; Hu et al., 2013). Arf1 is also shown to overexpress in several cancers and is suggested to promote resistance to chemotherapeutic agents (Haines et al., 2015; Xie et al., 2016). Whether Arf1 is involved in DNA damage response pathway, whether it associates with GOLPH3 in this pathway, and whether it further promotes drug resistance through its regulation of the Golgi, requires further evaluation. Additionally, the role of the Golgi organization in DNA damage response cascades, which could be altered in cancers to promote the survival of cancer cells, is worth exploring.

IN CONCLUSION

Present work shows that adhesion-dependent regulation of the Golgi organization, to be variable in different cancers. We identify AXL as a novel regulator of the adhesion-independent Golgi organization and function in A549 cells. Results suggests, this role of AXL at the Golgi, to possibly be mediated by a regulatory crosstalk between AXL and the small GTPase Arf1, which needs further exploring. Additionally, we obtained a profile for changes in cell surface glycosylation levels along a gradient of BFA-mediated changes in Golgi organization and changes in glycan endocytosis. Using a comprehensive mathematical model, looking at Golgi trafficking, processing of glycans, their delivery to the plasma membrane and their endocytosis, our data was used to provide early mechanistic insights into how changes in Golgi organization could variably affect cell surface glycosylation. It is worth testing if this predicted understanding of Golgi structure-function dynamics holds in experimental outcomes.

REFERENCES

Abeliovich, A., & Gitler, A. D. (2016). Defects in trafficking bridge Parkinson's disease pathology and genetics. *Nature*, *539*(7628), 207–216. https://doi.org/10.1038/NATURE20414

Adarska, P., Wong-Dilworth, L., & Bottanelli, F. (2021). ARF GTPases and Their Ubiquitous Role in Intracellular Trafficking Beyond the Golgi. *Frontiers in Cell and Developmental Biology*, *9*. https://doi.org/10.3389/FCELL.2021.679046

Aebi, M. (2013). N-linked protein glycosylation in the ER. *Biochimica et Biophysica Acta*, *1833*(11), 2430–2437. https://doi.org/10.1016/j.bbamcr.2013.04.001

Aebi, M., & Hennet, T. (2001). Congenital disorders of glycosylation: genetic model systems lead the way. *Trends in Cell Biology*, *11*(3), 136–141. https://doi.org/10.1016/S0962-8924(01)01925-0

Agliarulo, I., & Parashuraman, S. (2022). Golgi apparatus regulates plasma membrane composition and function. *Cells*, *11*(3). https://doi.org/10.3390/cells11030368

Ahat, E., Bui, S., Zhang, J., da Veiga Leprevost, F., Sharkey, L., Reid, W., Nesvizhskii, A. I., Paulson, H. L., & Wang, Y. (2022). GRASP55 regulates the unconventional secretion and aggregation of mutant huntingtin. *The Journal of Biological Chemistry*, *298*(8), 102219. https://doi.org/10.1016/J.JBC.2022.102219

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., & Walter, P. (2002). *Transport from the ER through the Golgi Apparatus*. https://www.ncbi.nlm.nih.gov/books/NBK26941/

ALLFREY, V., DALY, M. M., & MIRSKY, A. E. (1953). Synthesis of protein in the pancreas. II. The role of ribonucleoprotein in protein synthesis. *The Journal of General Physiology*, *37*(2), 157–175. https://doi.org/10.1085/JGP.37.2.157

Anandi, L., Chakravarty, V., Ashiq, K. A., Bodakuntla, S., & Lahiri, M. (2017). DNA-dependent protein kinase plays a central role in transformation of breast epithelial cells following alkylation damage. *Journal of Cell Science*, *130*(21), 3749–3763. https://doi.org/10.1242/JCS.203034

Ángel Martínez-Menárguez, J., Tomás, M., Martínez-Martínez, N., & Martínez-Alonso, E. (2019). *cells Golgi Fragmentation in Neurodegenerative Diseases: Is There a Common Cause?* https://doi.org/10.3390/cells8070748

Arriagada, C., Silva, P., & Torres, V. A. (2018). Role of glycosylation in hypoxia-driven cell migration and invasion. *Cell Adhesion & Migration*, 1–10. https://doi.org/10.1080/19336918.2018.1491234

Auyez, A., Sayan, A. E., Kriajevska, M., & Tulchinsky, E. (2021a). AXL Receptor in Cancer Metastasis and Drug Resistance: When Normal Functions Go Askew. *Cancers 2021, Vol. 13, Page 4864*, *13*(19), 4864. https://doi.org/10.3390/CANCERS13194864

Auyez, A., Sayan, A. E., Kriajevska, M., & Tulchinsky, E. (2021b). Axl receptor in cancer metastasis and drug resistance: when normal functions go askew. In *Cancers* (Vol. 13, Issue 19). MDPI. https://doi.org/10.3390/cancers13194864

Ayala, I., & Colanzi, A. (2022). Structural organization and function of the golgi ribbon during cell division. *Frontiers in Cell and Developmental Biology*, *10*, 925228. https://doi.org/10.3389/fcell.2022.925228

Ayala, I., Mascanzoni, F., & Colanzi, A. (2020). The Golgi ribbon: mechanisms of maintenance and disassembly during the cell cycle. *Biochemical Society Transactions*, *48*(1), 245–256. https://doi.org/10.1042/BST20190646

Bajaj, R., Warner, A. N., Fradette, J. F., & Gibbons, D. L. (2022). Dance of the golgi: understanding golgi dynamics in cancer metastasis. *Cells*, *11*(9). https://doi.org/10.3390/cells11091484

Banfield, D. K. (2011). Mechanisms of protein retention in the Golgi. *Cold Spring Harbor Perspectives in Biology*, *3*(8), a005264. https://doi.org/10.1101/cshperspect.a005264

Barbara Mullock, A. M., & Paul Luzio, J. (2000). *A service of the National Library of Medicine, National Institutes of Health. Madame Curie Bioscience Database*. Landes Bioscience.

Barretina, J., Caponigro, G., Stransky, N., Venkatesan, K., Margolin, A. A., Kim, S., Wilson, C. J., Lehár, J., Kryukov, G. V., Sonkin, D., Reddy, A., Liu, M., Murray, L., Berger, M. F., Monahan, J. E., Morais, P., Meltzer, J., Korejwa, A., Jané-Valbuena, J., … Garraway, L. A. (2012). The Cancer Cell Line Encyclopedia enables predictive modeling of anticancer drug sensitivity. *Nature*, *483*(7391), 603. https://doi.org/10.1038/NATURE11003

Barretta, M. L., Spano, D., D'Ambrosio, C., Cervigni, R. I., Scaloni, A., Corda, D., & Colanzi, A. (2016). Aurora-A recruitment and centrosomal maturation are regulated by a Golgi-activated pool of Src during G2. *Nature Communications 2016 7:1*, *7*(1), 1–13. https://doi.org/10.1038/ncomms11727

Baschieri, F., & Farhan, H. (2012). Crosstalk of small GTPases at the Golgi apparatus. *Small GTPases*, *3*(2), 80–90. https://doi.org/10.4161/sgtp.19842

Bassagañas, S., Carvalho, S., Dias, A. M., Pérez-Garay, M., Ortiz, M. R., Figueras, J., Reis, C. A., Pinho, S. S., & Peracaula, R. (2014). Pancreatic cancer cell glycosylation regulates cell adhesion and invasion through the modulation of α2β1 integrin and E-cadherin function. *Plos One*, *9*(5), e98595. https://doi.org/10.1371/journal.pone.0098595

Batisse, C., Marquet, J., Greffard, A., Fleury-Feith, J., Jaurand, M.-C., & Pilatte, Y. (2004). Lectin-based three-color flow cytometric approach for studying cell surface glycosylation changes that occur during apoptosis. *Cytometry. Part A: The Journal of the International Society for Analytical Cytology*, *62*(2), 81–88. https://doi.org/10.1002/cyto.a.20094

Bekier, M. E., Wang, L., Li, J., Huang, H., Tang, D., Zhang, X., & Wang, Y. (2017). Knockout of the Golgi stacking proteins GRASP55 and GRASP65 impairs Golgi structure and function. *Molecular Biology of the Cell*, *28*(21), 2833–2842. https://doi.org/10.1091/mbc.E17-02-0112

Bellouze, S., Schä fer, M. K., Buttigieg, D., Baillat, G., Rabouille, C., & Haase, G. (n.d.). *Golgi fragmentation in pmn mice is due to a defective ARF1/TBCE cross-talk that coordinates COPI vesicle formation and tubulin polymerization*. https://doi.org/10.1093/hmg/ddu320

Berrier, A. L., & Yamada, K. M. (2007). Cell-matrix adhesion. *Journal of Cellular Physiology*, *213*(3), 565–573. https://doi.org/10.1002/jcp.21237

Bhat, G., Hothpet, V.-R., Lin, M.-F., & Cheng, P.-W. (2017). Shifted Golgi targeting of glycosyltransferases and α-mannosidase IA from giantin to GM130-GRASP65 results in formation of high mannose N-glycans in aggressive prostate cancer cells. *Biochimica et Biophysica Acta*, *1861*(11 Pt A), 2891–2901. https://doi.org/10.1016/j.bbagen.2017.08.006

Bi, S., Wang, C., Li, Y., Zhang, W., Zhang, J., Lv, Z., & Wang, J. (2017). LncRNA-MALAT1-mediated Axl promotes cell invasion and migration in human neuroblastoma. *Tumour Biology : The Journal of the International Society for Oncodevelopmental Biology and Medicine*, *39*(5). https://doi.org/10.1177/1010428317699796

Bor, G., Mó Rotz, M., De Vos, K. J., Vagnoni, A., Ackerley, S., Shaw, C. E., & Miller, C. C. J. (n.d.). *Amyotrophic lateral sclerosis-associated mutant VAPBP56S perturbs calcium homeostasis to disrupt axonal transport of mitochondria*. https://doi.org/10.1093/hmg/dds011

B.R., R., Shah, N., Joshi, P., Madhusudan, M. S., & Balasubramanian, N. (2023). Kinetics of Arf1 inactivation regulates Golgi organisation and function in non-adherent fibroblasts. *Biology Open*, *12*(4). https://doi.org/10.1242/BIO.059669

Brownhill, K., Wood, L., & Allan, V. (2009). Molecular motors and the Golgi complex: Staying put and moving through. In *Seminars in Cell and Developmental Biology* (Vol. 20, Issue 7, pp. 784–792). Elsevier Ltd. https://doi.org/10.1016/j.semcdb.2009.03.019

Bui, S., Mejia, I., Díaz, B., & Wang, Y. (2021). Adaptation of the Golgi Apparatus in Cancer Cell Invasion and Metastasis. *Frontiers in Cell and Developmental Biology*, *9*. https://doi.org/10.3389/FCELL.2021.806482

Burkhardt, J. K. (n.d.). *The role of microtubule-based motor proteins in maintaining the structure and function of the Golgi complex*.

