

SIGNIFICANCE OF ALTERED GOLGI APPARATUS AND CELL FUSION EVENTS IN OVARIAN CANCER CELLS

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ABSTRACT

Ovarian cancer is one of the most lethal gynaecological cancer. Heterogeneity associated with this cancer can be partially attributed to cancer cells being highly multinucleated and polyploid in nature. Stress-induced cytotoxic effects of cisplatin - a chemotherapy drug used to treat ovarian cancer, on subcellular organelles including the ER, mitochondria, nucleus and the cytoskeleton are well known, however, its effect on the structural integrity of the Golgi apparatus and associated alterations in functions are not reported. In this study, the effect of cisplatin on the structure and function of the Golgi apparatus are examined and a novel pathway for cisplatin-induced changes in the Golgi is also reported. Additionally, a mechanism is also proposed for formation of multinucleated ovarian cancer cells and a role for *transcription adapter-3* (TADA3)-mediated multinucleation has been suggested.

Treatment of ovarian cancer cells with cisplatin resulted in significant fragmentation of the Golgi apparatus with cisplatin-induced Golgi fragmentation detected before cisplatin-induced DNA damage. Inhibition of cisplatin-stress induced activation of c-Jun-N-terminal kinase (JNK) significantly reduced fragmentation of the Golgi in ovarian cancer cells while extracellular signal-regulated kinase (ERK1/2) inhibition significantly increased fragmentation in A2780 cells but had no effect on other cell lines (OVCAR3 and SKOV3) tested. Cells exposed to cisplatin during migration showed significantly depolarised Golgi, reduced cell motility and wound healing capacity. Protein trafficking was also significantly reduced in response to cisplatin. Cell-line specific effect was found on the expression of terminal GalNAc glycans in response to cisplatin. Moreover, ovarian cancer cells were observed to fuse with each other following cell division producing multinucleated cells. Gene expression correlation analysis identified *TADA3* as a candidate gene associated with multinuclearity. *TADA3* knockdown significantly reduced the number of multinucleated ovarian cancer cells, suggesting a role in cancer cell fusion.

In conclusion, this study proposes a novel mode of action for cisplatin-induced cytotoxicity in ovarian cancer cells. Understanding its biology further can open avenues for future treatment options. This study also identifies a novel mechanism for cancer cell fusion. Further investigation in this area can help unravel issues of drug resistance and associated tumour relapse.

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ABBREVIATIONS

17-DMAG	17-dimethylaminoethylamino-17-demethoxygeldanamycin
Abcb1a	ATP-binding cassette, sub-family B, member 1A
Abcb1b	ATP-binding cassette, sub-family B, member 1B
ActB	β actin
Ada3	alteration/deficiency in activation
AF	alexa fluor
AnxA5	annexin A5
APAF-1	apoptosis promoting activating factor-1
ARF1	ADP-ribosylation factor-1
ARID1A	AT-rich interaction domain 1A
ASCT2	Alanine, Serine, Cystein Transporter 2
ATP	adenosine triphosphate
ATP7A	ATPase copper transporting alpha
ATP7B	ATPase copper transporting beta
BFA	brefeldin A
BMDC	bone marrow-derived dendritic cells
BRCA1	breast cancer gene 1
BRCA2	breast cancer gene 2
BSA	bovine serum albumin
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
c-Abl	Abelson tyrosine kinase

CCL21/CCR7	chemokine (C-C motif) ligand 21/C-C chemokine receptor type 7
CD9	tetraspanin
Cdk1	cyclin-dependent kinase 1
CDKN1A	cyclin-dependent kinase inhibitor 1A
COPI	coat protein complex I
CtBP1-S/BARS	C-terminal-binding protein 1
CTNNB1	Catenin beta 1
CTR1	copper transporter 1
CXCR4	C-X-C chemokine receptor type 4
DAB	3,3'-diaminobenzidine
DISC	death inducing signaling complex
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DNA-PKcs	catalytic subunit of DNA-PK
DPBS	Dulbecco's phosphate buffered saline
Duf	Dumbfounded
DYRK1B	Dual-specificity Y-phosphorylation regulated kinase 1B
<i>E. coli</i>	Escherichia coli
EFF-1	epithelial fusion failure 1
EGFP	enhanced green fluorescent protein
EHP	external hydrophobic peptide
EMT	epithelial to mesenchymal transition
Env	envelope protein

EOC	epithelial ovarian cancer
ER	endoplasmic reticulum
ERBB2	Erb-B2 receptor tyrosine kinase 2
ERK	extracellular signal-regulated kinase
EXO1	exonuclease 1
FCS	fetal calf serum
FRAP	fluorescence recovery after photobleaching
G2	Gap 2
GADD45	growth arrest and DNA damage
GalNAc	α -N-acetylgalactosamine
GALNT	N-acetylgalactosaminyltransferase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GGR	global genome repair
GlcNAc	N-acetylglucosamine
GM130	130 KDa cis-Golgi matrix protein
GOLPH3	Golgi phosphoprotein 3
GOLPH3L	Golgi phosphoprotein-3-like
GRASP55	Golgi reassembly and stacking protein 55 KDa
GRASP65	Golgi reassembly and stacking protein 65 KDa
GSH	glutathione
HA	hemagglutinin
Hbs	Hibris
HIV-1	human immunodeficiency virus type 1
HPA	helix pomatia agglutinin

HRAS	Harvey rat sarcoma viral oncogene homolog
IC50	half-maximal inhibitory concentration
ICR	interstrand crosslink repair
IMS	industrial methylated spirit
JNK	c-Jun N-terminal kinase
KRAS	Kirsten rat sarcoma viral oncogene homolog
LB	Luria broth
LMP	lysosome membrane permeabilisation
MAP	mitogen activated protein
Mdm2	mouse double minute 2
MEK1	MAP kinase kinase 1
MFR/CD47	leukocyte surface antigen
MLH1	mutL homolog 1
MMR	mismatch repair
MSH6	MutS homolog 6
mTOR	mammalian target of rapamycin
MUC1	mucin 1
MUC16	mucin 16
MutL	DNA mismatch repair protein
MutSa/MSH2	DNA mismatch repair protein
MYO18A	myosin XVIII A
NER	nucleotide excision repair
NHE1	sodium-hydrogen antiporter 1
NTC	no template control

OC	ovarian cancer
p115	general vesicular transport factor
p21	cyclin dependent kinase inhibitor
p73	tumour protein 73
PAQR11	progesterin and adipoQ 11 receptors
PBS	phosphate buffered saline
PI4P	phosphatidylinositol 4-phosphate
PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase
Plk1	polo-like kinase 1
POLH	DNA polymerase eta
Pol δ	DNA polymerase delta
Pol ϵ	DNA polymerase ϵ
PS	phosphatidylserine
PTEN	phosphatase and tensin homolog deleted on chromosome 10
PTPNC1	phosphatidylinositol transfer protein cytoplasmic 1
qPCR	quantitative polymerase chain reaction
RAB1B	Ras-related protein Rab-1B
RAF1	Raf-1 proto-oncogene, serine/threonine kinase
REV3	reversionless 3
RNA	ribonucleic acid
RNAi	RNA interference
ROI	region of interest
ROS	reactive oxygen species
Rst	Roughest

SAC	spindle assembly checkpoint
SCID	severely compromised immunodeficient
SFM	serum free media
siRNA	short interfering RNA
Sns	Sticks and stones
STn	sialyl-Tn antigen
Syn1	syncytin-1
TADA3	transcriptional adaptor 3
TADA3L	transcriptional adaptor 3-like
TBS	tris buffered saline
TCR	transcription-coupled repair
TE	Tris-EDTA
TFIIH	transcription and DNA repair factor IIH
TNF α	tumour necrosis factor- α
TP53	tumour antigen p53
ts045-EGFP-VSVG	temperature sensitive mutant of vesicular stomatitis G envelope glycoprotein fused to green fluorescent protein
UPR	unfolded protein response
VSVG	vesicular stomatitis viral G protein
WGA	wheat germ agglutinin
XPB	xeroderma pigmentosum group B protein complex
XPC-HR23B	xeroderma pigmentosum group C protein complex
XPD	xeroderma pigmentosum group D protein complex
XPF/ERCC1	DNA repair endonuclease
XPG	xeroderma pigmentosum group G protein complex

γ H2A.X gamma histone 2 A phosphorylated

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INTRODUCTION

CHAPTER 1

1.1 OVARIAN CANCER

1.1.1 Overview

Ovarian cancer (OC) is the seventh most common cause of cancer related deaths in women worldwide and the most common gynaecologic malignancy (Sun *et al.*, 2017). It is estimated that around 230,000 women will be diagnosed and 150,000 will die of epithelial ovarian cancer (EOC), the most common form, annually worldwide (Ferlay *et al.*, 2012). The 5-year survival rate of the patients is 46% despite the advances in chemotherapeutic agents and surgery (Doherty *et al.*, 2017). This is attributed mostly to majority of the cases being diagnosed at an advanced stage. The 5-year survival rate for patients diagnosed at an early stage is 92% compared to 29% for late-stage diagnosis (Reid, Permuth and Sellers, 2017). Thus, constant effort is made to detect the cancer in high-risk patients. However, EOC is highly heterogeneous with histologically defined subtypes such as serous, endometrioid, mucinous and clear cell (Bonome *et al.*, 2005). Each subtype involves high and low grade tumours, and is comprised of different underlying molecular signatures and clinical manifestations, which remain poorly understood (Saad, Hu and Sood, 2010).

1.1.2 Origin of ovarian cancer

For decades the general notion has been that ovarian cancer originates from three main cell types that make up the ovary: (1) germ cells, derived from the endoderm, proliferate and differentiate into oocytes, (2) endocrine and interstitial cells, that produce estrogen and progesterone, and (3) epithelial cells, derived from the Mullerian duct, line the ovary and inclusion cysts beneath the ovarian surface (Romero and Bast Jr, 2012). During ovulation, oocytes are released from the mature follicles of the ovary and enter the Fallopian tube, where fertilisation takes place (Romero and Bast Jr, 2012). Each of the three cell types can reportedly form benign and malignant tumours, with germ cell tumours accounting for 3-5% of ovarian cancers and frequently arise in the second and third decade (Smith *et al.*, 2006). Sex-cord stromal tumours arising from hormone-

producing cells, account for approximately 7% of ovarian malignancies and have been reported to affect women of all ages (Horta and Cunha, 2015). Epithelial ovarian cancers account for 90% of all malignant ovarian tumours and is generally known to affect women over the age of 40 (Romero and Bast Jr, 2012).

1.1.3 Clinical management of the disease

The past three decades has seen a rise in 5-year survival rate of ovarian cancer patients from 37% to 45%, and that is attributed to consistent use of cytoreductive surgery and combination chemotherapy using platinum-based compounds and taxanes (Jemal *et al.*, 2009).

1.1.3.1 Cytoreductive surgery

Cytoreductive surgery or more commonly known as ‘debulking’ involves containment of the disseminated ovarian tumour masses within the peritoneal cavity – the lining of the abdomen (Matulonis *et al.*, 2016). The cytoreductive surgery is performed in patients with early and late stage cancer, although the likelihood of successful cytoreduction is substantially diminished in patients at late stage due to widespread nature of metastatic lesions which prevent complete cytoreduction (Jayson *et al.*, 2014). The primary course of treatment for patients with high grade serous OC is most often a cytoreductive surgery followed by removal of gross tumour tissue, the reproductive organs and the sigmoid colon. In some cases peritonectomy – the removal of part or full lining of the abdomen, and omentectomy – removal of the fatty lining of the omentum, may also be performed (Matulonis *et al.*, 2016). A surgical outcome with no macroscopic residual disease is considered successful and is associated with greater overall survival. In clinical practice, although residual cancer <1cm is considered optimal, in advanced stage cancer, ‘complete’ clearance of the abdominal cavity is recommended (Matulonis *et al.*, 2016; Pomel *et al.*, 2007).

1.1.3.2 Cytotoxic chemotherapy

Adjuvant chemotherapy is mostly recommended to patients with advanced ovarian cancer following cytoreductive surgery (Matulonis *et al.*, 2016). The type of chemotherapy received is the same irrespective of EOC subtype.

Ovarian cancer was one of the first malignancies to be successfully treated with chemotherapy drugs (Markman, 2008). The ‘platinum-based therapy’ which has been widely used in recent decades as standard of care for ovarian cancer was introduced in late 1970s following the emergence of results from clinical trials that validated its effectiveness in the context of recurrence disease resistant to alkylating agents and doxorubicin (Young *et al.*, 1978). The first such drug to be approved for clinical use was cisplatin. Briefly, cisplatin forms DNA adducts that distorts DNA conformation and inhibits subsequent replication and transcription (Figure 1.1; Peng *et al.*, 2010). This increases the DNA damage response and results in the activation of multiple signalling pathways that induce cell-cycle arrest and apoptosis (Galluzzi *et al.*, 2012). Cisplatin was incorporated into primary chemotherapy either on its own or in combination with other drugs such as cyclophosphamide, doxorubicin or hexamethylmelamine (Aabo *et al.*, 1998). Carboplatin was introduced in late 1980s and was administered in combination with cyclophosphamide following clinical trial results that showed more favourable toxicity profile compared to cisplatin. The results prompted clinicians to replace cisplatin based treatments to carboplatin (Alberts *et al.*, 1992; Swenerton *et al.*, 1992).

The taxanes were also introduced in late 1980s after being isolated from the bark of the pacific yew tree (*Taxus brevifolia*) (McGuire *et al.*, 1989). Paclitaxel (Taxol), the prototype for taxanes, stabilises the microtubular cytoskeleton by inhibiting tubulin depolymerisation which results in dysregulation of the cell cycle, mitotic failure and ultimately, cell death (Bukowska, Gajek and Marczak, 2015). Early reports indicated that paclitaxel treatment resulted in objective response rates (e.g. reduced tumour size) in women with platinum-resistant ovarian cancer (Thigpen *et al.*, 1994; McGuire *et al.*, 1989; McGuire *et al.*, 1996)

Combination chemotherapy involving platinum-based drugs and taxanes was introduced in 1996 following results from a landmark clinical trial that evaluated the effectiveness of cisplatin-paclitaxel versus cisplatin-cyclophosphamide (McGuire *et al.*, 1996). Results showed that cisplatin-paclitaxel combination therapy significantly improved objective response rate, progression free survival and overall survival in patients compared to cisplatin-cyclophosphamide (McGuire *et al.*, 1996). Due to the toxic side-effects of cisplatin, carboplatin has since been used along with paclitaxel following several clinical trial results that showed comparable results (International Collaborative Neoplasm Group, 2002; Bookman, Greer and Ozols, 2003). This combination has thus been the standard of care for ovarian cancer patients for the last 20 years (Lisio *et al.*, 2019).

1.2 CISPLATIN

1.2.1 Overview

Cisplatin or cis-diamminedichloroplatinum is one of several platinum compounds that have been used as anticancer drugs to treat several cancers including ovarian and have been shown to improve survival outcomes in women with OC in the last 25 years (Aabo *et al.*, 1998). Although its biochemical mechanisms of action are not entirely known, research indicates that by covalently binding to N-7 atoms of purines, cisplatin forms DNA adducts that distort DNA conformation and inhibits subsequent replication and transcription (Peng *et al.*, 2010). This increases the DNA damage response and results in the activation of multiple signalling pathways that induce cell-cycle arrest and apoptosis (Galluzzi *et al.*, 2012). However, the emergence of cisplatin resistance in OC cells poses a major obstacle in the treatment and clinical management of the disease (Cannistra, 2004). Therefore, cisplatin is often used as a first-line chemotherapy drug because research has shown that 20% of ovarian tumours treated with cisplatin are either intrinsically resistant to this drug while others have the tendency to acquire resistance towards cisplatin, thus increasing the rate of relapse in these patients (Kelland, 2007).

1.2.2 Biology and mode of action

Once administered in the bloodstream, 65-98% of cisplatin is bound to proteins found in blood such as human serum albumin that contain thiol group and amino acid cysteine and is also associated with the severe side effects observed in response to cisplatin treatment (Ivanov *et al.*, 1998; Lempers and Reedijk, 1991; Andrews, Wung and Howell, 1984; Dolman, Deacon and Hambley, 2002; Borch and Pleasants, 1979). Beck and Brubaker (1973) and Fraval, Rawlings and Roberts (1978) showed that prokaryotic and eukaryotic cells deficient in DNA repair pathway proteins were more sensitive to cisplatin. Their work further highlighted DNA as a critical binding target of cisplatin.

Cisplatin is transported through copper membrane transporter CTR1 into the tumour cell (Ishida *et al.*, 2002). Due to low chloride concentration in the cytosol, cisplatin undergoes the process of aquation, whereby, one or two chloride atoms on cisplatin are displaced by water molecules (Figure 1.1; Rocha *et al.*, 2018). This hydrolysis step activates cisplatin and makes it a potent electrophile (electron acceptor). The sulfhydryl groups on proteins and nitrogen donor atoms on nucleic acids act as the perfect nucleophiles (electron donors) for the hydrolysed cisplatin (Rocha *et al.*, 2018). Consequently, cisplatin binds to N7 atoms on purines and causes DNA adducts such as 1,2-intrastrand cross-links of purine bases (when purines are located on the same strand): 1,2-intrastrand d(GpG) account for 90% of adducts and 1,2-intrastrand d(ApG) account for the remaining 10%, or alternatively, when purines are on opposite strands, an interstrand crosslink is formed (Siddik, 2003). These distort DNA conformation and inhibit subsequent replication and transcription (Peng *et al.*, 2010). This increases the DNA damage response and results in the activation of multiple signalling pathways that induce cell-cycle arrest and apoptosis (Galluzzi *et al.*, 2012). Cells prevent cisplatin from damaging DNA by decreasing cisplatin uptake, increasing cisplatin efflux and inducing drug detoxification by covalently binding to glutathione or metalloproteins (Zisowsky *et al.*, 2007; Rocha *et al.*, 2014). But once cisplatin has caused DNA damage, the major DNA repair mechanisms nucleotide excision repair (NER) and mismatch repair (MMR) are activated (Martin, Hamilton and Schilder, 2008). These are described briefly below.

Nucleotide excision repair (NER):

Nucleotide excision repair (NER) pathway mainly repairs the bulky DNA adducts caused by cisplatin binding, and is composed of two sub-pathways namely, global genome repair (GGR) and transcription-coupled repair (TCR) (Menck and Munford, 2014). GGR involves recognition and repair of damage throughout the genome, while TCR addresses DNA lesions at actively transcribing genes and is induced by stalling of RNA polymerase II caused by intrastrand breakage. The DNA lesion is recognised by the heterodimer XPC- HR23B of GGR along with the stalling of RNA polymerase II induced TCR.

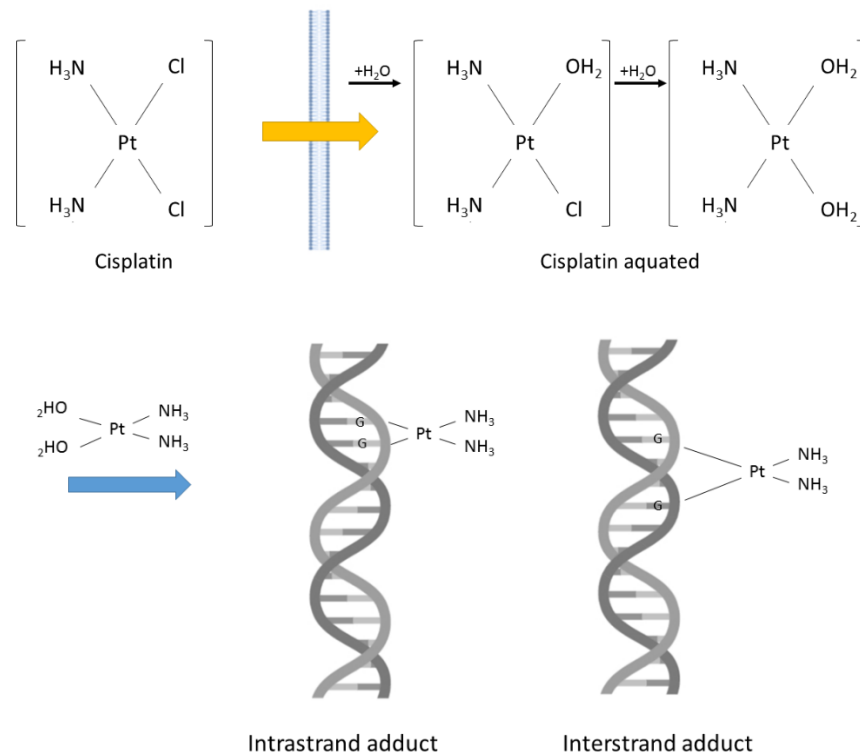


Figure 1.1. Cisplatin-induced DNA adduct formation. Image adapted from Rocha *et al.* (2018).

Helicases XPB and XPD of TFIIH complex separate the double helix. Endonucleases XPF/ERCC1 and XPG then cleave the damaged strand on both sides of the lesion with XPF/ERCC1 cleaving the phosphodiester chain at the 5' while XPG at 3' end. The gap caused by excised oligonucleotide is filled by DNA polymerase ϵ using the intact strand as a template (Menck and Munford, 2014).

Mismatch repair pathway:

The mismatch repair (MMR) pathways corrects single strand DNA errors such as mismatches and insertions or deletions generated during replication (Martin, Lord and Ashworth, 2010). The heterodimers MutS α (formed by MSH2-MSH6) and MutS β (MSH2-MSH3) form a complex with MutL that then helps locate the replication machinery proteins by sliding up and down the double strand DNA in a process regulated by ATP. The DNA daughter strand with nucleotide mismatches is cleaved by exonuclease

EXO1, and the gap is filled by high fidelity replicative polymerases (Pol δ and Pol ϵ). The DNA ligase finally rejoins the DNA sequence. MMR, however, fails to fully repair the damage and the process starts again (Martin, Lord and Ashworth, 2010).

1.2.3 *Cisplatin resistance*

The main limitation to clinical use of cisplatin as an anticancer drug is the high rate of chemoresistance (Ozols 1991; Giaccone, 2000; Koberle *et al.*, 2010). Several other platinum based compounds were developed such as carboplatin and oxaliplatin, with the hopes to reduce the cytotoxic effects of cisplatin, however, clinical data suggest that cisplatin resistant tumours are also resistant to other platinum drugs (Harrap, 1985; Kidani *et al.*, 1978). Even more platinum based drugs such as picoplatin and satraplatin were introduced into clinical trials, but they did not provide with any significant advantage over cisplatin. Cisplatin being the most prominent therapeutic option in specific clinical settings such as for ovarian cancer (Armstrong *et al.*, 2006), it is important to understand the molecular mechanisms that account for cisplatin resistant phenotype in tumours. Galluzzi *et al.* (2012) in their extensive review have systematically categorised these mechanisms in four key groups/stages, which are described below.

(i) *Pre-target resistance*

Pre-target resistance is attained when cancer cells elude cisplatin-cytotoxicity even before cisplatin has interacted with DNA and other cytoplasmic targets. Several cisplatin-resistant cancer cells have shown reduced uptake and accumulation of cisplatin intracellularly (Loh *et al.*, 1992; Mellish, Kelland and Harrap, 1993). Copper transporter 1 (CTR1) is responsible for copper homeostasis and cisplatin uptake. CTR1 deficient mouse embryonic fibroblasts accumulated less cisplatin and were resistant to its cytotoxic effects (Ishida *et al.*, 2002; Katano *et al.*, 2002). Moreover, pre-treatment with copper protected cells from cisplatin cytotoxicity, while copper chelators made cells more sensitive (Ishida *et al.*, 2010). Cisplatin resistant cells also showed increased efflux of cisplatin and that was attributed to expression levels of multidrug resistance protein

(MRP) 2 (Koike *et al.*, 1997; Cui *et al.*, 1999; Liedert *et al.*, 2003). Copper-extruding P-type ATPases ATP7A and ATP7B are reportedly upregulated in cisplatin resistant cancer cells, with ATP7B expression levels predicting cisplatin sensitivity in ovarian and endometrial cancers (Nakayama *et al.*, 2002; Nakayama *et al.*, 2004; Aida *et al.*, 2005). Furthermore, oxidative stress observed in response to cisplatin treatment is a result of aquated cisplatin avidly binding to cysteine-rich proteins such as glutathione (GSH), methionine and metallothioneins (Slater *et al.*, 1995). Elevated levels of GSH, γ -glutamylcysteine synthetase and glutathione S-transferase have been observed in cisplatin resistant cells (Lewis *et al.*, 1988).

(ii) *On-target resistance*

Cisplatin resistant cells often present with impaired DNA repair mechanisms whereby the DNA adducts are either not recognised or they are repaired at an increased pace or in some instances the cells become tolerant towards unrepaired DNA lesions (translesion synthesis), ultimately generating impaired apoptotic signal (Galluzzi *et al.*, 2012). Expression levels of one of the key NER proteins, ERCC1, has been negatively correlated with survival and responsiveness to cisplatin treatment in several cancers including ovarian at clinical level (Dabholkar *et al.*, 1992). Interstrand crosslink repair (ICR), DNA repair pathway that ERCC1 plays a role in, is reduced in cisplatin sensitive cells while it is augmented in cisplatin resistant cells (Zhen *et al.*, 1992; Usanova *et al.*, 2010). Although increased expression levels of ERCC1 does not necessarily correspond to increased NER (and ICR), it has proven to be a promising biomarker for cisplatin resistance and responsiveness in patients (Olaussen, 2009). Moreover, MMR-related proteins MLH1 and MSH2 are involved in recognition of GpG interstrand adducts (Mello *et al.*, 1996; Vaisman *et al.*, 1998). However in case of cisplatin resistance, these proteins fail to repair these adducts and *MLH1* and *MSH2* are either often mutated or under expressed (Aebi *et al.*, 1996; Drummond *et al.*, 1996; Brown *et al.*, 1997). Defects in MMR proteins MLH1 and MSH6 are also associated with increased translesion synthesis (Bassett *et al.*, 2002). Defects in DNA polymerases POLH and REV3 involved in mediating this replicative bypass of GpG adducts are linked with increased sensitivity to cisplatin, while overexpression of REV3 increases cisplatin resistance (Wittschieben *et*

et al., 2006; Roos *et al.*, 2009; Wang *et al.*, 2009). Cancer cells with mutations or deficiencies in homologous recombination repair pathway genes are often more sensitive to cisplatin (Bryant *et al.*, 2005; Farmer *et al.*, 2005). BRCA1/2 deficient ovarian cancers although more metastatic in nature, are also more responsive to platinum treatment and are associated with better prognosis (Ben David *et al.*, 2002; Gourley *et al.*, 2010). It has also been shown that cisplatin resistance can be acquired in cisplatin sensitive tumours in cases where DNA damage repair pathway homologous recombination is re-established as a result of mutations that try to compensate for BRCA1/2 deficiency (Edwards *et al.*, 2008; Sakai *et al.*, 2008) Thus, mutations in DNA repair pathway genes and associated proteins are one of the determining factors for cisplatin resistance.

(iii) *Post-target resistance*

Post-target resistance to cisplatin can result from defects in signaling pathways that elicit apoptosis following DNA damage, and have the potential to influence cisplatin sensitivity (Galluzzi *et al.*, 2012). One of the most important mechanisms involves inactivation of TP53 (Vousden and Lane, 2007). Ovarian cancer patients with wild-type *TP53* reportedly are more sensitive to cisplatin and thus have higher chances to benefit from cisplatin treatment than patients with *TP53* mutations (Gadducci *et al.*, 2002; Feldman *et al.*, 2008). Moreover, DNA content or ploidy could also account for cisplatin resistance with tetraploid cancer cells being more than 10-fold tolerant to cisplatin and oxaliplatin than diploid cancer cells, and is further attributed to mutations in TP53 (Castedo *et al.*, 2006; Vitale *et al.*, 2007).

(iv) *Off-target resistance*

Off-target resistance refers to role played by signaling pathways not directly activated or inactivated in response to cisplatin but indirectly interrupt cisplatin-induced death signals thereby conferring resistance (Galluzzi *et al.*, 2012). The proto-oncogene *ERBB2*, overexpressed in ovarian cancer, leads to nuclear exclusion of cyclin-dependent kinase inhibitor 1A (CDKN1A) and associated cell cycle arrest (Zhou *et al.*, 2001). This

suggestively contributes to cisplatin resistance because CDKN1A-mediated cell cycle arrest exerts antiapoptotic function by allowing cells to repair cisplatin-induced DNA damage (Galluzzi *et al.*, 2012). Dual-specificity Y-phosphorylation regulated kinase 1B (DYRK1B) generally upregulated in several cancers including ovarian, increases expression of antioxidant enzymes thereby exerting prosurvival effect (Friedman, 2007; Deng *et al.*, 2009). Depletion of DYRK1B leads to increased cytotoxicity in response to subapoptotic concentrations of cisplatin in ovarian cancer and leads to oxidative stress (Hu and Friedman, 2010). Off target resistance is conferred by rendering cells less sensitive cisplatin-induced apoptotic signals (Galluzzi *et al.*, 2012).

1.2.4 Role of protein kinases in cisplatin-induced cell death

Cisplatin induces cell death by initiating the apoptosis response, defect in which can confer cisplatin resistance (Basu and Krishnamurthy, 2010). Two major cell death pathways have been described: extrinsic and intrinsic pathway. Briefly, extrinsic pathway is initiated following binding of ligands to tumour necrosis factor- α (TNF α) on the plasma membrane which in turn recruits and activates caspase-8 and ultimately forms death inducing signaling complex (DISC) (Kischkel *et al.*, 1995). Intrinsic pathway is initiated in response to DNA damage and leads to Bcl-2-regulated release of cytochrome c from the mitochondria (Nunez *et al.*, 1998; Gupta, *et al.*, 2012). Cytochrome c interacts with apoptosis promoting activating factor-1 (APAF-1) and causes activation of procaspase-9 and formation of active apoptosome complex (Gupta, *et al.*, 2012). Multiple signaling pathways are activated including extracellular signal-regulated kinase (ERK1/2) and c-Jun N-terminal kinase (JNK) in response to cisplatin-induced DNA damage which contribute to apoptosis (Figure 1.2; Basu and Krishnamurthy, 2010).

1.2.4.1 Extracellular signal regulated kinase (ERK)

In ovarian cancer, ERK functions as a prosurvival protein in response to cisplatin (Persons, Yazlovitskaya and Pelling, 2000; Hayakawa *et al.*, 2000). Following activation by cisplatin, ERK directly phosphorylates p53 and causes upregulation of Mdm2,

GADD45 and p21 (DeHaan, Yazlovitskaya and Persons, 2001). This leads to cell cycle arrest and allows repair of damaged DNA by p53 (DeHaan, Yazlovitskaya and Persons, 2001). Persons *et al.* (1999) found that inhibiting ERK activity increased cisplatin cytotoxicity in ovarian cancer cells further suggesting that activation of ERK in response to cisplatin protects cells from cisplatin cytotoxicity. However, there are also contradicting evidences that suggest that down-regulation of ERK inhibits cisplatin-induced apoptosis in cervical cancer HeLa cells (Wang, Martindale and Holbrook, 2000). Moreover, ovarian cancer cells grown in three-dimensional cultures reportedly acquired resistance to apoptosis mediated by ERK1/2 signaling when exposed to cisplatin (Tang *et al.*, 2010). These evidences suggest that more concrete evidence is required to determine ERK function in response to cisplatin.

1.2.4.2 c-Jun-N-terminal kinase (JNK)

JNK, also known as stress activated protein kinase, is activated in response to stress including cisplatin-induced DNA damage (Basu and Krishnamurthy, 2010). The role of JNK in inducing apoptosis was first identified when cells defective in JNK pathway were also cisplatin-resistant (Zanke *et al.*, 1996). JNK induces apoptosis following binding and activation by p73, a proapoptotic member of the p53 family (Jones, Dickman and Whitmarsh, 2007). Mutations in binding site for p73 resulted in reduced apoptosis (Jones, Dickman and Whitmarsh, 2007). JNK, along with c-Abl, reportedly also functions as a signal transducer for DNA damage repair system MMR in response to cisplatin and induces cell death (Nehme *et al.*, 1997).

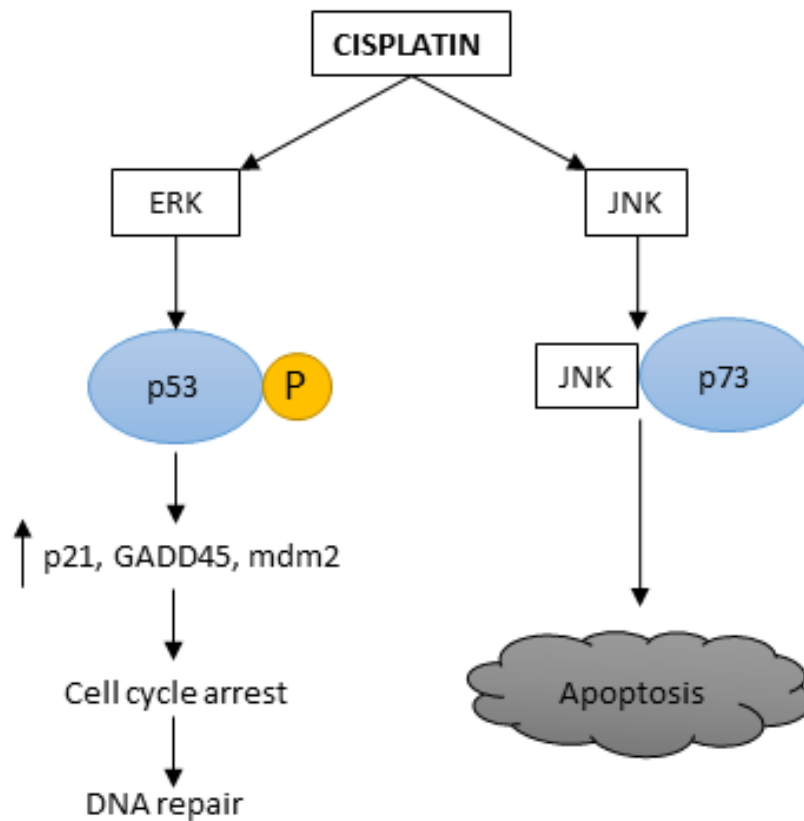


Figure 1.2. Cisplatin-induced activation of ERK and JNK signaling pathways. Activated ERK directly phosphorylates p53 that results in upregulation of p21, GADD45 and mdm2 which regulate cell cycle, DNA repair and p53 activity, respectively. This results in cell cycle arrest and allows repair of damaged DNA by p53. Activated JNK directly binds to and activates p73, a proapoptotic member of p53 family, and results in apoptosis.

1.2.5 Effect of cisplatin on cell organelles

Cisplatin is believed to enter organelles through endosomal route and accumulate in subcellular membranes (Blair *et al.*, 2009; Shen *et al.*, 2004). In addition to causing DNA damage in the nucleus and inducing the DNA damage response, cisplatin also causes injury, alterations and stress in other cell organelles, which are described briefly below.

1.2.5.1 Plasma membrane and Cytoskeleton

Cisplatin reacts with the plasma membrane by forming strong bonds with plasmalemmal proteins and weaker bonds with negatively charged phospholipids on the plasma membrane (Jensen *et al.*, 2010; Speelmans *et al.*, 1997; Taylor *et al.*, 1995). It reduces activity of ion channels and transporters including Na/K ATPase, the sodium-proton exchanger NHE1 and the NA/Pi and Na/glucose co-transporters (Grunicke and Hofmann, 1992; Rebillard *et al.*, 2008; Courjault-Gautier *et al.*, 1995). Treatment with cisplatin also leads to destabilisation of plasmalemma and increased fluidity, and has been suggested to be related to: (i) its direct interaction with phospholipids, (ii) activation of sphingomyelinase by intracellular acidification by NHE1 inhibition and (iii) alterations in cholesterol metabolism (Huang *et al.*, 2003; Liang *et al.*, 2004; Rebillard *et al.*, 2007). Membrane fluidity activates distribution of death receptor Fas which further activates the extrinsic pathway of apoptosis by formation of death inducing signaling complex (DISC) (Ducoroy *et al.*, 2003; Gajate *et al.*, 2009).

Cisplatin also interacts with the cytoskeleton (Rebillard *et al.*, 2008). Low doses of cisplatin interact with and prevent actin polymerisation while higher doses lead to g- and f-actin depolymerisation (Zeng *et al.*, 1996). Cisplatin also inactivates ezrin, involved in structural interaction between the cytoskeleton and the cell membrane, and causes cytoskeleton destabilisation and appearance of membrane ruffles (Zeidan *et al.*, 2008). Cytoskeleton disruption causes Bcl-2 family members to mediate pro-apoptotic signaling and ultimately lead to cell death (Knipling and Wolff, 2006).

1.2.5.2 Endoplasmic reticulum

The endoplasmic reticulum (ER) is responsible for post-translational modifications of proteins including appropriate protein folding and maturing, and transport and secretion (Ellgaard, Mollinari and Helenius, 1999). It is also important for cellular biosynthesis and intracellular calcium homeostasis (Gorlach, Klappa and Kietzmann, 2006; Paschen, 1996). Alterations or injury to the ER can lead to accumulation of misfolded proteins or deregulation of calcium storage further leading to ER stress and triggering the unfolded protein response (UPR) (Linder and Shoshan, 2005; Treiman, 2002). UPR is known to activate apoptosis as a coping-mechanism under excessive ER stress (Mandic *et al.*, 2003; Patil and Walter, 2001; Zhang *et al.*, 2006). Cisplatin treatment has shown to activate ER stress markers (Grp78, GADD153/CHOP, calpain, caspase 12/human caspase 4, cytosolic calcium increase, etc.) in cancer cells and tumour tissue (Gately *et al.*, 1994; Gately *et al.*, 1996; Kim, Kim and Kim, 2011; Liu *et al.*, 2008). ER stress preconditioning has also shown to significantly reduce cisplatin toxicity (Feng *et al.*, 2011; Peyrou and Cribb, 2007). Moreover, cisplatin accumulates in the ER and binds and disrupts proper folding of proteins including albumin (Ahmed-Ouameur *et al.*, 2006). Cisplatin-induced ER stress is also suggested to result from cross-talk with other subcellular organelles (Sancho-Martinez *et al.*, 2012). Cytosolic pro-apoptotic Bcl-2 family members activate ER resident Bax and Bak to form pores and increase permeability to calcium which further activate different deadly mechanisms (Sancho-Martinez *et al.*, 2012). Cisplatin-induced DNA damage-activated p53 further activate pro-apoptotic proteins in ER membrane (Sancho-Martinez *et al.*, 2012). ER resident Bcl-2 also activate caspase cascades including caspase 2 which further attacks mitochondria and causes the release of proapoptotic factors (Sancho-Martinez *et al.*, 2012).

1.2.5.3 Mitochondria

Cisplatin treatment causes alterations in mitochondria that activate the intrinsic pathway of apoptosis (Cullen *et al.*, 2007). Exposure to cisplatin makes the outer mitochondrial membrane more permeable causing rapid release of Bcl-2-regulated cytochrome c

ultimately inducing apoptosis (Yang *et al.*, 2006). Experimental studies on isolated mitochondria showed that cisplatin induces depolarisation of mitochondrial membrane potential, mitochondrial swelling and increases reactive oxygen species (ROS) production (Custodio *et al.*, 2009; Santandreu *et al.*, 2010). Increased ROS lowers Bcl-2 levels in intermembrane space and causes mitochondrial permeability transition pore to open and release cytochrome c (Chen and Lesnefsky, 2011). Cisplatin also inhibits several antioxidant enzymes in kidney tissues thereby further increasing ROS production (Antunes *et al.*, 2000; Hannemann *et al.*, 1991). Evidence also indicates that cisplatin binds more effectively to mitochondrial DNA than nuclear DNA and that is attributed to lower DNA repair in mitochondria (Murata *et al.*, 1990; Olivero *et al.*, 1995; Singh and Maniccia-Bozzo, 1990). More importantly, mitochondrial DNA binding is suggested to affect expression of antioxidant enzymes further increasing ROS production (Sancho-Martinez *et al.*, 2012). Interestingly, cells lacking mitochondrial DNA are significantly more resistant to cisplatin (Yang *et al.*, 2006). Mitochondria injury can also result from cumulative effect of cisplatin on other cellular targets. For instance, upon apoptotic signaling activated pro-apoptotic proteins relocate to mitochondria, increase permeability and release of cytochrome c (Crawford *et al.*, 2011; Kim, Ryu and Song, 2006).