Buschman, M. D., Xing, M., & Field, S. J. (2015a). The GOLPH3 pathway regulates Golgi shape and function and is activated by DNA damage. *Frontiers in Neuroscience*, *9*, 362. https://doi.org/10.3389/fnins.2015.00362

Buschman, M. D., Xing, M., & Field, S. J. (2015b). The GOLPH3 pathway regulates Golgi shape and function and is activated by DNA damage. *Frontiers in Neuroscience*, *9*, 362. https://doi.org/10.3389/fnins.2015.00362

Carlton, J. G., Jones, H., & Eggert, U. S. (2020). Membrane and organelle dynamics during cell division. *Nature Reviews. Molecular Cell Biology*, *21*(3), 151–166. https://doi.org/10.1038/s41580-019-0208-1

Casalou, C., Faustino, A., & Barral, D. C. (2016a). Arf proteins in cancer cell migration. *Small GTPases*, *7*(4), 270. https://doi.org/10.1080/21541248.2016.1228792

Casalou, C., Faustino, A., & Barral, D. C. (2016b). Arf proteins in cancer cell migration. *Small GTPases*, *7*(4), 270–282. https://doi.org/10.1080/21541248.2016.1228792

Casalou, C., Ferreira, A., & Barral, D. C. (2020). The role of ARF family proteins and their regulators and effectors in cancer progression: A therapeutic perspective. *Frontiers in Cell and Developmental Biology*, *8*, 217. https://doi.org/10.3389/fcell.2020.00217

Cervigni, R. I., Bonavita, R., Barretta, M. L., Spano, D., Ayala, I., Nakamura, N., Corda, D., & Colanzi, A. (2015). JNK2 controls fragmentation of the Golgi complex and the G2/M transition through phosphorylation of GRASP65. *Journal of Cell Science*, *128*(12), 2249–2260. https://doi.org/10.1242/JCS.164871/VIDEO-2

Chakrabarti, R., Lee, M., & Higgs, H. N. (2021). Multiple roles for actin in secretory and endocytic pathways. In *Current Biology* (Vol. 31, Issue 10, pp. R603–R618). Cell Press. https://doi.org/10.1016/j.cub.2021.03.038

Chen, F., Song, Q., & Yu, Q. (2018). Axl inhibitor R428 induces apoptosis of cancer cells by blocking lysosomal acidification and recycling independent of Axl inhibition. In *Am J Cancer Res* (Vol. 8, Issue 8). www.ajcr.us/ISSN:2156-6976/ajcr0080858

Chia, J., Goh, G., Racine, V., Ng, S., Kumar, P., & Bard, F. (2012a). RNAi screening reveals a large signaling network controlling the Golgi apparatus in human cells. *Molecular Systems Biology*, *8*, 629. https://doi.org/10.1038/msb.2012.59

Chia, J., Goh, G., Racine, V., Ng, S., Kumar, P., & Bard, F. (2012b). RNAi screening reveals a large signaling network controlling the Golgi apparatus in human cells. *Molecular Systems Biology*, *8*, 629. https://doi.org/10.1038/msb.2012.59

Chia, J., Wang, S.-C., Wee, S., Gill, D. J., Tay, F., Kannan, S., Verma, C. S., Gunaratne, J., & Bard, F. A. (n.d.). *Src activates retrograde membrane traffic through phosphorylation of GBF1*. https://doi.org/10.1101/2020.08.02.233353

Chiu, V. K., Bivona, T., Hach, A., Sajous, J. B., Silletti, J., Wiener, H., Johnson, R. L., Cox, A. D., & Philips, M. R. (2002). Ras signalling on the endoplasmic reticulum and the Golgi. *Nature Cell Biology 2002 4:5*, *4*(5), 343–350. https://doi.org/10.1038/ncb783

Cluett, E. B., Kuismanen, E., & Machamer, C. E. (1997). Heterogeneous Distribution of the Unusual Phospholipid Semilysobisphosphatidic Acid through the Golgi Complex. *Molecular Biology of the Cell*, *8*, 2233–2240.

Cockcroft, S. (2021). Mammalian lipids: structure, synthesis and function. *Essays in Biochemistry*, *65*(5), 813–845. https://doi.org/10.1042/EBC20200067

Colanzi, A., & Corda, D. (2007). Mitosis controls the Golgi and the Golgi controls mitosis. In *Current Opinion in Cell Biology* (Vol. 19, Issue 4, pp. 386–393). https://doi.org/10.1016/j.ceb.2007.06.002

Colanzi, A., Suetterlin, C., & Malhotra, V. (2003). Cell-cycle-specific Golgi fragmentation: how and why? *Current Opinion in Cell Biology*, *15*(4), 462–467. https://doi.org/10.1016/S0955-0674(03)00067-X

Colley, K. J., Varki, A., & Kinoshita, T. (2015). Cellular organization of glycosylation. In A. Varki, R. D. Cummings, J. D. Esko, P. Stanley, G. W. Hart, M. Aebi, A. G. Darvill, T. Kinoshita, N. H. Packer, J. H. Prestegard, R. L. Schnaar, & P. H. Seeberger (Eds.), *Essentials of Glycobiology* (3rd ed.). Cold Spring Harbor Laboratory Press. https://doi.org/10.1101/glycobiology.3e.004

Dagamajalu, S., Rex, D. A. B., Palollathil, A., Shetty, R., Bhat, G., Cheung, L. W. T., & Prasad, T. S. K. (2021). A pathway map of AXL receptor-mediated signaling network. *Journal of Cell Communication and Signaling*, *15*(1), 143. https://doi.org/10.1007/S12079-020-00580-5

Dalton, A. J., & Felix, M. D. (n.d.). *A COMPARATIVE STUDY OF THE GOLGI COMPLEX*.

Damke, H., Klumperman, J., Von Figura, K., & Braulke, T. (1991). Effects of brefeldin A on the endocytic route. Redistribution of mannose 6-phosphate/insulin-like growth factor II receptors to the cell surface. *Journal of Biological Chemistry*, *266*(36), 24829–24833. https://doi.org/10.1016/S0021-9258(18)54303-9

Day, K. J., Staehelin, L. A., & Glick, B. S. (2013). A Three-Stage Model of Golgi Structure and Function. *Histochemistry and Cell Biology*, *140*(3), 239. https://doi.org/10.1007/S00418-013-1128-3

Debruyne, D. N., Bhatnagar, N., Sharma, B., Luther, W., Moore, N. F., Cheung, N. K., Gray, N. S., & George, R. E. (2016). ALK inhibitor resistance in ALKF1174L-driven neuroblastoma is associated with AXL activation and induction of EMT. *Oncogene*, *35*(28), 3681. https://doi.org/10.1038/ONC.2015.434

del Pozo, M. A., Balasubramanian, N., Alderson, N. B., Kiosses, W. B., Grande-García, A., Anderson, R. G. W., & Schwartz, M. A. (2005). Phospho-caveolin-1 mediates integrin-regulated membrane domain internalization. *Nature Cell Biology*, *7*(9), 901–908. https://doi.org/10.1038/ncb1293

Demetriou, M., Granovsky, M., Quaggin, S., & Dennis, J. W. (2001). Negative regulation of T-cell activation and autoimmunity by Mgat5 N-glycosylation. *Nature*, *409*(6821), 733–739. https://doi.org/10.1038/35055582

Donaldson, J. G., Honda, A., & Weigert, R. (2005). Multiple activities for Arf1 at the Golgi complex. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1744*(3), 364–373. https://doi.org/10.1016/J.BBAMCR.2005.03.001

Duan, Y., Hu, B., Qiao, C., Luo, L., Li, X., Wang, J., Liu, H., Zhou, T., Shen, B., Lv, M., & Feng, J. (2019a). Engineered axl-ecd-fc variants that abolish the axl/gas6 interaction suppress tumor cell migration. *Oncology Letters*, *17*(6), 5784–5792. https://doi.org/10.3892/ol.2019.10255

Duan, Y., Hu, B., Qiao, C., Luo, L., Li, X., Wang, J., Liu, H., Zhou, T., Shen, B., Lv, M., & Feng, J. (2019b). Engineered axl-ecd-fc variants that abolish the axl/gas6 interaction suppress tumor cell migration. *Oncology Letters*, *17*(6), 5784–5792. https://doi.org/10.3892/ol.2019.10255

Egea, G., Lázaro-Diéguez, F., & Vilella, M. (2006). Actin dynamics at the Golgi complex in mammalian cells. *Current Opinion in Cell Biology*, *18*(2), 168–178. https://doi.org/10.1016/j.ceb.2006.02.007

Egea, G., Serra-Peinado, C., Gavilan, M. P., & Rios, R. M. (2015). Cytoskeleton and Golgi-apparatus interactions: a two-way road of function and structure. *Cell Health and Cytoskeleton*, 37. https://doi.org/10.2147/CHC.S57108

Farber-Katz, S. E., Dippold, H. C., Buschman, M. D., Peterman, M. C., Xing, M., Noakes, C. J., Tat, J., Ng, M. M., Rahajeng, J., Cowan, D. M., Fuchs, G. J., Zhou, H., & Field, S. J. (2014). DNA damage triggers Golgi dispersal via DNA-PK and GOLPH3. *Cell*, *156*(3), 413–427. https://doi.org/10.1016/j.cell.2013.12.023

Farhan, H., & Hsu, V. W. (n.d.). *Cdc42 and cellular polarity: Emerging roles at the Golgi*. https://doi.org/10.1016/j.tcb.2015.11.003

FARQUHAR, M., & PALADE, G. (n.d.). *from Artifact to Center Stage*. https://rupress.org/jcb/article/91/3/77s/19588/The-Golgi-apparatus-complex-1954-1981-from

Feinstein, T. N., & Linstedt, A. D. (2007). Mitogen-activated Protein Kinase Kinase 1-dependent Golgi Unlinking Occurs in G2 Phase and Promotes the G2/M Cell Cycle Transition. *Molecular Biology of the Cell*, *18*(2), 594. https://doi.org/10.1091/MBC.E06-06-0530

Ferreira, I. G., Pucci, M., Venturi, G., Malagolini, N., Chiricolo, M., & Dall'Olio, F. (2018). Glycosylation as a main regulator of growth and death factor receptors signaling. *International Journal of Molecular Sciences*, *19*(2). https://doi.org/10.3390/ijms19020580

Fisher, P., Spencer, H., Thomas-Oates, J., Wood, A. J., & Ungar, D. (2019). Modeling Glycan Processing Reveals Golgi-Enzyme Homeostasis upon Trafficking Defects and Cellular Differentiation. *Cell Reports*, *27*(4), 1231-1243.e6. https://doi.org/10.1016/J.CELREP.2019.03.107

Fisher, P., & Ungar, D. (2016). Bridging the Gap between Glycosylation and Vesicle Traffic. *Frontiers in Cell and Developmental Biology*, *4*, 15. https://doi.org/10.3389/fcell.2016.00015

Fujita, Y., Watabe, K., Ikeda, K., Mizuno, Y., & Okamoto, K. (2011). Morphological changes of Golgi apparatus in adult rats after facial nerve injuries. *Neuropathology*, *31*(1), 42–47. https://doi.org/10.1111/J.1440-1789.2010.01123.X

Gallicchio, M., Mitola, S., Valdembri, D., Fantozzi, R., Varnum, B., Avanzi, G. C., & Bussolino, F. (2005). Inhibition of vascular endothelial growth factor receptor 2–mediated endothelial cell activation by Axl tyrosine kinase receptor. *Blood*, *105*(5), 1970–1976. https://doi.org/10.1182/BLOOD-2004-04-1469

Garnis, C., Buys, T. P. H., & Lam, W. L. (2004). Genetic alteration and gene expression modulation during cancer progression. *Molecular Cancer 2004 3:1*, *3*(1), 1–23. https://doi.org/10.1186/1476-4598-3-9

Gee, H. Y., Kim, J., & Lee, M. G. (2018). Unconventional secretion of transmembrane proteins. *Seminars in Cell & Developmental Biology*, *83*, 59–66. https://doi.org/10.1016/J.SEMCDB.2018.03.016

Gill, D. J., Chia, J., Senewiratne, J., & Bard, F. (2010). Regulation of O-glycosylation through Golgi-to-ER relocation of initiation enzymes. *The Journal of Cell Biology*, *189*(5), 843–858. https://doi.org/10.1083/jcb.201003055

Gjerdrum, C., Tiron, C., Høiby, T., Stefansson, I., Haugen, H., Sandal, T., Collett, K., Li, S., McCormack, E., Gjertsen, B. T., Micklem, D. R., Akslen, L. A., Glackin, C., & Lorens, J. B. (2010). Axl is an essential epithelial-to-mesenchymal transition-induced regulator of breast cancer metastasis and patient survival. *Proceedings of the National Academy of*

Sciences of the United States of America, *107*(3), 1124. https://doi.org/10.1073/PNAS.0909333107

Glick, B. S., & Nakano, A. (2009). Membrane traffic within the Golgi apparatus. *Annual Review of Cell and Developmental Biology*, *25*, 113–132. https://doi.org/10.1146/annurev.cellbio.24.110707.175421

Goldman, M. J., Craft, B., Hastie, M., Repečka, K., McDade, F., Kamath, A., Banerjee, A., Luo, Y., Rogers, D., Brooks, A. N., Zhu, J., & Haussler, D. (2020). Visualizing and interpreting cancer genomics data via the Xena platform. *Nature Biotechnology 2020 38:6*, *38*(6), 675–678. https://doi.org/10.1038/s41587-020-0546-8

Gonatas, N. K., Stieber, A., & Gonatas, J. O. (2006). Fragmentation of the Golgi apparatus in neurodegenerative diseases and cell death. *Journal of the Neurological Sciences*, *246*(1–2), 21–30. https://doi.org/10.1016/J.JNS.2006.01.019

Goodwin, J. S., Drake, K. R., Rogers, C., Wright, L., Lippincott-Schwartz, J., Philips, M. R., & Kenworthy, A. K. (2005). Depalmitoylated Ras traffics to and from the Golgi complex via a nonvesicular pathway. *The Journal of Cell Biology*, *170*(2), 261–272. https://doi.org/10.1083/jcb.200502063

Goud, B., Liu, S., & Storrie, B. (2018). Rab proteins as major determinants of the Golgi complex structure. *Small GTPases*, *9*(1–2), 66–75. https://doi.org/10.1080/21541248.2017.1384087

Graham, D. K., Deryckere, D., Davies, K. D., & Earp, H. S. (2014). The TAM family: phosphatidylserine-sensing receptor tyrosine kinases gone awry in cancer. *Nature Reviews Cancer 2014 14:12*, *14*(12), 769–785. https://doi.org/10.1038/nrc3847

Griffing, L. R. (1991). Comparisons of golgi structure and dynamics in plant and animal cells. *Journal of Electron Microscopy Technique*, *17*(2), 179–199. https://doi.org/10.1002/JEMT.1060170206

Guadamillas, M. C., Cerezo, A., & Del Pozo, M. A. (2011). Overcoming anoikis–pathways to anchorage-independent growth in cancer. *Journal of Cell Science*, *124*(Pt 19), 3189–3197. https://doi.org/10.1242/jcs.072165

Guizzunti, G., & Seemann, J. (2016). Mitotic Golgi disassembly is required for bipolar spindle formation and mitotic progression. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(43), E6590–E6599. https://doi.org/10.1073/PNAS.1610844113

Haase, G., & Rabouille, C. (2015). Golgi fragmentation in ALS motor neurons. new mechanisms targeting microtubules, tethers, and transport vesicles. *Frontiers in Neuroscience*, *9*, 448. https://doi.org/10.3389/fnins.2015.00448

Haines, E., Schlienger, S., & Claing, A. (2015). The small GTPase ADP-Ribosylation Factor 1 mediates the sensitivity of triple negative breast cancer cells to EGFR tyrosine kinase inhibitors. *Cancer Biology & Therapy*, *16*(10), 1535. https://doi.org/10.1080/15384047.2015.1071737

Harris, M. A., Clark, J., Ireland, A., Lomax, J., Ashburner, M., Foulger, R., Eilbeck, K., Lewis, S., Marshall, B., Mungall, C., Richter, J., Rubin, G. M., Blake, J. A., Bult, C., Dolan, M., Drabkin, H., Eppig, J. T., Hill, D. P., Ni, L., … White, R. (2004). The Gene Ontology (GO) database and informatics resource. *Nucleic Acids Research*, *32*(Database issue), D258. https://doi.org/10.1093/NAR/GKH036

Hennet, T., & Cabalzar, J. (2015). Congenital disorders of glycosylation: a concise chart of glycocalyx dysfunction. *Trends in Biochemical Sciences*, *40*(7), 377–384. https://doi.org/10.1016/j.tibs.2015.03.002

Hirschberg, K., Miller, C. M., Ellenberg, J., Presley, J. F., Siggia, E. D., Phair, R. D., & Lippincott-Schwartz, J. (1998a). Kinetic analysis of secretory protein traffic and

characterization of golgi to plasma membrane transport intermediates in living cells. *The Journal of Cell Biology*, *143*(6), 1485–1503. https://doi.org/10.1083/jcb.143.6.1485

Hirschberg, K., Miller, C. M., Ellenberg, J., Presley, J. F., Siggia, E. D., Phair, R. D., & Lippincott-Schwartz, J. (1998b). Kinetic Analysis of Secretory Protein Traffic and Characterization of Golgi to Plasma Membrane Transport Intermediates in Living Cells. *The Journal of Cell Biology*, *143*(6), 1485. https://doi.org/10.1083/JCB.143.6.1485

Holland, S. J., Pan, A., Franci, C., Hu, Y., Chang, B., Li, W., Duan, M., Torneros, A., Yu, J., Heckrodt, T. J., Zhang, J., Ding, P., Apatira, A., Chua, J., Brandt, R., Pine, P., Goff, D., Singh, R., Payan, D. G., & Hitoshi, Y. (2010). R428, a selective small molecule inhibitor of Axl kinase, blocks tumor spread and prolongs survival in models of metastatic breast cancer. *Cancer Research*, *70*(4), 1544–1554. https://doi.org/10.1158/0008-5472.CAN-09-2997/655769/P/R428-A-SELECTIVE-SMALL-MOLECULE-INHIBITOR-OF-AXL

Hong, C. C., Lay, J. D., Huang, J. S., Cheng, A. L., Tang, J. L., Lin, M. T., Lai, G. M., & Chuang, S. E. (2008). Receptor tyrosine kinase AXL is induced by chemotherapy drugs and overexpression of AXL confers drug resistance in acute myeloid leukemia. *Cancer Letters*, *268*(2), 314–324. https://doi.org/10.1016/J.CANLET.2008.04.017

Hong, J., Maacha, S., Pidkovka, N., Bates, A., Salaria, S. N., Washington, M. K., & Belkhiri, A. (2022). AXL Promotes Metformin-Induced Apoptosis Through Mediation of Autophagy by Activating ROS-AMPK-ULK1 Signaling in Human Esophageal Adenocarcinoma. *Frontiers in Oncology*, *12*, 903874. https://doi.org/10.3389/FONC.2022.903874/FULL

Hu, B. S., Hu, H., Zhu, C. Y., Gu, Y. L., & Li, J. P. (2013). Overexpression of GOLPH3 is associated with poor clinical outcome in gastric cancer. *Tumour Biology : The Journal of the International Society for Oncodevelopmental Biology and Medicine*, *34*(1), 515–520. https://doi.org/10.1007/S13277-012-0576-Z

Hua, Z., & Graham, T. R. (n.d.). *Madame Curie Bioscience Database [Internet]. Austin (TX): Landes Bioscience; The Golgi Apparatus*. https://www.ncbi.nlm.nih.gov/books/NBK6268/

Huang, S., & Wang, Y. (2017). Golgi structure formation, function, and post-translational modifications in mammalian cells. *F1000Research*, *6*, 2050. https://doi.org/10.12688/f1000research.11900.1

Huanna, T., Tao, Z., Xiangfei, W., Longfei, A., Yuanyuan, X., Jianhua, W., Cuifang, Z., Manjing, J., Wenjing, C., Shaochuan, Q., Feifei, X., Naikang, L., Jinchao, Z., & Chen, W. (2015). GALNT14 mediates tumor invasion and migration in breast cancer cell MCF-7. *Molecular Carcinogenesis*, *54*(10), 1159–1171. https://doi.org/10.1002/mc.22186

Hurtado, L., Caballero, C., Gavilan, M. P., Cardenas, J., Bornens, M., & Rios, R. M. (2011). Disconnecting the Golgi ribbon from the centrosome prevents directional cell migration and ciliogenesis. *Journal of Cell Biology*, *193*(5), 917–933. https://doi.org/10.1083/jcb.201011014

Ignashkova, T. I., Gendarme, M., Peschk, K., Eggenweiler, H.-M., Lindemann, R. K., & Reiling, J. H. (2017). Cell survival and protein secretion associated with Golgi integrity in response to Golgi stress-inducing agents. *Traffic*, *18*(8), 530–544. https://doi.org/10.1111/tra.12493

Iida, K., Sakai, R., Yokoyama, S., Kobayashi, N., Togo, S., Yoshikawa, H. Y., Rawangkan, A., Namiki, K., & Suganuma, M. (2017). Cell softening in malignant progression of human lung cancer cells by activation of receptor tyrosine kinase AXL. *Scientific Reports*, *7*(1). https://doi.org/10.1038/s41598-017-18120-4

Imamaki, R., Ogawa, K., Kizuka, Y., Komi, Y., Kojima, S., Kotani, N., Honke, K., Honda, T., Taniguchi, N., & Kitazume, S. (2018). Glycosylation controls cooperative PECAM-

VEGFR2-β3 integrin functions at the endothelial surface for tumor angiogenesis. *Oncogene*, *37*(31), 4287–4299. https://doi.org/10.1038/s41388-018-0271-7