1.2.5.4 Lysosome

Lysosome is a source of cell death signaling (Sancho-Martinez *et al.*, 2012). Alterations and injury to lysosomes such as exposure to cisplatin can result in lysosome membrane permeabilisation (LMP) and release of its content into the cytosol (Ivanova *et al.*, 2008). Lysosomal fluid contains several cathepsins (proteases) that either activate the intrinsic mitochondrial pathway of apoptosis by activating pro-apoptotic members of the Bcl-2 family or cause extensive proteolysis that trigger necrotic cell death (Foghsgaard *et al.*, 2001; Roberg and Ollinger, 1998; Wu *et al.*, 1998; Brunk and Svensson, 1999; Li *et al.*, 2000). In some instances depending on membrane permeabilisation, cathepsins can also directly cleave and activate executioner caspases 3 and 7, thus activating apoptosis (Chwieralski, Welte and Buhling, 2006). Thus, cisplatin can cause LMP followed by apoptosis by accumulating in the lysosomes and triggering primary death signals (Appelqvist *et al.*, 2011; Fehrenbacher *et al.*, 2008; Nilsson *et al.*, 2010). Studies showed

that inhibiting cathepsins reduced cell death in cisplatin treated cells (Nilsson *et al.*, 2010). Although it isn't exactly known how cisplatin causes LMP, it has been suggested that cisplatin accumulation destabilises lysosomal membrane structure (Sancho-Martinez *et al.*, 2012). The cells were further rescued from cisplatin toxicity when lysosomal membrane was protected by experimental accumulation of cholesterol into lysosomes (Appelqvist *et al.*, 2011). These results suggested that lysosomal death route played a certain role in cisplatin cytotoxicity. LMP can also be caused by signals originated from other subcellular organelles in response to cisplatin. Some of these signaling include ROS originated in the endoplasmic reticulum and mitochondria, p53 activated as a result of DNA damage that is further recruited to the lysosome and causes LMP, ER stress activated caspase-2, and activation of pro-apoptotic protein Bax and its relocalisation to lysosomal membrane (Yuan *et al.*, 2002; Chen *et al.*, 2005; Li *et al.*, 2007; Huang *et al.*, 2009).

1.3 THE GOLGI APPARATUS

1.3.1 Structural organisation of the Golgi apparatus

The Golgi apparatus is localised in the perinuclear region of the cytoplasm and consists of flattened membranous stacks, called cisternae (Figure 1.3; Ladinsky *et al.*, 1999). The defining feature of the Golgi apparatus is its polarisation in structure and function both since different regions within the Golgi are involved in different processing and sorting events that take place in an orderly fashion (Bisel *et al.*, 2008; Preisinger *et al.*, 2004). The cisternae are arranged such that the Golgi has three functionally distinct regions – *cis*, *medial* and *trans*-Golgi, and each play essential roles in cell physiology (Wei and Seemann, 2017). Proteins secreted from the ER are transported to the ER-Golgi intermediate compartment and enter the Golgi apparatus at the *cis*-Golgi (Brandizzi and Barlowe, 2013). The *cis*-Golgi is the point for first post-translational modifications of secretory proteins and also sends and receives carriers to and from the ER (Potelle, Klein and Foulquier, 2015). The modified proteins, lipids and polysaccharides are then transported to the medial and *trans*-Golgi compartments where most metabolic activities of the Golgi take place (Nakamura, Wei and Seemann, 2012). The *medial* and *trans* Golgi further modify the secretory cargo and deliver it to a meshwork of membrane called the trans-Golgi network (Nakamura, Wei and Seemann, 2012). The trans Golgi network acts as a sorting and distribution center, directing molecular traffic to lysosomes, the plasma membrane or the cell exterior (Nakamura, Wei and Seemann, 2012).

1.3.1.1 The Golgi matrix

In vertebrates the Golgi is a single copy structure usually found next to the centrosome. Most lower eukaryotes contain one or more discrete Golgi stacks per cells while in vertebrates the stacks are linked to form the Golgi ribbon (Lowe, 2011). The Golgi matrix is involved in scaffolding Golgi assembly and maintaining its structural integrity

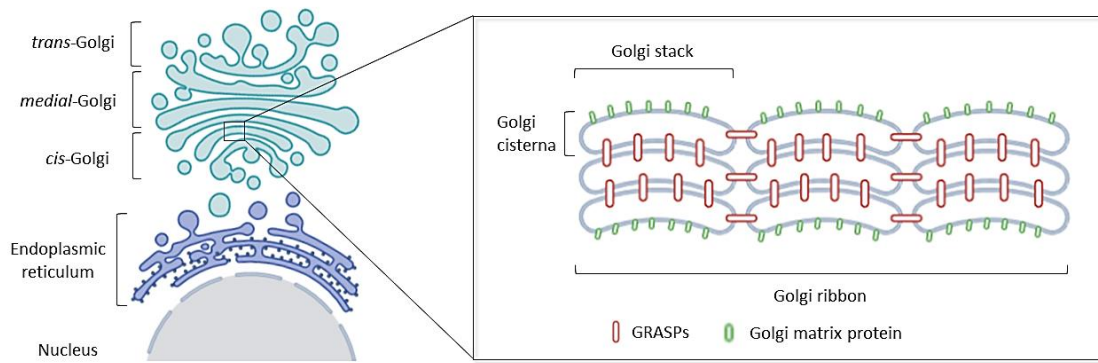


Figure 1.3. Structural organisation of the Golgi apparatus.

(Staehelin and Kang, 2008). Electron microscopy studies have shown that the matrix consists of ‘ribosome exclusion zone’ consisting of extended protein network surrounding the Golgi apparatus in the cytoplasm (Staehelin and Kang, 2008). Warren and colleagues in 1994 introduced the concept of ‘Golgi matrix’ proteins when they successfully isolated detergent and salt-resistant protein complex purified from Golgi membranes (Slusarewicz *et al.*, 1994). And in 1995, the first Golgi matrix protein GM130, an extended coiled-coil rod-like protein localised to *cis*-Golgi, was identified by Nakamura and colleagues (Nakamura *et al.*, 1995). GM130 binds the vesicle docking protein p115 at its N-terminus and also plays a role in membrane tethering at the *cis*-Golgi (Ramirez and Lowe, 2009). Several different Golgi matrix proteins including the GRASPs and golgins have been discovered since then and have proven vital in maintaining structural organisation of the Golgi and regulating protein trafficking through the Golgi stack (Xiang and Wang, 2011). Golgins are essentially members of Golgi-associated peripheral coiled-coil proteins anchored to the Golgi membrane necessary for vesicle docking and Golgi structure (Xiang and Wang, 2011). Because of their coiled-coil domains, they are excellent at tethering membranes over relatively long distances (Barr and Short, 2003). In fact, treatment with brefeldin A (BFA) – a fungal metabolite that inactivates the small GTPase ADP-ribosylation factor-1 (ARF1), blocked the assembly of COPI-coated vesicles and redistributed golgins and Golgi enzymes into the ER via tubular connections between the Golgi and the ER (Bascom, Srinivasan and Nussbaum, 1999; Klausner, Donaldson and Lippincott-Schwartz, 1992; Orci *et al.*, 1991). Some Golgi matrix proteins including GM130 and GRASPs remained in the tubulovesicular remnants of the Golgi distinct from the ER, and could also reform the perinuclear ribbon-like structure, suggesting a vital role for Golgi matrix proteins in maintaining the structure and identity of the Golgi apparatus

(Seemann *et al.*, 2000). The Golgi matrix proteins are thus also highly dynamic in nature, to be able to adapt to morphological changes during cell cycle progression, cargo trafficking and also during experimental treatments (Xiang and Wang, 2011).

1.3.1.2 The GRASPs and Golgi cisternal stacking

Golgi Reassembly And Stacking Proteins (GRASPs) are responsible for cross-linking adjacent Golgi cisternae (Mollenhauer, 1965; Franke *et al.*, 1972; Cluett and Brown, 1992). In vertebrate, GRASP65 and GRASP55 localise to *cis* and *medial/trans* cisternae respectively, while lower eukaryotes have only a single GRASP protein (Barr *et al.*, 1997; Shorter *et al.*, 1999; Behnia *et al.*, 2007; Kondylis, Spoorendonk and Rabouille, 2005). *In vitro* assay on rat liver Golgi stacks during post-mitotic assembly showed that both GRASP65 and 55 participate in the stacking of cisternae (Barr *et al.*, 1997; Shorter *et al.*, 1999). Furthermore, depletion of GRASP65 resulted in partial loss of stacking *in vivo* (Sutterlin *et al.*, 2005). However, other studies showed that depletion of GRASP65 or 55 instead resulted in a loss of Golgi ribbon continuity, suggesting that instead of stacking cisternae GRASPs laterally link adjacent cisternae in the Golgi ribbon (Puthenveedu *et al.*, 2006; Feinstein and Linstedt, 2008). Xiang and Wang (2010) showed using a high resolution quantitative electron microscopy analysis that depletion of either GRASP alone consistently reduced the number of cisternae within a stack, while depletion of both GRASPs completely abolished stack formation and instead resulted in vesiculated Golgi remnants. These results strongly supported the idea that GRASPs play an essential role in Golgi cisternal stacking. Briefly, GRASPs form homodimers that further oligomerise via the N-terminal PDZ domain. GRASP65 also interacts with GM130 which is required for GRASP65 membrane association (Barr, Nakamura and Warren, 1998). GRASPs are dual-anchored onto the membrane such that it forms trans-oligomers that then cross-links Golgi membranes during cisternal stacking (Bachert and Linstedt, 2010).

1.3.1.3 The Golgi ribbon

The cisternal stacks are further connected through tubular membranes to form a continuous structure named the Golgi ribbon (Lowe, 2011). Since the Golgi ribbon is often located adjacent to the nucleus around the centrosome, the microtubule cytoskeleton also plays a functional role in maintaining the Golgi ribbon (Burkhardt, 1998). Microtubule network and the minus end-directed microtubule motor cytoplasmic dynein are required to maintain an intact Golgi ribbon (Burkhardt, 1998). Dynein functions to form the Golgi ribbon through homotypic fusion of ER to Golgi carriers and laterally connecting cisternae (Lowe, 2011). Loss of either the microtubules or dynein results in dispersed Golgi stacks that accumulate next to ER (exit sites) (Cole *et al.*, 1996). Interestingly, disruption of the ribbon into mini-stacks through microtubule depolymerisation does not reportedly affect the secretory function of the Golgi and cargo trafficking to the cell surface (Cole *et al.*, 1996). Maintenance of the Golgi ribbon is thus dependent on both microtubules and dynein motor activity. The assembly of the Golgi ribbon is also dependent on Golgi-nucleated microtubules (Miller *et al.*, 2009). The Golgi apparatus also functions as a microtubule organiser where it is able to nucleate microtubules in its own right via a pool of γ -tubulin (Efimov *et al.*, 2007). During Golgi assembly, Golgi-derived microtubules bring individual Golgi stacks together from the cell periphery to the cell centre via dynein-mediated trafficking along the centrosomal microtubule array in a 'search and capture' fashion, and keep stacks in close proximity for linking by tubular connections (Efimov *et al.*, 2007). Golgi ribbon formation thus requires two independently formed microtubule networks acting in concert (Lowe, 2011).

In addition to Golgi matrix proteins, GRASPs and microtubules, actin cytoskeleton also plays an important role in maintaining the structural integrity of the Golgi apparatus. Egea and colleagues (2006) have shown that depolymerisation of actin resulted in altered Golgi morphology and trafficking, but the exact mechanism was not known at the time (Egea, Lazaro-Dieiguez and Vilella, 2006). Dippold *et al.* (2009) studied the interaction between Golgi-associated PI4P and the actin cytoskeleton, and found that reduced association between PI4P and actin cytoskeleton resulted in significant reduction of the Golgi size. Proteomic screening further identified GOLPH3 (*more on GOLPH3 in later section*) as the interaction partner of PI4P at the *trans*-Golgi (Dippold *et al.*, 2009). They further

discovered that unconventional myosin MYO18A directly interacted with GOLPH3. MYO18A was already known to localise at the Golgi and bind the actin filaments F-actin (Mori *et al.*, 2003; Isogawa *et al.*, 2005). Depletion of PI4P and siRNA knockdown of GOLPH3 and MYO18A resulted in compact Golgi with diluted *trans*-Golgi cisternae, suggesting that PI4P-GOLPH3-MYO18A-F-actin interaction is required for normal Golgi ribbon structure (Dippold *et al.*, 2009).

1.3.2 Functions of the Golgi apparatus

The Golgi apparatus plays a central role for an array of cellular responses including modulating microtubule network (Cole *et al.*, 1996; Trucco *et al.*, 2004), regulating calcium and pH homeostasis (Marra *et al.*, 2007), autophagy (Geng and Klionsky, 2010), apoptosis (Jaffe and Hall, 2005) and the stress response (Reiling *et al.*, 2013; Oku *et al.*, 2011; Jiang *et al.*, 2011; Farber-Katz *et al.*, 2014). More importantly, the Golgi apparatus plays an essential role in the biosynthetic pathway where it is primarily responsible for post-translational modifications and secretion of proteins (Wei and Seemann, 2017). The proteins undergo a variety of post-translational modifications including glycosylation.

1.3.2.1 Glycosylation

Glycosylation is one of the most important post-translational modification processes that the Golgi apparatus is responsible for (Fuster and Esko, 2005). Secreted and cell-surface glycoconjugates play an integral role in mediating key cellular processes including cell-to-cell communication, cell adhesion, motility and cell-signalling pathways during normal development (Marth, 1999). Glycosylation is an enzymatic process that involves post-translational modifications of proteins and lipids through synchronised action of glycosyltransferases and glycosidases. These enzymes add glycosidic linkages of carbohydrate molecules called glycans or saccharides to other saccharides, lipids or proteins to produce glycoconjugates such as glycoproteins, glycolipids and proteoglycans (Munkley and Elliott, 2016).

The two most common mechanisms by which glycans are linked to proteins are: O-linked glycosylation and N-linked glycosylation. In O-linked glycosylation, monosaccharides are added incrementally to the hydroxyl oxygen of serine or threonine residues (Kudelka *et al.*, 2015). A common type of O-linked glycosylation is initiated via addition of N-acetylgalactosamine (GalNAc), which can then be extended into various different structures, and occurs entirely in the Golgi (Kudelka *et al.*, 2015). On the other hand, N-linked glycosylation is initiated in the ER where preassembled blocks of 14 sugar molecules are added co-translationally on nascent polypeptide via the amide group of an asparagine residue, and completed in the Golgi (Schwarz and Aebi, 2011). N- and O-glycans are secreted and mostly found as cell-surface entities. These cell-surface glycans, an integral part of the extracellular matrix, mediate cell adhesion, motility and intracellular signalling (Fuster and Esko, 2005). Alterations in glycoconjugate profiles have also been associated with disease progression of cancer cells (Rambaruth, Greenwell and Dwek, 2012).

Altered cell-surface or secreted glycoproteins have been identified in almost every cancer type and although it is not officially considered as a hallmark of cancer, aberrant glycans are involved in every step of metastasis (Fuster and Esko, 2005; Pinho and Reis, 2015). Two principle mechanisms underlying the tumour-associated alterations of carbohydrate structures were first postulated by Hakomori and Kannagi – the so-called incomplete synthesis and the neo-synthesis processes (Hakomori and Kannagi, 1983). During the incomplete synthesis process, the normal synthesis of complex glycans in normal epithelial cells is impaired, resulting in truncated glycan structures and occurs often in early stages of cancer (e.g., STn expressions seen in several cancers) (Hakomori and Kannagi, 1983). On the contrary, during neo-synthesis, tumour cells induce expression of sialyl Lewis antigens and glycosyltransferases that further enhance expression of sialyl Lewis antigens, and mostly occurs in advanced stages of cancer (Kannagi *et al.*, 2008).

A number of N-glycosylated proteins have been shown to be differentially and aberrantly synthesised in different cancer types and are also used as predictive markers for cancer diagnosis (Tian and Zhang, 2013). Branching of N-glycans is involved in different cellular processes such as signal transduction, cell adhesion, proliferation, differentiation,

and programmed cell death (Zhao *et al.*, 2008). However, β 1,6-GlcNAc branching of N-glycans results in alterations of activated glycosyltransferases, that further influence malignant transformation, such as cancer cell invasion and metastasis (Zhao *et al.*, 2008). O-glycans, on the other hand, are found to be truncated in cancer cells (Brockhausen, 1999). MUC1 is a glycoprotein that is integrated in the plasma membrane of epithelial cells, downregulation of which leads to alterations associated with disease progression. Characterised as a major oncogene, MUC1 is aberrantly glycosylated and overexpressed in cancers of epithelial origin including ovarian, lung, colon and pancreatic carcinomas, and >90% of breast carcinomas (Singh and Bandyopadhyay, 2007). MUC16 (or CA125) is considered the most effective marker for early EOC detection and the monitoring of EOC reoccurrence after therapy (Gupta and Lis, 2009).

Lectin from *Helix pomatia*, Helix pomatia agglutinin (HPA) has a binding specificity for GalNAc epitopes (Brooks, Hall and Buley, 2001). Presence of HPA-binding glycans in tumour tissue samples have been associated with poor prognosis of patients. HPA-positive presenting tumour patients have lower 5-year cancer free survival rate compared to HPA-negative tumour patients (Rambaruth, Greenwell and Dwek, 2012). Study done by Brooks, Hall and Buley (2001) showed that HPA-binding increased significantly with increasing metastatic capacity of breast cancer cells, and could be used as a prognostic marker for breast cancer patients. More recently, through glyco-proteomic array analysis, it has been revealed that HPA recognises and binds to other glycosylation patterns including O-GlcNAc in breast cancer cells (Rambaruth, Greenwell and Dwek, 2012).

1.3.2.2 Golgi apparatus and the stress response

The Golgi apparatus has also been shown to sense and transduce death signals during stress-induced DNA damage (Nakagomi *et al.*, 2008). As a stress response, the cell upregulates the transcription of genes involved in mediating glycosylation, post-Golgi vesicular transport and maintenance of the Golgi structure (Oku *et al.*, 2011). Welch and Suhan (1985) had reported identifying elevated levels of proteins localised in the nucleus including the nucleolus and in the cytoplasm specifically the ER and the Golgi complex

in rat embryo fibroblast cells after heat-shock treatment. They further presented evidence for morphological alterations in the Golgi complex with an increase in the number of vesicularised membranes within the perinuclear region through electron microscopy after 3hr heat shock treatment, and suggested that disruption in morphology did not adversely affect its ability to glycosylate newly synthesised proteins (Welch and Suhan, 1985). Petrosyan and Cheng (2014) further demonstrated increased interaction between non-muscle myosin IIA and core 2 N-acetylglucosaminyltransferase-M as a result of heat shock of Panc1 cells which resulted in Golgi disorganisation.

DNA damage as a result of stress induces an array of cellular reactions that in addition to repairing the damaged DNA also lead to Golgi fragmentation and dispersal throughout the cytoplasm (Buschman, Rahajeng and Field, 2015). Farber-Katz and colleagues (2014) treated various cell lines (primary and immortalised) with chemotherapy drugs camptothecin, doxorubicin or ionizing radiation, and observed fragmented Golgi dispersed throughout the cytoplasm indicating a ubiquitous feature of the DNA damage response. The response was detected fairly early at 4h after exposure and reportedly lasted weeks. They showed that in response to DNA damage, DNA-PK phosphorylates GOLPH3. GOLPH3 also known as GPP34/GMx33/MIDAS, is a 34-kDa phosphorylated matrix protein that is localised in the *trans*-Golgi complex, and is involved in vesicular trafficking, protein glycosylation, receptor sorting, maintaining cell signalling pathways and Golgi architecture (Li *et al.*, 2014). Phosphorylation of GOLPH3 resulted in recruitment and increased interaction with unconventional myosin MYO18A to the Golgi where it binds to F-actin. This complex applied a tensile force to the Golgi resulting in excessive vesicle budding and deformation. DNA-damage induced Golgi dispersal also impaired Golgi to plasma membrane trafficking. Moreover, Golgi dispersal was inhibited when GOLPH3, MYO18A and DNA-PKcs were depleted after DNA damage, resulting in increased apoptosis and decreased cell survival and proliferation, suggesting Golgi fragmentation as a survival response to DNA damaging agents.

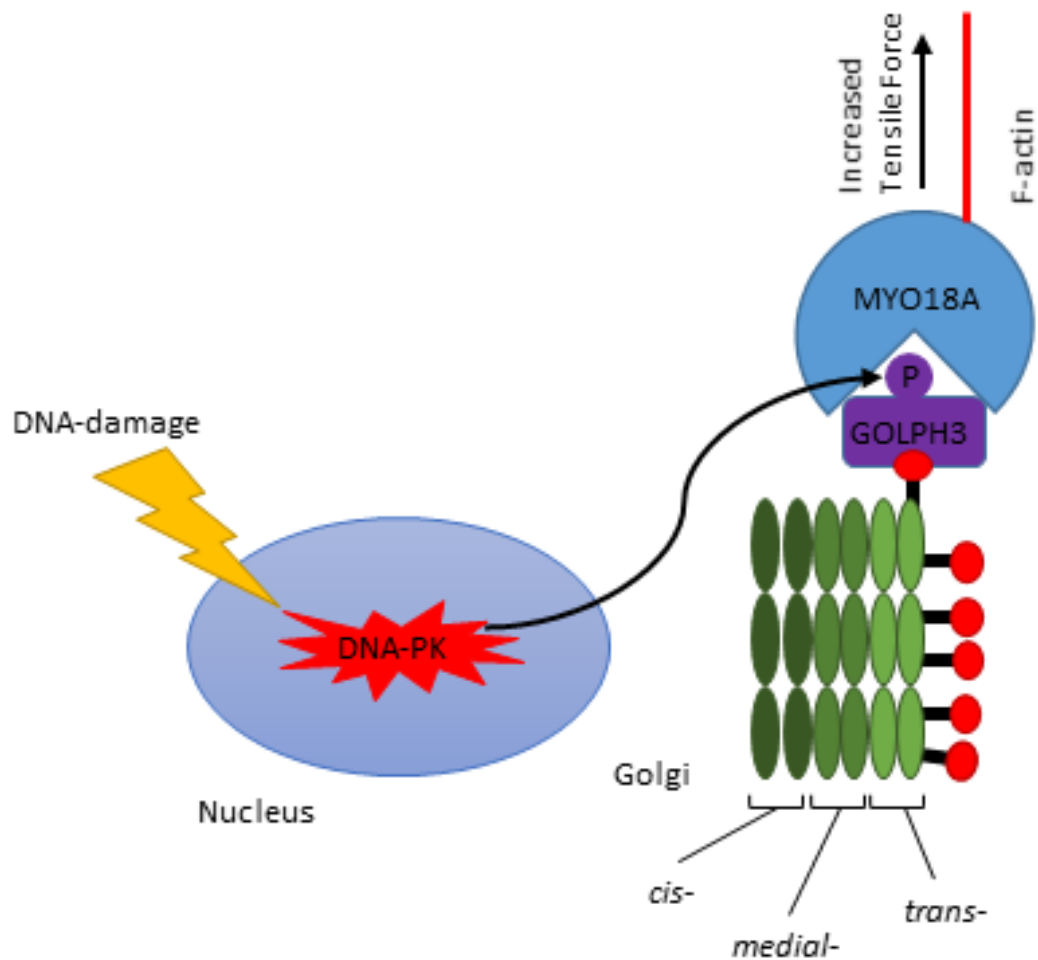


Figure 1.4. DNA-damage induced Golgi dispersal. DNA-PK activated in response to DNA damage phosphorylates overexpressed GOLPH3 localised at *trans*-Golgi. Phosphorylation of GOLPH3 results in increased recruitment and interaction with unconventional myosin MYO18A to *trans*-Golgi where it binds to F-actin. This complex applies a tensile force to the Golgi resulting in excessive vesicle budding and dispersal. Image adapted from Farber-Katz *et al.* (2014).

1.3.3 Golgi disassembly during mitosis

Golgi disassembly or fragmentation during mammalian cell division is a highly monitored, well characterised and studied cellular process. Interestingly, mitotic Golgi disassembly is not common to all organisms (Wei and Seemann, 2017). In plants and yeast, the stacked cisternae remain intact and functional throughout the cell cycle (Preuss, *et al.*, 1992; Segui-Simarro and Staehelin, 2006). In mammalian cells, the interconnected Golgi ribbon requires disassembling of the Golgi and segregation of its membranes into the daughter cells, where it then reassembles into a functional ribbon (Guizzunti and Seemann, 2016). In fact, Guizzunti and Seemann (2016) showed that Golgi vesiculation is a requirement for mitotic progression and acts as a ‘Golgi mitotic checkpoint’. They assembled inorganic 3,3’-diaminobenzidine (DAB) polymer into the lumen of the Golgi cisternae and found that although DAB did not prevent transitioning into metaphase, it inhibited Golgi vesiculation. As a consequence, cells preparing to undergo mitosis were arrested in metaphase with monopolar spindle and an active spindle assembly checkpoint (SAC). This novel cell cycle checkpoint thus senses the integrity of the Golgi complex and also highlights the regulatory interplay between the signalling pathways regulating cell division and organelle inheritance (Guizzunti and Seemann, 2016).

The process of Golgi segregation begins in the G2 phase of cell cycle when the Golgi ribbon is cut into individual Golgi stacks (Figure 1.5; Valente and Colanzi, 2015). During prophase/prometaphase, these are unstacked and undergo vesiculation to form small fragments that are dispersed throughout the cytoplasm. CtBP1-S/BARS (referred here as BARS) is a fission inducing protein that also severs Golgi tubular network (Colanzi *et al.*, 2007). Studies have shown that BARS phosphorylation and activation specifically occurs in G2 phase and promotes severing of Golgi ribbon by binding to other specific partners, and proves as a crucial step for G2/M transition (Colanzi *et al.*, 2007).

GRASP65 form *trans* homo-oligomers in non-mitotic cells to maintain Golgi stacks and Golgi ribbon and phosphorylation by kinases prevents this *trans*-oligomerisation formation properties of GRASP65 (Tang, Yuan and Wang, 2010). GRASP proteins also play an important role in mitotic Golgi fragmentation and mitotic entry. GRASP65 is phosphorylated by Cdk1 and Plk1 kinases at multiple sites (in its C-terminal

serine/proline-rich domain) during mitosis (Lin *et al.*, 2000; Wang *et al.*, 2003; Preisinger *et al.*, 2005; Yoshimura *et al.*, 2005). It has been suggested that phosphorylation of GRASP65 on C-terminal domain by Cdk1 creates a binding site for Plk1. Phosphorylation of GRASP65 by Cdk1 and Plk1 results in unlinking of adjacent stacks that form the ribbon as well as prevent stacking of the cisternae by abrogating *trans*-oligomerisation of GRASP65 (Wang *et al.*, 2003; Sengupta and Linstedt, 2010). Moreover, GRASP65 has also been shown to be phosphorylated by JNK2 kinase specifically in Ser227, and this phosphorylation is believed to be a crucial step for Golgi unlinking during mitosis (Cervigni *et al.*, 2015). Inhibiting JNK2 kinase either by RNAi or JNK inhibitor has shown to persistently block cell cycle in G2 phase (Cervigni *et al.*, 2015).

GRASP55 is also phosphorylated during mitosis. The most well characterised GRASP55 phosphorylation is the one mediated by ERK2 (Acharya *et al.*, 1998; Feinstein and Linstedt, 2007). Mitogen activated protein (MAP) kinase kinase 1 (MEK1) is activated by RAF1 and during late prophase it is recruited to the Golgi ribbon where it helps mediate Golgi fragmentation (Colanzi, Sutterlin and Malhotra, 2003). Activated MEK1 further activates ERK2 which then phosphorylates GRASP55 and induces Golgi disassembly during mitosis (Feinstein and Linstedt, 2008). Phosphorylation of GRASP55 by ERK2 has been shown to be required for Golgi ribbon unlinking and mitotic progression (Duran *et al.*, 2008; Feinstein and Linstedt, 2008; Xiang and Wang, 2010). Activation of RAF1/MEK1 is dependent on PKD1 and PKD2 (Kienzle *et al.*, 2013). MEK1b-activated ERK1c also phosphorylates GRASP55 (Shaul and Seger, 2006). Although it isn't known how these phosphorylation events assist in Golgi fragmentation during mitosis, it has been suggested that similar to GRASP65, phosphorylation by kinases prevents *trans*-oligomerisation of GRASP55 which possibly results in unlinking of adjacent Golgi stacks that form the ribbon (Valente and Colanzi, 2015).

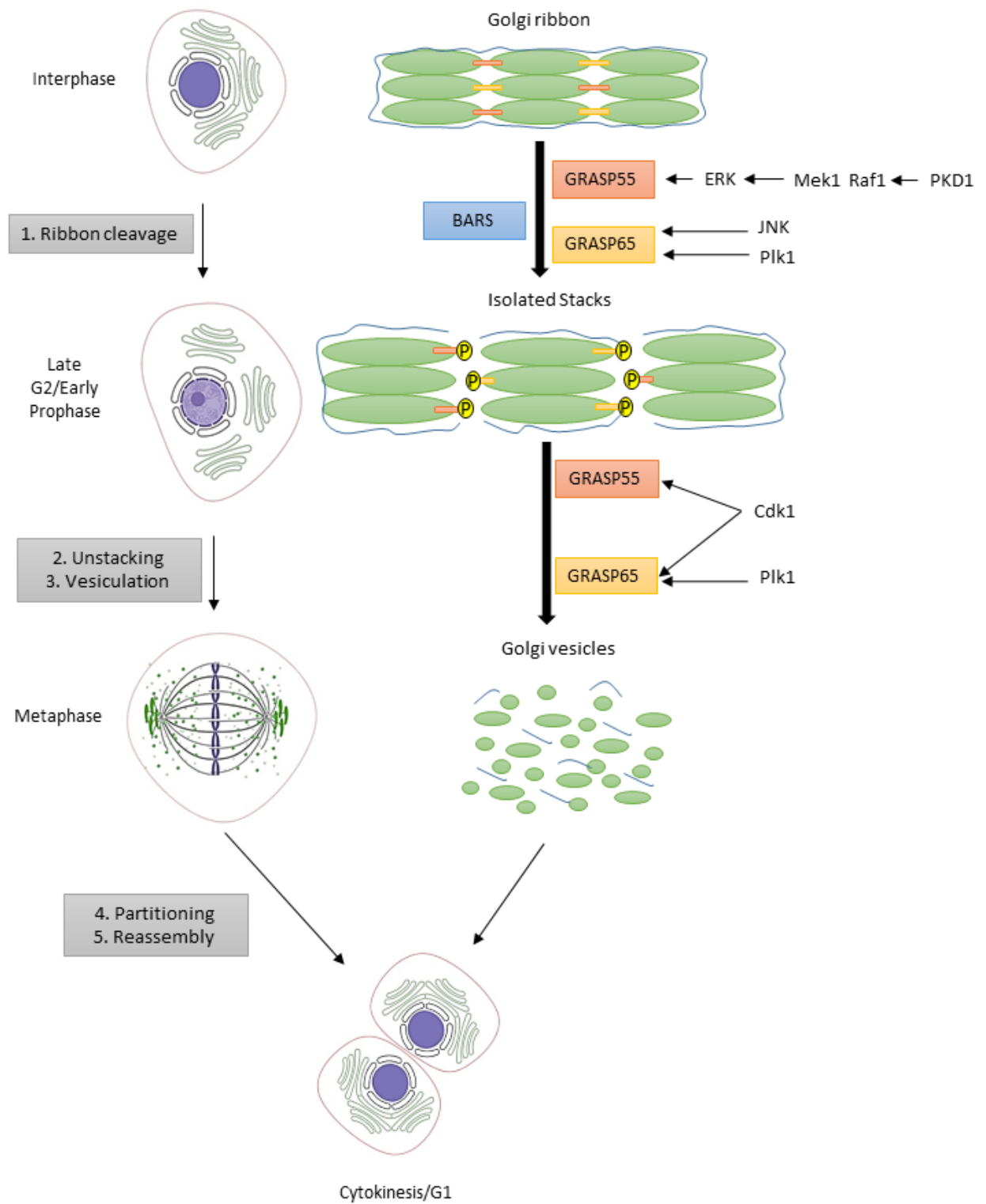


Figure 1.5. Golgi disassembly during mitosis. Image adapted from Valente and Colanzi (2015).

The additional fragmentation of the Golgi depends on unstacking of the cisterna followed by the vesiculation (Misteli and Warren, 1995). Golgi cisterna unstacking requires Cdk1-mediated phosphorylation of GRASP65 and GRASP55, which further inhibits the formation of *trans*-oligomers (Misteli and Warren, 1995). Disassembly of Golgi cisterna has been suggested to proceed via COPI-dependent and COPI-independent pathways, both of which lead to membrane budding and vesiculation (Sonnichsen *et al.*, 1996). During telophase, Golgi starts to reassemble gradually into stacks to eventually form a Golgi ribbon in each of the daughter cells (Shorter and Warren, 2002; Altan-Bonnet *et al.*, 2003; Colanzi and Corda, 2007).

1.3.4 Golgi-associated proteins and their role in cancer progression

Cell migration is a critical developmental biological process. When unregulated, it is the driving force behind cell invasion and metastasis – two phenomena that are hallmarks of cancer (Hanahan and Weinberg, 2011). The pericentriolar localisation of the Golgi apparatus plays an important role in establishing and maintaining cell polarity (Yadav, Puri and Linstedt, 2009). Its orientation further guides membrane traffic towards the plasma membrane which lays the cornerstones for directional protein secretion required for cell migration (Bisel *et al.*, 2008; Yadav, Puri and Linstedt, 2009; Preisinger *et al.*, 2004). Depletion of Golgin-160 or GMAP210 disrupted pericentriolar position of the Golgi and affected cell migration (Yadav, Puri and Linstedt, 2009). Recently, Golgi-associated proteins have also been implicated in cell migration and invasion of cancer cells. For instance, depletion of GM130 in several breast cancer cells abrogated cell polarity and subsequently increased cellular velocity and invasiveness (Baschieri *et al.*, 2015). Furthermore, GOLPH3 has been implicated as an oncogene upregulated in several cancers and associated with poor patient prognosis (Zhou *et al.*, 2013; Scott *et al.*, 2009; Zeng *et al.*, 2012; Hua *et al.*, 2012). Overexpressed GOLPH3 enhances cancer cell migration by promoting reorientation of the Golgi towards the leading edge during directional cell migration and drives forward trafficking (Xing *et al.*, 2016). *PITPNCl*, overexpressed in several metastatic cancers, promotes malignant secretion by binding Golgi resident protein PI4P and localising RAB1B to the Golgi. RAB1B localisation recruits GOLPH3 and enhances vesicular release of pro-invasive and pro-angiogenic

mediators (Halberg *et al.*, 2016). GOLPH3 has also been shown to enhance growth-factor induced mTOR signalling in human cancer cells and alters the response to mTOR inhibitor *in vivo* (Scott *et al.*, 2009). *GOLPH3* is also overexpressed in EOC and promoted migration and invasion of EOC cells by upregulating the expression of epithelial-to-mesenchymal transition (EMT) markers such as *N-cadherin* and *Snail* via activation of Wnt/ β -catenin signaling (Sun *et al.*, 2017). Moreover, during EMT, the Golgi was shown to attain a more compact morphology via upregulation of Golgi scaffolding protein PAQR11 (Tan *et al.*, 2017). High PAQR11 levels were correlated with EMT and shorter survival in human cancers, and was essential for tumour cell migration and metastasis of lung cancer. *GOLPH3L*, a gene highly homologous to *GOLPH3* and localised to the *trans*-Golgi, was also shown to be overexpressed in EOC cells and was associated with poor prognosis and aggressive phenotype in ovarian cancer patients (He *et al.*, 2017). Interestingly, overexpression of *GOLPH3L* also conferred cisplatin resistance in EOC cells, and its inhibition or silencing led to increased cellular cisplatin-sensitivity (He *et al.*, 2017).

1.4 CELL FUSION

1.4.1 *Cell fusion during development*

Fusion between cells (referred here as cell fusion) is an essential part of growth and development of tissues in eukaryotes. From the fusion of gametes and placental formation to development of tissues and organs, fusion events are a tightly regulated and controlled process (Brukman *et al.*, 2019). Studies done in nematode *C. elegans* show that about one third of the cells in this organisms are subsequently fused into 44 syncytia in a reproducible and stereotypic way with the side of the cell being fused predetermined (Podbilewicz and White, 1994), suggesting that only selected tissue types are prone to fusion and result in terminally differentiated multinuclear cells incapable of further proliferation.

1.4.1.1 *Mechanism of cell fusion*

Fusion process varies and depends widely on the biological context, fusing membranes and regulatory mechanisms. Essentially, there are two main fusion mechanisms: unilateral and bilateral (Brukman *et al.*, 2019). In unilateral fusion, fusion proteins or fusogens that mediate the fusion process are present on only one of the fusing membranes (Brukman *et al.*, 2019). While in bilateral fusion, same (homotypic) or different (heterotypic) fusogens are present on both fusing membranes (Brukman *et al.*, 2019). Experimental studies (mostly on viral fusion) have revealed a highly conserved model of membrane phospholipid rearrangement present in all membrane-fusion events (Chernomordik *et al.*, 1993; Chernomordik and Kozlov, 2005; Nakatogawa, Ichimura and Ohsumi, 2007; Podbilewicz *et al.*, 2006 and Xu *et al.*, 2005). According to this model, firstly, the distance between the two opposed biological membranes is maintained between 10-20nm and is controlled by specific cell-cell adhesion proteins (Dhanyasi *et al.*, 2015; Leikina *et al.*, 2004). The bilayers are then brought closer following

displacement of membrane proteins and overcoming strong repulsive thermal and hydration forces (Chernomordik and Kozlov, 2003). Following deformations such as bending of one or both bilayers elicits a reaction that involves stereotypic rearrangement of membrane lipids producing an intermediate hemifusion state. During this stage, the outer leaflets first fuse with each other resulting in lipid mixing (Chernomordik, Zimmerberg and Kozlov, 2006). Subsequent merging of the inner leaflets generates a nascent fusion pore that allow content mixing (Figure 1.6; Sapir *et al.*, 2008).

Investigating fusion of viruses with host cell membranes has provided with better mechanistic understanding of cell-cell fusion (Chen and Olson, 2005). Fusion of enveloped viruses to host cell membranes is mediated through transmembrane viral proteins. Class 1 viral fusion proteins, such as human immunodeficiency virus type 1 (HIV-1) envelope protein (Env) and influenza hemagglutinin (HA), contain hydrophobic fusion peptide (Skehel and Wiley, 2000; Eckert and Kim, 2001). In response to external signalling/stimuli such as low pH (for HA) or cell surface receptor binding (for Env), the fusion proteins are cleaved (Skehel and Wiley, 2000; Eckert and Kim, 2001). This exposes the hydrophobic fusion peptide and is inserted into the target membrane (Durrer *et al.*, 1996). Membrane fusion is then facilitated by conformational change in the fusion protein whereby the two well-separated α -helices fold to form hairpin-like α -helical bundle bringing the two membranes in close proximity, allowing membrane fusion (Eckert and Kim, 2001).

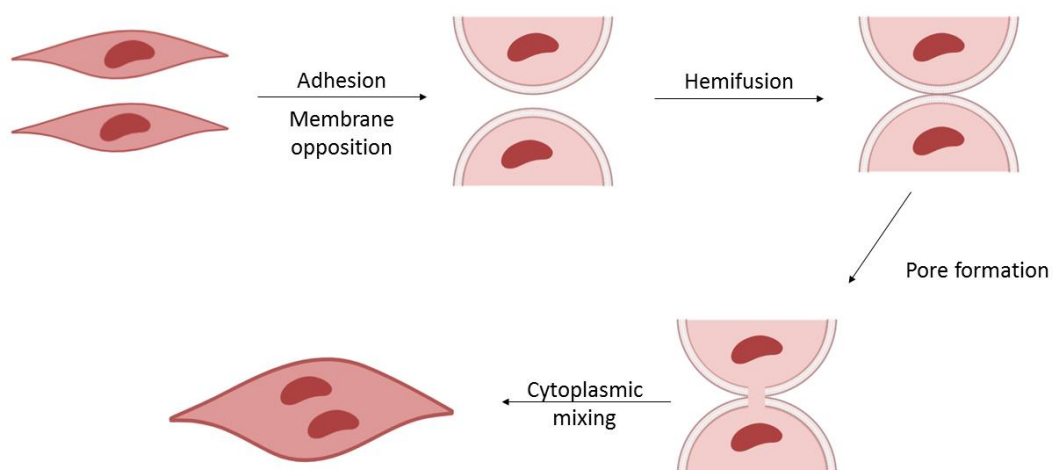


Figure 1.6. Schematic of cell fusion.

1.4.1.2 Characterised fusion proteins

Well-characterised fusogens are mostly identified through developmental studies in cell fusion of gametes (CD9), muscle cells (Myomaker/Myomerger; Hernandez and Podbilewicz, 2017; Sampath and Millay, 2018), the placental trophoblast of mammals (Syncytin) (Mi *et al.*, 2000), macrophage fusion (MFR/CD47) (Vignery, 2000) and the epithelia of nematode (Fusion Family EFF-1; Oren-Suissa and Podbilewicz, 2007), but mechanisms of only some are well understood and described briefly below.

Epidermal cell fusion:

Genetic screening in *C. elegans* identified *epithelial fusion failure 1 (eff-1)*, which is required for epidermal cell-cell fusion. Expression of *eff-1* is induced in cells predetermined to fuse (Menon and Chia, 2001) and encodes a transmembrane domain and an external hydrophobic peptide (EHP) that is required for the localisation of EFF-1 to sites of cell-to-cell contact prior to pore formation (del Campo *et al.*, 2005).

Fertilisation:

Tetraspanin family protein CD9 is required for fusion of sperm and egg in mammals (Chen and Olson, 2005). Unlike EFF-1, CD9 lacks a hydrophobic peptide, but is suggested to facilitate fusion by forming multiprotein fusion complex at the site of egg-sperm binding (Tarrant *et al.*, 2003). Syncytin, a single-pass transmembrane protein, induces fusion of placental trophoblasts to form syncytiotrophoblasts that acts as a barrier between maternal and fetal blood vessels (Mi *et al.*, 2000). It is suggested that syncytin functions as a class 1 fusion protein and may use viral protein to facilitate fusion (Mi *et al.*, 2000).

Myoblast fusion:

Muscle cells (or myoblasts) fuse to form elongated multinucleated myofibers. Genetic and developmental studies on *Drosophila melanogaster* have identified a model for myoblast fusion that includes two types of cells that are destined for fusion: founder cells that act as seeds for future muscle fibers and fusion competent cells that fuse with founder cells (Abmayr *et al.*, 2003; Chen and Olson, 2004). Immunoglobulin domain-containing transmembrane proteins Dumbfounded (Duf) and Roughest (Rst) are redundantly required in founder cells, while Sticks and stones (Sns) and Hibris (Hbs) are specifically expressed on fusion-competent cells for myoblast fusion (Ruiz-Gomez *et al.*, 2000; Strunkelnberg *et al.*, 2001; Bour *et al.*, 2000; Artero, Castanon and Baylies, 2001). Although these cell surface receptors lack hydrophobic fusion peptide, they mediate recognition and adhesion of muscle cells through direct interaction between Duf and Sns (Galletta *et al.*, 2004). In addition, extensive alignment of apposing membranes is mediated through several intracellular signaling proteins that function to remodel the cytoskeleton and the lipid bilayers (Chen and Olson, 2005). Vertebrate myoblast fusion is mediated by expression of Myomaker and Myomerger on cell surface (Sampath and Millay, 2018). Myomaker along with AnxA1 and AnxA5 facilitate the formation of hemifusion, while Myomerger aids the transition of myoblast hemifusion to multinucleation through pore formation (Gamage *et al.*, 2017; Leikina *et al.*, 2013; Leikina *et al.*, 2018). Notably, Myomaker and Myomerger function independently and do not depend on each other to complete the fusion process (Leikina *et al.*, 2018).

1.4.2 Cancer cell fusion

The idea of cancer cell fusion was put forth a century ago by Aichel (1911), when he proposed that cancer cell fusion was a mechanism of cancer metastasis. Works of Mekler (1971) and Warner (1975) further showed that fusion of tumor cells with lymphocytes produced metastatic cells with genetic and epigenetic diversity. These subpopulations of hybrid cells have the added advantage to avert chemotherapy treatments, develop drug resistance and metastasise (Noubissi and Ogle, 2016).

Fusion frequency in tumours, as suggested by *in vitro* and animal studies, is approximately 1% with only 1% of these hybrids hypothesised to survive and proliferate, suggest that only 0.01% of the tumour hybrid cells exhibit their novel properties (Wakeling, Greetham and Bennet, 1994; Fortuna, Dewey and Furmanski, 1989; Miller *et al.*, 1988; Dittmar and Zanker, 2015). However, recent research using xenograft assay shows that 6% of the tumour cells were hybrids, and when treated with chemotherapy agents, the fusion rate increased to as high as 12% (Yan, Wang and Liu, 2016). Tumour cells interact with the microenvironment (stroma, macrophages and fibroblasts) and the cells of the hematopoietic lineage, and produce hybrids with increased heterogeneity that further promote tumour progression (Yan, Wang and Liu, 2016).

1.4.2.1 Mechanism for cancer cell fusion

Fusogens required for other cellular processes including cancer cell fusion have not been fully described and warrants further investigation. Recently, Uygur *et al.* (2019) identified higher expression levels of placental fusogens Syn1 and AnxA5 in human prostate cancer cells compared to non-malignant tissues following co-culturing with primary skeletal or smooth muscle cells. Increased expression in prostate cancer cells also resulted in increased (10-20%) cancer cell fusion, which was then inhibited by blocking fusogenic refolding of Syn1. This fusion behaviour is suggestively triggered by higher concentrations of anti-inflammatory interleukins 4 and 13 as a result of interaction between the muscle and prostate cancer cells. Furthermore, fusion between breast cancer cells and endothelial cells is inhibited by knocking down Syn1 or Syn1 receptor, ASCT2 (Bjerregaard *et al.*, 2006). Cancer cell fusion is suggested to be triggered by hypoxia, inflammation and oxidative stress - processes associated with cell-surface expression of phosphatidylserine (PS), with several different cancer cells expressing unusually higher levels of PS (Sharma and Kanwar, 2018). However, more research is required to understand the process of cancer cell fusion. Perhaps investigating underlying genetic alterations that regulate expression of fusion proteins will provide with the necessary mechanistic detail.

1.4.2.2 Implications of cancer cell fusion

i. Increased heterogeneity

One of the consequences of cell-to-cell fusion of cancer cells is increased heterogeneity. Barski and Cornefert (1962) demonstrated that two different co-cultured mouse cell lines through cell fusion were overgrown by a third new cell line that had combined genetic composition of its parental genes. Goldenberg *et al.* (2012) demonstrated that when glioblastoma cells were added to normal hamster host cells, seven human genes were transferred to normal hamster host cells through heterokaryon formation. These progenies were malignant and expressed both human and hamster genes and gene products. Evidently, cell-to-cell fusion is a means of horizontal gene transfer and cancer cells adapt and survive in microenvironments through interactions with their stromal environment producing viable hybrid progeny (Goldenberg *et al.*, 2012). Fusion of cancer cells also results in formation of polyploid cells and have more chromosomal instability compared to diploid cancers (Coward and Harding, 2014; Dewhurst *et al.*, 2014).

ii. Increased metastatic capacity

In vitro studies involving breast cancer cells have shown spontaneous fusion between normal breast epithelia and breast cancer cells (Berndt *et al.*, 2013; Dittmar *et al.*, 2011), between breast tumour cells (Lu and Kang, 2009), between breast cancer epithelia and cells of the endothelium (Mortensen *et al.*, 2004) and between breast cancer epithelium and stromal cells (Rappa, Mercapide and Lorico, 2012; Nagler, Zanker and Dittmar, 2011). Hybrids formed between normal breast epithelium (M12SV1-EGFP-Neo) and breast cancer cells (HS578T-Hyg) showed increased migratory capacity associated with altered CCL21/CCR7 signaling, previously linked to the metastatic spread of breast cancer cells to lymph nodes (Berndt *et al.*, 2013). Breast tumour cells also fuse with mesenchymal stem cells to produce hybrids with increased invasion and migratory capacity (Noubissi *et al.*, 2015; McArdle, Ogle and Noubissi, 2016).

In vivo studies have shown that subcutaneous injection of breast cancer cell variant (MDA-MB-231) with tropism for either lung or bone in nude mice resulted in hybrids

capable of metastasising to both organs (Lu and Kang, 2009). Another xenograft study involving subcutaneous injection of lung cancer cells and mesenchymal stem cells produced hybrids with epithelial to mesenchymal properties and increased motility and invasiveness (Xu *et al.*, 2014). In more recent study, hybrids formed from fusion between cancer cells (ovarian and lung) and hematopoietic cells of the myeloid lineage expressed significantly higher levels of promigratory marker CXCR4 (C-X-C chemokine receptor type 4) initially conferred only in hematopoietic cells (Ramakrishnan, Mathur and Mukhopadhyay, 2013).

Several clinical studies show the presence of tumour hybrids as a result of cell fusion. For instance, short-tandem repeat length-polymorphism and genetic techniques were used to show that metastatic melanoma lesion in a patient was the result of fusion between a tumor cell and a bone marrow derived cells that the patient received during bone marrow transplant (Lazova *et al.*, 2013). These results suggest that hybrids are equipped with increased migratory capacity and dissemination from primary tumour site to various secondary organs.

iii. Increased drug resistance

Cell-to-cell fusion of cancer cells can also contribute to emergence of drug resistance in hybrid subpopulations. Fusion of 5-fluorouracil-resistant cells and methotrexate-resistant cells *in vitro* produced a hybrid double-resistant cancer cell line resistant to both drugs (Miller, Mohamed and McEachern, 1989). In addition, the hybrid cells were also found to be resistant to drug melphalan that the parental cells were initially sensitive to (Miller, Mohamed and McEachern, 1989). Nagler *et al.* (2011) also demonstrated formation of dual resistant cells suggestively through cell fusion following co-cultivation of hygromycin-resistant murine mammary carcinoma cells and puromycin-resistant murine BMDCs. Interestingly, the dual-resistant hybrid cells also showed upregulation of multidrug transporters Abcb1a and Abcb1b which conferred them resistance towards 17-DMAG, etoposide, doxorubicin and paclitaxel (Nagler *et al.*, 2011). Emergence of multidrug-resistant hybrid cells provides an added advantage for survival and is a source for tumour relapse (Lu and Kang, 2009).

AIMS OF THIS STUDY

1. Structural alterations in Golgi apparatus in response to cisplatin-induced stress

Cisplatin has been used for decades for treatment of ovarian cancer despite the risk of resistance-related cancer relapse. Cisplatin has also been known to cause structural and functional alterations of several different cellular organelles. But its effect on Golgi apparatus is not yet known. Given the role played by the Golgi apparatus in mediating stress response and recent discovery of Golgi-related proteins functioning in cancer progression, it was important to study the effect of cisplatin-induced stress on the morphology (fragmentation state) of the Golgi. During mitosis, Golgi is required to disassemble, and this disassembly is mediated by two signalling pathways – ERK1/2 and JNK. Cisplatin-induced cell death signaling, amongst other signaling cascades, also activates ERK1/2 and JNK. Taking advantage of the fact that these two pathways coincide in two completely independent cellular processes, ERK1/2 and JNK pathways were tested for cisplatin-induced alterations of Golgi apparatus. Doing so, will also provide with a novel, previously unreported mechanism for cisplatin-induced stress response.

2. Altered Golgi function in response to cisplatin-induced stress

Effect of cisplatin on the functions of the Golgi apparatus were also investigated. Three key functions of Golgi were tested: (i) Golgi polarisation during cell migration. Golgi polarisation is vital to directional cell migration, and cell migration is one of the earlier steps to cancer progression. Performing *in vitro* cell migration assays to determine Golgi orientation in the presence of cisplatin will help further understand early response of cancer cells towards cisplatin. This will be further supported with experiments studying cell motility and wound healing capacity of cancer cells. Doing so will provide with more insight and highlight the importance of correct Golgi polarisation and the extent to which

cisplatin exerts its effect. (ii) Glycosylation profile. Alterations in glycosylation profile are extensively shown to play a role in cancer progression and associated with metastatic capacity of cancer cells. Determining the effect of cisplatin on glycosylation profile will provide insight into ability of cisplatin to render cells more metastatic (by altering cell surface expression of glycoconjugates) and could possibly also help understand the resistance-related relapse. And finally, (iii) ER/Golgi to plasma membrane trafficking. One of the main functions of the Golgi is protein secretion and trafficking. Studying the effect of cisplatin on protein trafficking will help understand the extent to which Golgi function is altered.

3. Cancer cell fusion and associated multinucleation

Fusion of cells predetermined to fuse and underlying fusion proteins that facilitate their fusion is widely studied and reported. Fusion of cancer cells with non-cancerous cells is also widely studied and reported. However, fusion of cancer cells with each other is not reported. Ovarian cancer cells in literature are reported to be highly multinucleated. In this study, through live time-lapse microscopy, the emergence of multinucleated ovarian cancer cells will be examined. It is suggested/hypothesised that multinucleated ovarian cancer cells are a result of cell-to-cell fusion, more so of daughter cells following mitosis. Through gene correlation expression analysis of multinucleation profile of ovarian cancer cell lines, a candidate gene's (*TADA3/TADA3L*) role will be investigated in mediating fusion of cancer cells

METHODS

CHAPTER 2

Methods

2.1 Cell culture

Human ovarian cancer cell lines A2780 (a gift from Professor Robert Brown, Imperial College, London, UK), IGROV-1, OVCAR3, OVCAR4, OVCAR5, OVCAR8 and SKOV3 (Table 2.1; National Cancer Institute) were put in growth phase by sub-culturing them using RPMI (RPMI Media 1640(1x) + 2.05mM L-Glutamine, HyClone) + 10% Fetal Calf Serum (FCS, Gibco by Life Technologies). Culture media and other cell culture consumables were obtained sterile and to prevent contamination were treated with extreme caution. Culture media and washing buffer were always warmed to 37°C using a dry beads bath. All tissue culture consumables, containers and gloves were thoroughly sprayed with 70% industrial methylated spirit (IMS) before placing them in the laminar flow hood. Cells were incubated in humidified incubators set at 37°C and 5% CO₂ levels. The cells were sub-cultured every five days using 75cm² flasks when they reached a confluency of 70-80% as viewed under the light microscope. Old media from 75cm² flask was aspirated and the cells were gently washed with 6ml Dulbecco's Phosphate Buffered Saline without Ca²⁺ and Mg²⁺ (DPBS, Gibco by Life Technologies). The cells then trypsinised using 2ml 1x trypsin-EDTA (Thermofisher) and incubated for 3-4mins at 37°C and 5% CO₂. Cells were suspended in 10ml RPMI + 10% FCS media and split 1:10 dilution, and incubated again at 37°C and 5% CO₂ until further sub-culturing. The media was replaced with fresh RPMI + 10% FCS media every third day or as required.

2.2 Cisplatin treatment

Cisplatin treatment of ovarian cancer cells was followed according to Mansouri *et al.* (2002) and Samuel *et al.* (2018). Fresh stock of 2mM cisplatin (Thermofisher) was prepared every time before treatment using DPBS as solvent. From the stock, cells were treated with pre-determined IC₅₀ dosage (see section 2.3) of cisplatin and incubated for

Table 2.1. Characteristics of ovarian cancer cell lines

Cell line	Age (y)	Tissue origin	p53 function	Mutations	Doubling time (hr)	Invasiveness (depicted by matrigel assay)	Migration (depicted by wound closure at 30hr)	Tumour formation in mice High (4/4 mice with tumors), Med (1–3/4), or Low (0/4) (Hernandez <i>et al.</i> (2016))	References
A2780	unspecified	Ovarian endometrioid adenocarcinoma	Wild type	<i>ARID1A; BRCA2; PTEN; PIK3CA</i>	18	low	<20%	2/4	Beaufort <i>et al.</i> , (2014); Hernandez <i>et al.</i> (2016); Hallas-Potts, Dawson and Herrington (2019)
IGROV-1	47	Cystadenocarcinoma	Mutant	<i>ARID1A; BRCA1; BRCA2; PIK3CA; PTEN</i>	27	Low	~20%	4/4	Beaufort <i>et al.</i> , (2014); Benard <i>et al.</i> (1985); Feng <i>et al.</i> (2019); Huang <i>et al.</i> (2020)
OVCAR3	60	Adenocarcinoma, ascites	Mutant	<i>Heterozygous loss of BRCA1, BRCA2</i>	34.7	moderate	~40%	0/4	CellMiner™; Domcke <i>et al.</i> (2013); Hamilton, Young and Ozols (1984); Hallas-Potts, Dawson and Herrington (2019); Stordal <i>et al.</i> (2013)
OVCAR4	42	Adenocarcinoma, ascites	Mutant	<i>BRCA2</i>	41.4	moderate	~40%	0/4	Bourgeois <i>et al.</i> (2015); CellMiner™; Hamilton, Young and Ozols (1984); Hayley <i>et al.</i> (2016)
OVCAR5	67	Adenocarcinoma, ascites	Mutant	<i>KRAS</i>	48.8	high	~60%	4/4	CellMiner™; Hamilton, Young and Ozols (1984); Hayley <i>et al.</i> (2016)
OVCAR8	64	Carcinoma	Mutant	<i>ERBB2; KRAS; CTNNB1</i>	26.1	high	~75%	4/4	CellMiner™; Domcke <i>et al.</i> (2013); Hamilton, Young and Ozols (1984); Hallas-Potts, Dawson and Herrington (2019)
SKOV3	64	Adenocarcinoma, ascites	Absent	<i>PIK3CA; HRAS; ARID1A; Heterozygous loss of BRCA1, BRCA2; CTNNB1</i>	27	high	>90%	4/4	Beaufort <i>et al.</i> , (2014); Domcke <i>et al.</i> (2013); Hernandez <i>et al.</i> (2016); Fogh and Trempe (1975); Hallas-Potts, Dawson and Herrington (2019)

3hr at 37°C and 5% CO₂. Media with cisplatin was removed and cells were gently washed with PBS. Fresh growth media (RPMI + 10% FCS) was added to cells, and incubated at 37°C and 5% CO₂ for 24hr or until further experimentation. Please note that during cisplatin treatments, cells were also treated with equal volumes of PBS as solvent and used as control unless otherwise stated

2.3 Determining IC₅₀ dosage

Half-maximal inhibitory concentration (IC₅₀) indicates how much of a pharmacologic agent is required to inhibit a given biological activity by half for a given cell line (Aykul and Martinez-Hackert, 2016). To determine the IC₅₀ dosage, A2780 (10,000 cells/100µl), IGROV-1 (10,000 cells/100µl), OVCAR3 (8,000 cells/100µl), OVCAR4 (10,000 cells/100µl), OVCAR5 (10,000 cells/100µl), OVCAR8 (10,000 cells/100µl) and SKOV3 (5,000 cells/100µl) were seeded in 96-well plates and treated with increasing concentrations of cisplatin (0 to 400µM) for 3 hours. The cells were washed with DPBS, and replaced with fresh growth media and incubated for 24 hours at 37°C and 5% CO₂. After 24 hours, cell viability was measured using the Alamar Blue/resazurin assay (see section 2.4). Using GraphPad Prism 7, cell viability data was analysed using in-built Nonlinear Regression model with variable slope (four parameters) that produced IC₅₀ values and inhibitory dose response curve was generated.

2.4 Cell viability assay

Cell viability was measured by performing Alamar Blue assay that uses resazurin, a weakly fluorescent non-toxic blue-purple dye. Metabolically active living cells through mitochondrial reductase reduce the non-fluorescent resazurin to strongly-fluorescent pink product resorufin. Fluorescence measured is proportional to the number of viable cells present. Stock resazurin solution (0.15mg/ml) was prepared by dissolving high purity resazurin (Sigma-Aldrich) was dissolved in sterile DPBS (pH 7.4). In a class 2 hood, the resazurin solution was filter-sterilised through a 0.2µm filter into a sterile light protected

container. For short term storage and frequent use, the solution was stored at 4°C, or at -20°C for long term storage. To determine cell viability, cells (control and treated) were seeded in black opaque 96-well plates with clear bottom. In a cat 2 hood, resazurin-containing growth media was prepared by adding 10% resazurin solution to the total growth media and mixed by vortexing. Complete growth media from the well was removed and replaced with resazurin-containing media. An extra row of well containing only growth media (no cells) was used for background subtraction and instrument gain adjustment. Cells were incubated in resazurin-containing media at 37°C and 5% CO₂ for 2-3 hr checking occasionally for a pink colour in the wells. Relative fluorescence was recorded using a plate reader with a 560nm excitation and 590nm emission filter set, and the wells were read from below.

2.5 Time-course of Golgi fragmentation and DNA damage

A2780 cells grown in 12-well plates for 24 hours were treated with IC₅₀ dosage of cisplatin and fixed at t=0hr (cisplatin just added), t=3hr, t=6hr, t=12hr, t=24hr and t=72hr post-treatment. Time point t=3hr represents the 3 hour cisplatin treatment performed; therefore, cisplatin was removed at t=3hr and replaced with fresh growth media. Cells were fixed and antibody labelled with anti-GM130 and γ H2A.X conjugated to FITC (see section 2.7) antibodies. Cells were imaged using confocal LSM 880 as described in section 2.12.2. Golgi fragmentation was analysed as described in section 2.13.2 and γ H2A.X labelled nuclear foci were analysed as described in section 2.13.4.

2.6 Maxi prep of VSVG plasmid

ts045-EGFP-VSVG (Addgene plasmid # 11912; <http://n2t.net/addgene:11912>; RRID: Addgene_11912, Figure 2.1) was received in bacteria as agar stab. Firstly, kanamycin resistant plates were created by aseptically pouring Luria broth (LB): Agar containing 50 μ g/mL kanamycin onto the plates and allowing them to cool under a blue flame. Using an aseptic spreader, bacteria from agar stab was evenly spread across the surface of the

kanamycin resistant plate. To prevent contamination the plate was allowed to dry for 5 mins under a blue flame and placed upside down in a 37°C incubator for overnight colony formation.

Single colonies were picked and transferred into 5mL LB containing 50µg/mL kanamycin and incubated in a shaker overnight at 37°C. Following overnight incubation, glycerol stocks were prepared by mixing a small aliquot of bacterial culture in a 1:1 ratio with glycerol and stored at -80°C for long term storage. The remaining bacterial culture was added to 250 mL LB containing kanamycin and further grown overnight at 37°C. The bacterial culture was inspected to form viscous liquid indicating confluent growth and transferred to two 50ml falcon tubes and centrifuged for 10 mins at 3,500 rpm at 4°C. The supernatant was discarded and the tubes were briefly left upside down to allow drying of the pellets. Qiagen Plasmid Midi Kit (25) (Cat. No. 12143) and supplied buffers were used from here onwards. The pellets were resuspended in 2.5ml cold Resuspension Buffer P1 and combined into tube 1. Then, 5mL Lysis Buffer P2 was added and the tube was inverted few times to allow mixing. 5mL Neutralisation Buffer P3 was added and the tube was inverted few times to allow mixing. The tube was incubated on ice for 10 mins. The tube was mixed again by inverting few times and centrifuged at 4°C 3,000 rpm for 30 mins. The supernatant was carefully transferred into a fresh tube and equal volume of room temperature isopropanol was added. The tube was mixed by inverting few times and incubated at -20°C for 30 mins. The tube was then centrifuged at 4°C 3,000rpm for 15 mins. The supernatant was discarded. The pellet was washed by adding 50µL 70% ethanol and centrifuging at 3,000 rpm for 10mins at 4°C; the supernatant was carefully pipetted and discarded. The pellet was resuspended in 1ml Tris-Cl/EDTA (TE) buffer.

To remove proteins and perform plasmid clean-up, the sample was split equally into two 500µL eppendorf tubes. In a fume hood, 500µL of phenol:chloroform:isoamyl alcohol (25:24:1) was added to each tube. The tubes were mixed vigorously and centrifuged at 13,000 rpm at 4°C for 2 mins. 400µL of aqueous phase from each tube was transferred to new tubes and 400µL of chloroform was added to it. The tubes were centrifuged at 13,000 rpm at 4°C for 2 mins. 300µL of the aqueous phase from each tube was transferred to new tubes. 30µL 3M sodium acetate anhydrous was added to the new tubes followed by 750µL of 100% ethanol. The tubes were mixed well and stored overnight at -20°C. The tubes were centrifuged at 13,000 rpm at 4°C for 30 mins. The pellets containing DNA was washed by adding 50µL 70% ethanol and centrifuging at 13,000 rpm for 5 mins. The

supernatant was carefully pipetted and discarded. The pellets were resuspended in 200 μ L TE buffer, recombined and quantified using Nanodrop One.

Created with SnapGene®

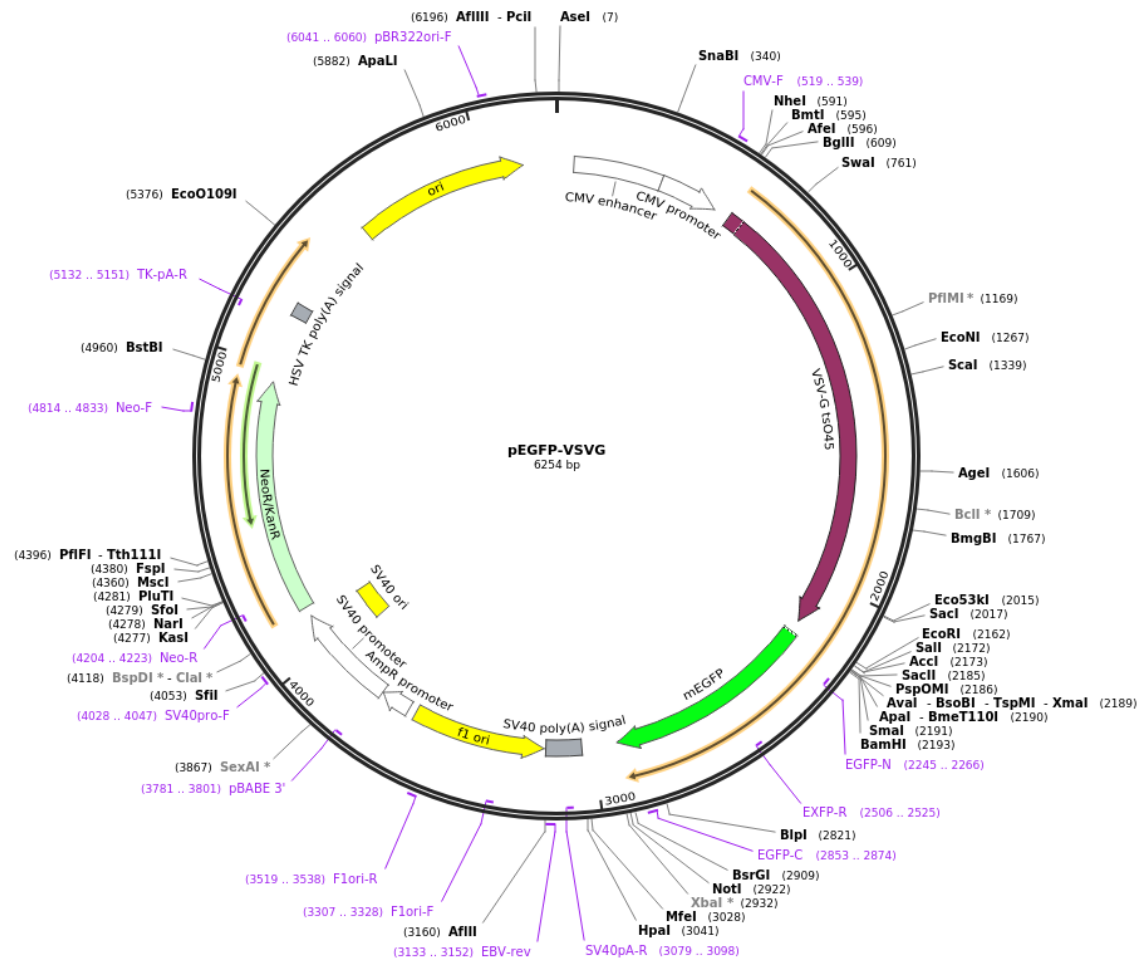


Figure 2.1. Vector map of ts045-EGFP-VSVG from Addgene.

2.7 Transient transfection

A2780 (2×10^5 cells/2mL), OVCAR3 (1.6×10^5 cells/2mL) and SKOV3 (1×10^5 cells/2mL) cells grown in glass bottom dishes for 24 hours were transiently transfected with ts045-EGFP-VSVG using FuGene HD (Promega). Per 2ml total volume, transfection reaction was prepared using 95 μ l serum free media (SFM), 4.5 μ L FuGene HD and 2 μ g of ts045-EGFP-VSVG. The mixture was gently mixed by pipetting up and

down few times and incubated at room temperature for 10 mins. The media in the dish was replaced with 1900 μ l fresh growth media. After 10mins, transfection reaction mixture was added slowly to the cells. The dish was gently swirled around to ensure homogenous distribution of the reaction mixture and incubated at 37°C and 5% CO₂. The cells were then confocal imaged 24 hours later. To study the effect of cisplatin on VSVG trafficking, the cells were incubated for 6-8 hours to allow incorporation of VSVG DNA and treated with cisplatin for 3 hours (see section 2.2). Cells were washed gently with DPBS, replaced with fresh growth media and incubated for 24 hours at 37°C and 5% CO₂ and then confocal imaged.

2.8 JNK pathway inhibition

BI-87G3 (Sigma-Aldrich), a chemical inhibitor of JNK, was dissolved in DMSO to create a 2mM stock solution according to manufacturer protocol. To inhibit the JNK pathway, protocol was followed according to Mansouri *et al.* (2003) and Sheng *et al.* (2018) with slight modifications. Firstly, a kill curve was generated for A2780, OVCAR3 and SKOV3 cells to determine the optimal concentration of the inhibitor. Another kill curve of the inhibitor with and without cisplatin was also generated. Cells (A2780–10,000cells/100 μ L, OVCAR3 – 8,000 cells/100 μ L and SKOV3 – 5,000cells/100 μ L) were grown in 96-well plates for 24 hours and pre-treated with increasing concentration of the JNK inhibitor (0 μ M to 100 μ M) for 3 hours (see Figure 2.2). Cells in cisplatin wells were then treated with IC₅₀ dosage of cisplatin for further 3 hours. Equivalent volumes of DMSO used as solvent for the inhibitor was used to treat cells and used as control. Cells were then washed with DPBS and replaced with fresh growth media. The plates were incubated for 24 hours at 37C and 5% CO₂, and cell viability was measured using the Alamar Blue assay (see Section 2.4).

From the kill curves generated, the following JNK inhibitor concentrations were considered optimal: A2780 - 5 μ M, OVCAR-3 - 20 μ M and SKOV-3 - 10 μ M. To determine the effect of JNK inhibition on Golgi fragmentation in cisplatin treated cells, cells grown in 12-well plates for 24 hours were treated with pre-determined JNK inhibitor

concentrations for 3 hours, like previously, followed by cisplatin treatment for further 3 hours (Figure 2.3). Equivalent volume of DMSO was used as control. Cells were washed with DPBS and replaced with fresh growth media and incubated for 24 hours at 37°C and 5% CO₂. After 24 hours, cells were fixed, antibody labelled, confocal imaged and Golgi fragmentation analysed.

Plate 1:

JNK Inhibitor concentration (μM)									
	0	5	10	20	30	40	50	100	
JNK Inhib only									Rep1
									Rep2
									Rep3
JNK inhib + Cis									Rep1
									Rep2
									Rep3
Media									

Plate 2:

JNK Inhibitor concentration (μM)									
	0	5	10	20	30	40	50	100	
DMSO only									Rep1
									Rep2
									Rep3
DMSO + Cis									Rep1
									Rep2
									Rep3
Media									

Figure 2.2. Experimental layout for generating JNK kill curves.

	Control	PBS	Cisplatin
+ JNK inhib			
- JNK inhib			
DMSO			

Figure 2.3. Schematic of JNK inhibitor, cisplatin and control treatments

2.9 ERK pathway inhibition

FR180201 (Sigma-Aldrich), a chemical inhibitor of ERK, was dissolved in DMSO to create a 2mM stock solution according to manufacturer protocol. To inhibit the ERK pathway, protocol was followed according to Persons *et al.* (1999) with slight modifications. Similar to JNK inhibition assay, firstly, a kill curve was generated for A2780, OVCAR3 and SKOV3 cells to determine the optimal concentration of the inhibitor. Another kill curve of the inhibitor with and without cisplatin was also generated. Cells (A2780 – 10,000cells/100µL, OVCAR3 – 8,000 cells/100µL and SKOV3 – 5,000cells/100µL) were grown in 96-well plates for 24 hours and pre-treated with increasing concentration of the ERK inhibitor (0µM to 100µM) for 3 hours (see Figure 2.4). Cells in cisplatin wells were then treated with IC50 dosage of cisplatin for further 3 hours. Equivalent volumes of DMSO used as solvent for the inhibitor was used to treat cells and used as control. Cells were then washed with DPBS and replaced with fresh growth media. The plates were incubated for 24 hours at 37C and 5% CO2, and cell viability was measured using the Alamar Blue assay (see Section 2.4).

Plate 1:

ERK Inhibitor concentration (μM)									
	0	5	10	20	30	40	50	100	
ERK Inhib only									Rep1
									Rep2
									Rep3
ERK inhib + Cis									Rep1
									Rep2
									Rep3
Media									

Plate 2:

ERK Inhibitor concentration (μM)									
	0	5	10	20	30	40	50	100	
DMSO only									Rep1
									Rep2
									Rep3
DMSO + Cis									Rep1
									Rep2
									Rep3
Media									

Figure 2.4. Experimental layout for generating ERK kill curves.

From the kill curves generated, 100 μM ERK inhibitor concentration was considered optimal for all three cell lines. To determine the effect of ERK inhibition on Golgi fragmentation in cisplatin treated cells, cells grown in 12-well plates for 24 hours were treated with pre-determined ERK inhibitor concentrations for 3 hours, like previously, followed by cisplatin treatment for further 3 hours. Equivalent volume of DMSO was used as control. Cells were washed with DPBS and replaced with fresh growth media and incubated for 24 hours at 37°C and 5% CO₂. After 24 hours, cells were fixed, antibody labelled, confocal imaged and Golgi fragmentation analysed.

	Control	PBS	Cisplatin
+ ERK inhib			
- ERK inhib			
DMSO			

Figure 2.5. Schematic of ERK inhibitor, cisplatin and control treatments

2.10 Scratch/Wound healing assay

The *in vitro* scratch/wound healing assay is an economical and straightforward method to study cell migration. In this method, a new artificial gap or ‘scratch’ is created on a confluent cell monolayer. The cells on the edges of the scratch then move towards the opening to close the gap. This process in theory mimics to some extent migration of cells *in vivo* (Liang, Park and Guan, 2007). Liang, Park and Guan’s (2007) methodology was followed while performing scratch/wound healing assay with some modifications. Firstly, the assay was performed using glass bottom dishes (Ibidi). The sterile dishes were coated with 0.1mg/ml poly-d-lysine for 1hr. The dishes were washed thoroughly with DPBS, and cell culture was added: A2780 – 4×10^5 cells/ml, OVCAR3 - 3×10^5 cells/ml, SKOV3 - 2×10^5 cells/ml. The dishes were incubated at 37°C and 5% CO₂ for 24-36hr until a 90-95% confluent monolayer was formed. For studying the effects of cisplatin treatment on cell migration, once a 90-95% confluent monolayer was formed, the cells were treated with cisplatin for 3hr and incubated at 37°C and 5% CO₂. A scratch was created using a sterile ruler and p20 pipette tip. The old growth media was discarded and cells were gently washed with warm DPBS followed by a wash with growth media to remove debris and floating dead cells. Cells were replaced with fresh growth media and incubated at 37°C

and 5% CO₂ for 12-24hr until the scratch was observed to be closing in. For immunofluorescence assays, the cells were fixed at this point in the dishes and antibody labelling (GM130, vimentin and HPA) was performed as described previously (see section 2.14).

2.11 Gene expression correlation analysis

Gene expression dataset by Reinhold *et al.* (2015) (Cell Miner GSE32474) comprising of NCI-60 ovarian cancer cell line panel was used to perform gene expression correlation analysis of ovarian cancer cells (IGROV-1, OVCAR3, OVCAR4, OVCAR5, OVCAR8 and SKOV3) and their multinucleation count. Corrected p-value was calculated by performing Benjamini-Hochberg correction:

$$p_{\text{Benjamini-Hochberg}} = \min (p \cdot \text{nbp} / j, 1)$$

p = uncorrected p-value

nbp = total number of computed p-values

j = rank of uncorrected p-value

TADA3 was identified as a candidate gene. Pearson's correlation test was performed using MS Excel to determine strength between expression of *TADA3* in ovarian cancer cells and percentage of multinucleated cells.

2.12 *TADA3/TADA3L* silencing

Transfection of short interfering RNA (siRNA) was used to determine the role of *TADA3/TADA3L* on cell to cell fusion of daughter cells following cell division. Briefly, siRNAs target complementary mRNA of the gene of interest for degradation and effectively silence gene expression (Dana *et al.*, 2017). siRNA_1 (SASI_Hs01_00186838, Sigma-Aldrich) and siRNA_2 (SASI_Hs01_00186837, Sigma-Aldrich) were chosen to silence all variants of *TADA3/TADA3L*. MISSION® siRNA Universal Negative Control #2 (Sigma-Aldrich, SIC002-1NMOL) was used as negative control (mimic). A2780, OVCAR3 and SKOV3 cells grown on coverslips for 24 hours in multi-well plates were transfected with siRNA_1 (final concentration 50nM), siRNA_2 (final concentration 50nM) and siRNA negative control (mimic; final concentration 5nM) using Dharmafect transfection reagent 3 (Thermo Fisher Scientific) and serum free media (SFM). Thermofisher recommends that RNA transfections should be performed in absence of serum because the presence of RNAses in serum can cause contamination and further interfere with silencing process. Essentially, reaction mixture per 2ml well (and scaled up as required) was first prepared in two different tubes – Tube 1 and Tube 2. In Tube 1, 50nM siRNA (20µl siRNA_1 or siRNA_2) or 5nM Mimic (10µl) was added to 180µl SFM or 190µl SFM. In Tube 2, 2µl transfection reagent 3 was added to 198µl SFM. Each tube was incubated separately at room temperature for 5mins. Add contents of Tube 2 into Tube 1 (both should be equal volume) and mix lightly, and incubate for 20mins at room temperature. Replace media in wells with 1600µl fresh SFM. Add 400µl of reaction mix to each well and incubate at 37°C and 5% CO₂ for 8hr. It takes 6-8hr for cells to incorporate siRNA. Following 8hr incubation, the media in wells was replaced with growth media and incubated further for 48hr. At this point, further experimentation was performed as required. To validate the silencing, qPCR was performed to determine *TADA3/TADA3L* mRNA levels. To determine the number of multinucleated cells following *TADA3/TADA3L* silencing, the cells were fixed, labelled, imaged and analysed.

2.13 Quantitative PCR

2.13.1 Lysate preparation

ISOLATE II RNA/DNA/Protein kit (Phenol free; BIOLINE Catalogue No. BIO-52085) was used to prepare lysates and extract RNA from cells grown in culture and protocol was following as provided with the kit. A2780, OVCAR3 and SKOV3 cells grown in 6-well plates and transfected with TADA3 siRNAs (siRNA1 and siRNA2), siRNA negative control/mimic and control cells were used to collect lysates at 24 hours and 48 hours after transfection. Media from the wells was aspirated and cells were washed with DPBS. Per 2mL well in 6-well plate, 300 μ L of Lysis Buffer TX was directly added to the cells. Cells were lysed by gently tapping the plate and swirling the buffer around plate surface for 5 min. Lysate was transferred using RNase-free microcentrifuge tube (supplied in the kit).