INCHANALKAR, S. (2014). Adhesion dependent regulation of aurora kinase a and its role in rala mediated anchorage independence. *University*. http://hdl.handle.net/10603/445078

Isaji, T., Im, S., Gu, W., Wang, Y., Hang, Q., Lu, J., Fukuda, T., Hashii, N., Takakura, D., Kawasaki, N., Miyoshi, H., & Gu, J. (2014). An oncogenic protein Golgi phosphoprotein 3 up-regulates cell migration via sialylation. *The Journal of Biological Chemistry*, *289*(30), 20694–20705. https://doi.org/10.1074/jbc.M113.542688

Ishihara, S., & Haga, H. (2022). Matrix Stiffness Contributes to Cancer Progression by Regulating Transcription Factors. *Cancers*, *14*(4). https://doi.org/10.3390/CANCERS14041049

Iyer, P., Bhave, M., Jain, B. K., RoyChowdhury, S., & Bhattacharyya, D. (2018). Vps74p controls Golgi size in an Arf1-dependent manner. *FEBS Letters*, *592*(22), 3720–3735. https://doi.org/10.1002/1873-3468.13266

Jaarsma, D., & Hoogenraad, C. C. (2015a). Cytoplasmic dynein and its regulatory proteins in Golgi pathology in nervous system disorders. In *Frontiers in Neuroscience* (Vol. 9, Issue OCT). Frontiers Media S.A. https://doi.org/10.3389/fnins.2015.00397

Jaarsma, D., & Hoogenraad, C. C. (2015b). Cytoplasmic dynein and its regulatory proteins in Golgi pathology in nervous system disorders. *Frontiers in Neuroscience*, *9*(OCT), 397. https://doi.org/10.3389/FNINS.2015.00397

Jamieson, J. D. (n.d.). *The Golgi complex: perspectives and prospectives*.

Janik, M. E., Lityńska, A., & Vereecken, P. (2010). Cell migration-the role of integrin glycosylation. *Biochimica et Biophysica Acta*, *1800*(6), 545–555. https://doi.org/10.1016/J.BBAGEN.2010.03.013

Janiszewska, M., Primi, M. C., & Izard, T. (2020). Cell adhesion in cancer: Beyond the migration of single cells. *The Journal of Biological Chemistry*, *295*(8), 2495–2505. https://doi.org/10.1074/jbc.REV119.007759

Jeggo, P. A., Pearl, L. H., & Carr, A. M. (2016). DNA repair, genome stability and cancer: a historical perspective. *Nature Reviews. Cancer*, *16*(1), 35–42. https://doi.org/10.1038/NRC.2015.4

Jiang, Y., Zhang, H., Wang, J., Liu, Y., Luo, T., & Hua, H. (2022). Targeting extracellular matrix stiffness and mechanotransducers to improve cancer therapy. *Journal of Hematology and Oncology*, *15*(1), 1–15. https://doi.org/10.1186/S13045-022-01252-0/TABLES/1

Kanlikilicer, P., Ozpolat, B., Aslan, B., Bayraktar, R., Gurbuz, N., Rodriguez-Aguayo, C., Bayraktar, E., Denizli, M., Gonzalez-Villasana, V., Ivan, C., Lokesh, G. L., Amero, P., Catuogno, S., Haemmerle, M., Yen-Yao Wu, S., Mitra, R., Gorenstein, D. G., Volk, D. E., de Franciscis, V., … Lopez-Berestein, G. (2017). Therapeutic Targeting of AXL Receptor Tyrosine Kinase Inhibits Tumor Growth and Intraperitoneal Metastasis in Ovarian Cancer Models. *Molecular Therapy: Nucleic Acid*, *9*, 251–262. https://doi.org/10.1016/j.omtn.2017.06.023

Kellokumpu, S., Sormunen, R., & Kellokumpu, I. (2002). Abnormal glycosylation and altered Golgi structure in colorectal cancer: dependence on intra-Golgi pH. *FEBS Letters*, *516*(1–3), 217–224. https://doi.org/10.1016/s0014-5793(02)02535-8

Khine, M. N., & Sakurai, K. (2023). Golgi-Targeting Anticancer Natural Products. *Cancers*, *15*(7). https://doi.org/10.3390/CANCERS15072086

Kim, J. J., Lipatova, Z., Majumdar, U., & Segev, N. (2016). Regulation of Golgi Cisternal Progression by Ypt/Rab GTPases. *Developmental Cell*, *36*(4), 440–452. https://doi.org/10.1016/J.DEVCEL.2016.01.016

Kim, N., Hwang, C. Y., Kim, T., Kim, H., & Cho, K. H. (2023). A Cell-Fate Reprogramming Strategy Reverses Epithelial-to-Mesenchymal Transition of Lung Cancer Cells While Avoiding Hybrid States. *Cancer Research*, *83*(6), 956. https://doi.org/10.1158/0008-5472.CAN-22-1559

Kimura, M., Takagi, S., & Nakashima, S. (2018). Aurora A regulates the architecture of the Golgi apparatus. *Experimental Cell Research*, *367*(1), 73–80. https://doi.org/10.1016/j.yexcr.2018.03.024

Klute, M. J., Melaṇon, P., & Dacks, J. B. (2011). Evolution and diversity of the Golgi. In *Cold Spring Harbor Perspectives in Biology* (Vol. 3, Issue 8, pp. 1–17). Cold Spring Harbor Laboratory Press. https://doi.org/10.1101/cshperspect.a007849

Kondylis, V., & Rabouille, C. (2009). The Golgi apparatus: Lessons from Drosophila. *FEBS Letters*, *583*(23), 3827–3838. https://doi.org/10.1016/J.FEBSLET.2009.09.048

Koorstra, J.-B. M., Karikari, C. A., Feldmann, G., Bisht, S., Rojas, P. L., Johan, G., Offerhaus, A., Alvarez, H., & Maitra, A. (n.d.). *The Axl receptor tyrosine kinase confers an adverse prognostic influence in pancreatic cancer and represents a new therapeutic target*.

Kreft, M. E., Mironov, A. A., & Hudoklin, S. (2022). The Golgi complex: An organelle that determines urothelial cell biology in health and disease. *Histochemistry and Cell Biology*, *158*, 229–240. https://doi.org/10.1007/s00418-022-02121-0

Křivohlavá, R., Grobárová, V., Neuhöferová, E., Fišerová, A., & Benson, V. (2018). Interaction of colon cancer cells with glycoconjugates triggers complex changes in gene expression, glucose transporters and cell invasion. *Molecular Medicine Reports*, *17*(4), 5508–5517. https://doi.org/10.3892/mmr.2018.8490

Kudo, M., Brem, M. S., & Canfield, W. M. (2006). Mucolipidosis II (I-cell disease) and mucolipidosis IIIA (classical pseudo-hurler polydystrophy) are caused by mutations in the GlcNAc-phosphotransferase alpha / beta -subunits precursor gene. *American Journal of Human Genetics*, *78*(3), 451–463. https://doi.org/10.1086/500849

Kulkarni-Gosavi, P., Makhoul, C., & Gleeson, P. A. (2019). Form and function of the Golgi apparatus: scaffolds, cytoskeleton and signalling. *FEBS Letters*, *593*(17), 2289–2305. https://doi.org/10.1002/1873-3468.13567

Läubli, H., & Borsig, L. (2019a). Altered cell adhesion and glycosylation promote cancer immune suppression and metastasis. *Frontiers in Immunology*, *10*, 2120. https://doi.org/10.3389/fimmu.2019.02120

Läubli, H., & Borsig, L. (2019b). Altered cell adhesion and glycosylation promote cancer immune suppression and metastasis. *Frontiers in Immunology*, *10*, 2120. https://doi.org/10.3389/fimmu.2019.02120

Lay, J. D., Hong, C. C., Huang, J. S., Yang, Y. Y., Pao, C. Y., Liu, C. H., Lai, Y. P., Lai, G. M., Cheng, A. L., Su, I. J., & Chuang, S. E. (2007). Sulfasalazine suppresses drug resistance and invasiveness of lung adenocarcinoma cells expressing AXL. *Cancer Research*, *67*(8), 3878–3887. https://doi.org/10.1158/0008-5472.CAN-06-3191

Lee, I. J., Lee, C.-W., & Lee, J.-H. (2015). CaMKKβ-AMPKα2 signaling contributes to mitotic Golgi fragmentation and the G2/M transition in mammalian cells. *Cell Cycle*, *14*(4), 598–611. https://doi.org/10.4161/15384101.2014.991557

Lenz, D., McClean, P., Kansu, A., Bonnen, P. E., Ranucci, G., Thiel, C., Straub, B. K., Harting, I., Alhaddad, B., Dimitrov, B., Kotzaeridou, U., Wenning, D., Iorio, R., Himes, R. W., Kuloğlu, Z., Blakely, E. L., Taylor, R. W., Meitinger, T., Kölker, S., … Staufner, C. (2018). SCYL1 variants cause a syndrome with low γ-glutamyl-transferase cholestasis, acute liver failure, and neurodegeneration (CALFAN). *Genetics in Medicine*, *20*(10), 1255. https://doi.org/10.1038/GIM.2017.260

Lev, S., Halevy, D. Ben, Peretti, D., & Dahan, N. (2008). The VAP protein family: from cellular functions to motor neuron disease. *Trends in Cell Biology*, *18*(6), 282–290. https://doi.org/10.1016/J.TCB.2008.03.006

Lewis, J. M., Truong, T. N., & Schwartz, M. A. (2002). Integrins regulate the apoptotic response to DNA damage through modulation of p53. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(6), 3627–3632. https://doi.org/10.1073/pnas.062698499

Lewis-Saravalli, S., Campbell, S., & Claing, A. (2013). ARF1 controls Rac1 signaling to regulate migration of MDA-MB-231 invasive breast cancer cells. *Cellular Signalling*, *25*(9), 1813–1819. https://doi.org/10.1016/J.CELLSIG.2013.05.011

Li, J., Jia, L., Ma, Z. H., Ma, Q. H., Yang, X. H., & Zhao, Y. F. (2012). Axl glycosylation mediates tumor cell proliferation, invasion and lymphatic metastasis in murine hepatocellular carcinoma. *World Journal of Gastroenterology : WJG*, *18*(38), 5369. https://doi.org/10.3748/WJG.V18.I38.5369

Li, L. Y., Guan, Y. Di, Chen, X. S., Yang, J. M., & Cheng, Y. (2021). DNA Repair Pathways in Cancer Therapy and Resistance. *Frontiers in Pharmacology*, *11*, 629266. https://doi.org/10.3389/FPHAR.2020.629266/BIBTEX

Linders, P. T. A., Peters, E., Ter Beest, M., Lefeber, D. J., & van den Bogaart, G. (2020). Sugary logistics gone wrong: membrane trafficking and congenital disorders of glycosylation. *International Journal of Molecular Sciences*, *21*(13). https://doi.org/10.3390/ijms21134654