2.13.2 Genomic DNA Purification

ISOLATE II DNA Column (with white/blue ring) was assembled with a Collection Tube (provided). 600 μ L of the lysate prepared from above were applied to the column and centrifuged at 14,000x g for 1 min. If the entire lysate was not passed through the column, then the column was spun for additional 1 min at 14,000x g. The flow through was retained for Total RNA Purification. It contains the RNA and protein and should be stored on ice or -20°C.

2.13.3 Total RNA extraction and purification

To every 100 μ L of RNA and protein flow through obtained from above, 60 μ L of 96-100% ethanol was added and mixed by vortexing. ISOLATE II RNA/Protein Column (black ring) with a Collection Tube was assembled. Up to 600 μ L of the ethanolic lysate was added to the column and centrifuged at 4000x g for 1 min. If the entire volume was

not passed through the column, then the column was spun for additional 1 min at 14,000x g. The flow through at this point contains protein and should be retained for protein extraction (protein extraction was not performed here). The Column was reassembled with the Collection Tube for RNA wash. First RNA wash was performed by applying 400µL of Wash Buffer W1 to the column and centrifuged at 14,000x g for 1 min. If the entire volume was not passed through the column, then the column was spun for additional 1 min at 14,000x g. The flow through was discarded and the column was reassembled with the Collection Tube. A second wash was performed by applying another 400µL of Wash Buffer W1 and centrifuging the column at 14,000x g for 1 min. The flow through was discarded and the column was reassembled with the Collection Tube. The column was washed for the third time by adding 400µL of Wash Buffer W1 and centrifuging at 14,000x g for 1 min. The flow through was discarded and the column was reassembled with the Collection Tube. The column was then thoroughly dried by centrifuging for 2 min at 14,000x g. The Collection Tube was discarded at this point. RNA elution was performed using the Elution Tube provided. The column was placed onto the 1.7mL Elution Tube. 50µL of RNA Elution Buffer was added to the column and centrifuged for 2 min at 200x g, followed by 1 min at 14,000x g. Inspect the column to ensure entire volume was eluted. If the entire volume was not eluted, the column was centrifuged for additional 1 min at 14,000x g. The eluate at this point contains the isolated RNA, and should be stored at -20°C for short term storage or -80°C for long term storage.

2.13.4 DNase treatment

Isolated RNA was further treated with DNase 1 (Sigma-Aldrich – AMPD1-1KT) that specifically degrades DNA to further ensure that it was free of genomic DNA contamination. 3.5µL of DNase 1 reaction and 3.5µL of DNase 1 were added to the isolated RNA, and incubated at room temperature for 15 mins. 4µL of stop solution (provided) was added to inactivate DNase and incubated at 70°C for 5mins. Using Nanodrop One, concentration of DNase 1 treated RNA was quantified and stored on ice for immediate use.

2.13.5 cDNA synthesis

RNA to cDNA reverse transcription was performed using High-Capacity RNA-to-cDNA Kit (Catalogue No. 4387406, Applied Biosystems) and protocol followed as provided with the kit. The components from the kit were thawed on ice. Using RNase-free PCR tubes, 20µL reactions were prepared on ice and components from the kit were added as shown in Table 2.1. For A2780 samples - 0.5µg of RNA, for OVCAR3 samples – 0.3µg of RNA, and for SKOV3 samples – 0.2µg of RNA was used. The tubes were sealed with appropriate RNase-free caps and briefly centrifuged to remove any air bubbles and spin down the contents. Using a thermal cycler, the reaction mix was incubated at 37°C for 60 min. The reaction was stopped by heating the reaction mix to 95°C for 5 min. The reaction tubes were stored at 4°C. +RT (with reverse transcriptase) cDNA and –RT (without reverse transcriptase) reactions were normalised to 100ng/µL using nuclease-free water.

2.13.6 Quantitative PCR

iTaq™ Universal SYBR® Green Supermix (Catalog No. 172-5121 Biorad) was used to perform quantitative PCR (qPCR) reactions. iTaq™ Universal SYBR® Green Supermix (2X) was thawed to room temperature. Protected from light, the supermix was mixed thoroughly and briefly centrifuged and stored on ice. Predesigned and prevalidated primers from Sigma-Aldrich were used and their sequences are shown in Table 2.6. β -actin (*ACTB*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were used as housekeeping genes because they are universally required for basic cellular functions and are stably expressed in varying physiological and experimental conditions (Panina *et al.*, 2018). Respective master mixes were prepared on ice except the DNA template: master mix for 30 +RT reactions for each set of primer pair was prepared as shown in Table 2.2. For –RT reactions, No Reverse Transcriptase (NRT) reaction master mix was prepared as shown in Table 2.3 using only ActB primer pair. No Template control (NTC) containing only the primers was prepared as shown in Table 2.4.

Table 2.2. Reaction mixture preparation for cDNA synthesis.

Component	Volume per reaction	
	+RT reaction	-RT reaction
2X RT Buffer Mix	10.0 μ L	10.0 μ L
20X RT Enzyme Mix	1.0 μ L	-
Nuclease-free water	-	1.0 μ L
Total per well	11.0μL	11.0μL
RNA sample	up to 9 μ L	up to 9 μ L
Nuclease-free water	Q.S. to 9 μ L	Q.S. to 9 μ L
Total per reaction	20.0μL	20.0μL

Q.S. = Quantity Sufficient; RT = reverse transcriptase

The master mixes were thoroughly mixed to ensure homogeneity and equal volume (19.0 μ L) was dispensed into each well of white opaque 96-well qPCR plate. Respective DNA samples (+RT cDNA, - RT cDNA or H₂O) were then added into respective wells containing the respective master mix. The plates were sealed with optically transparent film and vortexed for approximately 30 seconds to ensure thorough mixing of the components. The plates were spun down briefly to remove any air bubbles and to also collect the reaction components at the bottom. Using the Bio-Rad CFX real-time PCR, cDNA denaturation was performed by heating the plate to 95°C for 30 seconds followed by template denaturation at 95°C for 5 seconds thermal cycled to 40 times and primer annealing and extension at 60°C for 30 seconds. At the end of each extension cycle, fluorescence was recorded. A melt curve was also generated at the end of the cycles by heating the samples for 2 seconds at 65°C and gradually increasing the temperature to 95°C in 0.5°C increments, and measuring fluorescence at each increment. Data was recorded as arbitrary fluorescence at each thermal cycle and based on Bio-Rad CFX system defaults quantitative C_q value was generated.

Table 2.3. Mastermix preparation for reactions with reverse transcriptase (+RT).

	Volume per reaction	Volume per 30 reactions
iTaq TM Universal SYBR® Green Supermix (2X)	10.0µL	300.0µL
Forward Primer (10µM)	1.0µL	30.0µL
Reverse Primer (10µM)	1.0µL	30.0µL
H ₂ O	7.0µL	210.0µL
Per well	19.0µL	-
Add 1µL (100ng) +RT cDNA to each well		
Total reaction volume per well = 20.0µL		

Table 2.4. Mastermix preparation for reactions without reverse transcriptase (–RT).

	Volume per reaction	Volume per 30 reactions
iTaq TM Universal SYBR® Green Supermix (2X)	10.0µL	300.0µL
ActB Forward Primer (10µM)	1.0µL	30.0µL
ActB Reverse Primer (10µM)	1.0µL	30.0µL
H ₂ O	7.0µL	210.0µL
Per well	19.0µL	-
Add 1µL (100ng) +RT cDNA to each well		
Total reaction volume per well = 20.0µL		

Table 2.5. Mastermix preparation for no template control (NTC) reactions.

	Volume per reaction	Volume per 5 reactions
iTaq TM Universal SYBR® Green Supermix (2X)	10.0µL	50.0µL
ActB Forward Primer (10µM)	1.0µL	5.0µL
ActB Reverse Primer (10µM)	1.0µL	5.0µL
H ₂ O	7.0µL	35.0µL
Per well	19.0µL	-
Add 1µL H ₂ O to each well		
Total reaction volume per well = 20.0µL		

Table 2.6. Primers

Target Gene	Forward Primer	Reverse Primer	Expected product size
<i>TADA3</i> (1)	ATTCACTGATGACCCT ATCG	CTTAGTAACTCCTCAA GTG	230bp
<i>TADA3</i> (2)	GCATCATCTCTTAATC CTCAG	GTCTTTCAACTCACTC ATGG	802bp
<i>ACTB</i>	GACGACATGGAGAAA ATCTG	ATGATCTGGGTCATCT TCTC	131bp
<i>GAPDH</i>	ACAGTTGCCATGTAG ACC	TTGAGCACAGGGTAC TTTA	88bp

2.13.7 Thresholding and quality control

Samples with low gene expression (cycles above 35) were quality controlled and removed. No template control (NTC) and negative reverse transcription (-RT) reactions showed no gene amplification indicating the reverse transcription reaction had worked well. Melt curve analysis did not show any multiple peaks for samples tested indicating gene specific primer binding and amplification.

2.13.8 qPCR analysis

Change in *TADA3/TADA3L* expression was measured using the delta-delta method. Changes in *TADA3/TADA3L* in A2780, OVCAR3 and SKOV3 cells transfected with mimic/negative control, TADA3_siRNA1 and TADA3_siRNA2 were normalised to respective control cells. Cq values for *TADA3* were first normalised to respective *GAPDH* and *ACTB* Cq value (first delta). Experimental change in TADA3_siRNA1 and TADA3_siRNA2 transfected cells at t=24hr and t=48hr were then normalised to respective control (second delta). Gene expression was then calculated using the following formula:

Relative TADA3 gene expression in siRNA treated cells compared to control
$$= 2^{-\Delta\Delta Cq(\Delta Cq(TADA3_siRNA-HKG)-\Delta Cq(CONTROL-HKG))}$$

HKG = house keeping gene

2.14 Immunofluorescence

In a class 2 hood, coverslips were first sterilised by dipping them in absolute ethanol for 30 sec, and leaving them to dry in 6-well or 12-well plates. A2780 cells were noted to not form a homogenous monolayer and often appeared to grow on top of each other. Other

ovarian cancer cell lines (IGROV-1, OVCAR3 and OVCAR5) also often detached very readily during the different wash steps involved in antibody labelling. Thus following abcam's instructions, the coverslips were coated with 0.1mg/ml poly-d-lysine (Sigma-Aldrich) for 1hr at room temperature to assist homogenous monolayer formation and prevent cell loss from detaching. After 1hr the coverslips were washed thoroughly with sterile tissue culture grade DPBS 3 times. Cell culture was added to wells and experimental treatments were performed as needed.

Immunofluorescence protocol was followed according to abcam recommendations. Old growth media from the wells was removed and cells were washed gently with PBS warmed to 37°C twice. Cells were fixed using 4% Formaldehyde (37% Formaldehyde was diluted to 4% using DPBS) for 30mins at 4°C. Formaldehyde was removed and discarded in appropriate toxic waste container. Cells were gently washed with cold DPBS 3 times to ensure all the fixing agent was removed. They were then permeabilised using 0.1% Triton X-100 in DPBS for 10mins at room temperature. This step enables labelling of intracellular proteins/antigens. Cells were washed with cold DPBS with 3 times 5mins each to ensure permeabilising agent was removed completely. To prevent non-specific labelling, cells are blocked with 1% BSA in DPBS for 1hour at room temperature on a rocker. The rocking motion further facilitates the blocking process. Primary antibody cocktail is prepared as required (see Table 2.6) using the same blocking agent (1% BSA in DPBS) and cells are incubated at 4°C on a rocker overnight. Antibody labelling at 4°C overnight is reportedly better because lower temperature slows down the binding process and is thus more specific. Cells were washed with cold DPBS 3 times 5mins each on the rocker. Secondary antibody cocktail was prepared in dark as required (Table 2.6) using the same blocking agent. Because secondary antibodies are conjugated with fluorescent proteins, they are photosensitive and exposure to direct light can lead to photobleaching. As a consequence, secondary antibody cocktail preparation and labelling is done in dark. Cells were labelled with secondary antibody for 30mins in the dark on the rocker at room temperature. Cells were then washed thoroughly with cold DPBS 3 times 5mins each in the dark to ensure all the secondary antibody was removed and prevent non-specific binding and background fluorescence. Coverslips were then mounted on glass slides using Prolong Gold Antifade Dapi mountant by adding 10µl of the mountant onto the glass slide ensuring there were no bubbles present, and gently adding the coverslip on top with cell side facing down. Slides were stored in the dark at 4°C. They were sealed with

nail varnish after 24hr for long term storage at 4°C. For Golgi fragmentation studies, the Golgi apparatus was labelled using *cis*-Golgi marker anti-GM130 antibody. For migration assay, cells were labelled with anti-GM130 and anti-Vimentin antibodies with antibody cocktail prepared at the same time. To label nuclear foci, γ H2A.X labelling was performed according to manufacturer recommended protocol (Milipore). HPA labelling was done using lectin Helix pomatia agglutinin (HPA; Thermofisher) conjugated to Alexa fluor 647 prepared in 3% BSA-TBS. Plasma membrane was labelled using Wheat Germ Agglutinin (WGA) conjugated to Alexa fluor 594 (Thermofisher). Hoechst 33342 was used to label nuclei (Thermofisher).

Table 2.7. Antibody combinations and concentrations used for immunofluorescence assays.

Primary antibody	Concentration	Corresponding secondary antibody	Concentration
Anti-GM130 Rabbit monoclonal (ab52649)	1:500	Goat Anti-Rabbit IgG H&L (Alexa fluor 488) (ab150077)	1:1000
Anti-GM130 Mouse polyclonal (ab169276)	7 μ g/ml	Goat Anti-Mouse IgG H&L (Alexa Fluor 594) (ab150116)	1:1000
γH2A.x conjugated to FITC	2 μ g/ml	N/A	N/A
HPA conjugated to Alexa fluor 647	5 μ g/ml	N/A	N/A

2.15 Confocal microscopy

2.15.1 Imaging antibody-labelled fixed cells

Antibody (GM130, vimentin, HPA-647, γ H2A.X-FITC, WGA594/Hoechst 33342) labelled fixed cells were imaged using confocal LSM 880 (Zeiss). The cells were first located through the eyepiece using the bright field setting. Once the cells were in focus, laser scanning microscope (LSM) mode was used to allow excitation of fluorescent cells and resulting emission was captured. Using 40X oil immersion lens, image acquisition settings were made including pinhole (1.0-1.35 units), laser power (<5%), image resolution (1024x1024) and pixel dwell (0.76 μ s). Table 2.8 illustrates the emission and excitation wavelengths used for different lasers. Detector gain and offset (rarely used) were adjusted as required to maintain optimal saturation. These settings were made firstly using the control samples and only slight changes in detector gain were made in treated cells, if any. Also, the cells were always imaged from 'left to right' direction starting from left corner of the dish or coverslip to avoid bias and reimaging the same cells. Once the appropriate region was located, the complete Z-axis of the cell was captured: using the Z-stack feature, the lower and uppermost Z-axis were selected. The number of slices were set based on the overlap between each slice (10-15% overlap maintained to avoid risking of losing any valuable data) and also the thickness of each cell (which varied from cell line to cell line, and thus needed adjusting regularly). 3D Z-stacks were rendered and imported into appropriate image analysis software.

Table 2.8. Excitation and emission wavelengths for different lasers on confocal LSM 880.

LASER	EXCITATION	EMISSION
405	405	453
488	488	544
561	590	617
633	650	665

2.15.2 Live cell imaging

2.15.2.1 24hr time course to document cell fusion events

The confocal LSM 880 was also equipped with temperature and CO₂ controlled incubator. This feature was instrumental in facilitating imaging of live cells for longer time periods. Heated stage was used to hold dishes and multiwell chambers at 37°C. A2780, OVCAR3 and SKOV3 cells grown in glass bottomed dishes were used to capture cell fusion events. Like previously, the cells were first located through the eyepiece using the bright field setting. Once located, the LSM acquisition mode was used to capture the time course. Red laser 633 was used at 1% for excitation because it is least damaging and phototoxic. Using the 'Positions' feature, 5 different positions were selected and each position was imaged every 1 minute with definite focus turned on. Image resolution was set to 1024X1024, and 24hr time course was captured for each cell line

2.15.2.2 Scratch assay time course

Multiwell plate from ibidi was used to capture time course of scratch assay. A2780, OVCAR3 and SKOV3 were grown to confluency in multiwell plate. In cat 2 hood, a scratch was created using sterile p10 pipette tip. The cells were gently washed with growth media to remove debris. Cells were treated with IC50 dosage of cisplatin and nuclei were labelled with 1uM Hoechst 33342. The confocal incubator temperature and CO₂ was pre-set to 37°C and 5%, respectively. The temperature of the heated stage was also pre-set to 37°C. The multiwell plate was secured in the heated stage and cells at the edge of the scratch were located through the eyepiece. Using the LSM acquisition mode and DAPI 405 laser at 1%, positions were selected in this order: Position 1 – A2780 control, Position 2 – A2780 cisplatin, Position 3 – SKOV3 control, Position 4 – SKOV3 cisplatin, Position 5 – OVCAR3 control and Position 6 – OVCAR3 cisplatin. Definite focus was turned on for each position and each position was imaged every 30 sec for 6 hours. Single cell tracking was then used to analyse migration speed of control and

cisplatin treated cells. Position 5 and 6 (OVCAR3 control and cisplatin) went out of focus at some point during the time-course and excluded from the analysis.

2.15.3 Fluorescence recovery after photobleaching (FRAP)

As described in section 2.7 (transient transfection using FuGene HD) OVCAR3 and SKOV3 cells grown in glass bottomed dishes were transfected with 2µg of protein trafficking reporter ts045-VSVG-EGFP (VSVG; temperature sensitive mutant of vesicular stomatitis G envelope glycoprotein fused to green fluorescent protein) and incubated at 37°C and 5% CO₂ for 8 hours. The cells were then treated with IC50 dosage of cisplatin for 3 hours and incubated at 37°C and 5% CO₂. The cells were washed gently with DPBS and replaced with fresh growth media and incubated at 37°C and 5% CO₂ for further 24 hours. After 24 hours, fluorescence recovery after photobleaching (FRAP) was performed. On the confocal LSM 880, the temperature of the heated stage was set to 40°C. The temperature of the incubator was set to 32°C and 5%CO₂. The VSVG reporter is temperature sensitive; it is released from the endoplasmic reticulum and traffics through the Golgi to the plasma membrane upon a shift from the restrictive temperature (40°C) to the permissive temperature (32°C). Following the protocol by Presley *et al* (1997), control and cisplatin treated cells were held at the restrictive temperature (40°C) for 1 hour to allow VSVG to accumulate in the ER. The temperature of the heated stage was then reduced to 32°C. The cells were held at the permissive temperature (32°C) for 1 hour to allow trafficking of VSVG. While at 32°C, a region of interest was created between the ER/Golgi and the plasma membrane. Using the 'bleaching' feature, bleaching was set to start after 5 cycles/scans. These first 5 cycles/scans are used for normalisation. The number of iterations for bleaching was set to 50, but this can vary depending on signal. A sample region is always tested first to determine the number of iterations needed to achieve considerable bleaching before performing the experiment. Because VSVG was tagged to EGFP, 488 laser was used at 80% to perform bleaching. Image resolution was set to 1024x1024 and definite focus was also turned on because FRAP was performed in two dimension, and there is a possibility that cells might drift away over time. The time-series was set to 100 cycles with 5 second intervals and captured at 40X objective with

488 excitation laser set at 3-5%. The time series was then imported to FIJI and analysed for fluorescence recovery.

2.16 Image analysis

2.16.1 Image analysis software's – Volocity and FIJI

Volocity is an image analysis software provided by Perkin Elmer. FIJI (or ImageJ) is an open source image analysis program with built-in plugins.

2.16.2 Golgi apparatus fragmentation quantification

Z-stacks created using confocal microscope were analysed using Volocity where anti-GM130 antibody labelled Golgi apparatus were analysed in 3D. A region of interest (ROI) was created around the individual cell, and the number of 'objects' (referring to Golgi fragments) in that ROI were identified through the signal detected through the fluorescence channel. The threshold limit was set to distinguish between the fluorescence emitted by the Golgi and the background signals, and also between bright and weak signals in control cells first, and the same thresholding was applied on treated cells. Intensity and volume measurements of each fragment were recorded for each Golgi analysed and the corresponding number of total Golgi fragments was tabulated. Approximately 100 cells were analysed for each treatment per replicate unless otherwise stated.

2.16.3 Golgi orientation angle measurement

Golgi apparatus polarisation was determined by measuring orientation or angle of the Golgi with respect to the nucleus and wound edge/scratch front as described by Xing *et*

al. (2016). Z-stacks of anti-GM130 antibody labelled cells at the wound edge/scratch front created using confocal microscope, were analysed using FIJI image analysis software. The Z-stacks were first converted into maximal intensity projected images. Then the angle formed between the Golgi and the scratch front was measured. Essentially, first line was drawn from the center of the nucleus to the wound edge and a second line was drawn from the center of the nucleus to the center of the Golgi. The angle formed between the lines was measured and termed Golgi orientation angle. The centres of the nucleus and Golgi were estimated by eye. Intuitively, smaller angles ($\sim 60^\circ$) mean that Golgi is polarised and oriented towards the wound edge, while larger angles ($> 60^\circ$) mean that Golgi is depolarised and oriented away from the wound edge.

2.16.4 Nuclear foci count

Number of nuclear foci per cell were measured for control and cisplatin treated A2780 cells. Nuclei of cells were labelled with γ H2A.X conjugated to FITC and DAPI, and Z-stacks were created using confocal microscopy. Protocol provided by Duke University's Light Microscopy Core Facility (Count Nuclear Foci – ImageJ, <https://microscopy.duke.edu/guides/count-nuclear-foci-ImageJ>) was used to measure nuclear foci per cell. Using FIJI, the channels were split into FITC and DAPI channel. Selecting the DAPI channel, the image was thresholded so that each nucleus was highlighted as a single region. Using Analyse Particles feature, minimum size of the nuclei was set – default setting (> 200 pixels) suggested by FIJI was used, and 'Overlay Outlines' option was selected to further highlight the boundaries of the nuclei. After pressing OK, ROI manager window opened which highlighted all the nuclei. This was helpful in verifying the accuracy of the process and making sure all the nuclei were identified. Using the FITC channel, nuclear foci within each nuclei were identified based on maximum intensity of FITC-labelled foci. Firstly, from the Process $>$ 'Find Maxima' feature, background noise was removed by setting a threshold for noise tolerance. Several control images were tested to determine the best threshold level, and the same was then applied to treated images. Selecting output type as 'Single Points' produced a new binary image with each maxima (or foci) highlighted within each nuclei. ROI manager was then opened which accesses nuclei shapes made by Analyse Particles earlier. 'Show all' option in ROI

manager was selected which put together the ROI depicting the nuclei and maxima identifying foci within each nuclei. ‘Measure’ option was clicked and that produced Results window showing each ROI, the area of the ROI, Integrated Density and Raw Integrated Density – which is the sum of all pixels in the region. These options can also be set by going to Analyse > Set Measurements. Each pixel represents a foci (or maxima) and has a pixel intensity value of 255. Dividing the value in Raw Integrated Density column by 255 gave the number of detected foci in respective region of interest (or nuclei).

2.16.5 FRAP analysis

FRAP data were analysed according to the protocol suggested by Webster and Wachsmuth (2014). Once the FRAP data was acquired, files were imported into FIJI. Using the square tool, the first ROI was drawn around the bleached area (BL) and stored in ROI Manager. The dimension of the box was kept the same as that of the bleach region during microscopy. Second ROI was drawn to detect background non-target fluorescence (BG) and stored in ROI Manager. Finally, third ROI was drawn around a region of fluorescence that was not bleached and used as reference (REF). Using the Multi Measure feature in ROI Manager, mean gray value was measured for all 100 cycles (also select One Row Per Slice option here). The data were then extracted for all regions of interest: BL, BG and REF.

Using MS Excel, background noise signal from the data was eliminated as follows:

$$BL_{corrected}(t) = BL(t) - BG(t)$$

$$REF_{corrected}(t) = REF(t) - BG(t)$$

Normalised corrected Bleach to corrected Reference as followed:

$$BL_{norm}(t) = \frac{BL_{corrected}(t)}{REF_{corrected}(t)}$$

A second normalisation step is performed to the mean pre-bleach intensity. This is the maximum intensity to which the bleached region could possibly recover. During FRAP acquisition, 5 pre-bleach cycles were obtained. The mean gray value for 5 pre-bleach cycles was averaged (hence, mean pre-bleach) and used to perform second normalisation as follows:

$$BL_{norm2}(t) = \frac{BL_{norm}(t)}{BL_{norm}(prebleach)}$$

Performing second normalisation produces data in the range of 1 and 0 or 100% signal intensity and 0% signal intensity, and can be used to demonstrate recovery of control and treated cells in a graphical format. Using the Curve Fitting function in FIJI is then used to model a fit of data. Using only the post-bleach section of the normalised recovery curve, ‘Exponential Recovery’ fit is performed. Exponential recovery provides following information:

$$y = a * (1 - \exp(-b * x)) + c,$$

a = slowly recovery fraction

c = rapidly diffusing fraction

b = recovery rate

Complete recovery according to the fit parameters suggest that $a + c = 1$. Fraction of mobile or immobile protein can also be calculated as follows:

$$f_{mobile} = a + c \text{ or } f_{immobile} = 1 - a - c$$

Above mentioned process was repeated for >5 cells per treatment for OVCAR3 and SKOV3, and repeated twice. For control and cisplatin treated cells, mean mobile fraction of VSVG was determined.

2.16.6 Multinucleate cell count

Cells grown on coverslips to 60-70% confluency were labelled with plasma membrane marker WGA594 and nucleus marker Hoechst 33342. The cells were fixed as described previously and Z stacks were created using confocal LSM 880. Using plasma membrane labelling as a guide especially for cells in cluster and Hoechst 33342 labelling, number of nuclei per cell were manually counted by following the single cell approach.

2.16.7 Counting cell fusion events

Cell fusion events were quantified from 24-hour time series captured using confocal microscopy on A2780, OVCAR3 and SKOV3 cells. Essentially, cells going through cell division (from prophase to cytokinesis) were quantified using the following criteria: (i) whether it was a multipolar or bi-polar division indicating whether the parent cell was multinucleated or single-nucleated and (ii) whether daughter cells fused or not after cell division.

2.16.8 Measuring migration speed

Migration speed of control and cisplatin treated A2780 and SKOV3 cells was measured from 6 hour time series created. Nuclei were labelled with Hoechst 33342 and was used to detect and track single cells using Volocity. Once the individual cells were detected, length of the track and total time span for each cell was measured. Migration of speed of each cell was calculated using the following formula:

$$Speed = \frac{distance (\mu m)}{time (hr)}$$

2.16.9 Percentage wound closure

Percentage of wound closed in control and cisplatin treated A2780, OVCAR3 and SKOV3 cells was measured to determine the effect of cisplatin on wound healing capacity of cells. After creating a scratch on a confluent glass bottom dish, multiple bright field images of the entire scratch area were captured at t=0hr, when the scratch was created, until the scratch appeared to be closing in at t=14hr for control and cisplatin treated cells using light microscope. Using the region of interest tool in FIJI, total empty area in the scratch was measured at t=0hr and t=14hr, and percentage wound closure was calculated using the following equation:

$$\text{Percentage wound closure} = \frac{(\text{total area at } t_0 - \text{total area at } t_{14})}{(\text{total area at } t_0)} \times 100$$

2.18 Statistics

Outliers were detected and removed using Rout's method (Q=1%). D'Agostino-Pearson's normality test was performed to determine distribution of data. For data with normal distribution, Student's t-test and ANOVA followed by Tukey's test for multiple comparison was used to determine significance. For non-parametric data, Mann Whitney U test or Kruskal-Wallis test followed by Dunn's multiple comparison test was used to determine significance. p<0.05 was considered significant

CISPLATIN TREATMENT ALTERS MORPHOLOGY OF THE GOLGI APPARATUS

RESULTS | CHAPTER 3

3 Cisplatin treatment alters morphology of the Golgi apparatus

3.1 Overview

The main aim of the project was to determine whether chemotherapy drug treatment, specifically cisplatin, alters the morphology of the Golgi apparatus. Cisplatin is a chemotherapy drug used to treat several cancers including ovarian, and has shown significant improvement in patient survival rates. The main obstacle in using this drug however is the emergence of cisplatin resistance and subsequent tumour relapse (Cannistra, 2004). And, although the damaging effects of cisplatin on several cell organelles including altered molecular mechanisms in mitochondria, ER, nucleus, cytoskeleton (actin and microtubules) are known (Sancho-Martinez *et al.*, 2012), its effect on the structural integrity of the Golgi apparatus and associated function is not reported. Farber-Katz *et al.* (2014) had shown in their study that treatment of several different cell lines with chemotherapy drugs (doxorubicin and camptothecin) or ionizing radiation resulted in significant dispersal of the Golgi apparatus at its *trans* end in a time and concentration dependent manner, suggesting that Golgi dispersal is a common response to DNA damage in mammalian cells. It was decided to determine whether the same was true in the case of ovarian cancer cells and the drug most commonly used to treat them: cisplatin.

To determine the effect of cisplatin on Golgi apparatus, firstly the IC-50 dosage of cisplatin for seven different ovarian cancer cell lines was determined. The cells were then treated with their respective IC-50 dose of cisplatin and Golgi fragmentation was measured. The time-course for Golgi fragmentation and DNA damage accrual was also established to determine whether Golgi fragmentation precedes or succeeds DNA damage. Finally, ERK and JNK signaling pathways have been shown to play a role in Golgi fragmentation during mitosis; therefore, these signaling pathways were also investigated in cisplatin-induced Golgi fragmentation.

3.2 Determining IC-50 dosage levels of cisplatin for ovarian cancer cell lines

Half-maximal inhibitory concentration (IC-50) indicates how much of a pharmacologic agent or drug is required to inhibit a given biological activity by half for a given cell line (Aykul and Martinez-Hackert, 2016). For this project, seven different ovarian cancer cell lines were used to determine their sensitivity towards cisplatin: A2780, IGROV-1, OVCAR-3, OVCAR-4, OVCAR-5, OVCAR-8 and SKOV-3. To determine the IC-50 dosage for each cell line, cells were treated with different concentrations of cisplatin (0 to 400 μ M) and cell viability assay was performed using the Alamar Blue assay. IC-50 was then measured from the kill curves produced using GraphPad Prism 7.0. Each experiment involved three technical replicates and each experiment was repeated three times. Results obtained are shown in Table 3.1 and Figure 3.1. OVCAR5 and OVCAR4 cells were found to be the most sensitive (IC-50: 34.5 μ M $\pm$ 5.4 and 45.5 μ M $\pm$ 2.4, respectively) while OVCAR8 and SKOV3 were most resistant (IC-50: 205.3 μ M $\pm$ 57.5 and 96.9 μ M $\pm$ 40.3, respectively). These results show that each cell line presents with different level of sensitivity or resistance towards cisplatin.

Table 3.1. Cisplatin IC-50 dosage measured for ovarian cancer cell lines. Cells were treated with increasing concentration of cisplatin (0-400 μ M) for 3hr. Cell viability was measured using the Alamar Blue assay after 24hr with absorbance recorded at 590nm. Mean and SD was measured from the mean of three biological replicates.

Cell line	Mean IC-50 for cisplatin (μ M) $\pm$ SD*
A2780	57.8 $\pm$ 14.5
IGROV-1	66.4 $\pm$ 18.2
OVCAR-3	81.3 $\pm$ 7.2
OVCAR-4	45.5 $\pm$ 2.4
OVCAR-5	34.5 $\pm$ 5.4
OVCAR-8	205.3 $\pm$ 57.5
SKOV-3	96.9 $\pm$ 40.3

*SD = Standard Deviation

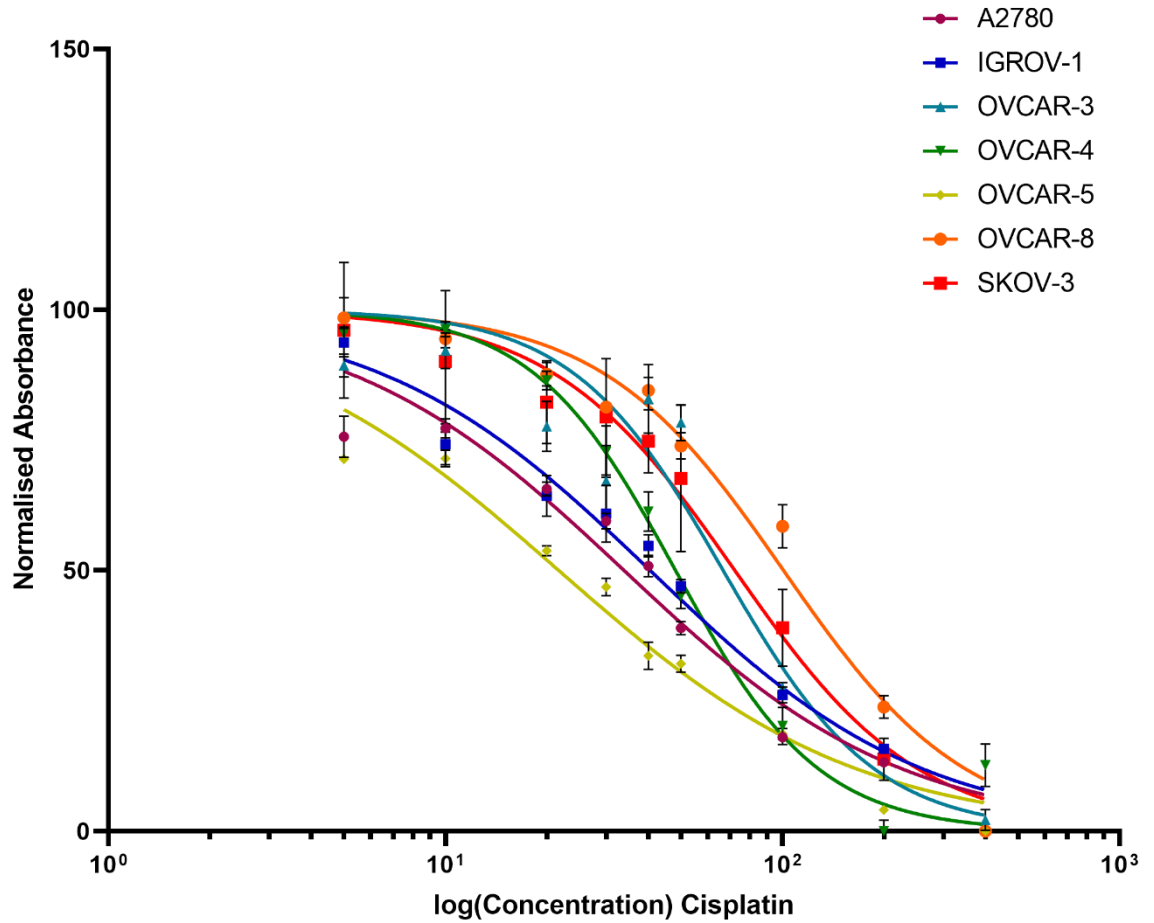


Figure 3.1. Cisplatin kill curves generated for ovarian cancer cell lines. Each curve represents average of three independent biological experiments with three technical replicates each. Cells were treated with increasing concentration of cisplatin (0-400 μ M) for 3hr. Cell viability was measured using the Alamar Blue assay after 24hr with absorbance recorded at 590nm. IC-50 measured for each cell line: A2780 – 57.8 μ M, IGROV-1 – 66.35 μ M, OVCAR3 – 81.27 μ M, OVCAR4 – 45.55 μ M, OVCAR5 – 34.53 μ M, OVCAR8 – 205.3 μ M and SKOV3 – 96.9 μ M. Error bars represent $\pm$ SEM.

3.3 Altered Golgi morphology in response to cisplatin treatment

DNA-damaging effects of cisplatin on cancer cells are known, however, its effect on the Golgi apparatus morphology has not been reported. To determine whether cisplatin treatment has an effect on the structure of the Golgi apparatus, ovarian cancer cells A2780 were first tested. A2780 cells were treated with their respective inhibitory concentration (IC-50) of cisplatin for 3 hours and Golgi structure was analysed after 24 hours. Golgi apparatus was labelled with antibody to the *cis*-Golgi marker, Golgi matrix protein GM130. Z-projected images of cisplatin-treated and control cells were created using the Zeiss LSM880 confocal microscope.

To measure changes in Golgi morphology, overall Golgi fragmentation per cell was determined using the image analysis software Volocity. Cells in interphase as judged by DAPI staining were used throughout the study while mitotic cells were excluded from analyses. Figure 3.2 illustrates the process of analysis employed for cisplatin treated A2780 cells. Firstly, 3D Z-stacks created on confocal microscope were imported into the image analysis software (Figure 3.2 (a)). Using the 'Extended Focus' feature on Volocity the 3D Z-stacks were converted into projected images (Figure 3.2 (b)). Then, using the transmitted light channel as a guide to determine the outline of the cell, region of interest (ROI) around the selected cell was created (Figure 3.2 (c)). Individual fragments were identified using 'Find Objects' within ROI feature. Thresholding was done using software-provided default 'Percentage' settings. The thresholding slider was adjusted based on fluorescent objects detected by eye. For every experiment, the thresholding was done on control cells first and the same settings were then applied to cisplatin treated cells. Since image acquisition settings while performing confocal microscopy were also kept the same for control and cisplatin treated cells, threshold levels for analysis between the two experimental groups were also found to be the same. Minimum object size was set to $0.1\mu\text{m}^3$. The smallest of object (or fragment) detected in control cells from different cell lines based on preliminary analysis was found to be approximately $0.1\mu\text{m}^3$ and was kept constant throughout the analyses. Once the settings were made, all the objects or fragments were then identified by the software in the selected ROI or cell (Figure 3.2 (e)).

Single cell analysis approach was employed to identify number of Golgi fragments in each cell for control and cisplatin treated A2780 cells.

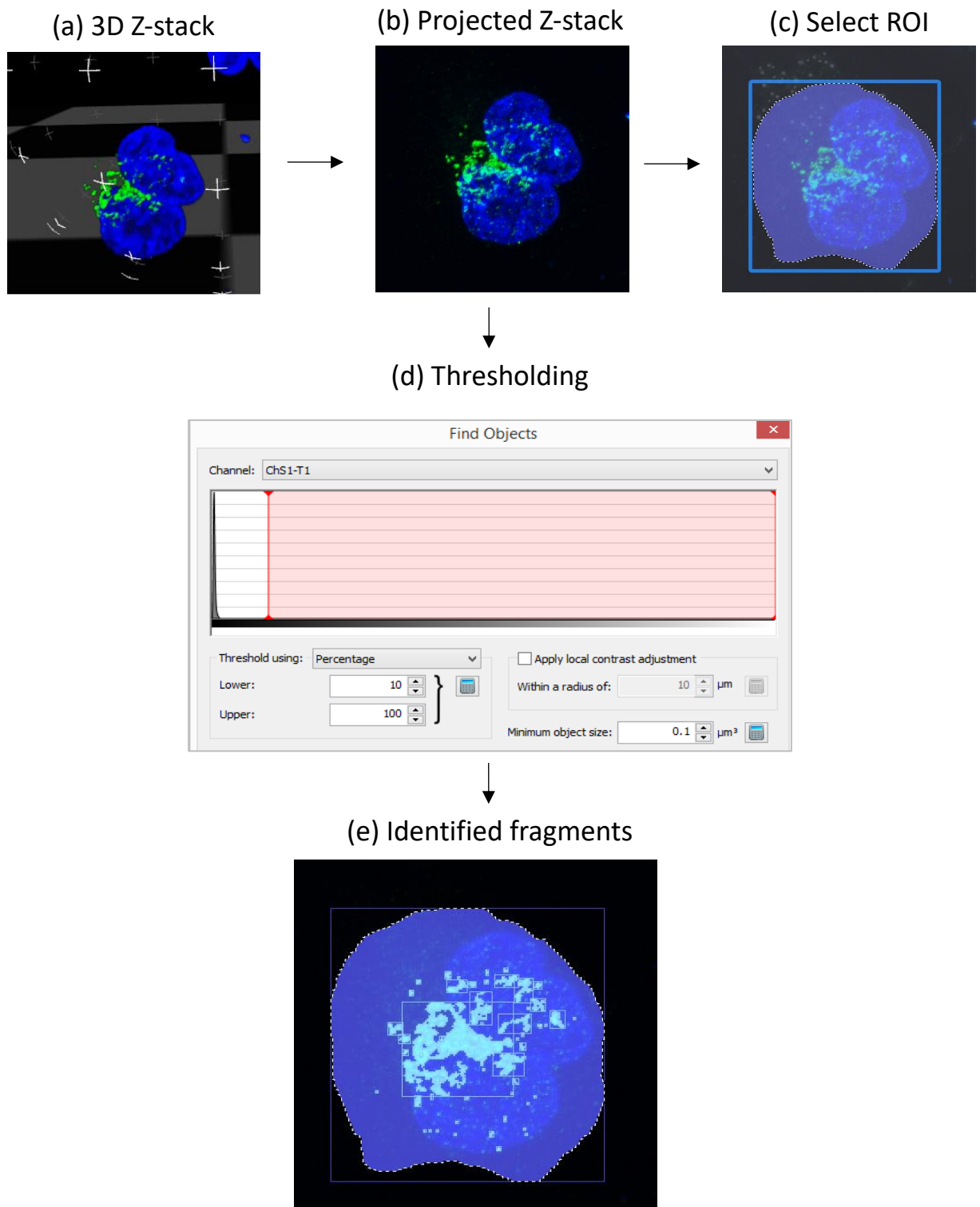


Figure 3.2. Illustrating the process for identifying Golgi fragments using Volocity.
See the main text for detailed step-by-step process.

Figure 3.3 illustrates the effect of cisplatin on *cis*-Golgi (referred as Golgi from here on) structure in A2780 cells. Significantly more number of Golgi fragments were counted in cisplatin treated cells compared to control ($p < 0.0001$). Although it wasn't quantified, the Golgi in cisplatin treated cells also appeared to be more dispersed throughout the cytoplasm whereas Golgi in control cells retained a more compact morphology.

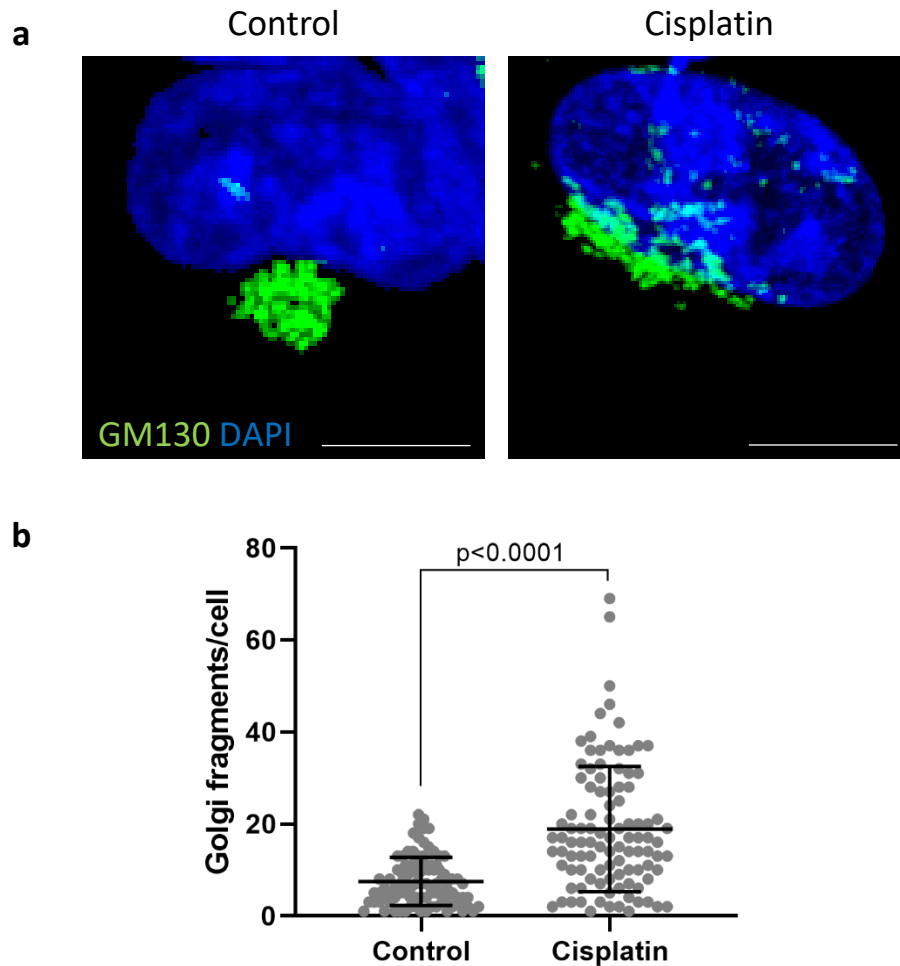


Figure 3.3. Effect of cisplatin treatment on Golgi fragmentation in A2780 cells. A2780s were treated with their IC-50 dose of cisplatin for 3hr. Antibody labelling using anti-GM130 (green) was performed to label the Golgi apparatus 24hr after treatment. Nucleus labelled with DAPI (blue). Z-projected images of control and cisplatin treated cells were created using confocal microscope, and Golgi fragmentation was measured using Volocity image analysis software. (a) Representative confocal images of control and cisplatin-treated cells labelled. (b) Cisplatin treated cells were significantly more fragmented than control cells (Mann-Witney U test, $p < 0.01$). 100 cells in total were counted from two biological replicates. Error bars represent mean $\pm$ 1 standard deviation. Scale bar = 10 μ m.

To ensure the effect observed in response to cisplatin was not cell line specific, the same experiment was performed using other ovarian cancer cell lines namely, IGROV-1, OVCAR3, OVCAR4, OVCAR5, OVCAR8 and SKOV3, and Golgi structure of cells in interphase was analysed as described previously. Figure 3.4 illustrates the effect of cisplatin on Golgi structure in ovarian cancer cell lines. Cisplatin treated cells were found to have significantly more fragmentation of the Golgi than control ($p<0.01$) for all the cell lines tested. This suggests that cisplatin treatment alters overall structure of the Golgi apparatus in ovarian cancer cells.

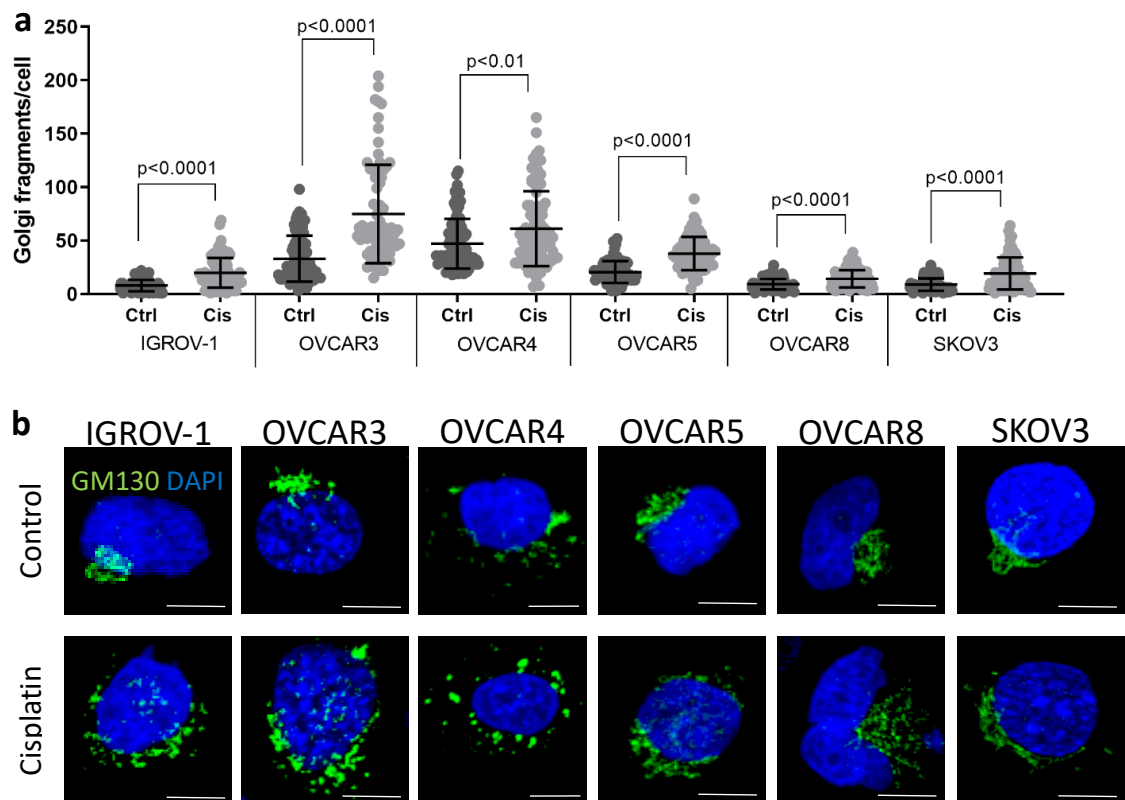


Figure 3.4. Effect of cisplatin treatment on Golgi fragmentation in ovarian cancer cells. Cell lines were treated with cisplatin for 3hr. After 24hr, antibody labelling using anti-GM130 (green) was performed to label the Golgi apparatus. Nucleus labelled with DAPI (blue). (a) Cisplatin treated cells showed significantly more Golgi fragments than control in all the cell lines tested (Mann-Witney U test, $p<0.01$). (b) Representative confocal images. 100 cells in total were counted from two biological replicates. Error bars represent mean $\pm$ 1 standard deviation. Ctrl = control, Cis = cisplatin. Scale bar = 10 μ m.

3.4 Golgi fragmentation precedes detectable levels of cisplatin-induced DNA damage

Treatment of seven different ovarian cancer cell lines with cisplatin consistently resulted in significant fragmentation of the Golgi apparatus. Farber-Katz *et al.* (2014) had noted in their study that Golgi dispersal following stress (chemotherapy drug or irradiation) was detected as early as 4hr post treatment. Knowing that Golgi plays a role in stress sensing and effecting (Nakagomi *et al.*, 2008; Oku *et al.*, 2011), it was important to establish at what time Golgi starts to fragment following cisplatin treatment. To achieve this, A2780 cells were treated with their IC-50 dosage of cisplatin and cells were fixed at t=0hr (cisplatin just added) to t=72hr post treatment. Cells were antibody labelled, imaged and analysed as described in 3.2. Cells in interphase as judged by DAPI staining were used for analyses while mitotic cells were excluded. Figure 3.5 illustrates the time course of Golgi fragmentation following cisplatin treatment. There was a trend towards significant increase in the number of Golgi fragments by 3 hours post cisplatin treatment ($P=0.06$). Significantly more Golgi fragmentation compared to control was observed 6 hours post treatment ($P=0.02$) and this significant increase in number of fragments remained throughout the rest of the tested time and was very pronounced by 72 hours post drug treatment ($p<0.001$).

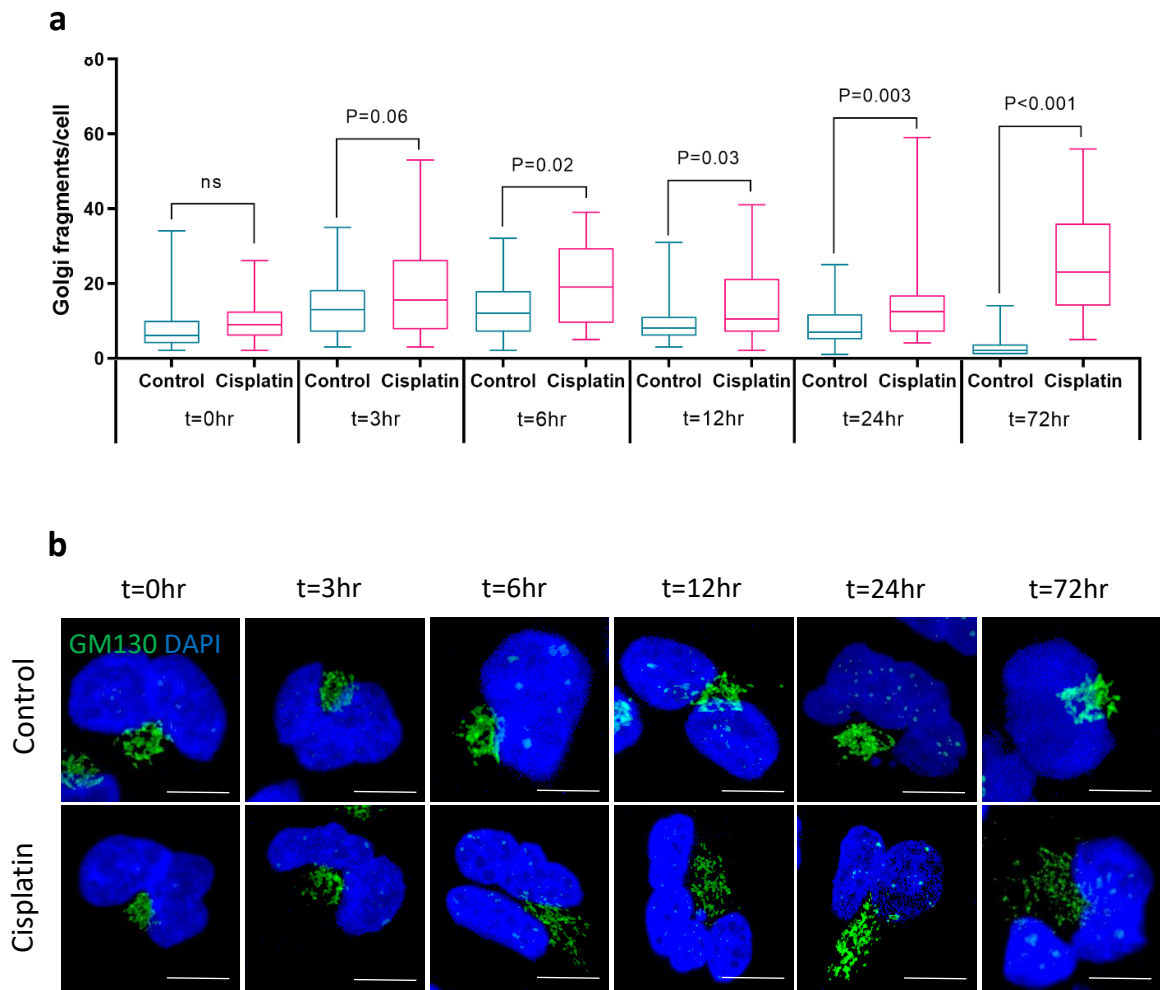


Figure 3.5. Time-course of Golgi fragmentation in response to cisplatin treatment in A2780 cells. Cells were treated with cisplatin and fixed at indicated time points post treatment. Antibody labelling using anti-GM130 (green) was performed to label the Golgi apparatus. Nuclei were labelled with DAPI (blue). (a) Non-significant increase in Golgi fragmentation was observed 3 hours post treatment, with fragmentation found to be significant after 6 hours. The Golgi continued to significantly fragment more until 72 hours post treatment (Mann-Witney U test, $p < 0.05$). (b) Representative confocal images. Experiment was performed twice and 30 cells were counted in total. Scale bar = $10\mu\text{m}$.

Farber-Katz *et al.* (2014) presented evidence in their study that dispersed Golgi morphology was a downstream effect and a result of the DNA damage response. In an attempt to investigate whether Golgi fragmentation in response to cisplatin treatment was detected before or after DNA damage caused by cisplatin, it was important to also determine the time-course for DNA-damage in ovarian cancer cells in response to cisplatin treatment. To study this, again A2780 cells were treated with cisplatin and fixed at different time points (t=0hr, t=3hr, t=6hr, t=12hr and t=24hr) like previously. To detect DNA damage, cells were labelled with DNA damage marker γ H2A.X conjugated with FITC and the number of nuclear foci (indicative of DNA damage) per cell were measured for cells in interphase as judged by DAPI staining. Figure 3.6 illustrates the time course for DNA damage in A2780 cells following cisplatin treatment. No significant difference in DNA damage as shown by number of nuclear foci per cell was observed at 3hr and 6hr post treatment. Maximal DNA damage in A2780 cells was recognised at 12hr after cisplatin treatment ($p<0.001$) and increased up to 24hr post treatment ($p<0.001$). These results indicate that cisplatin-induced DNA damage is detected after Golgi fragmentation.

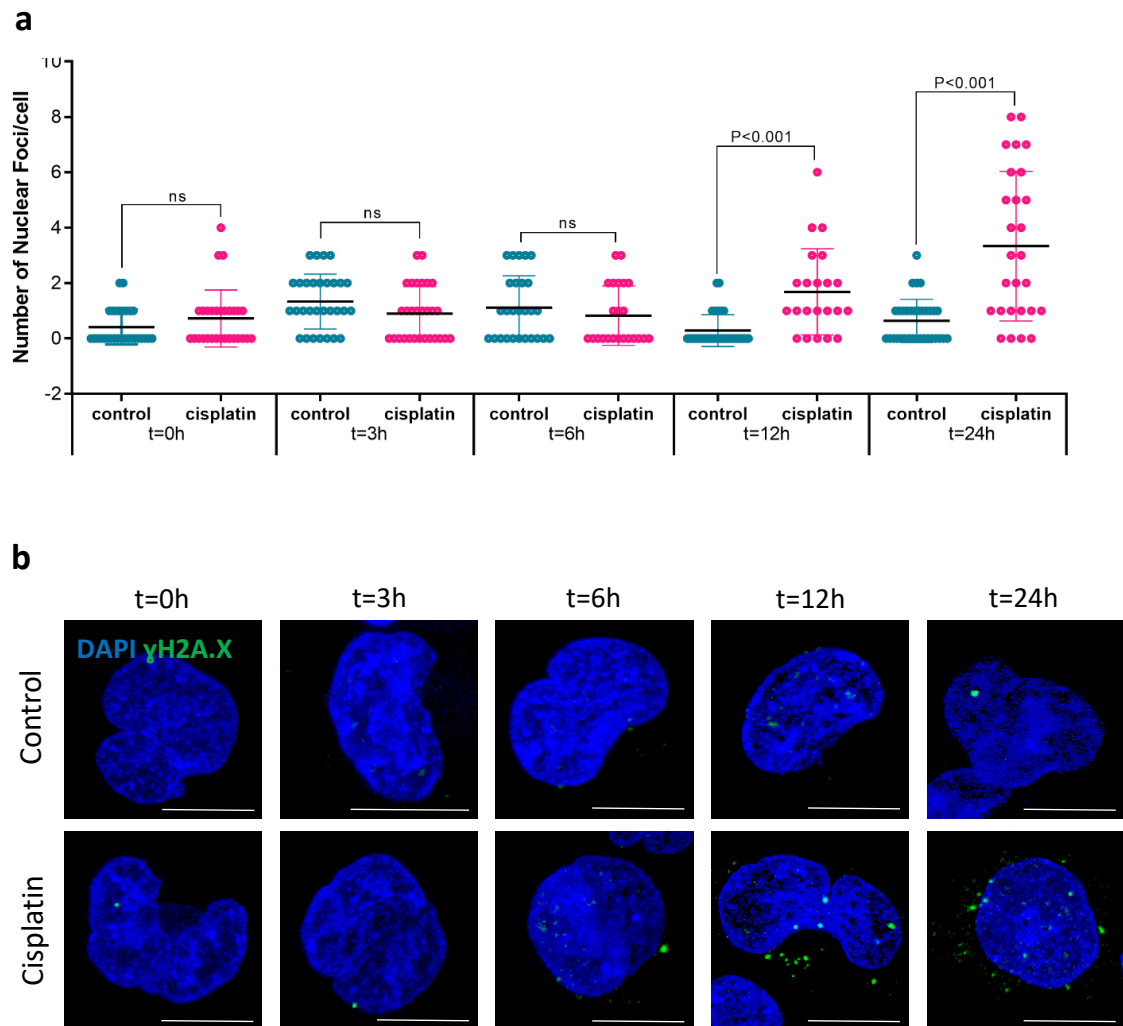


Figure 3.6. Time-course of DNA damage following cisplatin treatment in A2780 cells. Cells were treated with cisplatin and fixed at indicated time points post treatment. yH2A.x conjugated to FITC (green) was used to label nuclear foci. Nucleus was labelled with DAPI (blue). Number of nuclear foci per cell indicating DNA damage were calculated using FIJI. (a) Significant DNA damage was detected at 12hr post treatment and increased at 24hr. (Mann-Witney U test, $p<0.001$). (b) Representative confocal images. Experiment was performed twice and 30 cells were counted in total. Error bars represent mean $\pm$ 1 SD. Scale = 10 μ m.

3.5 Role of ERK in cisplatin-induced Golgi fragmentation

The extracellular signal-regulated kinases (ERK), one of the subfamilies of mitogen-activated protein kinases (MAPK), is activated in response to cisplatin treatment (Wei, Yan and Tang, 2011). In normal cells, ERK functions to regulate cell growth and differentiation, and is highly induced in response to growth factors, cytokines and phorbol esters (Johnson and Vaillancourt, 1994). In cancer cells, there is mixed evidence that suggests that while ERK activation enhances survival of cisplatin-treated cells (Persons *et al.*, 1999), it also mediates cisplatin-induced apoptosis by initiating the apoptotic signal (Wang, Martindale and Holbrook, 2000). Coincidentally, ERK is also activated and plays a role in Golgi fragmentation during mitosis (Cervigni *et al.*, 2015). Hence, the role of ERK signaling in cisplatin-induced Golgi fragmentation in ovarian cancer cells was studied by inhibiting the ERK pathway using a chemical inhibitor FR180204. FR180204 is a selective ATP competitive inhibitor of ERK1/2 (Ohori *et al.*, 2005; Cicenaz *et al.*, 2017).

Firstly, kill curves were generated for A2780, OVCAR-3 and SKOV-3 cells in response to the ERK inhibitor with and without IC-50 dosage of cisplatin predetermined for each cell line: A2780 - ~35 μ M, OVCAR-3 - ~150 μ M, SKOV-3 - ~90 μ M. This was done to identify an optimal sublethal concentration of the inhibitor in the presence and absence of cisplatin. Cells seeded in 96-well plates were pre-treated with increasing concentration of the ERK inhibitor (0 μ M to 200 μ M) for 3hr followed by treatment with IC-50 dosage of cisplatin in 'cisplatin-wells' for further 3hr. Cell viability was measured after 24hr using the Alamar Blue assay. Figures 3.7a-c illustrates the kill curves generated for A2780, OVCAR3 and SKOV3 cells, respectively. Based on the kill curves, ERK inhibitor concentration that produced relatively high cell viability and low toxicity in the presence of cisplatin was considered optimal for each cell line, and was further used to test whether cisplatin mediated Golgi fragmentation through ERK pathway: A2780 - 100 μ M, OVCAR-3 - 100 μ M and SKOV-3 - 100 μ M.

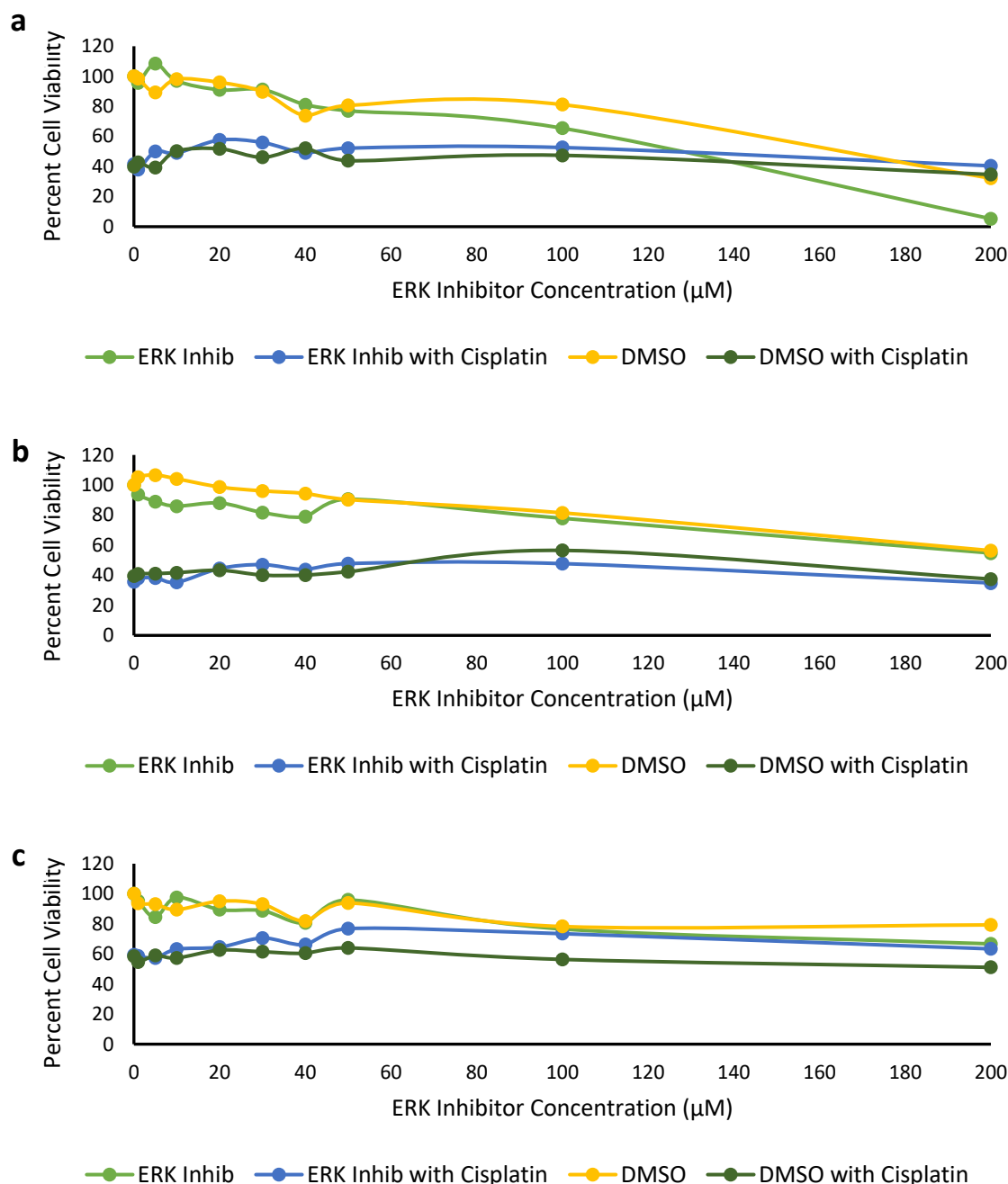


Figure 3.7. Kill curves generated for ERK inhibitor. Cells were pre-treated with increasing concentration of ERK inhibitor FR180204 for 3hr followed by cisplatin for additional 3hr. Cell viability was measured using Alamar Blue assay after 24hr. ERK inhibitor concentration that produced relatively high cell viability in the presence of cisplatin was considered optimal for each cell line. Each point on the curve represents average of three independent experiments performed in triplicates. (a) A2780, (b) OVCAR3 and (c) SKOV3.

In order to investigate whether cisplatin-induced Golgi fragmentation as observed in ovarian cancer cell lines was mediated through the ERK pathway, the effect of ERK inhibition on Golgi fragmentation was first tested using A2780 cells. The cells were pretreated with 100 μ M ERK inhibitor for 3hrs followed by treatment with IC-50 dosage of cisplatin for another 3hrs. It should be noted that cells were also treated with equivalent volumes of PBS and DMSO used as solvents for cisplatin and ERK inhibitor, respectively. The cells were fixed and antibody labelled at 24hrs and Golgi fragmentation was quantified as described in 3.2 using Volocity image analysis software. Cells in interphase as judged by DAPI staining were used for analyses while mitotic cells were excluded. Figure 3.8 illustrates the effect of ERK inhibition on cisplatin-induced Golgi fragmentation in A2780 cells. Cisplatin treated cells showed significantly more fragmented Golgi (20.49 ± 15.34 fragments/cell; $p < 0.0001$) than control (10.68 ± 5.17 fragments/cell) and PBS (10.99 ± 7.25 fragments/cell), further supporting the finding from 3.2. Strikingly, effect of ERK inhibition in cisplatin treated cells showed non-significant increase in number of Golgi fragments compared to cisplatin treated cells without ERK inhibition (cisplatin + ERK inhibitor: 31.63 ± 23.55 fragments/cell vs cisplatin alone: 20.49 ± 15.34 fragments/cell; $P = 0.07$). These results suggest that inhibiting ERK pathway possibly further increases Golgi fragmentation in response to cisplatin.

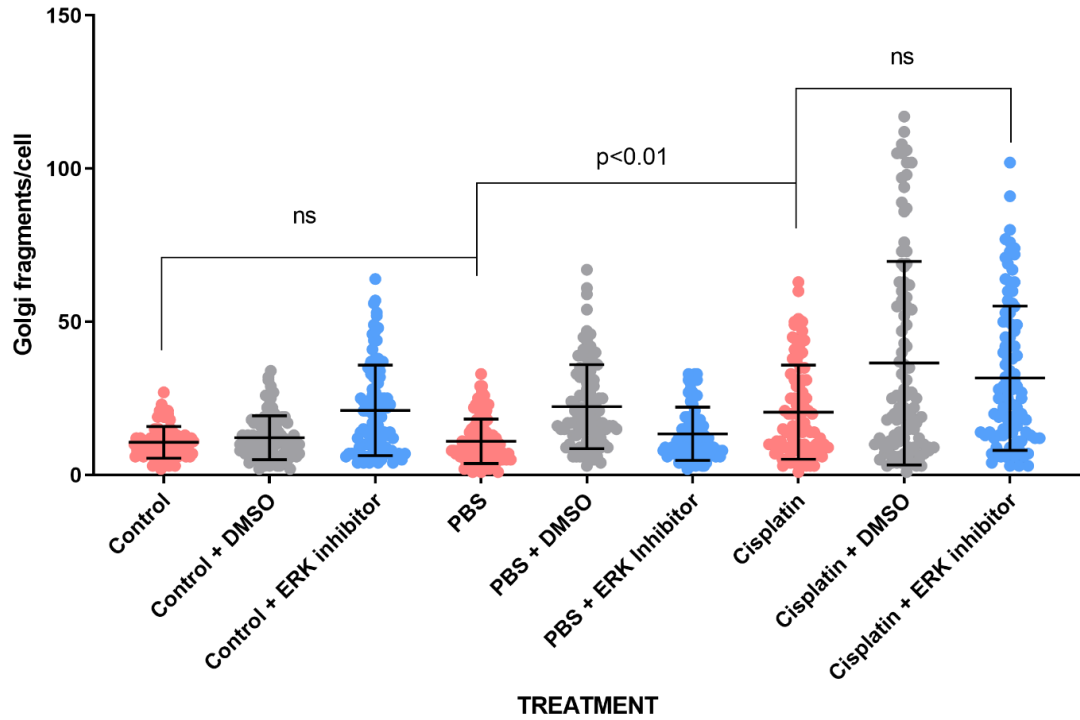


Figure 3.8. Effect of ERK inhibition on cisplatin-induced Golgi fragmentation in A2780 cells. Cells were pre-treated with 100 μ M ERK inhibitor for 3hr followed by cisplatin treatment for additional 3hr. Significant fragmentation of Golgi was observed in cisplatin (20.49 $\pm$ 15.34 fragments/cell) treated cells compared to control (10.68 $\pm$ 5.17 fragments/cell) and PBS (10.99 $\pm$ 7.25 fragments/cell). Following ERK inhibition, Golgi fragmentation non-significantly increased in cisplatin treated cells (31.63 $\pm$ 23.55 fragments/cell; $P=0.07$). 100 cells from two experiments were analysed. Kruskal-Wallis test followed by Dunn's multiple comparison test was performed to determine significance ($p<0.05$). Error bars represent mean $\pm$ 1 SD.

Based on the results observed for A2780 cells, the effect of ERK inhibition was also investigated in OVCAR3 and SKOV3 cells to determine whether ERK plays a role in cisplatin-induced Golgi fragmentation in other ovarian cancer cells. Like previously, cells in interphase were used for analyses while mitotic cells as judged by DAPI staining were excluded. Figures 3.9 and 3.10 illustrate the effect of ERK inhibition on cisplatin-induced Golgi fragmentation in OVCAR3 and SKOV3 cells, respectively. Similar to A2780 cells, cisplatin treated OVCAR3 and SKOV3 cells also showed significantly more number of Golgi fragments than control and PBS ($p < 0.05$). Inhibition of ERK in OVCAR3 and SKOV3 cells treated with cisplatin showed no significant difference in number of Golgi fragments than cisplatin cells alone. These results indicate that inhibiting ERK pathway has no effect on cisplatin-induced Golgi fragmentation in ovarian cancer cells.

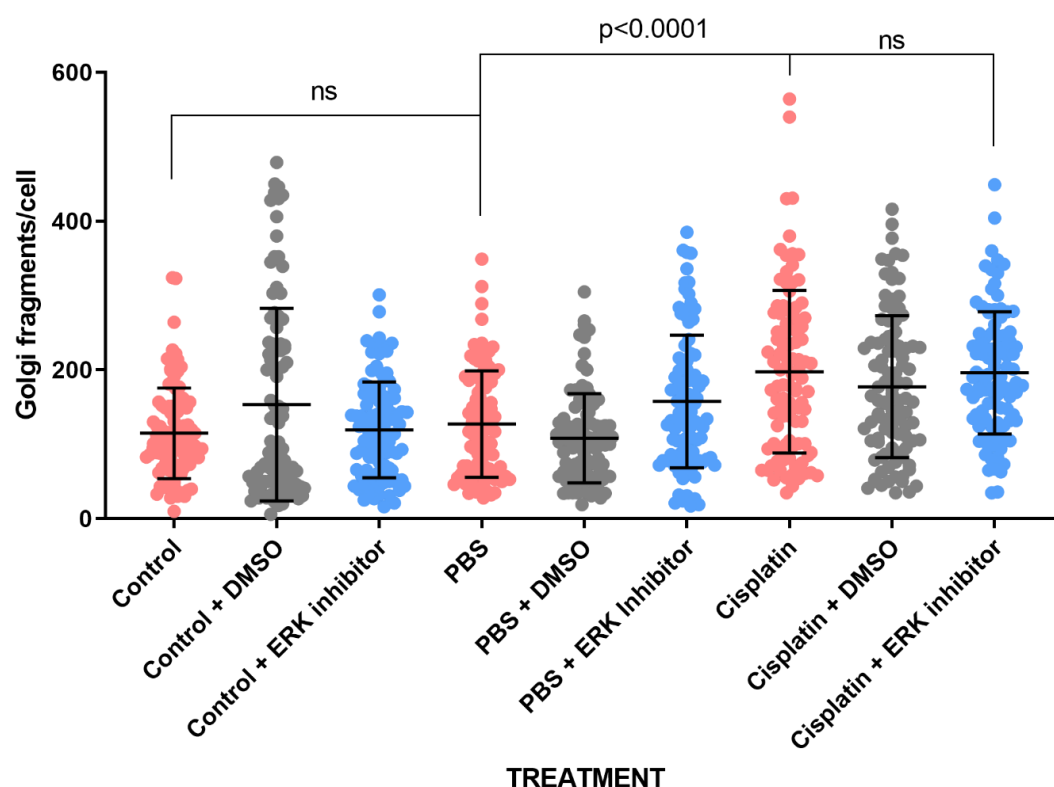


Figure 3.9. Effect of ERK inhibition on cisplatin-induced Golgi fragmentation in OVCAR3 cells. Cells were pre-treated with 100 μ M ERK inhibitor for 3hr followed by cisplatin treatment for additional 3hr. Significant fragmentation of Golgi was observed in cisplatin (197.5 $\pm$ 109.2 fragments/cell) treated cells compared to control (114.9 $\pm$ 60.85 fragments/cell) and PBS (127.3 $\pm$ 71.46 fragments/cell). Following ERK inhibition, no difference in Golgi fragmentation was found in cisplatin treated cells (196.1 $\pm$ 82.2 fragments/cell). 100 cells from two experiments were analysed. Kruskal-Wallis test followed by Dunn's multiple comparison test was performed to determine significance ($p<0.05$). Error bars represent mean $\pm$ 1 SD.

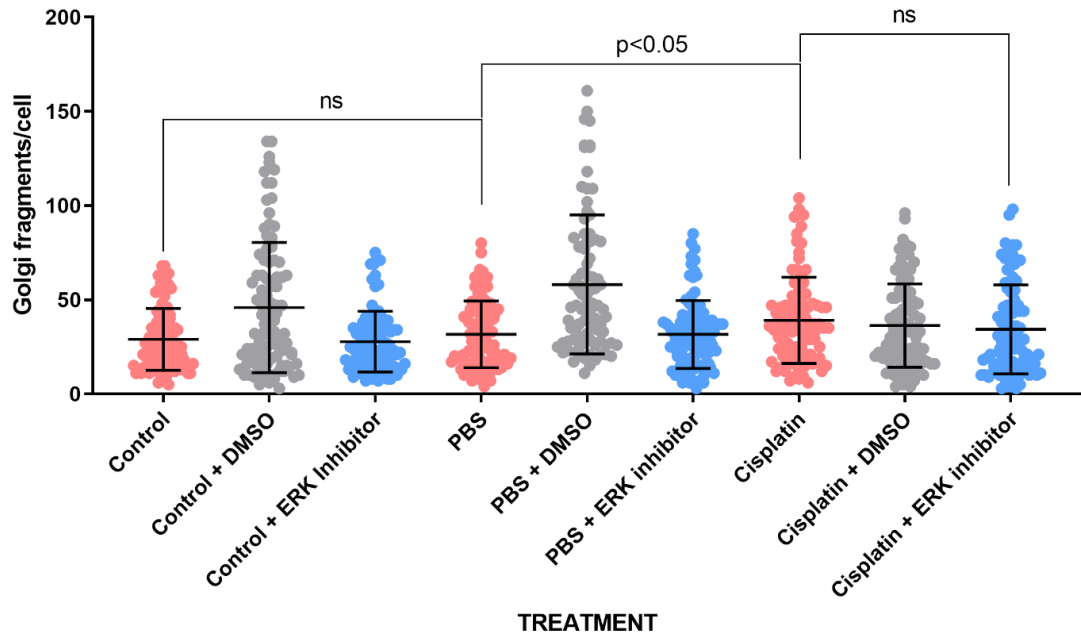


Figure 3.10. Effect of ERK inhibition on cisplatin-induced Golgi fragmentation in SKOV3 cells. Cells were pre-treated with 100 μ M ERK inhibitor for 3hr followed by cisplatin treatment for additional 3hr. Significant fragmentation of Golgi was observed in cisplatin (41.82 $\pm$ 27.6 fragments/cell) treated cells compared to control (30.12 $\pm$ 18.25 fragments/cell) and PBS (31.64 $\pm$ 17.71 fragments/cell). Following ERK inhibition, no difference in Golgi fragmentation was found in cisplatin treated cells (39.15 $\pm$ 32.04 fragments/cell). N=100 cells from two experiments were analysed. Kruskal-Wallis test followed by Dunn's multiple comparison test was performed to determine significance ($p<0.05$). Error bars represent mean $\pm$ 1 SD.

3.6 Role of JNK in cisplatin-induced Golgi fragmentation

Cisplatin-induced DNA damage activates an array of nuclear and cytoplasmic signalling pathways (involving induction of apoptosis) that further activate JNK signalling cascade and determine the fate of cells treated with cisplatin (Basu and Krishnamurthy, 2010). As a member of the MAPK family, JNK regulates several important cellular functions including cell proliferation, differentiation, survival and apoptosis (Yan, An and Kuo, 2016). Interestingly, JNK signaling pathway is also activated and plays a role in Golgi fragmentation during mitosis (Cervigni *et al.*, 2015). Hence, the role of JNK signaling pathway in cisplatin-induced Golgi fragmentation in ovarian cancer cells was studied by inhibiting the JNK pathway using a chemical inhibitor BI-87G3. BI-87G3 inhibits JNK by targeting JIP-JNK interaction site (De *et al.*, 2009).