Linger, R. M. A., Fellow, P., Keating, A. K., Earp, H. S., & Graham, D. K. (2010). Taking aim at Mer and Axl receptor tyrosine kinases as novel therapeutic targets in solid tumors. *Expert Opin Ther Targets*, *14*(10), 1073–1090. https://doi.org/10.1517/14728222.2010.515980

Liu, J., Huang, Y., Li, T., Jiang, Z., Zeng, L., & Hu, Z. (2021). The role of the Golgi apparatus in disease (Review). *International Journal of Molecular Medicine*, *47*(4). https://doi.org/10.3892/ijmm.2021.4871

Lodish, H., Berk, A., Zipursky, S. L., Matsudaira, P., Baltimore, D., & Darnell, J. (2000). *Protein Glycosylation in the ER and Golgi Complex*. https://www.ncbi.nlm.nih.gov/books/NBK21744/

Lopez Sambrooks, C., Baro, M., Quijano, A., Narayan, A., Cui, W., Greninger, P., Egan, R., Patel, A., Benes, C. H., Saltzman, W. M., & Contessa, J. N. (2018). Oligosaccharyltransferase inhibition overcomes therapeutic resistance to EGFR tyrosine kinase inhibitors. *Cancer Research*, *78*(17), 5094–5106. https://doi.org/10.1158/0008-5472.CAN-18-0505

Lowe, M. (2002). Golgi Complex: Biogenesis de novo? *Current Biology*, *12*(5), R166–R167. https://doi.org/10.1016/S0960-9822(02)00732-7

Lowe, M. (2011). Structural organization of the Golgi apparatus. *Current Opinion in Cell Biology*, *23*(1), 85–93. https://doi.org/10.1016/j.ceb.2010.10.004

Lu, Z., Joseph, D., Bugnard, E., Zaal, K. J. M., & Ralston, E. (2001). Golgi Complex Reorganization during Muscle Differentiation: Visualization in Living Cells and Mechanism. *Molecular Biology of the Cell*, *12*(4), 795. https://doi.org/10.1091/MBC.12.4.795

Luchsinger, C., Aguilar, M., Burgos, P. V, Ehrenfeld, P., & Mardones, G. A. (2018). Functional disruption of the Golgi apparatus protein ARF1 sensitizes MDA-MB-231 breast cancer cells to the antitumor drugs Actinomycin D and Vinblastine through ERK and AKT signaling. *Plos One*, *13*(4), e0195401. https://doi.org/10.1371/journal.pone.0195401

Luini, A. (2011). A brief history of the cisternal progression-maturation model. *Cellular Logistics*, *1*(1), 6. https://doi.org/10.4161/CL.1.1.14693

Luo, C. L., Xu, X. C., Liu, C. J., He, S., Chen, J. R., Feng, Y. C., Liu, S. Q., Peng, W., Zhou, Y. Q., Liu, Y. X., Wei, P. P., Li, B., Mai, H. Q., Xia, X. J., & Bei, J. X. (2021). RBFOX2/GOLIM4 Splicing Axis Activates Vesicular Transport Pathway to Promote Nasopharyngeal Carcinogenesis. *Advanced Science*, *8*(16). https://doi.org/10.1002/ADVS.202004852

Machamer, C. E. (2015a). The Golgi complex in stress and death. *Frontiers in Neuroscience*, *9*, 421. https://doi.org/10.3389/fnins.2015.00421

Machamer, C. E. (2015b). The Golgi complex in stress and death. *Frontiers in Neuroscience*, *9*, 421. https://doi.org/10.3389/fnins.2015.00421

Majumder, A., Hosseinian, S., Stroud, M., Adhikari, E., Saller, J. J., Smith, M. A., Zhang, G., Agarwal, S., Creixell, M., Meyer, B. S., Kinose, F., Bowers, K., Fang, B., Stewart, P. A., Welsh, E. A., Boyle, T. A., Meyer, A. S., Koomen, J. M., & Haura, E. B. (2022). Integrated Proteomics-Based Physical and Functional Mapping of AXL Kinase Signaling Pathways and Inhibitors Define Its Role in Cell Migration. *Molecular Cancer Research*, *20*(4), 542–555. https://doi.org/10.1158/1541-7786.MCR-21-0275

Makhoul, C., Gosavi, P., & Gleeson, P. A. (2019). Golgi dynamics: the morphology of the mammalian golgi apparatus in health and disease. *Frontiers in Cell and Developmental Biology*, *7*, 112. https://doi.org/10.3389/fcell.2019.00112

Mani, S., & Thattai, M. (2016). Stacking the odds for golgi cisternal maturation. *ELife*, *5*(AUGUST). https://doi.org/10.7554/ELIFE.16231

Manolea, F., Chun, J., Chen, D. W., Clarke, I., Summerfeldt, N., Dacks, J. B., & Melançon, P. (2010). Arf3 is activated uniquely at the trans-Golgi network by brefeldin A-inhibited guanine nucleotide exchange factors. *Molecular Biology of the Cell*, *21*(11), 1836–1849. https://doi.org/10.1091/mbc.e10-01-0016

Manser, E. (2002). Small GTPases take the stage. *Developmental Cell*, *3*(3), 323–328. https://doi.org/10.1016/s1534-5807(02)00268-x

Mao, L., Li, N., Guo, Y., Xu, X., Gao, L., Xu, Y., Zhou, L., & Liu, W. (2013). AMPK phosphorylates GBF1 for mitotic Golgi disassembly. *Journal of Cell Science*, *126*(Pt 6), 1498–1505. https://doi.org/10.1242/jcs.121954

Marra, P., Salvatore, L., Mironov, A., Campli, A. Di, Tullio, G. Di, Trucco, A., Beznoussenko, G., & De Matteis, M. A. (2007). The Biogenesis of the Golgi Ribbon: The Roles of Membrane Input from the ER and of GM130 □ D □ V. *Molecular Biology of the Cell*, *18*, 1595–1608. https://doi.org/10.1091/mbc.E06

Marsh, B. J., & Howell, K. E. (2002). The mammalian Golgi–complex debates. *Nature Reviews. Molecular Cell Biology*, *3*(10), 789–795. https://doi.org/10.1038/nrm933

Martino, F., Perestrelo, A. R., Vinarský, V., Pagliari, S., & Forte, G. (2018). Cellular Mechanotransduction: From Tension to Function. *Frontiers in Physiology*, *9*(JUL). https://doi.org/10.3389/FPHYS.2018.00824

Mascanzoni, F., Iannitti, R., & Colanzi, A. (2022a). Functional Coordination among the Golgi Complex, the Centrosome and the Microtubule Cytoskeleton during the Cell Cycle. In *Cells* (Vol. 11, Issue 3). NLM (Medline). https://doi.org/10.3390/cells11030354

Mascanzoni, F., Iannitti, R., & Colanzi, A. (2022b). Functional Coordination among the Golgi Complex, the Centrosome and the Microtubule Cytoskeleton during the Cell Cycle. *Cells*, *11*(3). https://doi.org/10.3390/CELLS11030354

Maziveyi, M., Alahari, S. K., Maziveyi, M., & Alahari, S. K. (2017). Cell matrix adhesions in cancer: The proteins that form the glue. *Oncotarget*, *8*(29), 48471–48487. https://doi.org/10.18632/ONCOTARGET.17265

Mazzarello, P., Garbarino, C., & Calligaro, A. (2009). How Camillo Golgi became 'the Golgi'. *FEBS Letters*, *583*(23), 3732–3737. https://doi.org/10.1016/j.febslet.2009.10.018

Millarte, V., & Farhan, H. (2012). The Golgi in Cell Migration: Regulation by Signal Transduction and Its Implications for Cancer Cell Metastasis. *The Scientific World Journal*, *2012*, 11. https://doi.org/10.1100/2012/498278

Miller, V. J., & Ungar, D. (2012). Re'cog'nition at the golgi. *Traffic*, *13*(7), 891–897. https://doi.org/10.1111/j.1600-0854.2012.01338.x

Miyamoto, T., Oshiro, N., Yoshino, K. I., Nakashima, A., Eguchi, S., Takahashi, M., Ono, Y., Kikkawa, U., & Yonezawa, K. (2008). AMP-activated protein kinase phosphorylates Golgi-specific brefeldin A resistance factor 1 at Thr1337 to induce disassembly of Golgi apparatus. *Journal of Biological Chemistry*, *283*(7), 4430–4438. https://doi.org/10.1074/jbc.M708296200

Montealegre, S., & Van Endert, P. M. (2019). Endocytic recycling of MHC class I molecules in non-professional antigen presenting and dendritic cells. *Frontiers in Immunology*, *10*(JAN), 423526. https://doi.org/10.3389/FIMMU.2018.03098/BIBTEX

Mowbrey, K., & Dacks, J. B. (2009). Evolution and diversity of the Golgi body. *FEBS Letters*, *583*(23), 3738–3745. https://doi.org/10.1016/J.FEBSLET.2009.10.025

Mukherjee, S., Chiu, R., Leung, S. M., & Shields, D. (2007). Fragmentation of the Golgi Apparatus: An Early Apoptotic Event Independent of the Cytoskeleton. *Traffic*, *8*(4), 369–378. https://doi.org/10.1111/J.1600-0854.2007.00542.X

Munro, S. (2005). The Golgi apparatus: defining the identity of Golgi membranes. *Current Opinion in Cell Biology*, *17*(4), 395–401. https://doi.org/10.1016/J.CEB.2005.06.013

Nakamura, N., Wei, J.-H., & Seemann, J. (2012). Modular organization of the mammalian Golgi apparatus. *Current Opinion in Cell Biology*, *24*(4), 467–474. https://doi.org/10.1016/j.ceb.2012.05.009

Ohashi, Y., Okamura, M., Katayama, R., Fang, S., Tsutsui, S., Akatsuka, A., Shan, M., Choi, H.-W., Fujita, N., Yoshimatsu, K., Shiina, I., Yamori, T., & Dan, S. (2018). Targeting the Golgi apparatus to overcome acquired resistance of non-small cell lung cancer cells to EGFR tyrosine kinase inhibitors. *Oncotarget*, *9*(2), 1641–1655. https://doi.org/10.18632/oncotarget.22895

Ohtsubo, K., & Marth, J. D. (2006). Glycosylation in cellular mechanisms of health and disease. *Cell*, *126*(5), 855–867. https://doi.org/10.1016/j.cell.2006.08.019

Okura, N., Nishioka, N., Yamada, T., Taniguchi, H., Tanimura, K., Katayama, Y., Yoshimura, A., Watanabe, S., Kikuchi, T., Shiotsu, S., Kitazaki, T., Nishiyama, A., Iwasaku, M., Kaneko, Y., Uchino, J., Uehara, H., Horinaka, M., Sakai, T., Tanaka, K., … Takayama, K. (2020). ONO-7475, a Novel AXL Inhibitor, Suppresses the Adaptive Resistance to Initial EGFR-TKI Treatment in EGFR-Mutated Non-Small Cell Lung Cancer. *Clinical Cancer Research*, *26*(9), 2244–2256. https://doi.org/10.1158/1078-0432.CCR-19-2321