Similar to ERK inhibitor, kill curves were generated for A2780, OVCAR-3 and SKOV-3 cells in response to the JNK inhibitor with and without IC-50 dosage of cisplatin predetermined for each cell line: A2780 - $\sim 35\mu\text{M}$, OVCAR-3 - $\sim 150\mu\text{M}$, SKOV-3 - $\sim 90\mu\text{M}$. This was done to identify an optimal sublethal concentration of the inhibitor in the presence and absence of cisplatin. Cells seeded in 96-well plates were pre-treated with increasing concentration of the JNK inhibitor ($0\mu\text{M}$ to $100\mu\text{M}$) for 3hr followed by treatment with IC-50 dosage of cisplatin in 'cisplatin-wells' for further 3hr. Cells were also treated with equivalent volumes of DMSO used as solvent for the inhibitor. Cell viability was measured after 24hr using the Alamar Blue assay. Figure 3.11 (a, b and c) show kill curves generated for A2780, OVCAR-3 and SKOV-3 cells, respectively. Based on the kill curves, a JNK inhibitor concentration that produced relatively high cell viability in the presence of cisplatin was considered optimal for each cell line, and was further used to test whether cisplatin mediated Golgi fragmentation through JNK pathway: A2780 - $5\mu\text{M}$, OVCAR-3 - $20\mu\text{M}$ and SKOV-3 - $10\mu\text{M}$.

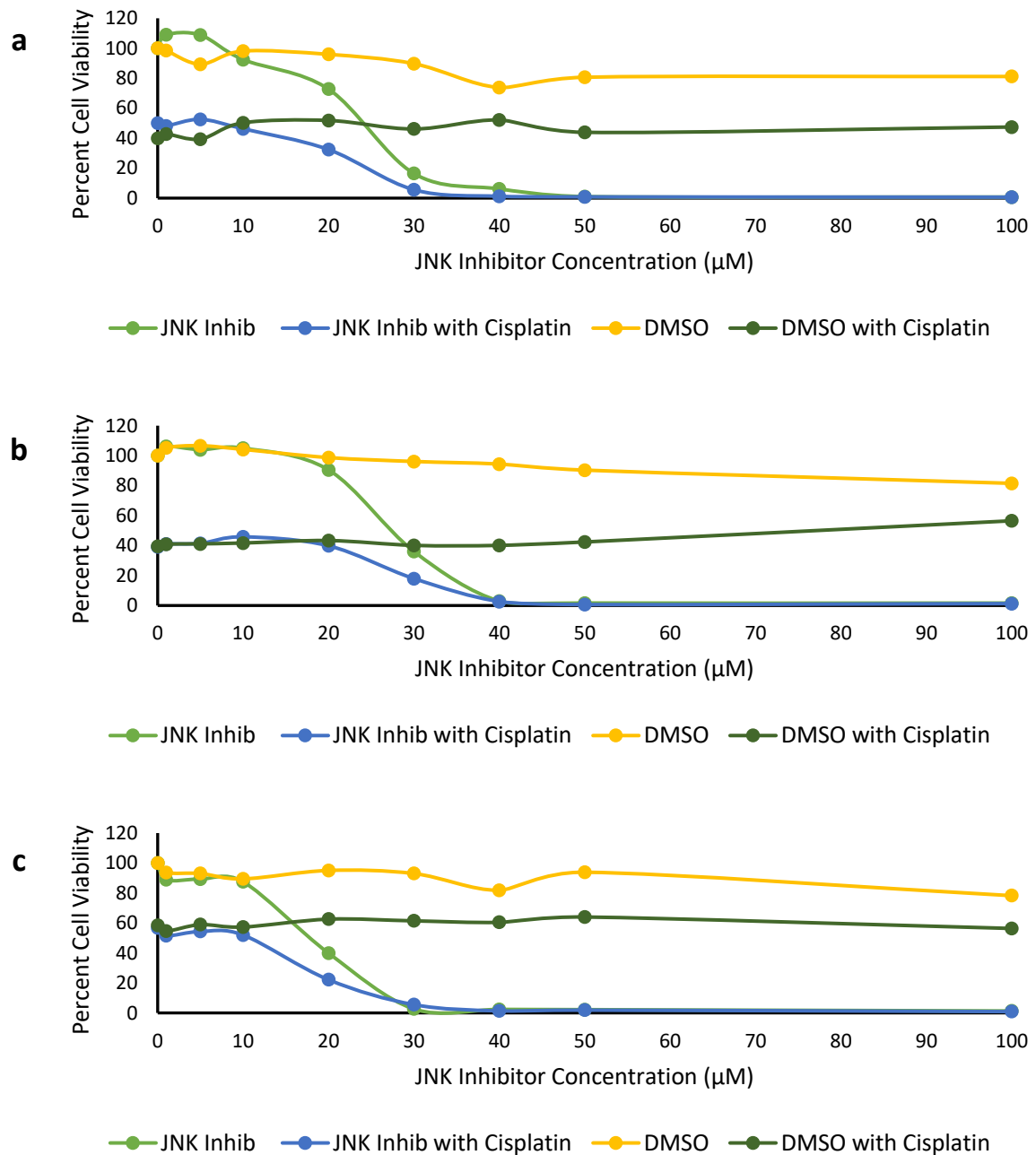


Figure 3.11. Kill curves generated for JNK inhibitor. Cells were pre-treated with increasing concentration of JNK inhibitor BI-87G3 for 3hr followed by cisplatin for additional 3hr. Cell viability was measured using Alamar Blue assay after 24hr. The JNK inhibitor concentration that produced relatively high cell viability in the presence of cisplatin was considered optimal for each cell line. Each point on the curve represents average of three independent experiments performed in triplicates. (a) A2780, (b) OVCAR3 and (c) SKOV3.

To test whether cisplatin-induced Golgi fragmentation as observed in ovarian cancer cell lines was mediated through the JNK pathway, the effect of JNK inhibition on Golgi fragmentation was first tested using A2780 cells. The cells were pretreated with 5 μ M JNK inhibitor for 3hrs followed by treatment with IC-50 dosage of cisplatin for another 3hrs. It should be noted that as a control cells were also treated with equivalent volumes of PBS and DMSO which are used as solvents for cisplatin and JNK inhibitor, respectively. The cells were fixed and antibody labelled at 24hrs and Golgi fragmentation was quantified as described in 3.2 using Volocity image analysis software. Cells in interphase were used for analyses while mitotic cells as judged by DAPI staining were excluded from the study. Figure 3.12 illustrates the effect of JNK inhibition on cisplatin-induced Golgi fragmentation in A2780 cells. Cisplatin alone showed significantly more fragmentation of the Golgi (10.41 ± 6.71 fragments/cell; $p < 0.0001$) than control (2.71 ± 1.54 fragments/cell) and PBS (2.59 ± 1.54 fragments/cell), further supporting the finding from 3.2. Interestingly, JNK inhibition in cisplatin treated cells resulted in significantly reduced number of Golgi fragments than cisplatin only cells (cisplatin + JNK inhibitor: 7.32 ± 4.68 fragments/cell vs cisplatin alone: 10.64 ± 7.06 fragments/cell; $p < 0.01$). These results suggest that JNK pathway possibly plays a role in cisplatin-induced Golgi fragmentation in A2780 cells.

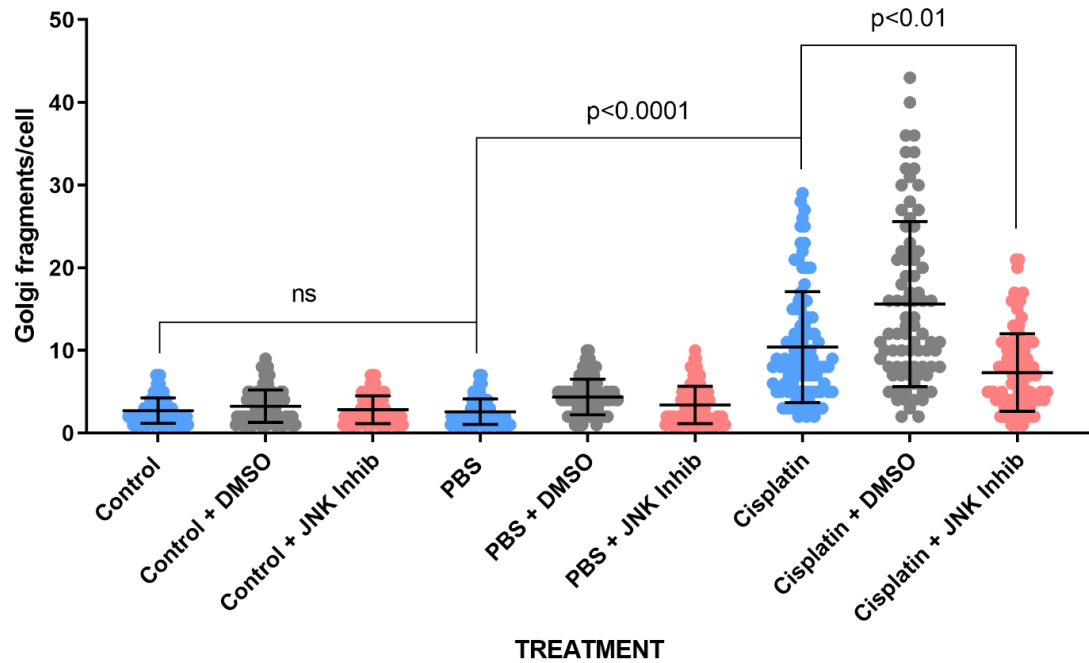


Figure 3.12. Effect of JNK inhibition on cisplatin-induced Golgi fragmentation in A2780 cells. Cells were pre-treated with JNK inhibitor for 3hr followed by cisplatin treatment for additional 3hr. Significant fragmentation of Golgi was observed in cisplatin (10.41±6.71 fragments/cell) treated cells compared to control (2.71±1.54 fragments/cell) and PBS (2.59±1.54 fragments/cell). Following JNK inhibition, Golgi fragmentation was significantly reduced in cisplatin treated cells (7.32±4.68 fragments/cell). 100 cells from two experiments were analysed. Kruskal-Wallis test followed by Dunn's multiple comparison test was performed to determine significance ($p<0.05$). Error bars represent mean $\pm$ 1 SD.

Based on the results observed for A2780 cells, the effect of JNK inhibition was also investigated in OVCAR3 and SKOV3 cells in interphase to determine whether JNK-mediated cisplatin-induced Golgi fragmentation also occurs in other ovarian cancer cells. Figures 3.13 and 3.14 illustrate the effect of JNK inhibition on cisplatin-induced Golgi fragmentation in OVCAR3 and SKOV3 cells, respectively. Similar to A2780 cells, cisplatin treated OVCAR3 and SKOV3 cells also showed significantly more number of Golgi fragments than control and PBS ($p < 0.0001$). In OVCAR3 cells, inhibition of JNK in the presence of cisplatin resulted in significantly reduced number of Golgi fragments compared to only cisplatin treated cells (68.71 ± 33.85 vs 130 ± 105.3 ; $p < 0.05$). SKOV3 cells showed similar effect with results trending towards significance; inhibition of JNK in cisplatin treated cells resulted in fewer Golgi fragments compared to cells treated with cisplatin only (48.98 ± 28.23 vs 63.84 ± 37.52 ; $P = 0.20$). These results reinforce the idea that JNK pathway plays a role in cisplatin-induced Golgi fragmentation in ovarian cancer cells.

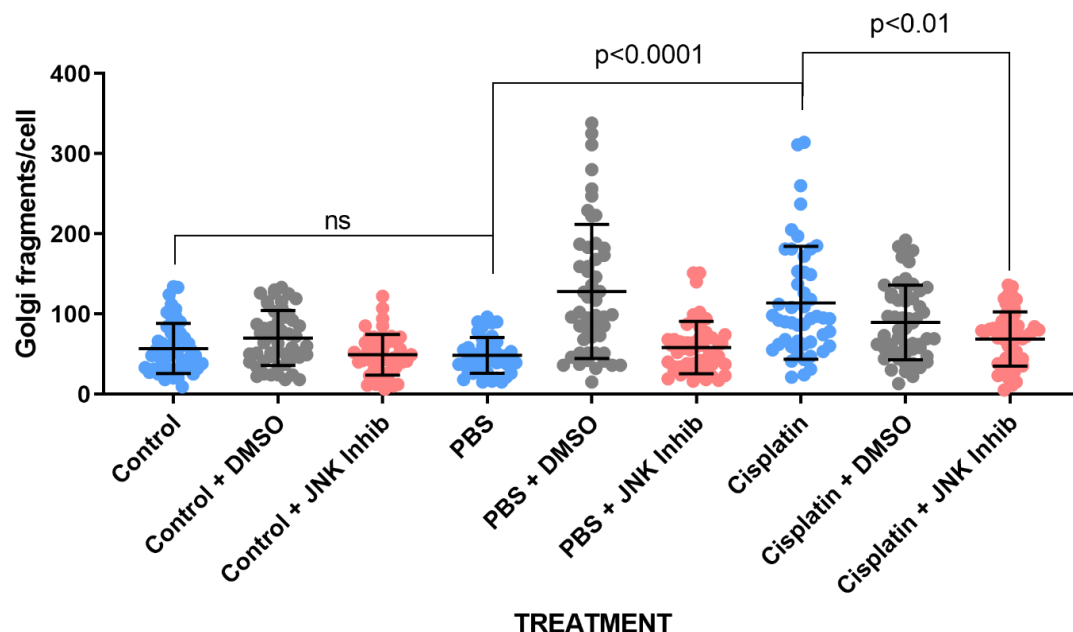


Figure 3.13. Effect of JNK inhibition on cisplatin-induced Golgi fragmentation in OVCAR3 cells. Cells were pre-treated with 20 μ M JNK inhibitor for 3hr followed by cisplatin treatment for additional 3hr. Significant fragmentation of Golgi was observed in cisplatin-treated cells (113 $\pm$ 70.41 fragments/cell) compared to control (56.92 $\pm$ 31.22 fragments/cell) and PBS (48.41 $\pm$ 22.41 fragments/cell) treatments. Following JNK inhibition, Golgi fragmentation was significantly reduced in cisplatin treated cells (68.71 $\pm$ 33.85 fragments/cell, p <0.01). N =50 cells from two experiments were analysed. Kruskal-Wallis test followed by Dunn's multiple comparison test was performed to determine significance (p <0.05). Error bars represent mean $\pm$ 1 SD.

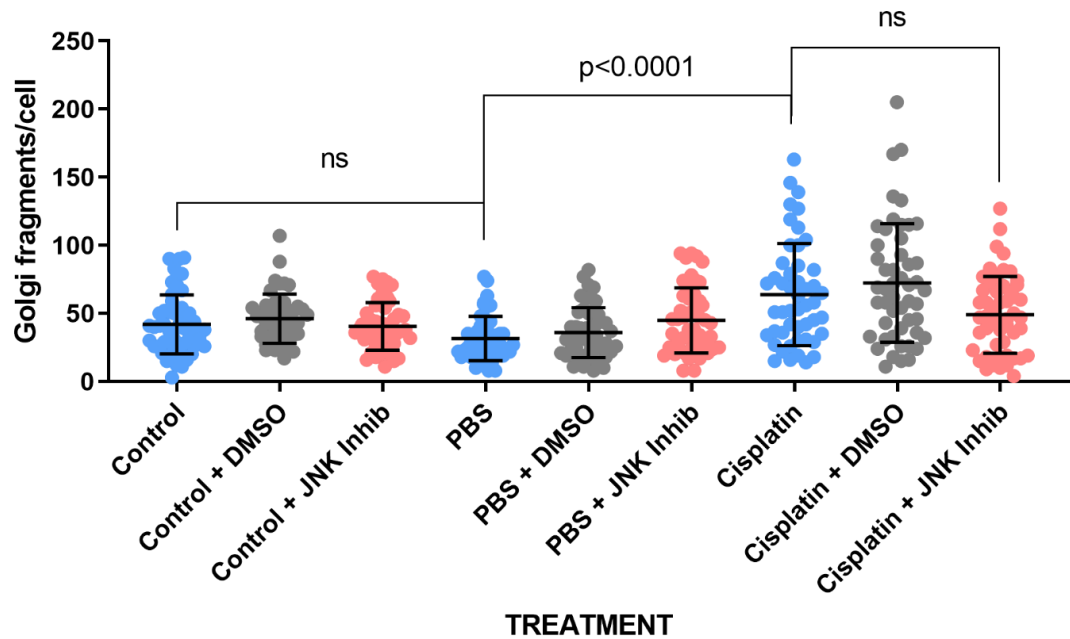


Figure 3.14. Effect of JNK inhibition on cisplatin-induced Golgi fragmentation in SKOV3 cells. Cells were pre-treated with 10 μ M JNK inhibitor for 3hr followed by cisplatin treatment for additional 3hr. Significant fragmentation of Golgi was observed in cisplatin-treated cells (63.84 $\pm$ 37.52 fragments/cell) compared to control (42 $\pm$ 21.61 fragments/cell) and PBS (32.92 $\pm$ 18.9 fragments/cell) treatments. Following JNK inhibition, Golgi fragmentation was considerably reduced in cisplatin treated cells (48.98 $\pm$ 28.23 fragments/cell; $P=0.20$). $N=50$ cells from two experiments were analysed. Kruskal-Wallis test followed by Dunn's multiple comparison test was performed to determine significance ($p<0.0001$). Error bars represent mean $\pm$ 1 SD.

3.7 Discussion

Cisplatin is a chemotherapy drug used to treat several cancers including ovarian and has shown significant improvement in patient survival rates (Aabo *et al.*, 1998; Kelland, 2007; Peng *et al.*, 2010). The main obstacle in using this drug however is the emergence of cisplatin resistance and subsequent tumour relapse (Cannistra, 2004). And, although the damaging effects of cisplatin on molecular function of several cell organelles including mitochondria, ER, nucleus, cytoskeleton (actin and microtubules) are known (Gately *et al.*, 1994; Marullo *et al.*, 2013; Saad, Najjar and Alashari, 2004; Sancho-Martinez *et al.*, 2012; Zeng *et al.*, 1996), its effect on the structural integrity of the Golgi apparatus and associated function is not reported. Farber-Katz *et al.* (2014) demonstrated that treatment of several different cell lines with chemotherapy drugs (doxorubicin and camptothecin) or ionizing radiation resulted in significant dispersal of the Golgi apparatus at its *trans* end, further suggesting that Golgi vesiculation is a common response to DNA damage in mammalian cells, and was reportedly time and concentration dependent. The main aim of the project was thus to determine whether chemotherapy drug treatment, specifically cisplatin, altered the morphology of the Golgi apparatus.

Firstly, to determine the effect of cisplatin treatment on Golgi apparatus in ovarian cancer cells, seven different human ovarian cancer cell lines were used. Ovarian cancer is highly heterogeneous in nature. Determining the half-maximal inhibitory concentration (IC-50) for cisplatin for different ovarian cancer cell lines further elaborated on the heterogeneous nature of this cancer (Figure 3.1, Table 3.1). Some cell lines were found to be highly sensitive towards cisplatin while others were relatively more resistant, indicating different level of sensitivity that each cell line presents with towards cisplatin.

Cisplatin treatment results in altered Golgi morphology

Farber-Katz *et al.* (2014) reported that treatment of cells with doxorubicin, camptothecin or ionizing radiation resulted in significant vesiculation of the *trans*-Golgi. It was previously reported that treatment of ovarian cancer cells A2780 with cisplatin resulted in significantly more fragmented *trans*-Golgi (Pradhan, 2015). To determine whether

cisplatin-induced structural alterations and fragmentation are also observed in the *cis* region, different ovarian cancer cell lines were treated with cisplatin and fragmentation in *cis* region was measured by labelling *cis*-Golgi with the *cis*-Golgi marker GM130. Results obtained showed that cisplatin treatment resulted in significant fragmentation of *cis*-Golgi in all seven ovarian cancer cell lines tested (Figures 3.3 and 3.4). Farber-Katz *et al.* (2014) have suggested that in response to DNA damage caused by stress (chemotherapy or radiation), DNA-Pk phosphorylated overexpressed GOLPH3 and the phosphorylation resulted in recruitment of unconventional myosin MYO18A and increased interaction with F-actin at *trans*-Golgi. This complex applied a tensile force to the Golgi resulting in excessive vesicle budding from *trans*-Golgi. One of the possible reasons for observing fragmentation in *cis*-Golgi in response to cisplatin, could be that since cisplatin also induces DNA damage response as a result of stress it also activated the DNA-Pk/GOLPH3/MYO18A/F-actin cascade (Figure 3.15). Although Farber-Katz *et al.* (2014) did not mention about deformation at *cis*-Golgi, the tensile force exerted at *trans*-Golgi is further extended to *cis*-Golgi and excessive budding observed in *trans*-Golgi may have also resulted in unlinking of stacks and cisternae in *cis*-Golgi resulting in its fragmentation.

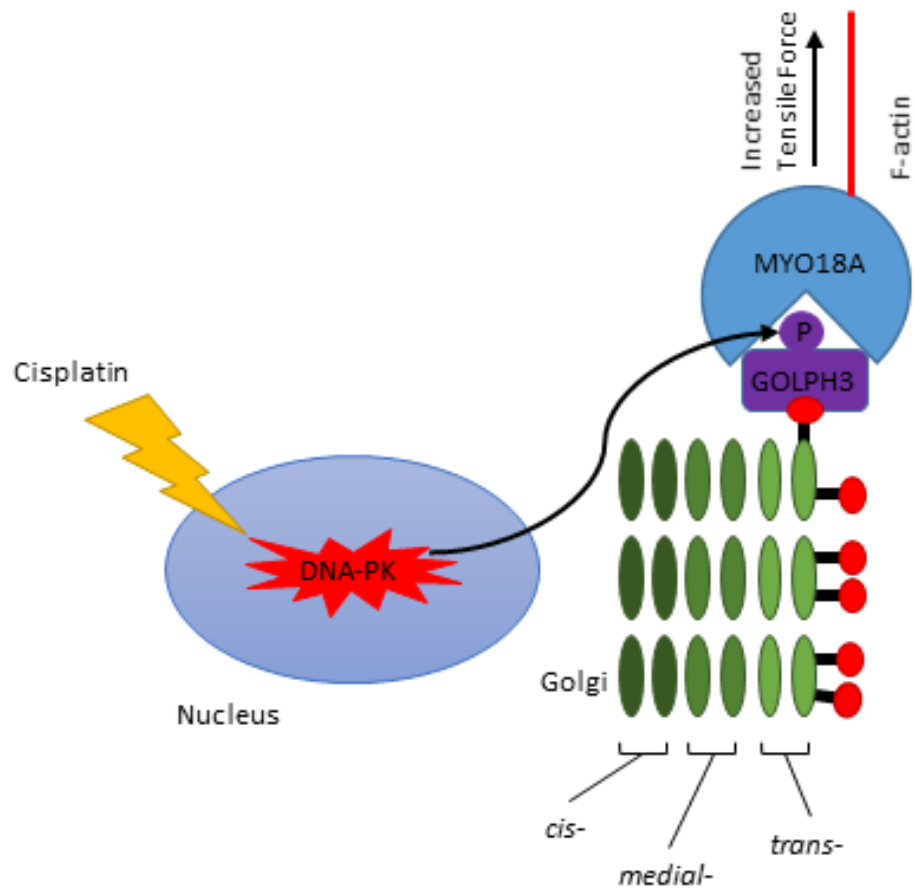


Figure 3.15. Cisplatin-stress induced Golgi fragmentation. Activation of DNA-Pk/GOLPH3/MYO18A/F-actin cascade results in vesiculation of trans-Golgi. Excessive budding at trans-Golgi result in unlinking of stacks and cisternae through medial- and cis-Golgi resulting in its fragmentation. Image adapted and modified from Farber-Katz et al. (2014).

More importantly, it has been reported that only 1% of cisplatin that enters the cells actually binds to DNA and causes DNA damage (Yu, Megyesi and Price, 2008). The rest of the unbound cisplatin hydrolyses and is retained in the cytoplasm where it binds to proteins and exerts its effects on other cellular organelles (Yu, Megyesi and Price, 2008). This offers a possibility that cisplatin could directly bind to a ‘substrate’ on the Golgi itself and induce structural alterations. Caspase-2 was shown to be activated in response to cisplatin in renal tubal epithelial cells (Kaushal *et al.*, 2001), and procaspase-2 is localised on the cytoplasmic side of the Golgi complex (Mancini *et al.*, 2000). Golgin-160 implicated in Golgi structure was shown to act as a substrate for caspase-2 and interact with upstream apoptotic regulators that results in its cleavage (Mancini *et al.*, 2000). It is possible that one of the ways cisplatin induces Golgi fragmentation is by activating apoptotic signaling that may result in cleaving (direct or indirect) of Golgi structure proteins (Figure 3.16). More experimentation in this area is needed to understand this in more detail.

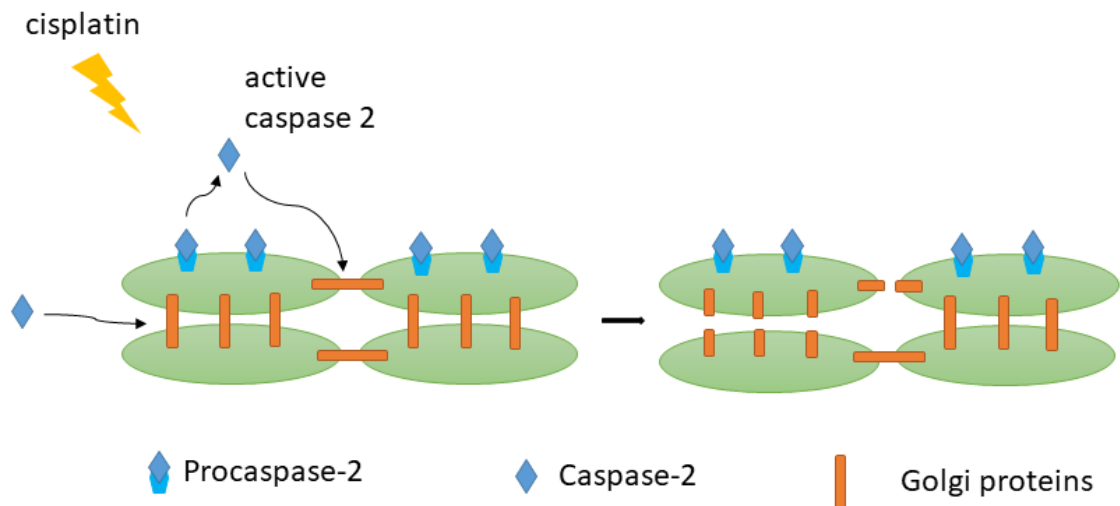


Figure 3.16. Cisplatin-induced caspase-2 activation and cleaving of Golgi proteins.

Golgi fragmentation observed could also be a combined effect of stress-induced apoptotic response from other organelles such as the mitochondria, the ER, lysosomes or the cytoskeleton. Golgi is a highly dynamic organelle and its efficient function relies heavily on other subcellular organelles. Cisplatin directly interacts with mitochondria inducing oxidative stress and activates them for apoptosis (Cullen *et al.*, 2007; Marullo *et al.*, 2013). Oxidative stress initiates apoptotic response and activates caspase-2 localised at the Golgi resulting in its fragmentation (Mancini *et al.*, 2000). Several other apoptotic regulatory components also localise at the Golgi including Fas, tumour necrosis factor receptor-1, Hippo protein and Bcl-2 family members (Bennett *et al.*, 1998; Bradley, Thiru and Pober, 1995; Gervais *et al.*, 2002; Hauser *et al.*, 1998). Cisplatin treatment has also shown to cause ER stress triggering the unfolded protein response and also activate pro-apoptotic proteins in ER in response to DNA damage-activated p53 (Gately *et al.*, 1994). Lysosomes also release apoptotic signaling as a result of cisplatin-induced lysosome membrane permeabilisation (Appelqvist *et al.*, 2011). The fragmentation observed in response to cisplatin could be the sensing and initial activation of apoptotic response from other subcellular organelles. Several Golgi proteins such as GRASP65 (Gervais *et al.*, 2002), GM130 (Chiu *et al.*, 2002; Walker *et al.*, 2004), giantin (Lowe *et al.*, 2004) and golgin-160 (Mancini *et al.*, 2000) are cleaved during apoptosis that result in apoptotic Golgi disassembly. Farber-Katz *et al.* (2014) had shown that Golgi dispersal they observed was independent of apoptosis, and though it was not tested here, it would be interesting to examine in the future.

Cisplatin-induced Golgi fragmentation is detected before cisplatin-induced DNA damage

The time-course of Golgi fragmentation in response to cisplatin was created using A2780 cells. The Golgi was found to be fragmented (trending to significance) 3 hours post treatment, and the fragmentation was found to be significant 6 hours post cisplatin treatment. The Golgi continued to be significantly more fragmented 12, 24 and 72 hours post treatment. This suggests that Golgi had started to fragment between 3 and 6 hours post treatment. These results were further supported by observations reported by Farber-Katz *et al.* (2014). They had observed Golgi dispersal as early as 4hr post treatment and fragmentation was observed for a month. Farber-Katz *et al.* (2014) assumed that Golgi

fragmentation was a downstream effect of DNA damage, and therefore, did not report the time-course for DNA damage. Since only 1% of cisplatin is effectively bound to nuclear DNA (Yu, Megyesi and Price, 2008), it was imperative to determine the time-course for DNA damage in response to cisplatin to help understand better the relationship between Golgi fragmentation and DNA damage in response to cisplatin. DNA damage was measured as number of γ H2A.x labelled nuclear foci per cell. Following DNA damage, a member of the histone H2A family, H2AX, is extensively phosphorylated (γ H2A.x) within minutes (1-3min) of DNA damage and forms foci at double strand break sites (Paull *et al.*, 2000). Antibody directed against γ H2A.x conjugated with FITC enabled quantification of nuclear foci in cisplatin treated cells. Results showed that although nuclear foci were observed in control and cisplatin treated cells at 0, 3 and 6hr post treatment, no significant difference was found between the two groups. Number of nuclear foci per cell was found to be significantly greater 12 hours post cisplatin treatment and it consistently increased at 24 hours. This suggests that double strand breaks caused by cisplatin in ovarian cancer cells are first detected 12 hours post treatment and is also consistent with that reported by Oliver *et al.* (2010). Oliver *et al.* (2010) observed maximal γ H2A.x labelling in lung tumours 12 to 24 hours post cisplatin treatment. DNA crosslinks suggestively form 2 to 4hr post treatment and these lesions are repaired between 10 and 24hr (Almeida *et al.*, 2004). During DNA damage, mismatch repair (MMR) and nucleotide excision repair (NER) repair pathways are both activated (Martin, Hamilton and Schilder, 2008). Although MMR only corrects single strand breaks, it also prevents NER proteins from correcting double strand breaks (Martin, Lord and Ashworth, 2010; Rocha *et al.*, 2018). This allows the lesions to persist for longer and in turn increases cisplatin lethality. Increased number of nuclear foci between 6 and 12 hours post treatment indicates the existence and persistence of DNA double strand breaks caused further by MMR. It also highlights that activation of the MMR pathway leads to more double strand breaks (it is an additive effect). Golgi fragmentation time-course analyses suggested that 3 hours post treatment there was considerably more fragmentation in cisplatin-treated cells than in control cells, while considerable DNA damage was observed from 12 hours post treatment. Because Golgi fragmentation was observed at an earlier time point than DNA damage, fragmentation is most likely a cumulative effect of general cellular stress or direct cisplatin interaction with the Golgi rather than an effect of cisplatin-induced DNA damage.

Cisplatin treatment activates an array of signalling pathways, including JNK and ERK. JNK and ERK both stem from the larger MAPK family (Basu and Krishnamurthy, 2010). While JNK is involved in regulating expression of pro-apoptosis and pro-survival factors in normal cells (Yan, An and Kuo, 2016), ERK regulates cell growth and differentiation (Wang, Martindale and Holbrook, 2000). Both pathways, however, are activated following cisplatin treatment. Activation of JNK activates the apoptotic response (Yan, An and Kuo, 2016) while ERK activation can either enhance survival of cisplatin treated cells (Persons *et al.*, 1999) or can also mediate cisplatin-induced apoptosis by initiating the apoptotic signal (Wang, Martindale and Holbrook, 2000). Coincidentally, these two pathways are also activated during mitosis and play a role in Golgi vesiculation and dispersal (Cervigni *et al.*, 2015). Therefore, their role in cisplatin-induced Golgi fragmentation in ovarian cancer cells was examined. JNK and ERK pathways were independently inhibited by pre-treating cells with a chemical inhibitor, and Golgi fragmentation in response to cisplatin treatment was measured. Golgi fragmentation was significantly reduced in cisplatin treated A2780 and OVCAR3 cells pre-treated with JNK inhibitor. Although significant difference was not found for cisplatin treated SKOV3 cells pre-treated with JNK inhibitor, Golgi fragmentation was still considerably reduced in the presence of JNK inhibitor. These results indicate that Golgi fragmentation could possibly be mediated by JNK activation following cisplatin treatment. During mitosis, activated JNK phosphorylates GRASP65 which in turn prevents *trans*-oligomerisation of Golgi stacks and results in unlinking of adjacent Golgi stacks that form the Golgi ribbon (Figure 3.17; Cervigni *et al.*, 2015). It is thus suggested that JNK mediates Golgi fragmentation following activation by cisplatin treatment by phosphorylating GRASP65. Although this was not tested here, further investigation would provide with mechanistic detail and would be worth testing in the future. Moreover, since it was previously suggested that Golgi fragmentation could be a combined effect of apoptotic responses activated by other cellular organelles in response to cisplatin, and since JNK pathway is involved in mediating the apoptotic response following cisplatin treatment, inhibiting this pathway also seems to have inhibited the combined apoptotic response, and thus prevented overall Golgi fragmentation.

Cisplatin treatment activates ERK which in turn provides cells with a survival benefit and counteracts pro-apoptotic effects associated with JNK activation (Guyton *et al.*, 1996; Wang *et al.*, 1998; Xia *et al.*, 1995). Persons *et al.* (1999) showed that inhibiting ERK activity increased cisplatin cytotoxicity in SKOV3 and UCI101 cells, further supporting the notion that activation of ERK in response to cisplatin protects cells from cisplatin cytotoxicity. Similar results were also reported by Hayakawa *et al.* (2000) in A2780 cells. However, there is also contradicting evidence which suggests that down-regulating ERK inhibits cisplatin-induced apoptosis while enhancing ERK activity further increases cell death in HeLa cells (Wang, Martindale and Holbrook, 2000). Golgi was significantly more fragmented in cisplatin-treated A2780 cells that were also pre-treated with ERK inhibitor compared to cisplatin only cells. This indicates that inhibiting ERK increases cisplatin cytotoxicity and sensitivity in A2780 cells, and that ERK activation provides cancer cells with a survival effect in the presence of cisplatin which further supports the results of Persons *et al.* (1999) and Hayakawa *et al.* (2000). Moreover, since ERK activation counteracts apoptotic effects of JNK in response to cisplatin, the increased fragmentation observed in cisplatin treated A2780 cells following ERK inhibition indicates that inhibiting ERK further accentuates the effects of JNK activation as indicated by the increased Golgi fragmentation observed. This further strengthens the argument that cisplatin-induced Golgi fragmentation is mediated by JNK, and not ERK, pathway.

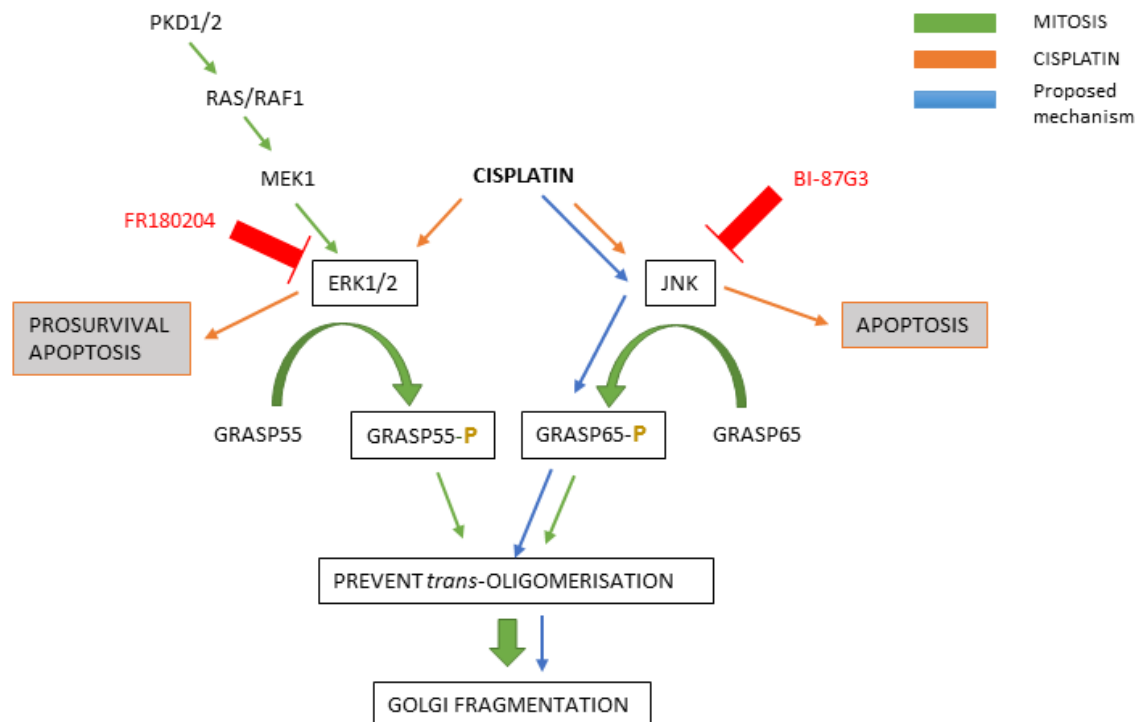


Figure 3.17. Activation of ERK and JNK pathways in response to cisplatin-induced stress and during mitosis. It is known that ERK and JNK activation during mitosis results in phosphorylation of GRASPs that prevent trans-oligomerisation and result in Golgi fragmentation (green arrows). ERK and JNK activation in response to cisplatin-induced stress is also known to activate survival and apoptosis responses (orange arrows). In this study, ERK and JNK were inhibited using FR180204 and BI-87G3, respectively. It is proposed that cisplatin-induced Golgi fragmentation is mediated through JNK activation resulting in phosphorylation of GRASP65 and prevention of trans-oligomerisation and subsequent fragmentation (blue arrows).

The effect of ERK inhibition on cisplatin sensitivity is cell line specific. Inhibiting ERK in cisplatin-treated cells further increased cisplatin cytotoxicity in A2780 cells as reflected in increased Golgi fragmentation. Golgi fragmentation was slightly reduced, although not significantly, in ERK-inhibited OVCAR3 and SKOV3 cells treated with cisplatin. There remains a possibility that Golgi fragmentation in response to cisplatin is a cumulative effect of apoptotic signaling (amongst other signaling events like the DNA damage response) from various cellular organelles including the nucleus, Wang, Martindale and Holbrook (2000) suggested that inhibiting ERK inhibits cisplatin-induced apoptosis, so it is possible that the slight reduction in Golgi fragmentation observed in ERK-inhibited OVCAR3 and SKOV3 cells could be due to inhibition of apoptotic signaling. It is also possible that ERK pathway wasn't effectively inhibited. Performing western blot analysis in the future to validate pathway inhibition could help understand this. It is also possible that the concentration of ERK inhibitor used in this study was fairly high and could have proven lethal to cells. Persons *et al.* (1999) also used similar ERK inhibitor concentration (100 μ M) for their experiments. Kielbik *et al.* (2018) used 50 μ M of ERK inhibitor to inhibit the pathway in A2780 cells. Using a lower concentration in the future could help determine the effect of ERK inhibition better. In summary, the fact that inhibiting ERK did not decrease overall Golgi fragmentation in all three cell lines eliminates the possibility that cisplatin-induced Golgi fragmentation is mediated by ERK signaling.

In conclusion, this study shows that Golgi fragmentation is an early response to cisplatin-induced stress in ovarian cancer cells. Detection of cisplatin-induced Golgi fragmentation before DNA damage further highlights the role played by the Golgi in mediating the stress response. Cisplatin-induced Golgi fragmentation in ovarian cancer cells is suggested to be mediated through JNK pathway.