Onken, J., Torka, R., Korsing, S., Radke, J., Krementeskaia, I., Nieminen, M., Bai, X., Ullrich, A., Heppner, F., & Vajkoczy, P. (2016). Inhibiting receptor tyrosine kinase AXL with small molecule inhibitor BMS-777607 reduces glioblastoma growth, migration, and invasion in vitro and in vivo. *Oncotarget*, *7*(9), 9876. https://doi.org/10.18632/ONCOTARGET.7130

Pally, D., Pramanik, D., Hussain, S., Verma, S., Srinivas, A., Kumar, R. V., Everest-Dass, A., & Bhat, R. (2021). Heterogeneity in 2,6-Linked Sialic Acids Potentiates Invasion of Breast Cancer Epithelia. *ACS Central Science*, *7*(1), 110–125. https://doi.org/10.1021/ACSCENTSCI.0C00601

Papanikou, E., & Glick, B. S. (2009). The yeast Golgi apparatus: Insights and mysteries. *FEBS Letters*, *583*(23), 3746–3751. https://doi.org/10.1016/J.FEBSLET.2009.10.072

Park, S. Y., Yang, J. S., Schmider, A. B., Soberman, R. J., & Hsu, V. W. (2015). Coordinated regulation of bidirectional COPI transport at the Golgi by CDC42. *Nature 2015 521:7553*, *521*(7553), 529–532. https://doi.org/10.1038/nature14457

Pawar, A., Meier, J. A., Dasgupta, A., Diwanji, N., Deshpande, N., Saxena, K., Buwa, N., Inchanalkar, S., Schwartz, M. A., & Balasubramanian, N. (2016). Ral-Arf6 crosstalk regulates Ral dependent exocyst trafficking and anchorage independent growth signalling. *Cellular Signalling*, *28*(9), 1225–1236. https://doi.org/10.1016/j.cellsig.2016.05.023

Pennauer, M., Buczak, K., Prescianotto-Baschong, C., & Spiess, M. (2022). Shared and specific functions of Arfs 1-5 at the Golgi revealed by systematic knockouts. *The Journal of Cell Biology*, *221*(1). https://doi.org/10.1083/jcb.202106100

Peretti, D., Dahan, N., Shimoni, E., Hirschberg, K., & Lev, S. (2008). Coordinated Lipid Transfer between the Endoplasmic Reticulum and the Golgi Complex Requires the VAP Proteins and Is Essential for Golgi-mediated Transport. *Molecular Biology of the Cell*, *19*, 3871–3884. https://doi.org/10.1091/mbc.E08

Petrosyan, A. (2015). Onco-Golgi: Is Fragmentation a Gate to Cancer Progression? *Biochemistry & Molecular Biology Journal*, *1*(1). https://doi.org/10.21767/2471-8084.100006

Petrosyan, A. (2019). Unlocking Golgi: Why Does Morphology Matter? *Biochemistry. Biokhimiia*, *84*(12), 1490. https://doi.org/10.1134/S0006297919120083

Petrosyan, A., Ali, M. F., & Cheng, P.-W. (2012). Glycosyltransferase-specific Golgi-targeting mechanisms. *The Journal of Biological Chemistry*, *287*(45), 37621–37627. https://doi.org/10.1074/jbc.C112.403006

Petrosyan, A., Holzapfel, M. S., Muirhead, D. E., & Cheng, P.-W. (2014). Restoration of compact Golgi morphology in advanced prostate cancer enhances susceptibility to galectin-1-induced apoptosis by modifying mucin O-glycan synthesis. *Molecular Cancer Research*, *12*(12), 1704–1716. https://doi.org/10.1158/1541-7786.MCR-14-0291-T

Pinato, D. J., Chowdhury, S., & Stebbing, J. (2015). TAMing resistance to multi-targeted kinase inhibitors through Axl and Met inhibition. *Oncogene 2016 35:21*, *35*(21), 2684–2686. https://doi.org/10.1038/onc.2015.374

Pinho, S. S., & Reis, C. A. (2015). Glycosylation in cancer: mechanisms and clinical implications. *Nature Reviews. Cancer*, *15*(9), 540–555. https://doi.org/10.1038/nrc3982

Piyush, T., Rhodes, J. M., & Yu, L.-G. (2017). MUC1 O-glycosylation contributes to anoikis resistance in epithelial cancer cells. *Cell Death Discovery*, *3*, 17044. https://doi.org/10.1038/cddiscovery.2017.44

Porat-Shliom, N., Milberg, O., Masedunskas, A., & Weigert, R. (2013). Multiple roles for the actin cytoskeleton during regulated exocytosis. In *Cellular and Molecular Life Sciences* (Vol. 70, Issue 12, pp. 2099–2121). https://doi.org/10.1007/s00018-012-1156-5

Pothukuchi, P., Agliarulo, I., Russo, D., Rizzo, R., Russo, F., & Parashuraman, S. (2019a). Translation of genome to glycome: role of the Golgi apparatus. *FEBS Letters*. https://febs.onlinelibrary.wiley.com/doi/pdfdirect/10.1002/1873-3468.13541@10.1002/(I)1873-3468.reviews

Pothukuchi, P., Agliarulo, I., Russo, D., Rizzo, R., Russo, F., & Parashuraman, S. (2019b). Translation of genome to glycome: role of the Golgi apparatus. *FEBS Letters*. https://febs.onlinelibrary.wiley.com/doi/pdfdirect/10.1002/1873-3468.13541@10.1002/(I)1873-3468.reviews

Prydz, K., Hansen, S. H., Sandvig, K., & Van Deurs, B. (1992). Effects of brefeldin A on endocytosis, transcytosis and transport to the Golgi complex in polarized MDCK cells. *The Journal of Cell Biology*, *119*(2), 259. https://doi.org/10.1083/JCB.119.2.259

Puthenveedu, M. A., Bachert, C., Puri, S., Lanni, F., & Linstedt, A. D. (2006). GM130 and GRASP65-dependent lateral cisternal fusion allows uniform Golgi-enzyme distribution. *Nature Cell Biology*, *8*(3), 238–248. https://doi.org/10.1038/ncb1366

Rabouille, C., & Linstedt, A. D. (2016). GRASP: A Multitasking Tether. *Frontiers in Cell and Developmental Biology*, *4*(JAN), 1. https://doi.org/10.3389/FCELL.2016.00001

Radulescu, A. E., Mukherjee, S., & Shields, D. (2011). *The Golgi Protein p115 Associates with-Tubulin and Plays a Role in Golgi Structure and Mitosis Progression * □ S.* https://doi.org/10.1074/jbc.M110.209460

Rambourg, A., & Clermont, Y. (1997). Three-dimensional structure of the Golgi apparatus in mammalian cells. In E. G. Berger & J. Roth (Eds.), *The Golgi Apparatus* (pp. 37–61). Birkhäuser Basel. https://doi.org/10.1007/978-3-0348-8876-9_2

Ramkumar, K., Stewart, C. A., Cargill, K. R., Della Corte, C. M., Wang, Q., Shen, L., Diao, L., Cardnell, R. J., Peng, D. H., Leticia Rodriguez, B., Fan, Y.-H., Heymach, J. V, Wang, J., Gay, C. M., Gibbons, D. L., & Byers, L. A. (2021). AXL inhibition induces DNA damage and replication stress in non-small cell lung cancer cells and promotes sensitivity to ATR inhibitors HHS Public Access. *Mol Cancer Res*, *19*(3), 485–497. https://doi.org/10.1158/1541-7786.MCR-20-0414

Reddig, P. J., & Juliano, R. L. (2005). Clinging to life: cell to matrix adhesion and cell survival. *Cancer Metastasis Reviews*, *24*(3), 425–439. https://doi.org/10.1007/s10555-005-5134-3

Rendón, W. O., Martínez-Alonso, E., Tomás, M., Martínez-Martínez, N., & Martínez-Menárguez, J. A. (2013). Golgi fragmentation is Rab and SNARE dependent in cellular models of Parkinson's disease. *Histochemistry and Cell Biology*, *139*(5), 671–684. https://doi.org/10.1007/S00418-012-1059-4/FIGURES/7

Reticker-Flynn, N. E., & Bhatia, S. N. (2015). Aberrant glycosylation promotes lung cancer metastasis through adhesion to galectins in the metastatic niche. *Cancer Discovery*, *5*(2), 168–181. https://doi.org/10.1158/2159-8290.CD-13-0760

Ribatti, D. (2018). An historical note on the cell theory. In *Experimental Cell Research* (Vol. 364, Issue 1, pp. 1–4). Elsevier Inc. https://doi.org/10.1016/j.yexcr.2018.01.038

Rios, R. M. (2014). The centrosome-Golgi apparatus nexus. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *369*(1650). https://doi.org/10.1098/rstb.2013.0462

Roeckel, N., Woerner, S. M., Kloor, M., Yuan, Y. P., Patsos, G., Gromes, R., Kopitz, J., & Gebert, J. (2009). High Frequency of LMAN1 Abnormalities in Colorectal Tumors with Microsatellite Instability. *Cancer Research*, *69*(1), 292–299. https://doi.org/10.1158/0008-5472.CAN-08-3314

Romani, P., Brian, I., Santinon, G., Pocaterra, A., Audano, M., Pedretti, S., Mathieu, S., Forcato, M., Bicciato, S., Manneville, J. B., Mitro, N., & Dupont, S. (2019). Extracellular matrix mechanical cues regulate lipid metabolism through Lipin-1 and SREBP. *Nature Cell Biology 2019 21:3*, *21*(3), 338–347. https://doi.org/10.1038/s41556-018-0270-5

Sanders, A. A. W. M., & Kaverina, I. (2015). Nucleation and Dynamics of Golgi-derived Microtubules. *Frontiers in Neuroscience*, *9*, 431. https://doi.org/10.3389/fnins.2015.00431

Scaltriti, M., Elkabets, M., & Baselga, J. (2016). Molecular pathways: AXL, a membrane receptor mediator of resistance to therapy. *Clinical Cancer Research*, *22*(6), 1313–1317. https://doi.org/10.1158/1078-0432.CCR-15-1458

Schlienger, S., Campbell, S., & Claing, A. (2014). ARF1 regulates the Rho/MLC pathway to control EGF-dependent breast cancer cell invasion. *Molecular Biology of the Cell*, *25*(1), 17. https://doi.org/10.1091/MBC.E13-06-0335

Schmidt, W. M., Rutledge, S. L., Schüle, R., Mayerhofer, B., Züchner, S., Boltshauser, E., & Bittner, R. E. (2015). *Disruptive SCYL1 Mutations Underlie a Syndrome Characterized by Recurrent Episodes of Liver Failure, Peripheral Neuropathy, Cerebellar Atrophy, and Ataxia*. https://doi.org/10.1016/j.ajhg.2015.10.011