FUNCTIONAL SIGNIFICANCE OF ALTERED GOLGI MORPHOLOGY

RESULTS | CHAPTER 4

4. Functional significance of altered Golgi apparatus morphology

4.1 Overview

It is evident from previous section that treatment of ovarian cancer cells with cisplatin alters the overall structure of the Golgi apparatus. However, it is not evident how structural alterations in the Golgi apparatus are reflected in their functional abilities in response to cisplatin treatment. Specifically, some of the most important Golgi apparatus functions are investigated in this including: (i) Golgi polarisation during cell migration, (ii) alterations in cell surface glycosylation and, (iii) protein secretion from the ER/Golgi to the plasma membrane and protein trafficking in response to cisplatin treatment in ovarian cancer cells.

4.2 Golgi apparatus polarisation during cancer cell migration

4.2.1 Cisplatin treatment interferes with Golgi orientation during cell migration

During epithelial to mesenchymal transition (EMT), the Golgi apparatus is known to obtain a compact morphology (Tan *et al.*, 2016). The pericentriolar localisation of the Golgi apparatus plays an important role in establishing and maintaining cell polarity during cell migration (Yadav, Puri and Linstedt, 2009). Its orientation further guides membrane traffic towards the plasma membrane which lays the cornerstones for directional protein secretion required for cell migration (Bisel *et al.*, 2008; Preisinger *et al.*, 2004). Thus, to determine whether cisplatin treatment alters Golgi polarity during cell migration, scratch/wound healing assay was first performed using A2780 cells to study migration of cells into the scratch area. Briefly, cells seeded in coverglass-bottomed dishes were grown to 90-95% confluency. Cells were then treated with predetermined IC-50 dosage of cisplatin for 3 hours. Control cells were treated with equivalent volumes of PBS used as solvent for cisplatin. A scratch was made using a sterile p10 tip and the cells

were incubated for further 14 hours until scratch was starting to close. The cells from control and cisplatin-treated groups were fixed and Golgi was labelled with *cis*-Golgi marker GM130 and also mesenchymal marker vimentin to label cells undergoing migration at t=0hr and t=14hr. For t=0hr, Z-projected images of cells in interphase, as judged by DAPI staining, at the edge of the scratch from 5 different locations on the dish were imaged using confocal microscope. Similarly, for t=14hr cells in interphase that had migrated in the scratch area and appeared positive for vimentin labelling were imaged. Mitotic cells as indicated by DAPI staining were excluded from analyses in this study.

Golgi apparatus polarisation was determined by measuring orientation or angle of the Golgi with respect to the nucleus and wound edge/scratch front as described by Xing *et al.* (2016) and Figure 4.1. Essentially, for each cell using FIJI image analysis software, a first line was drawn from the center of the nucleus to the wound edge and a second line was drawn from the center of the nucleus to the center of the Golgi. The angle formed between the lines was measured and termed Golgi orientation angle. Intuitively, smaller angles ($\sim 60^\circ$) mean that Golgi is polarised and oriented towards the wound edge, while larger angles ($>60^\circ$) mean that Golgi is depolarised and oriented away from the wound edge.

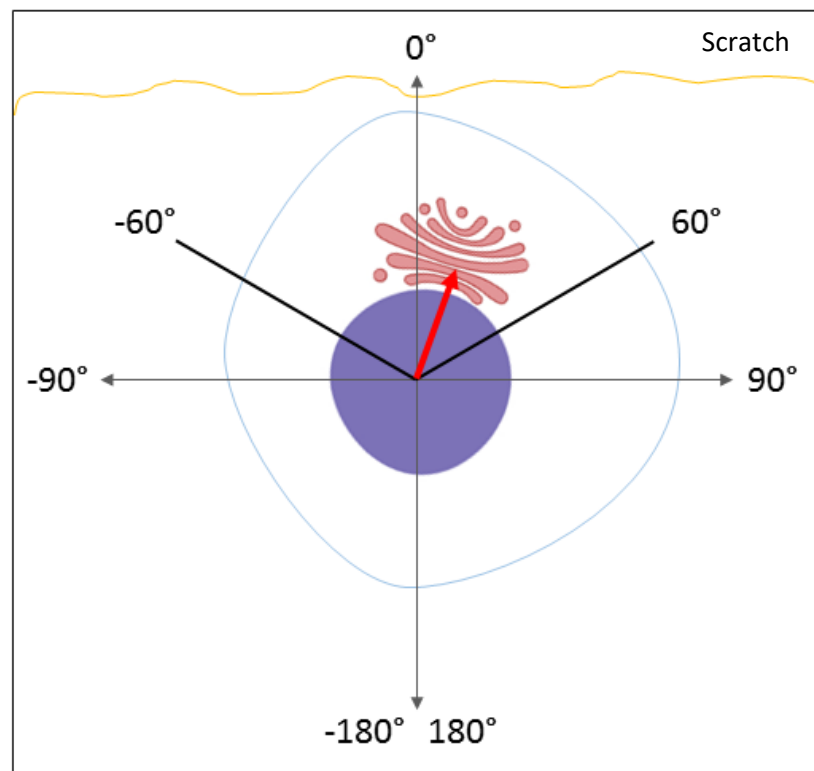
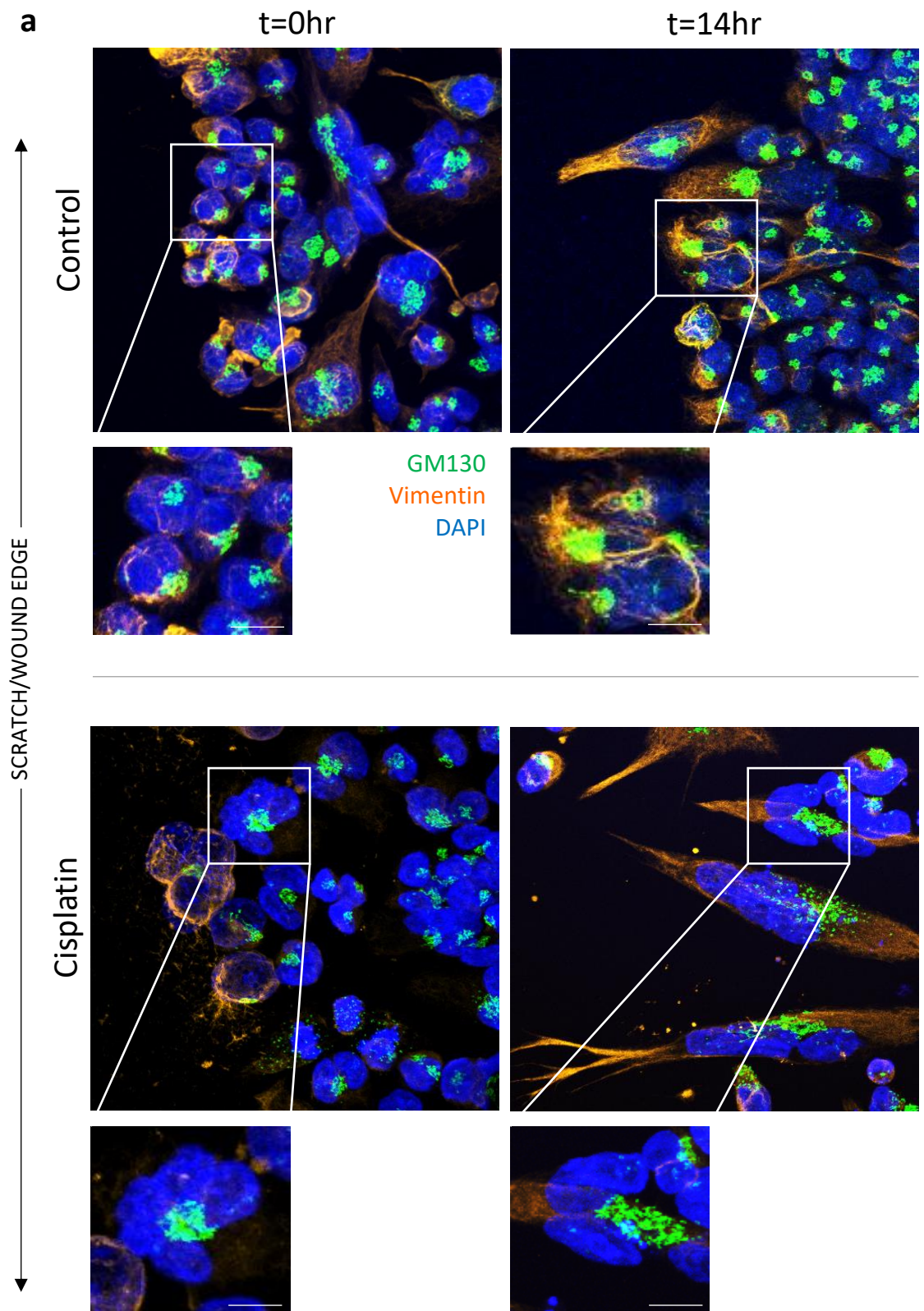


Figure 4.1. Schematic showing Golgi angle measurement.

Golgi orientation angle was measured for A2780 cells during wound-healing/scratch assay in response to cisplatin (Figure 4.2). In control cells, at t=14hr mean Golgi angle measured (59.01 ± 45) was significantly smaller than at t=0hr (96.93 ± 56.37 ; $p < 0.01$). In cisplatin treated cells, there was no significant difference in Golgi angle at t=0hr (89.57 ± 45.16) and t=14hr (98.42 ± 50.91). Golgi angle formed by cisplatin treated cells (98.42 ± 50.91) was significantly greater than in control cells (59.01 ± 45 ; $p < 0.0001$) at t=14hr. These results indicate that cisplatin treatment results in Golgi depolarisation during migration of A2780 cells.



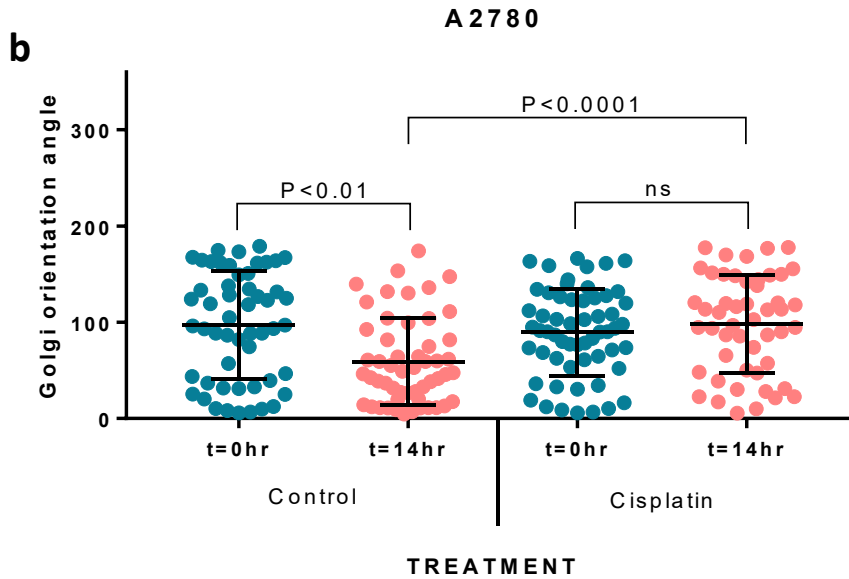
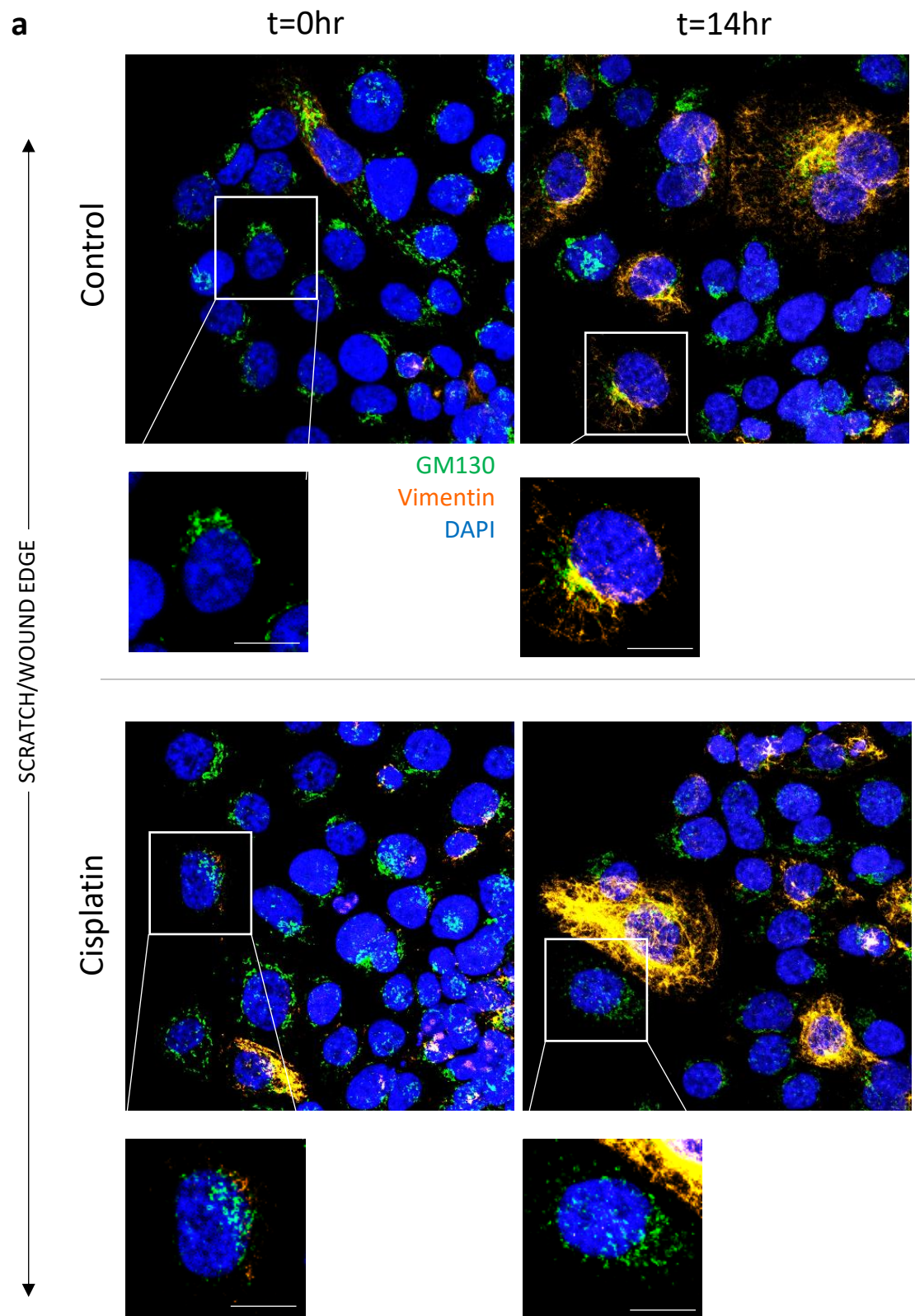


Figure 4.2. Golgi orientation angle measured for A2780 cells during cell migration. Wound healing assay was performed on control and cisplatin treated cells. Cells were fixed at t=0hr and t=14hr after creating wound. Golgi was labelled with GM130 (green), nuclei labelled with DAPI (blue) and cells labelled with vimentin (orange). Z-projected images of cells at the edge of the wound at t=0hr and t=14hr were created, and Golgi orientation angle was measured using FIJI. (a) Representative confocal images of cells at the wound edge. (b) In control cells, at t=14hr mean Golgi angle measured (59.01 ± 45) was significantly smaller than at t=0hr (96.93 ± 56.37). In cisplatin treated cells, there was no significant difference in Golgi angle at t=0hr (89.57 ± 45.16) and t=14hr (98.42 ± 50.91). Golgi angle formed by cisplatin treated cells (98.42 ± 50.91) was significantly greater than in control cells (59.01 ± 45) at t=14hr. 50 cells from two experiments were analysed. Outliers were identified using Rout's method ($Q=1\%$) and removed accordingly and Kruskal-Wallis test followed by Dunn's multiple comparison test was performed to determine significance ($p < 0.05$). Error bars represent mean $\pm$ 1 SD.

Based on the results observed for A2780 cells, scratch/wound healing assay was performed on OVCAR3 cells to determine whether Golgi depolarisation in response to cisplatin occurs in other ovarian cancer cell lines. Figure 4.3 shows Golgi orientation angle formed during wound-healing/scratch assay of OVCAR3 cells in response to cisplatin. In control cells, at t=14hr mean Golgi angle measured (56.04 ± 37.52) was significantly smaller than at t=0hr (109.8 ± 49.18 ; $p < 0.0001$). In cisplatin treated cells, there was no significant difference in Golgi angle at t=0hr (92.76 ± 49.38) and t=14hr (99.02 ± 54.13). Golgi angle formed by cisplatin treated cells (99.02 ± 54.13) was significantly greater than in control cells (56.04 ± 37.52 ; $p < 0.001$) at t=14hr. These results indicate that cisplatin treatment results in Golgi depolarisation during migration of ovarian cancer cells.



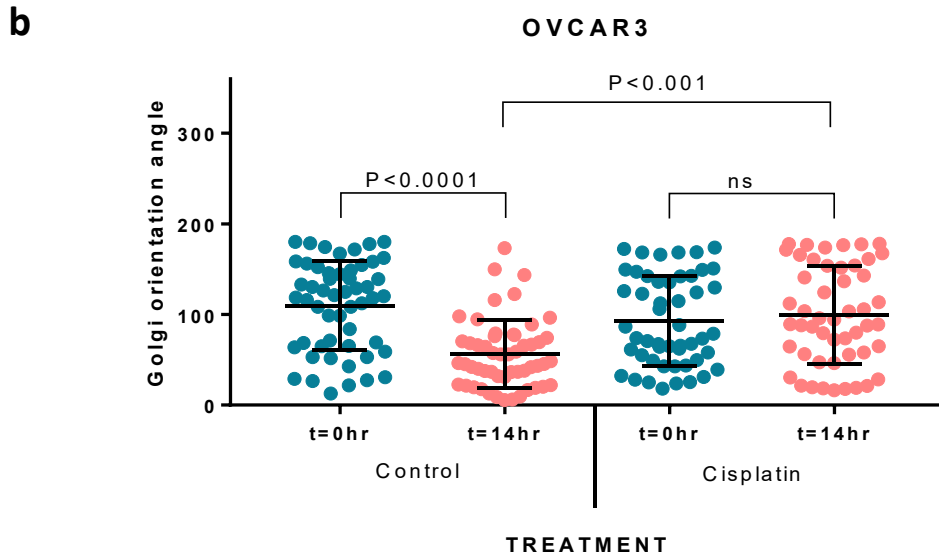
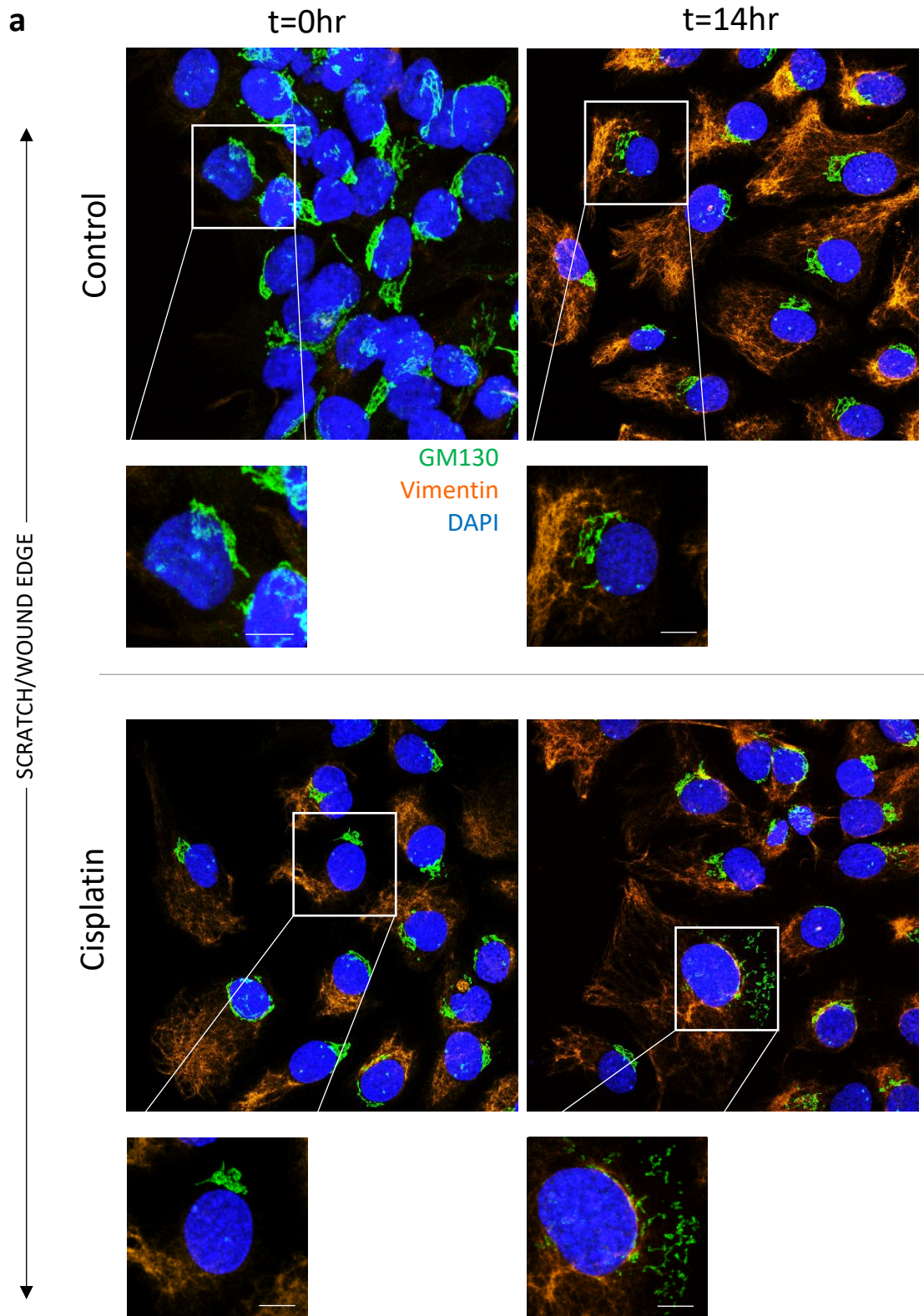


Figure 4.3. Golgi orientation angle measured for OVCAR3 cells during cell migration. Wound healing assay was performed on control and cisplatin treated cells. Cells were fixed at $t=0\text{hr}$ and $t=14\text{hr}$ after creating wound. Golgi was labelled with GM130 (green), nuclei labelled with DAPI (blue) and cells labelled with vimentin (orange). Z-projected images of cells at the edge of the wound at $t=0\text{hr}$ and $t=14\text{hr}$ were created, and Golgi orientation angle was measured using FIJI. (a) Representative confocal images of cells at the wound edge. (b) In control cells, at $t=14\text{hr}$ mean Golgi angle measured (56.04 ± 37.52) was significantly smaller than at $t=0\text{hr}$ (109.8 ± 49.18). In cisplatin treated cells, there was no significant difference in Golgi angle at $t=0\text{hr}$ (92.76 ± 49.38) and $t=14\text{hr}$ (99.02 ± 54.13). Golgi angle formed by cisplatin treated cells (99.02 ± 54.13) was significantly greater than in control cells (56.04 ± 37.52) at $t=14\text{hr}$. 50 cells from two experiments were analysed. Outliers were identified using Rout's method ($Q=1\%$) and removed accordingly and Kruskal-Wallis test followed by Dunn's multiple comparison test was performed to determine significance ($p<0.05$). Error bars represent mean $\pm$ 1 SD.

The wound-healing/scratch assay was also performed using SKOV3 cells and Golgi orientation angle was measured in response to cisplatin treatment. Figure 4.4 shows Golgi orientation angle formed during wound-healing/scratch assay of SKOV3 cells in response to cisplatin. In control cells, at t=14hr mean Golgi angle measured (60.04 ± 49.04) was significantly smaller than at t=0hr (96.62 ± 48 ; $p < 0.05$). In cisplatin treated cells, there was no significant difference in Golgi angle at t=0hr (107.1 ± 42.16) and t=14hr (81.3 ± 56.79). Cisplatin treated cells showed non-significant increase in Golgi angle (81.3 ± 56.79) compared to control cells (60.04 ± 49.04 ; $P = 0.12$) at t=14hr. These results suggest that cisplatin treatment may result in Golgi depolarisation during migration of SKOV3 cells.



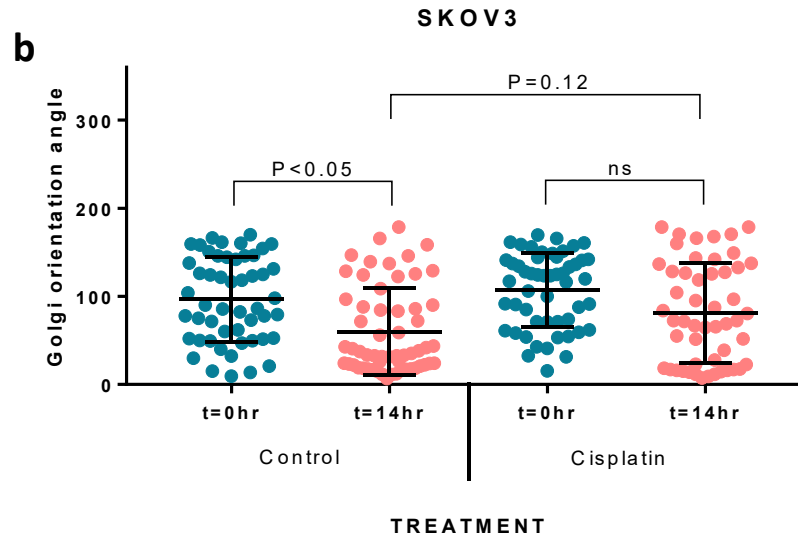


Figure 4.4. Golgi orientation angle measured for SKOV3 cells during cell migration. Wound healing assay was performed on control and cisplatin treated cells. Cells were fixed at t=0hr and t=14hr after creating wound. Golgi was labelled with GM130 (green), nuclei labelled with DAPI (blue) and cells labelled with vimentin (orange). Z-projected images of cells at the edge of the wound at t=0hr and t=14hr were created, and Golgi orientation angle was measured using FIJI. (a) Representative confocal images of cells at the wound edge. (b) In control cells, at t=14hr mean Golgi angle measured (60.04 ± 49.04) was significantly smaller than at t=0hr (96.62 ± 48). In cisplatin treated cells, there was no significant difference in Golgi angle at t=0hr (107.1 ± 42.16) and t=14hr (81.3 ± 56.79). Cisplatin treated cells showed non-significant increase in Golgi angle (81.3 ± 56.79) compared to control cells (60.04 ± 49.04 ; $P=0.12$) at t=14hr. 50 cells from two experiments were analysed. Outliers were identified using Rout's method ($Q=1\%$) and removed accordingly and Kruskal-Wallis test followed by Dunn's multiple comparison test was performed to determine significance ($p < 0.05$). Error bars represent mean $\pm$ 1 SD.

4.2.2 Cisplatin treatment reduces cell migration speed

Cisplatin treatment resulted in significant depolarisation of the Golgi apparatus in ovarian cancer cells. And, correct polarisation of the Golgi plays a role in directional cell migration (Bisel *et al.*, 2008; Preisinger *et al.*, 2004). Hence, it is hypothesised that cisplatin-induced Golgi depolarisation could also impact migration speed of cells. To test this, migration speed of control and cisplatin treated A2780 and SKOV3 cells was measured through single cell tracking from time-lapse images created following wounding. Like previously, cells were grown to 90-95% confluency in coverglass-bottomed multiwell plate and treated with IC-50 dosage of cisplatin for 3hr. A scratch was created using p10 pipette tip. Nuclei of cells were labelled with Hoechst 33342 and time-lapse images were created for 6hr post cisplatin treatment using confocal LSM880 with incubation chamber set at 37°C and 5% CO₂. Cell speed was measured through single cell tracking using Volocity.

Figure 4.5 shows the migration speed recorded for A2780 and SKOV3 cells treated with control and cisplatin. In response to cisplatin, A2780 cell speed was found to be significantly slower compared to control cells ($1.78 \pm 0.58 \mu\text{m}/\text{hour}$ vs $2.04 \pm 0.59 \mu\text{m}/\text{hour}$; $p < 0.0001$). SKOV3 cells treated with cisplatin also displayed significantly slower cell speed compared to control cells ($1.97 \pm 0.63 \mu\text{m}/\text{hour}$ vs $2.33 \pm 0.64 \mu\text{m}/\text{hour}$; $p < 0.0001$). These results indicate that cisplatin treatment reduces migration speed of cancer cells.

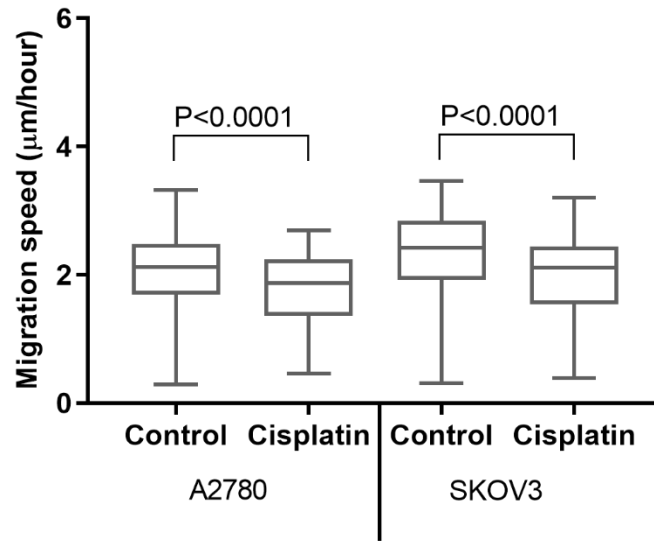


Figure 4.5. Migration speed of A2780 and SKOV3 cells treated with cisplatin. Wound healing assay was performed on control and cisplatin treated A2780 and SKOV3 cells. Nuclei of cells were labelled with Hoechst 33342 and time-lapse images were created for 6hr post cisplatin treatment using confocal LSM880 with incubation chamber set at 37°C and 5% CO₂. Cell speed was measured through single cell tracking using Volocity. In response to cisplatin, A2780 cell speed was found to be significantly slower compared to control cells ($1.78 \pm 0.58 \mu\text{m/hr}$ vs $2.04 \pm 0.59 \mu\text{m/hr}$; $p < 0.0001$). SKOV3 cells treated with cisplatin also reported significantly slower cell speed compared to control cells ($1.97 \pm 0.63 \mu\text{m/hr}$ vs $2.33 \pm 0.64 \mu\text{m/hr}$; $p < 0.0001$). >500 cells were analysed from one experiment and Mann Witney U test was performed to determine significance ($p < 0.05$).

4.2.3 *Cisplatin treatment affects normal wound healing*

Additionally, to determine whether cisplatin affected normal wound healing capacity of ovarian cancer cells percentage of wound closed following a scratch/wound was also measured for A2780, OVCAR3 and SKOV3 cells in response to cisplatin treatment. To determine this, cells grown to 90-95% confluency in coverglass-bottomed dishes were treated with IC-50 dosage of cisplatin (like previously). A scratch/wound was created like before and cells were incubated at 37°C and 5% CO₂ for further 24hr to allow cells to close the wound. Bright field images of the entire scratch region in the dish were captured at t=0hr and t=24hr, and percent wound closure was measured relative to t=0hr. Figure 4.6 illustrates percent wound closure for A2780, OVCAR3 and SKOV3 cells in response to cisplatin. A2780 cells treated with cisplatin showed non-significant reduction in wound closure compared to control cells (31.4 ± 3.36 vs 39.77 ± 13 ; $P=0.26$). OVCAR3 cells treated with cisplatin showed significantly reduced wound closure compared to control cells (12.73 ± 2.36 vs 38.6 ± 12.72 ; $p<0.05$). SKOV3 cells treated also showed significantly reduced wound closure in response to cisplatin compared to control cells (76.18 ± 5.58 vs 92.62 ± 1.03 ; $p<0.01$). These results indicate that cisplatin treatment reduces wound healing capacity of ovarian cancer cells.

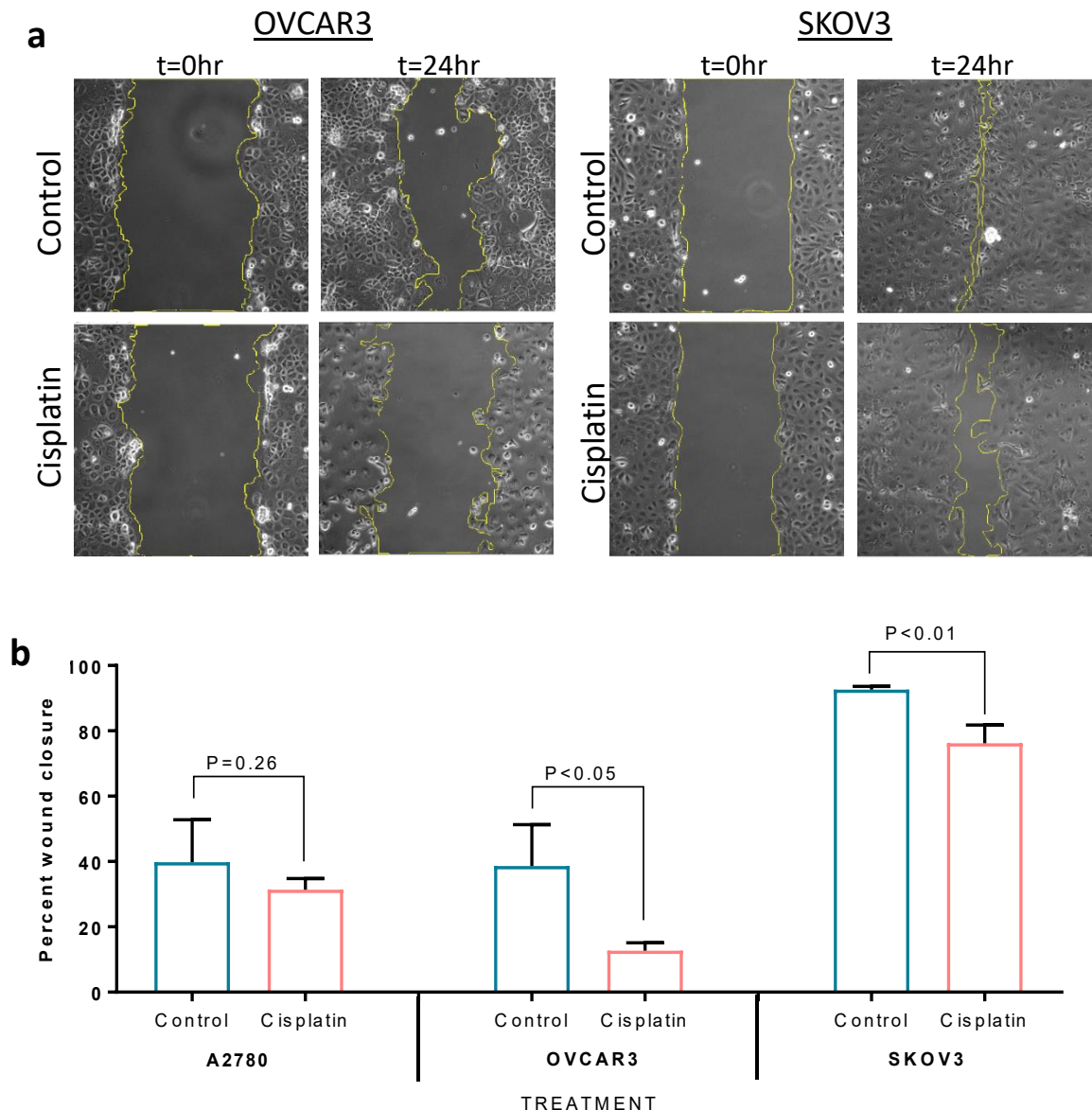


Figure 4.6. Wound closure in cisplatin treated ovarian cancer cells. Wound healing assay was performed on control and cisplatin treated A2780, OVCAR3 and SKOV3 cells. (a) Bright field images of the entire scratch region were captured at t=0hr and t=24hr, and percent wound closure was measured relative to t=0hr. (b) A2780 cells treated with cisplatin showed non-significant reduction wound closure compared to control cells (31.4 ± 3.36 vs 39.77 ± 13 ; $P=0.26$). OVCAR3 cells treated with cisplatin showed significantly reduced wound closure compared to control cells (12.73 ± 2.36 vs 38.6 ± 12.72). SKOV3 cells treated also showed significantly reduced wound closure in response to cisplatin compared to control cells (76.18 ± 5.58 vs 92.62 ± 1.03). Two independent experiments with three technical replicates were performed. Error bars represent mean $\pm$ 1 SD. Student's t-test was performed to determine significance

4.3 Aberrant glycosylation in ovarian cancer cells

4.3.1 Cisplatin treatment alters glycosylation profile of ovarian cancer cells

Lectin *Helix pomatia agglutinin* (HPA) is extensively used to identify the presence of aberrantly glycosylated proteins, specifically α -N-acetylgalactosamine (GalNAc), on cell surface of metastatic cancer cells (Schumacher *et al.*, 1996; Brooks, Hall and Buley, 2001; Rambaruth, Greenwell and Dwek, 2012). Since the Golgi is responsible for post-translational modifications of proteins, it has been hypothesized here that alterations in glycosylation as indicated by GalNAc expression on cell surface in response to cisplatin could be linked to structural changes in the Golgi apparatus itself. Moreover, since cells overexpressing GalNAc have been correlated with metastatic capacity of cancer cells, it was imperative to determine the proportion of cells synthesising aberrant glycans (HPA positive) and the ones not synthesising these glycans (HPA negative) in response to cisplatin.

To determine whether cisplatin treatment alters glycosylation profile of ovarian cancer cells, cells treated with control and IC-50 dosage of cisplatin were labelled with Alexa Fluor 647 conjugated HPA. Projected Z-stacks were collected using the LSM880 confocal microscope and individual cells in interphase, as judged by DAPI staining, were analysed for the presence of cell surface HPA by going through the projected Z-series and scored positive or negative, employing the traditional scoring method used for HPA labelled histochemical slides (Brooks, Hall and Buley, 2001). Mitotic cells also judged by DAPI staining were excluded from analyses. Figure 4.7 illustrates HPA binding profile of ovarian cancer cells treated with and without cisplatin. Percentage of HPA positive OVCAR3 cells increased from 38.67% in control cells to 45.33% in cisplatin treated cells. Percentage of HPA positive OVCAR4 cells also increased from 18% in control cells to 54% in cisplatin treated cells. OVCAR8 cells showed a slight increase in proportion of HPA positive cells in response to cisplatin from 78.65% in control to 80.68% in cisplatin treated cells. IGROV-1 cells showed a decrease in proportion of HPA positive cells following cisplatin treatment from 34.07% in control cells to 21.05% in cisplatin treated cells. SKOV3 cells also showed a decrease in proportion of HPA positive cells from 26.67% in control cells to 6.67% in cisplatin treated cells. OVCAR5 showed almost no

difference in HPA profile in control (72.84%) and cisplatin (72.73) treated cells. These results indicate that cisplatin differentially alters expression of cell surface GalNAc in ovarian cancer cell lines.