Sciaky, N., Presley, J., Smith, C., Zaal, K. J., Cole, N., Moreira, J. E., Terasaki, M., Siggia, E., & Lippincott-Schwartz, J. (1997a). Golgi tubule traffic and the effects of brefeldin A visualized in living cells. *The Journal of Cell Biology*, *139*(5), 1137–1155. https://doi.org/10.1083/jcb.139.5.1137

Sciaky, N., Presley, J., Smith, C., Zaal, K. J., Cole, N., Moreira, J. E., Terasaki, M., Siggia, E., & Lippincott-Schwartz, J. (1997b). Golgi tubule traffic and the effects of brefeldin A visualized in living cells. *The Journal of Cell Biology*, *139*(5), 1137–1155. https://doi.org/10.1083/jcb.139.5.1137

Sciaky, N., Presley, J., Smith, C., Zaal, K. J. M., Cole, N., Moreira, J. E., Terasaki, M., Siggia, E., & Lippincott-Schwartz, J. (1997c). Golgi Tubule Traffic and the Effects of Brefeldin A Visualized in Living Cells. *The Journal of Cell Biology*, *139*(5). http://www.jcb.org

Scott, K. L., Kabbarah, O., Liang, M. C., Ivanova, E., Anagnostou, V., Wu, J., Dhakal, S., Wu, M., Chen, S., Feinberg, T., Huang, J., Saci, A., Widlund, H. R., Fisher, D. E., Xiao, Y., Rimm, D. L., Protopopov, A., Wong, K. K., & Chin, L. (2009). GOLPH3 modulates mTOR signaling and rapamycin sensitivity in cancer. *Nature*, *459*(7250), 1085. https://doi.org/10.1038/NATURE08109

Sengupta, D., & Linstedt, A. D. (2010). Mitotic Inhibition of GRASP65 Organelle Tethering Involves Polo-like Kinase 1 (PLK1) Phosphorylation Proximate to an Internal PDZ Ligand. *Journal of Biological Chemistry*, *285*(51), 39994–40003. https://doi.org/10.1074/JBC.M110.189449

Sengupta, P., Satpute-Krishnan, P., Seo, A. Y., Burnette, D. T., Patterson, G. H., & Lippincott-Schwartz, J. (2015). ER trapping reveals Golgi enzymes continually revisit the ER through a recycling pathway that controls Golgi organization. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(49), E6752-61. https://doi.org/10.1073/pnas.1520957112

Sharma, V., Ichikawa, M., & Freeze, H. H. (2014). MANNOSE METABOLISM: MORE THAN MEETS THE EYE. *Biochemical and Biophysical Research Communications*, *453*(2), 220. https://doi.org/10.1016/J.BBRC.2014.06.021

Shima, D. T., Haldar, K., Pepperkok, R., Watson, R., & Warren, G. (1997). Partitioning of the Golgi Apparatus during Mitosis in Living HeLa Cells. In *The Journal of Cell Biology* (Vol. 137, Issue 6).

Singh, V., Erady, C., & Balasubramanian, N. (2018). Cell-matrix adhesion controls Golgi organization and function through Arf1 activation in anchorage-dependent cells. *Journal of Cell Science*, *131*(16). https://doi.org/10.1242/jcs.215855

Small, S. A., Kent, K., Pierce, A., Leung, C., Kang, M. S., Okada, H., Honig, L., Vonsattel, J. P., & Kim, T. W. (2005). Model-guided microarray implicates the retromer complex in Alzheimer's disease. *Annals of Neurology*, *58*(6), 909–919. https://doi.org/10.1002/ANA.20667

Smits, P., Bolton, A. D., Funari, V., Hong, M., Boyden, E. D., Lu, L., Manning, D. K., Dwyer, N. D., Moran, J. L., Prysak, M., Merriman, B., Nelson, S. F., Bonafé, L., Superti-Furga, A., Ikegawa, S., Krakow, D., Cohn, D. H., Kirchhausen, T., Warman, M. L., … David, the. (2010). Lethal Skeletal Dysplasia in Mice and Humans Lacking the Golgin GMAP-210 A bs tr ac t. In *N Engl J Med* (Vol. 362).

Spang, A., Shiba, Y., & Randazzo, P. A. (2010). Arf GAPs: gatekeepers of vesicle generation. *FEBS Letters*, *584*(12), 2646–2651. https://doi.org/10.1016/j.febslet.2010.04.005

Stalder, D., & Gershlick, D. C. (2020a). Direct trafficking pathways from the Golgi apparatus to the plasma membrane. *Seminars in Cell & Developmental Biology*, *107*, 112–125. https://doi.org/10.1016/j.semcdb.2020.04.001

Stalder, D., & Gershlick, D. C. (2020b). Direct trafficking pathways from the Golgi apparatus to the plasma membrane. *Seminars in Cell & Developmental Biology*, *107*, 112–125. https://doi.org/10.1016/j.semcdb.2020.04.001

Stanley, P. (2011). Golgi glycosylation. *Cold Spring Harbor Perspectives in Biology*, *3*(4). https://doi.org/10.1101/cshperspect.a005199

Stanley, P., Taniguchi, N., & Aebi, M. (2015). N-Glycans. In A. Varki, R. D. Cummings, J. D. Esko, P. Stanley, G. W. Hart, M. Aebi, A. G. Darvill, T. Kinoshita, N. H. Packer, J. H. Prestegard, R. L. Schnaar, & P. H. Seeberger (Eds.), *Essentials of Glycobiology* (3rd ed.). Cold Spring Harbor Laboratory Press. https://doi.org/10.1101/glycobiology.3e.009

Stowell, S. R., Ju, T., & Cummings, R. D. (2015). Protein glycosylation in cancer. *Annual Review of Pathology*, *10*, 473–510. https://doi.org/10.1146/annurev-pathol-012414-040438

Stringer, C., Wang, T., Michaelos, M., & Pachitariu, M. (2020). Cellpose: a generalist algorithm for cellular segmentation. *Nature Methods 2020 18:1*, *18*(1), 100–106. https://doi.org/10.1038/s41592-020-01018-x

Strous, G. J., Van Kerkhof, P., Van Meer, G., Rijnboutt, S., & Stoorvogel, W. (1993). Differential effects of brefeldin A on transport of secretory and lysosomal proteins. *Journal of Biological Chemistry*, *268*(4), 2341–2347. https://doi.org/10.1016/S0021-9258(18)53781-9

Sü, C., Hsu, P., Mallabiabarrena, A., & Malhotra, V. (2002). Fragmentation and Dispersal of the Pericentriolar Golgi Complex Is Required for Entry into Mitosis in Mammalian Cells been shown that these small Golgi fragments undergo fragmentation into small vesicles or fuse with the ER (Kano et al. In *Cell* (Vol. 109).

Sun, X., Mahajan, D., Chen, B., Song, Z., & Lu, L. (2021). A quantitative study of the Golgi retention of glycosyltransferases. *Journal of Cell Science*, *134*(20). https://doi.org/10.1242/JCS.258564/2105361/JCS258564.PDF

Sundararaman, A., Amirtham, U., & Rangarajan, A. (2016). Calcium-Oxidant Signaling Network Regulates AMP-activated Protein Kinase (AMPK) Activation upon Matrix Deprivation. *The Journal of Biological Chemistry*, *291*(28), 14410–14429. https://doi.org/10.1074/JBC.M116.731257

Sütterlin, C., Polishchuk, R., Pecot, M., & Malhotra, V. (2005). The Golgi-associated Protein GRASP65 Regulates Spindle Dynamics and Is Essential for Cell Division. *Molecular Biology of the Cell*, *16*(7), 3211. https://doi.org/10.1091/MBC.E04-12-1065

Szklarczyk, D., Franceschini, A., Wyder, S., Forslund, K., Heller, D., Huerta-Cepas, J., Simonovic, M., Roth, A., Santos, A., Tsafou, K. P., Kuhn, M., Bork, P., Jensen, L. J., & Von Mering, C. (2015). STRING v10: protein-protein interaction networks, integrated over the tree of life. *Nucleic Acids Research*, *43*(Database issue), D447–D452. https://doi.org/10.1093/NAR/GKU1003

Tajadura-Ortega, V., Gambardella, G., Skinner, A., Schjoldager, K. T.-B. G., Beatson, R., Graham, R., Achkova, D., Taylor-Papadimitriou, J., Ciccarelli, F. D., & Burchell, J. M. (2019). O-linked mucin-type glycosylation regulates the transcriptional programme downstream of EGFR in breast cancer. *BioRxiv*. https://doi.org/10.1101/714675

Tan, X., Banerjee, P., Guo, H. F., Ireland, S., Pankova, D., Ahn, Y. H., Nikolaidis, I. M., Liu, X., Zhao, Y., Xue, Y., Burns, A. R., Roybal, J., Gibbons, D. L., Zal, T., Creighton, C. J., Ungar, D., Wang, Y., & Kurie, J. M. (2017). Epithelial-to-mesenchymal transition drives a pro-metastatic Golgi compaction process through scaffolding protein PAQR11. *The Journal of Clinical Investigation*, *127*(1), 117. https://doi.org/10.1172/JCI88736

Tang, D., Zhang, X., Huang, S., Yuan, H., Li, J., & Wang, Y. (2016). Mena-GRASP65 interaction couples actin polymerization to Golgi ribbon linking. *Molecular Biology of the Cell*, *27*(1), 137–152. https://doi.org/10.1091/mbc.E15-09-0650

Taniguchi, H., Yamada, T., Wang, R., Tanimura, K., Adachi, Y., Nishiyama, A., Tanimoto, A., Takeuchi, S., Araujo, L. H., Boroni, M., Yoshimura, A., Shiotsu, S., Matsumoto, I., Watanabe, S., Kikuchi, T., Miura, S., Tanaka, H., Kitazaki, T., Yamaguchi, H., … Yano, S. (2019). AXL confers intrinsic resistance to osimertinib and advances the emergence of tolerant cells. *Nature Communications*, *10*(1). https://doi.org/10.1038/s41467-018-08074-0

Thomas, L. L., & Fromme, J. C. (2020). Extensive GTPase crosstalk regulates Golgi trafficking and maturation. *Current Opinion in Cell Biology*, *65*, 1–7. https://doi.org/10.1016/j.ceb.2020.01.014

Torgovnick, A., & Schumacher, B. (2015). DNA repair mechanisms in cancer development and therapy. *Frontiers in Genetics*, *6*(APR). https://doi.org/10.3389/FGENE.2015.00157

Valenzuela, H. F., Pace, K. E., Cabrera, P. V., White, R., Porvari, K., Kaija, H., Vihko, P., & Baum, L. G. (2007). O-Glycosylation Regulates LNCaP Prostate Cancer Cell Susceptibility to Apoptosis Induced by Galectin-1. *Cancer Research*, *67*(13), 6155–6162. https://doi.org/10.1158/0008-5472.CAN-05-4431

Varki, A. (2017). Biological roles of glycans. *Glycobiology*, *27*(1), 3. https://doi.org/10.1093/GLYCOB/CWW086

Very, N., Lefebvre, T., & El Yazidi-Belkoura, I. (2018). Drug resistance related to aberrant glycosylation in colorectal cancer. *Oncotarget*, *9*(1), 1380–1402. https://doi.org/10.18632/oncotarget.22377

Volpicelli-Daley, L. A., Li, Y., Zhang, C. J., & Kahn, R. A. (2005). Isoform-selective effects of the depletion of ADP-ribosylation factors 1-5 on membrane traffic. *Molecular Biology of the Cell*, *16*(10), 4495–4508. https://doi.org/10.1091/MBC.E04-12-1042

Walton, K., Leier, A., & Sztul, E. (2020). Regulating the regulators: role of phosphorylation in modulating the function of the GBF1/BIG family of Sec7 ARF-GEFs. *FEBS Letters*. https://doi.org/10.1002/1873-3468.13798

Wang, F., Liu, X., Bartholdy, B. A., Cheng, H., & Halmos, B. (2019). Blockade of AXL activation overcomes acquired resistance to EGFR tyrosine kinase inhibition in non-small cell lung cancer. *Translational Cancer Research*, *8*(6), 2425–2438. https://doi.org/10.21037/tcr.2019.09.61

Ward, T. H., Polishchuk, R. S., Caplan, S., Hirschberg, K., & Lippincott-Schwartz, J. (2001). Maintenance of Golgi structure and function depends on the integrity of ER export. *The Journal of Cell Biology*, *155*(4), 557–570. https://doi.org/10.1083/jcb.200107045

Waring, P., & Müllbacher, A. (1999). Cell death induced by the Fas/Fas ligand pathway and its role in pathology. *Immunology and Cell Biology*, *77*(4), 312–317. https://doi.org/10.1046/J.1440-1711.1999.00837.X

Wei, J. H., & Seemann, J. (2010). Unraveling the Golgi ribbon. In *Traffic* (Vol. 11, Issue 11, pp. 1391–1400). https://doi.org/10.1111/j.1600-0854.2010.01114.x

Weller, S. G., Capitani, M., Cao, H., Micaroni, M., Luini, A., Sallese, M., & McNiven, M. A. (2010). Src kinase regulates the integrity and function of the Golgi apparatus via activation of dynamin 2. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(13), 5863–5868. https://doi.org/10.1073/pnas.0915123107

Wheeler, D. B., Zoncu, R., Root, D. E., Sabatini, D. M., & Sawyers, C. L. (2015). Identification of an Oncogenic RAB Protein. *Science (New York, N.Y.)*, *350*(6257), 211. https://doi.org/10.1126/SCIENCE.AAA4903

Witkos, T. M., Chan, W. L., Joensuu, M., Rhiel, M., Pallister, E., Thomas-Oates, J., Mould, A. P., Mironov, A. A., Biot, C., Guerardel, Y., Morelle, W., Ungar, D., Wieland, F. T.,

Jokitalo, E., Tassabehji, M., Kornak, U., & Lowe, M. (2019). GORAB scaffolds COPI at the trans-Golgi for efficient enzyme recycling and correct protein glycosylation. *Nature Communications*, *10*(1), 127. https://doi.org/10.1038/s41467-018-08044-6

Witkos, T. M., & Lowe, M. (2015). The Golgin Family of Coiled-Coil Tethering Proteins. *Frontiers in Cell and Developmental Biology*, *3*, 86. https://doi.org/10.3389/fcell.2015.00086

Wium, M., Ajayi-smith, A. F., Paccez, J. D., & Zerbini, L. F. (2021a). The role of the receptor tyrosine kinase axl in carcinogenesis and development of therapeutic resistance: An overview of molecular mechanisms and future applications. In *Cancers* (Vol. 13, Issue 7). MDPI AG. https://doi.org/10.3390/cancers13071521

Wium, M., Ajayi-smith, A. F., Paccez, J. D., & Zerbini, L. F. (2021b). The role of the receptor tyrosine kinase axl in carcinogenesis and development of therapeutic resistance: An overview of molecular mechanisms and future applications. In *Cancers* (Vol. 13, Issue 7). MDPI AG. https://doi.org/10.3390/cancers13071521

Wium, M., Ajayi-Smith, A. F., Paccez, J. D., & Zerbini, L. F. (2021). The role of the receptor tyrosine kinase axl in carcinogenesis and development of therapeutic resistance: an overview of molecular mechanisms and future applications. *Cancers*, *13*(7). https://doi.org/10.3390/cancers13071521

Xiang, Y., & Wang, Y. (2011). New components of the Golgi matrix. *Cell and Tissue Research*, *344*(3), 365–379. https://doi.org/10.1007/s00441-011-1166-x

Xiang, Y., Zhang, X., Nix, D. B., Katoh, T., Aoki, K., Tiemeyer, M., & Wang, Y. (2013a). Regulation of protein glycosylation and sorting by the Golgi matrix proteins GRASP55/65. *Nature Communications*, *4*, 1659. https://doi.org/10.1038/ncomms2669

Xiang, Y., Zhang, X., Nix, D. B., Katoh, T., Aoki, K., Tiemeyer, M., & Wang, Y. (2013b). Regulation of protein glycosylation and sorting by the Golgi matrix proteins GRASP55/65. *Nature Communications*, *4*, 1659. https://doi.org/10.1038/ncomms2669

Xie, X., Tang, S.-C., Cai, Y., Pi, W., Deng, L., Wu, G., Chavanieu, A., Teng, Y., Xie, X., Tang, S.-C., Cai, Y., Pi, W., Deng, L., Wu, G., Chavanieu, A., & Teng, Y. (2016). Suppression of breast cancer metastasis through the inactivation of ADP-ribosylation factor 1. *Oncotarget*, *7*(36), 58111–58120. https://doi.org/10.18632/ONCOTARGET.11185

Xu, C., & Ng, D. T. W. (2015). Glycosylation-directed quality control of protein folding. *Nature Reviews. Molecular Cell Biology*, *16*(12), 742–752. https://doi.org/10.1038/nrm4073

Xu, M., Motabar, O., Ferrer, M., Marugan, J. J., Zheng, W., & Ottinger, E. A. (n.d.). *Disease models for the development of therapies for lysosomal storage diseases*. https://doi.org/10.1111/nyas.13052

Xu, M. Z., Chan, S. W., Liu, A. M., Wong, K. F., Fan, S. T., Chen, J., Poon, R. T., Zender, L., Lowe, S. W., Hong, W., & Luk, J. M. (2011). AXL receptor kinase is a mediator of YAP-dependent oncogenic functions in hepatocellular carcinoma. *Oncogene*, *30*(10), 1229. https://doi.org/10.1038/ONC.2010.504

Yadav, A., Vagne, Q., Sens, P., Iyengar, G., & Rao, M. (2022). Glycan processing in the Golgi: optimal information coding and constraints on cisternal number and enzyme specificity. *ELife*, *11*. https://doi.org/10.7554/ELIFE.76757

Yadav, S., Puthenveedu, M. A., & Linstedt, A. D. (2012). Golgin160 Recruits the Dynein Motor to Position the Golgi Apparatus. *Developmental Cell*, *23*(1), 153–165. https://doi.org/10.1016/j.devcel.2012.05.023

Yarwood, R., Hellicar, J., Woodman, P. G., & Lowe, M. (2020). Membrane trafficking in health and disease. *Disease Models & Mechanisms*, *13*(4). https://doi.org/10.1242/DMM.043448

Ye, X., Li, Y., Stawicki, S., Couto, S., Eastham-Anderson, J., Kallop, D., Weimer, R., Wu, Y., & Pei, L. (2010). An anti-Axl monoclonal antibody attenuates xenograft tumor growth and enhances the effect of multiple anticancer therapies. *Oncogene*, *29*(38), 5254–5264. https://doi.org/10.1038/onc.2010.268

Yin, Y. X., Shen, F., Pei, H., Ding, Y., Zhao, H., Zhao, M., & Chen, Q. (2012). Increased expression of Rab25 in breast cancer correlates with lymphatic metastasis. *Tumor Biology*, *33*(5), 1581–1587. https://doi.org/10.1007/S13277-012-0412-5/METRICS

Zajac, O., Leclere, R., Nicolas, A., Meseure, D., Marchiò, C., Vincent-Salomon, A., Roman-Roman, S., Schoumacher, M., & Dubois, T. (n.d.). *AXL Controls Directed Migration of Mesenchymal Triple-Negative Breast Cancer Cells*. https://doi.org/10.3390/cells9010247

Zajac, O., Leclere, R., Nicolas, A., Meseure, D., Marchiò, C., Vincent-Salomon, A., Roman-Roman, S., Schoumacher, M., & Dubois, T. (2020). AXL Controls Directed Migration of Mesenchymal Triple-Negative Breast Cancer Cells. *Cells*, *9*(1). https://doi.org/10.3390/CELLS9010247

Zhai, X., Pu, D., Wang, R., Zhang, J., Lin, Y., Wang, Y., Zhai, N., Peng, X., Zhou, Q., & Li, L. (2023). Gas6/AXL pathway: immunological landscape and therapeutic potential. *Frontiers in Oncology*, *13*. https://doi.org/10.3389/FONC.2023.1121130

Zhang, X., & Wang, Y. (2015). GRASPs in Golgi Structure and Function. *Frontiers in Cell and Developmental Biology*, *3*, 84. https://doi.org/10.3389/fcell.2015.00084

Zhang, X., & Wang, Y. (2016). Glycosylation quality control by the golgi structure. *Journal of Molecular Biology*, *428*(16), 3183–3193. https://doi.org/10.1016/j.jmb.2016.02.030

Zhang, Z., Lee, J. C., Lin, L., Olivas, V., Au, V., Laframboise, T., Abdel-Rahman, M., Wang, X., Levine, A. D., Rho, J. K., Choi, Y. J., Choi, C. M., Kim, S. W., Jang, S. J., Park, Y. S., Kim, W. S., Lee, D. H., Lee, J. S., Miller, V. A., … Bivona, T. G. (2012). Activation of the AXL kinase causes resistance to EGFR-targeted therapy in lung cancer. *Nature Genetics*, *44*(8), 852–860. https://doi.org/10.1038/ng.2330

Zhong, W. (2011). Golgi during Development. *Cold Spring Harbor Perspectives in Biology*, *3*(9), 1–17. https://doi.org/10.1101/CSHPERSPECT.A005363

Zhou, C., Cunningham, L., Marcus, A. I., Li, Y., & Kahn, R. A. (2006). Arl2 and Arl3 Regulate Different Microtubule-dependent Processes □ V. *Molecular Biology of the Cell*, *17*, 2476–2487. https://doi.org/10.1091/mbc.E05-10

Zhu, C., Wei, Y., & Wei, X. (2019). AXL receptor tyrosine kinase as a promising anti-cancer approach: functions, molecular mechanisms and clinical applications. *Molecular Cancer 2019 18:1*, *18*(1), 1–22. https://doi.org/10.1186/S12943-019-1090-3