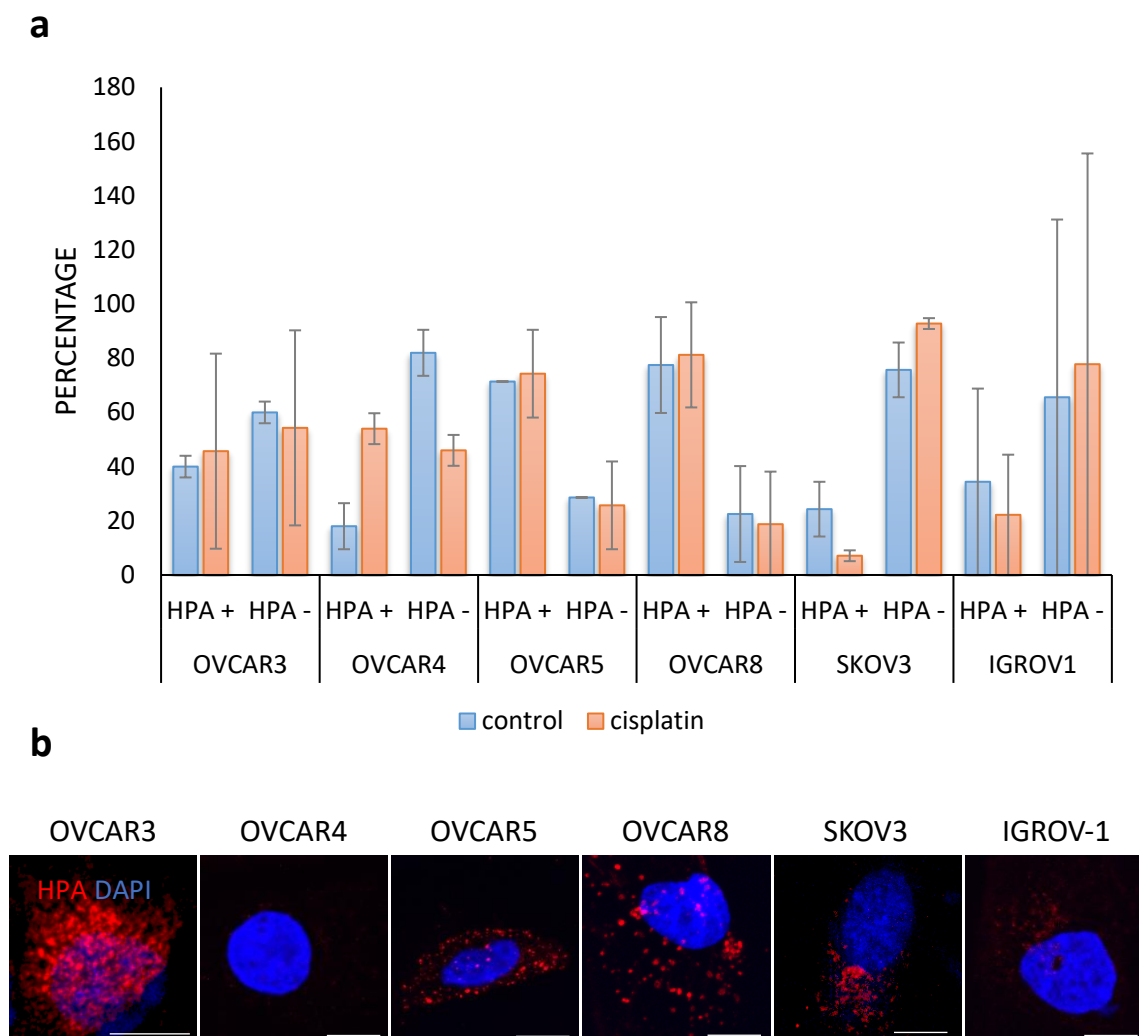


Figure 4.7. HPA binding profile of control and cisplatin treated ovarian cancer cells. Control and cisplatin treated ovarian cancer cell lines were fixed and labelled with 5 μ g/ml HPA conjugated with AF647 (red) and DAPI (blue). Z-projected images of cells were created using confocal microscope and cells were scored HPA positive or HPA negative. (a) In response to cisplatin, percentage of HPA positive cells increased in OVCAR3, OVCAR4 and OVCAR8 cells. In response to cisplatin, percentage of HPA positive cells decreased in IGROV-1 and SKOV3 cells. In response to cisplatin, there was no change in percentage of HPA positive OVCAR5 cells. (b) Representative confocal images of HPA labelled cells. 70 cells were analysed from one experiment involving two technical replicates. Errors bars = ± 1 SD. Scale = 10 μ m

4.3.2 *Altered glycosylation in migrating cancer cells in response to cisplatin*

Cancer cell migration is an essential step in cancer metastasis, and synthesis of aberrant glycans has been associated with and shown to influence different stages of metastasis including cell migration (Hauselmann and Borsig, 2014). Moreover, HPA positive cells have been shown to form secondary tumours and metastasise in mouse models (Schumacher *et al.*, 1996), while HPA negative cells are reportedly more invasive in nature (Beaman, 2017). Earlier it was reported that cisplatin treatment interferes with Golgi orientation during cancer cell migration, which seemingly also had an impact on migration speed and wound healing capacity of the cells. Thus, it is important to understand whether alterations in HPA profile in response to cisplatin treatment is further associated with Golgi polarisation during cell migration.

Control and cisplatin treated cells analysed for scratch assay performed on A2780, OVCAR3 and SKOV3 cells from section 4.2.1, were also labelled with lectin HPA conjugated with AF647 and used to determine HPA binding profile of ovarian cancer cells during migration. Like previously, cells at the wound edge/scratch front were scored either positive or negative for HPA by going through the Z-stack. Vimentin labelling was used to further confirm migrating cells. Cells in interphase were used for analyses while mitotic cells as judged by DAPI staining were excluded.

HPA binding profile of ovarian cancer cells treated with control and cisplatin at t=0hr and t=14hr post wounding is shown in Figure 4.8. In control A2780 cells at t=0hr, 60% cells labelled positive for HPA and 40% labelled negative (Figure 4.8a). At t=14hr, 82% cells labelled positive for HPA and 18% labelled negative, suggesting that migration of A2780 cells was associated with HPA positive cells. In response to cisplatin treatment, at t=0hr, there were 68% HPA positive cells and 32% HPA negative cells. During cell migration at t=14hr, 50% cells labelled positive for HPA and 50% labelled negative. This shows that in response to cisplatin, migration of these cells was associated equally with HPA positive and HPA negative.

In OVCAR3 cells, at t=0hr when the scratch was created, there were 10% HPA positive cells and 90% HPA negative cells at the wound edge (Figure 4.8b). At t=14hr, 36% cells labelled positive for HPA while 64% labelled negative for HPA during cell migration.

This shows that migration of OVCAR3 cells was associated with HPA negative cells. In response to cisplatin treatment, at t=0hr, there were 26% HPA positive cells and 74% HPA negative cells at the wound edge. During cell migration at t=14hr, 36% cells labelled positive for HPA and 64% labelled negative indicating that migration of OVCAR3 cells was associated with HPA negative cells in response to cisplatin. This also shows that cisplatin treatment did not affect overall percentage of HPA positive and negative OVCAR3 cells at the wound edge. In SKOV3 cells, at t=0hr at the wound edge, 43% cells labelled positive for HPA and 57% labelled negative (Figure 4.8c). At t=14hr, 22% cells labelled positive for HPA while 78% labelled negative for HPA during cell migration. This suggests that migration of SKOV3 cells was associated with HPA negative cells. In response to cisplatin treatment, at t=0hr, there were 6% HPA positive cells and 94% HPA negative cells at the wound edge. During cell migration at t=14hr, 20% cells labelled positive for HPA while 80% labelled negative, suggesting that migration of SKOV3 cells in response to cisplatin was also associated with HPA negative cells. This shows that similar to OVCAR3 cells, cisplatin treatment did not affect overall percentage of HPA positive and negative SKOV3 cells at the wound edge.

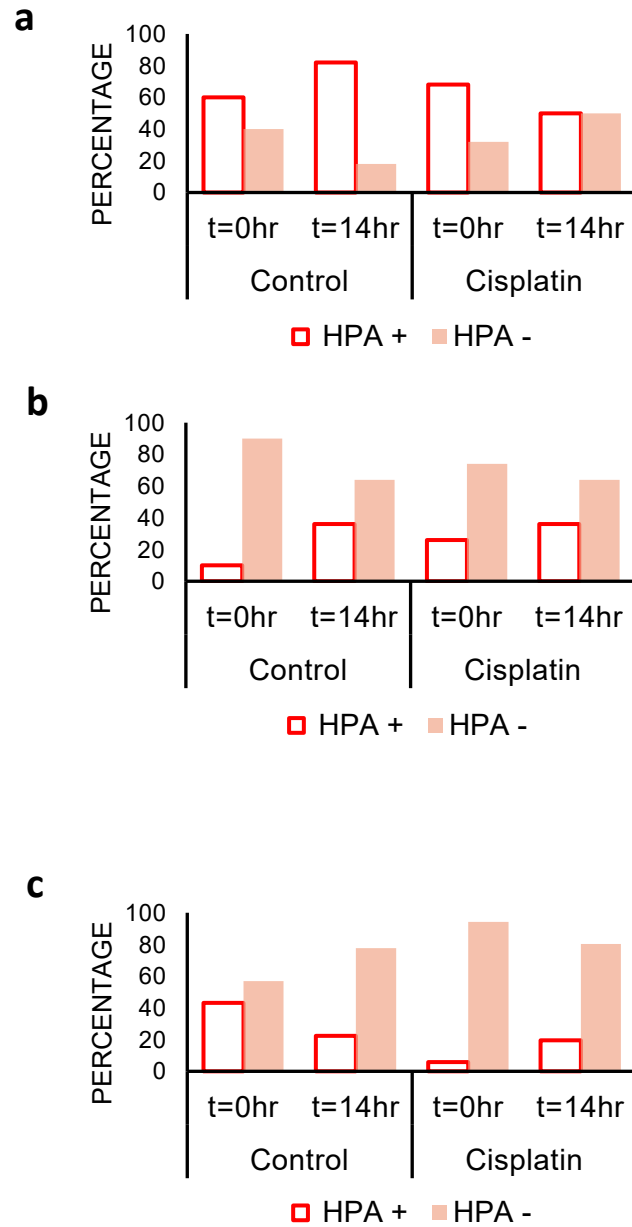
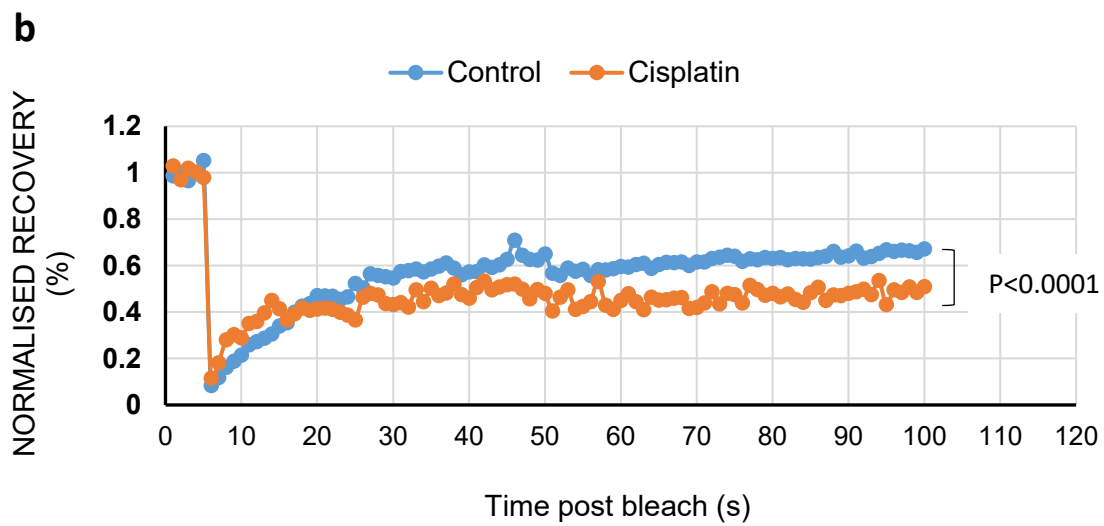
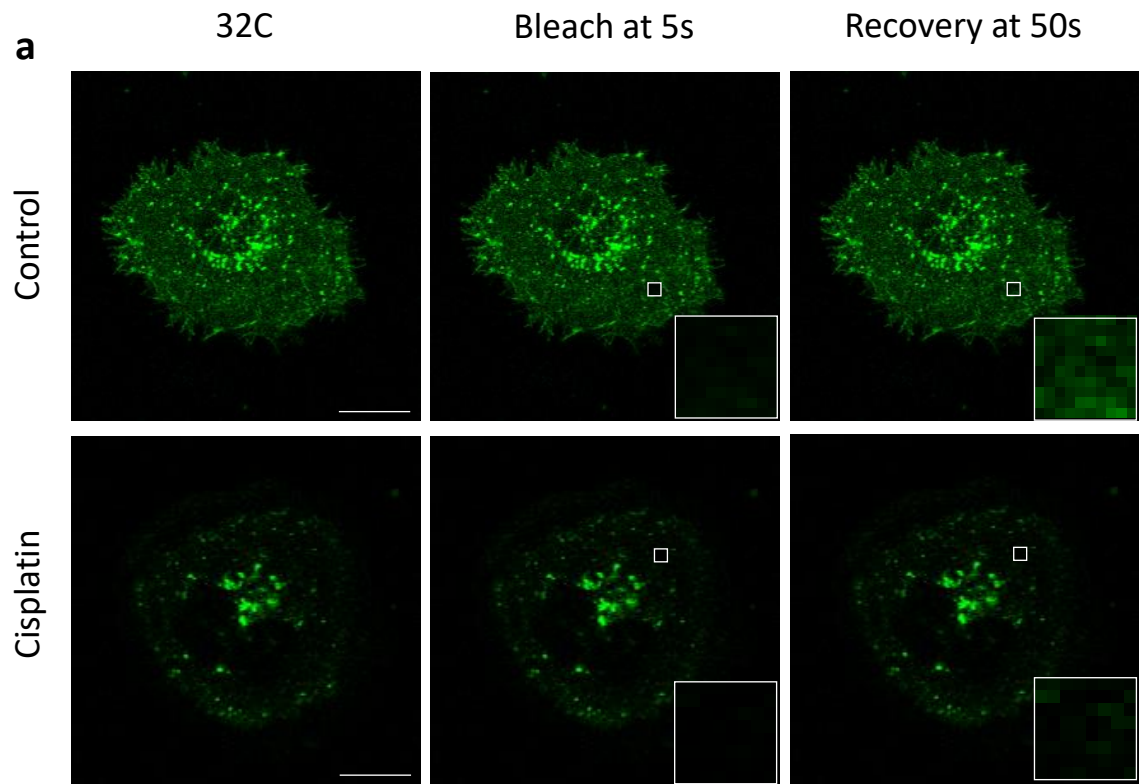


Figure 4.8. HPA binding profile of ovarian cancer cells treated with cisplatin during cell migration. Wound healing assay was performed on control and cisplatin treated A2780, OVCAR3 and SKOV3 cells. Cells at t=0hr and t=14hr were labelled with 5 μ g/ml HPA conjugated with AF647. Projected Z-stack images of cells at the wound edge created were used to score as HPA positive or HPA negative. (a) Migration of control A2780 cells was driven by HPA positive cells while in response to cisplatin, migration was driven by equal number of HPA positive and negative cells. (b and c) Migration of OVCAR3 and SKOV3 cells was driven by HPA negative cells regardless of cisplatin treatment. 50 cells were analysed from one experimental repeat for each cell line.

4.4 ER/Golgi to plasma membrane trafficking in response to cisplatin treatment

One of the main functions of the Golgi apparatus is to ensure efficient transfer of modified protein cargo coming from the ER and its secretion to the plasma membrane (Wei and Seemann, 2017). Given that treatment of cells with cisplatin resulted in fragmented Golgi, it was imperative to examine whether structural changes in the Golgi also affected protein trafficking in ovarian cancer cells in response to cisplatin. In order to study this, fluorescence recovery after photobleaching (FRAP) experiments of OVCAR3 and SKOV3 cells transfected with protein trafficking reporter ts045-VSVG-EGFP (VSVG; temperature sensitive mutant of vesicular stomatitis G envelope glycoprotein fused to green fluorescent protein) and treated with IC-50 dosage of cisplatin (like previously) were performed. The temperature sensitive aspect of the VSVG reporter control its sequestration and release. At 40°C, termed restrictive temperature, VSVG is sequestered in the ER; the protein is released from the ER and is transported through the Golgi to the plasma membrane when the temperature is reduced to 32°C, termed permissive temperature (Presley *et al.*, 1997). Several studies have also used this model to study Golgi trafficking (Farber-Katz *et al.*, 2014; Presley *et al.*, 1997; Xing *et al.*, 2016). Control and cisplatin treated cells were held at the restrictive temperature for 1hr to allow VSVG to sequester in the ER, followed by 1hr at the permissive temperature to allow trafficking of VSVG through the Golgi and to the plasma membrane. While at 32°C, a region of interest created between the ER/Golgi and the plasma membrane was bleached and recovery of fluorescent VSVG in the bleached area was measured 100s post photobleaching. OVCAR3 cells were transfected with VSVG and FRAP recovery curves of control and cisplatin treated cells were created as shown in Figure 4.9b. More VSVG trafficking was observed at permissive temperature in control cells while VSVG appeared to be restricted in ER/Golgi region in cisplatin-treated cells (Figure 4.9a). Control cells also showed relatively faster recovery of proteins in the bleached area while cisplatin treated cells showed significantly slower recovery (Student's paired *t*-test, $p < 0.05$). This is further reflected in the percentage of mobile protein fraction calculated for these cells with control cells showing 76.62% of mobile protein fraction while cisplatin treated cells showing significantly reduced mobile protein fraction (51.14%; $p < 0.05$, Figure 4.9c). These results together indicate that in response to cisplatin-induced stress, secretion of

VSVG protein from the ER/Golgi and its transport to the plasma membrane were reduced in OVCAR3 cells.



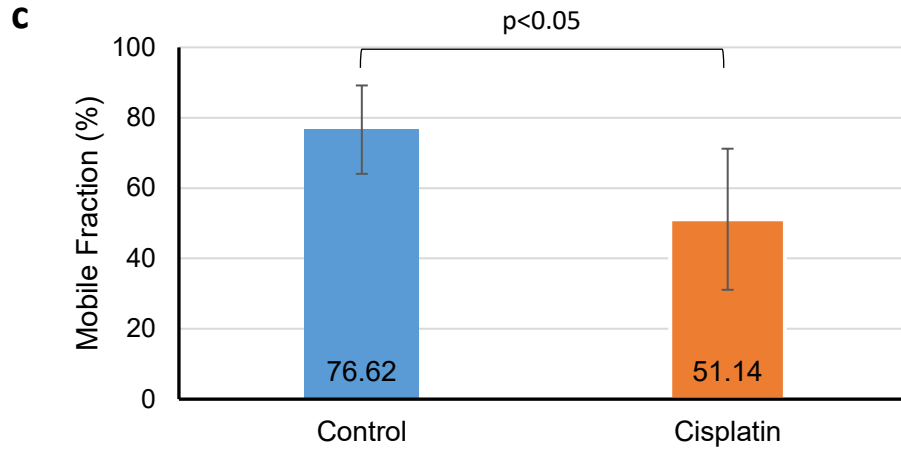


Figure 4.9. VSVG-EGFP recovery following photobleaching and mobile protein fraction in OVCAR3 cells. (a) Representative confocal images of control and cisplatin treated cells transfected with VSVG-EGFP (green). Cells were held at the restrictive temperature for 1hr to allow VSVG to accumulate in the ER, followed by 1hr at the permissive temperature to allow trafficking of VSVG. While at 32C, a region of interest created between the ER/Golgi and the plasma membrane was bleached and recovery of fluorescent VSVG in the bleached area was measured 100s post photo-bleaching. (b) Control cells showed significantly faster recovery of proteins in the bleached area than cisplatin treated cells (Student's paired t-test, $p < 0.05$). (c) Percentage of mobile protein fraction calculated for control cells showed 76.62% mobile fraction while it was significantly reduced in cisplatin treated cells (51.14% $p < 0.05$). 10 cells from two independent experiments were analysed. Student's t-test was performed to determine significance ($p < 0.05$). Scale bar = 20 μ m

VSVG recovery curves in response to cisplatin were also created for SKOV3 cells (Figure 4.10a). Similar to OVCAR3 cells, SKOV3 cells in response to cisplatin also showed significantly slower recovery than control cells (Student's paired t-test, $p < 0.05$). Strikingly, percentage of mobile fraction calculated for these cells did not reflect that. No significant difference in mobile fraction for control (67.11%) and cisplatin (66.47%) treated SKOV3 cells was found (Figure 4.10b). These results suggest that cisplatin-induced stress in SKOV3 cells effectively reduced secretion of VSVG from the ER/Golgi however it did not affect transport of VSVG from the ER/Golgi to the plasma membrane.

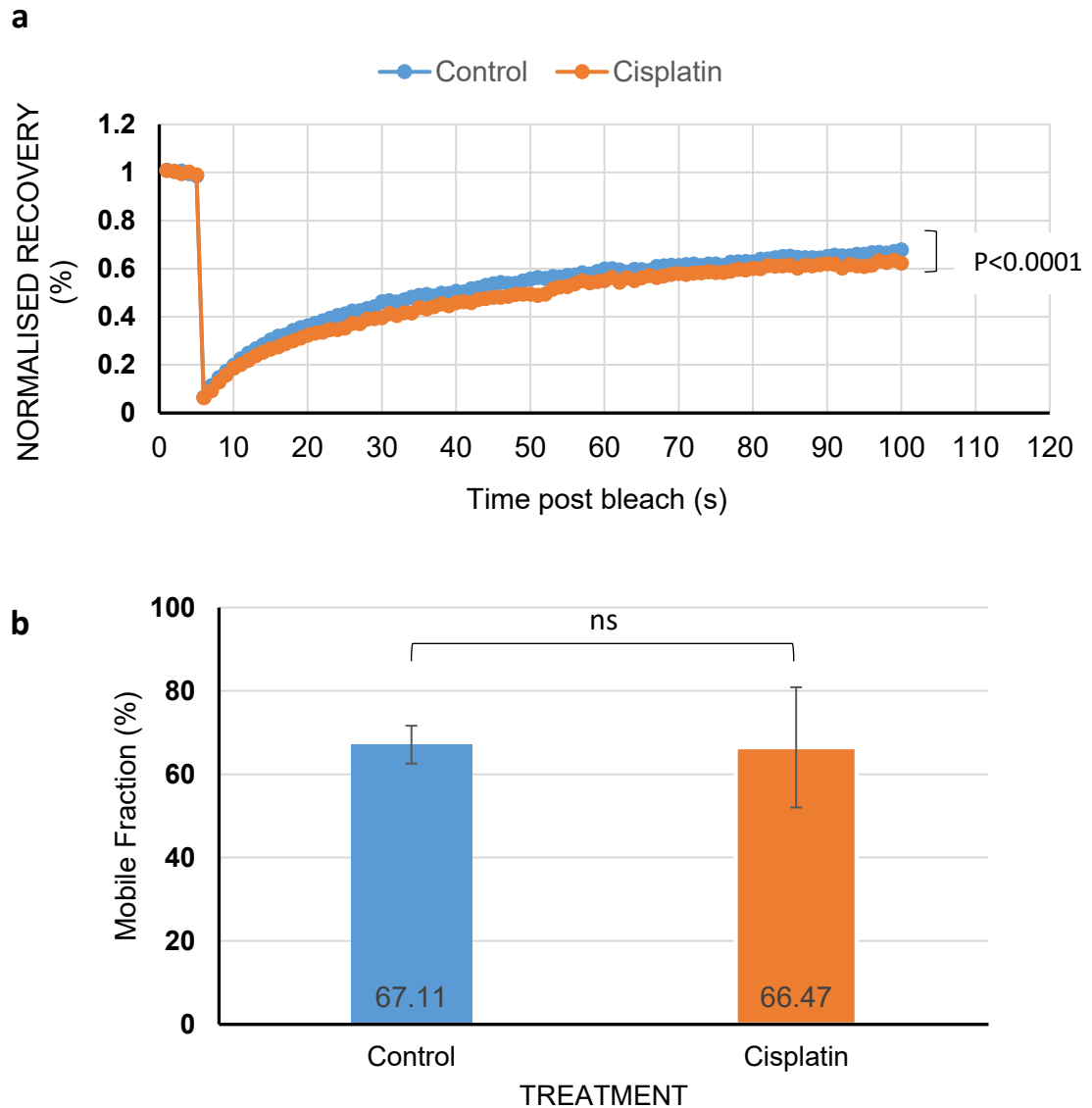


Figure 4.10. VSVG-EGFP recovery following photobleaching and mobile protein fraction in SKOV3 cells. Control and cisplatin treated cells were transfected with VSVG-EGFP. Cells were held at the restrictive temperature for 1hr to allow VSVG to accumulate in the ER, followed by 1hr at the permissive temperature to allow trafficking of VSVG. While at 32C, a region of interest created between the ER/Golgi and the plasma membrane was bleached and recovery of fluorescent VSVG in the bleached area was measured 100s post photo-bleaching. (a) Control cells showed significantly faster recovery of proteins in the bleached area than cisplatin treated cells (Student's paired t-test, $p < 0.05$). (b) Percentage of mobile protein fraction calculated for control and cisplatin treated cells showed no difference (67.11% vs 66.47%). 8 cells from two independent experiments were analysed. Student's t-test was performed to determine significance ($p < 0.05$).

4.5 Discussion

Cisplatin-induced stress affects functions of several cellular organelles including the ER, mitochondria, lysosomes and the cytoskeleton (Gately *et al.*, 1994; Marullo *et al.*, 2013; Sancho-Martines *et al.*, 2012; Zeng *et al.*, 1996). It was established that cisplatin treatment alters the morphology of the Golgi apparatus, and the result was consistently observed in seven different ovarian cancer cell lines. However, it is not known how structural alterations, specifically the fragmented state of the Golgi, further impact its functional abilities in response to cisplatin treatment. With that objective, key functions of the Golgi, namely, Golgi polarisation during cell migration, alterations in cell surface glycosylation and protein secretion and trafficking from the ER/Golgi to plasma membrane in response to cisplatin were investigated.

Golgi depolarisation during cell migration in response to cisplatin

The first Golgi function tested was its polarisation during cancer cell migration in response to cisplatin treatment. During epithelial to mesenchymal transition (EMT), the Golgi apparatus obtains a compact morphology and oriented towards the migratory front (Tan *et al.*, 2016). The pericentriolar localisation of the Golgi apparatus plays an important role in establishing and maintaining cell polarity during cell migration (Yadav, Puri and Linstedt, 2009). Its orientation further guides membrane traffic towards the plasma membrane which lays the cornerstones for directional protein secretion required for cell migration (Bisel *et al.*, 2008; Preisinger *et al.*, 2004). Thus, to determine whether cisplatin-induced stress alters Golgi polarity and orientation during cell migration, a simple scratch/wound healing assay was performed to study migration of cells into the scratch area. Cells in interphase were analysed while mitotic cells as judged by DAPI staining were excluded. During ovarian cancer cell migration the Golgi was polarised and oriented towards the wound ($\leq 60^\circ$). In the presence of cisplatin, the cells that had migrated in the wound area showed that the Golgi was oriented away from the wound with orientation angle measured being far greater than 60° in A2780 and OVCAR3 cells. Although no statistically significant difference was found between the mean Golgi angle

formed in migrating SKOV3 cells in cisplatin and control cells, the Golgi was still oriented away from the wound in the presence of cisplatin. These results indicate that cisplatin interfered with Golgi's ability to orientate and polarise during cell migration.

If the Golgi is unable to polarise and drive cell migration, it was hypothesised that that in turn would affect cell's migration speed. Testing the migration speed of A2780 and SKOV3 cells treated with cisplatin showed that cisplatin treated cells were significantly slower than their control counterpart. These results further support the recent work by Raudenska *et al.* (2019) who demonstrated that the presence of cisplatin in prostate cancer cells resulted in accumulation of stress-fibers and changes in actin organisation which led to increased cell stiffness. This in turn also affected their migration speed and invasive capacity (Raudenska *et al.*, 2019). Moreover, the wound healing capacity of cells as measured by percentage of wound closed, showed that cisplatin treated cells were less able to close the wound compared to control cells – the difference was found to be significant in OVCAR3 and SKOV3 cells, while A2780 showed non-significant decrease in wound closure in cisplatin treated cells. These results indicate that cisplatin treatment does indeed affect Golgi polarisation and migration of cancer cells.

Golgi orientation during cell migration depends heavily on actin and cytoskeletal network and associated motor proteins whereby they position Golgi by generating necessary forces and mechanical tension (Egea *et al.*, 2013). Low concentration of cisplatin has been shown to directly interact with and prevent actin polymerisation, while higher concentrations induce g- and f-actin depolymerisation (Zeng *et al.*, 1996). It also causes cell stiffness by inducing stress-fibre accumulation and actin disorganisation (Raudenska *et al.*, 2019). Cisplatin also destabilises cytoskeleton, by inactivating ezrin, a protein involved in maintaining structural interaction between the cytoskeleton and the cell membrane, and activates proapoptotic signaling (Gourlay and Ayscough, 2005; Neisch and Fehon, 2011; Zeidan, Jenkins and Hannun, 2008; Knipling and Wolff, 2006). It is thus believed that treatment of cells with cisplatin induces an array of cellular responses including disorganisation or depolymerisation of actin and destabilisation of the cytoskeleton network. Depolymerised or disorganised actin fails to generate necessary forces required for efficient orientation of the Golgi during cell migration. This is further reflected in reduced cell motility tested as migration speed of cells in the presence of cisplatin. Golgi orientation also guides membrane traffic and directional protein secretion (Bisel *et al.*, 2008; Preisinger *et al.*, 2004; Yadav, Puri and Linstedt, 2009). Inability to

polarise means disruption in directional protein secretion, and ultimately that hinders cell motility. This theory is further strengthened by cancer cells' inability to close the wound in the presence of cisplatin. It would be interesting to directly examine the effect of cisplatin-induced actin-cytoskeletal disruption on Golgi polarisation to understand the mechanism in more detail.

Cisplatin differentially alters glycosylation

The second Golgi function tested here was alteration in glycosylation in response to cisplatin treatment. Glycosylation is one of the most important post-translational modification processes that the Golgi apparatus is responsible for. It is an enzymatic process involving synchronised action of glycosyltransferases and glycosidases (Munkley and Elliott, 2016). Secreted and cell-surface glycoconjugates play an integral role in mediating key cellular processes including cell-to-cell communication, cell adhesion, motility and cell-signalling pathways during normal development (Fuster and Esko, 2005). Alterations in glycoconjugate profiles, specifically overexpression of cytoplasmic and secreted GalNAc epitopes have been associated with disease progression of cancer cells (Rambaruth, Greenway and Dwek, 2012). Lectin from *Helix pomatia*, Helix pomatia agglutinin (HPA) has a binding specificity for GalNAc epitopes (Brooks, Hall and Buley, 2001). Study done by Brooks, Hall and Buley (2001) showed that HPA-binding increased significantly with increasing metastatic capacity of breast cancer cells and presence of HPA-binding glycans in tumour tissue samples were associated with poor patient prognosis. Based on these evidences, different ovarian cancer cell lines were labelled with HPA conjugated with Alexa fluor 647. These cells were also treated with cisplatin to examine changes in HPA binding profiles in response to chemotherapy treatment. Results obtained showed that OVCAR8 cells were generally more HPA positive suggesting an overexpression of GalNAc epitopes. Cisplatin treatment further increased the overall proportion of HPA positive cells for OVCAR8 cells, indicating that drug treatment further increased expression of GalNAc epitopes. Control OVCAR3 and OVCAR4 were found to be more HPA negative, indicating that these two cell lines express comparatively less GalNAc epitopes. In response to cisplatin, the proportion of HPA positive cells increased for both the cell lines; however, overall HPA binding profile

showed that OVCAR3 were still more HPA negative while OVCAR4 were more HPA positive following drug treatment. This indicates that cisplatin treatment also increased expression of GalNAc epitopes for OVCAR3 and OVCAR4 cells, with OVCAR4 showing a more drastic shift in HPA binding profile. The overall HPA binding profile for IGROV-1 and SKOV3 showed that both the cell lines were more HPA negative suggesting comparatively reduced expression of GalNAc epitope in control cells. In response to cisplatin, the proportion of HPA negative cells further increased. This indicates that in response to cisplatin, expression of GalNAc epitope was further reduced for IGROV-1 and SKOV3 cells. HPA binding profile for OVCAR5 showed these cells were overall more HPA positive. Almost no change in binding profile was found for OVCAR5 following cisplatin treatment. This suggests that GalNAc epitopes were overexpressed in OVCAR5 cells and cisplatin did not seem to alter its expression further. Krushkal *et al.* (2017) reported that expression of *GALNT1*, *GALNT2*, *GALNT7* and *GALNT14* encoding multiple N-acetylgalactosaminyltransferase was downregulated by cisplatin treatment. In this study, cisplatin seemed to differentially alter expression of GalNAc – for some cell lines it induced overexpression while for other cell lines, it reduced its expression; and for OVCAR5, there was no change. This possibly indicates that effect of cisplatin is cell line specific. Each cell line has originated from different tumour sites and have different metastatic and invasive capacity. It is also possible that although cisplatin reduced expression of *GALNT*, further alterations in glycosylation process including activity of N-acetylgalactosaminyltransferase could amount to increased or decreased GalNAc expression. Performing further experimentation involving invasion assays will provide with in depth understanding of effect of cisplatin on alterations in glycosylation and GalNAc expression levels.

Alterations in HPA binding profile during cancer cell migration were also examined in response to cisplatin. Ample evidence in literature reports that HPA positive cells are more invasive, form metastatic tumours when injected in SCID mice and are associated with poor patient prognosis (Schumacher *et al.*, 1996). Based on these evidences, HPA binding of ovarian cancer cell lines A2780, OVCAR3 and SKOV3 cells during cell migration were studied using a scratch/wound healing assay. In control A2780 cells, more HPA positive cells were found at the wound edge at t=0hr. During migration at t=14hr, proportion of HPA positive cells at the wound edge had further increased. When treated with cisplatin, at t=0hr proportion of HPA positive cells found at the wound edge further

increased compared to control at t=0hr. During migration at t=14hr, proportion of HPA positive cells decreased and that of HPA negative cells increased. This suggests that possibly less GalNAc epitope is expressed during cancer cell migration. In response to cisplatin, its expression level is altered: increased when cells are not migrating at the wound edge but reduced during migration. Hernandez *et al.* (2016) characterised A2780 cells to have medium intraperitoneal invasion capacity and ranked medium on scale for subcutaneous tumour formation in mice. HPA positivity of cells is generally associated with increased risk of local invasion (Parameswaram *et al.*, 2018), suggests that for A2780 cells, the increased number of HPA positive cells during cell migration further supports its invasion and tumour forming capacity. In the presence of cisplatin, it seems that its migration and, by extension, its invasion capacity is reduced and is also supported by Golgi depolarisation during migration in the presence of cisplatin.

In control OVCAR3 cells, more HPA negative cells were found at the wound edge at t=0hr. During migration at t=14hr, although proportion of HPA positive cells had increased there were still more HPA negative cells at the wound edge. This is in agreement with other HPA studies which show that HPA positive cells are more invasive and metastatic, and the increase in HPA positive cells at the wound edge indicates that more HPA positive cells have entered the migration front or the expression of GalNAc in these cells is increased/upregulated. When treated with cisplatin, at t=0hr, there were considerably more HPA positive cells but comparatively more HPA negative cells were found at the wound edge, like previously. During migration at t=14hr, proportion of HPA positive cells had slightly increased although there were still comparatively more HPA negative cells at the wound edge. It is unclear what caused this effect since the proportion of HPA positive and negative cells at the wound edge in cisplatin and control cells at t=14hr was exactly the same, but it seems that in the presence of cisplatin alone, there were more cells expressing GalNAc at the wound edge, and during migration, the proportion of GalNAc-expressing cells increased further. Hernandez *et al.* (2016) characterised OVCAR3 to have low intraperitoneal invasion capacity and these cells failed to form subcutaneous tumour in mice. This is in agreement with cell migration results of control OVCAR3 cells which were mostly HPA negative at the wound edge. These results however do not completely support the Golgi depolarisation data: based on evidences about HPA positive cells, it was expected that more HPA positive cells would be at the wound edge during cell migration of control cells, but for OVCAR3 cells it

seems to be led by HPA negative cells. In the presence of cisplatin, the presence of more HPA negative cells at the wound edge during migration supports Golgi depolarisation results: cisplatin reduces migration and by extension its invasion capacity, as also indicated by Golgi depolarisation during migration in the presence of cisplatin.

In control SKOV3 cells, more HPA negative cells were found at the wound edge at t=0hr. During migration at t=14hr, proportion of HPA positive cells further decreased thus increasing the proportion of HPA negative cells at the wound edge. In response to cisplatin, at t=0hr, there were mostly HPA negative cells at the wound edge. During migration at t=14hr, proportion of HPA positive cells slightly increased although there were still more HPA negative cells at the wound edge. Hernandez *et al.* (2016) characterised SKOV3 to have high intraperitoneal invasion capacity and also ranked high for subcutaneous tumour formation in mice. Also, based on Golgi polarisation results of migrating control SKOV3 cells, it was expected that greater proportion of HPA positive cells would be at the wound edge. Here, however, completely opposite results were observed with more HPA negative cells being at the wound edge. It is unclear what would cause this discrepancy in results. Moreover, in the presence of cisplatin, more HPA negative cells were observed at the wound edge. This is in agreement with Golgi depolarisation in the presence of cisplatin during migration, and similar to OVCAR3 cells, this suggests that cisplatin reduced migration and invasion capacity of SKOV3 cells.

It should be noted that HPA labelling protocol used for these experiments was different than what has been done traditionally: immunofluorescence labelling followed by imaging through confocal microscopy technique was employed. The traditional method has involved immunohistochemistry on cancer cells involving streptavidin-biotin based HPA labelling followed by light microscopy. The differences in sample preparation and imaging could possibly account for the discrepancy and inconsistency observed in results here.

ER/Golgi to plasma membrane trafficking reduced in response to cisplatin

Finally, the third Golgi function tested was protein trafficking from the ER/Golgi to the plasma membrane. One of the main functions of the Golgi apparatus is to ensure efficient

transfer of modified protein cargo coming from the ER and its secretion to the plasma membrane. Given that treatment of cells with cisplatin resulted in (fragmented and) depolarised Golgi, it was imperative to examine its effect on protein trafficking in ovarian cancer cells to understand whether cisplatin treatment also affected Golgi function. OVCAR3 and SKOV3 cells were transfected with protein trafficking reporter ts045-VSVG-EGFP (VSVG; Presley *et al.*, 1997) and its recovery at permissive temperature following photobleaching was measured. Cisplatin treated OVCAR3 and SKOV3 cells showed significantly reduced recovery of VSVG compared to control cells. Moreover, at permissive temperature VSVG appeared to be still restricted in ER/Golgi region in cisplatin treated cells while more VSVG trafficking was observed in control cells. This was further reflected in percentage of mobile protein fraction measured with cisplatin treated OVCAR3 cells again showing smaller mobile fraction than control cells. No difference in mobile fraction was found between cisplatin and control SKOV3 cells. Reduced recovery of VSVG was also observed by Farber-Katz and colleagues in response to camptothecin treatment, further supporting the theory that chemotherapy-induced alterations in Golgi morphology also affect its functional abilities. Fluorescent labelling of cisplatin showed that it is sequestered in *trans*-Golgi and Golgi-derived vesicles of the secretory pathway (Safaei *et al.*, 2005). Cisplatin also causes ER stress (Mandic *et al.*, 2003) which activates the unfolded protein response and apoptotic signaling. These observations suggest that activation of ER stress combined with sequestration of cisplatin in Golgi, together interfere with VSVG reporter (and on larger scale other proteins) traffic. Reduced protein trafficking thus seems to be one of the ways that cisplatin exerts its cytotoxic effect on cancer cells.

In conclusion, alterations in Golgi morphology caused by cisplatin also has an affect on its functions. Specifically, cisplatin interferes with Golgi polarisation and orientation during cancer cell migration. This further affected cells' migration speed and their wound healing capacity. Furthermore, cisplatin differentially alters expression of cell surface glycans in ovarian cancer cells indicating alterations in glycosylation process. Finally, protein secretion and trafficking is also reduced in the presence of cisplatin.

CELL-TO-CELL FUSION OF OVARIAN CANCER CELLS

RESULTS | CHAPTER 5

5 Formation of multinucleated ovarian cancer cells

5.1 Overview

Ovarian cancer cells are sometimes reportedly highly multinucleated and polyploid *in vivo* (Hsu *et al.*, 2005; Niu *et al.*, 2017; Telleria, 2013). Several possible theories have been proposed including endoreplication, asynchronous mitosis, cytokinesis failure and syncytin-mediated cell to cell fusion producing a syncytium (Diaz-Carballo *et al.*, 2018; Fox and Duronio, 2013; Larsson *et al.*, 2007; Sheehy *et al.*, 1974). These possible mechanisms have been mostly proposed for cells subjected to some kind of mutagenic treatment such as chemotherapy or radiation. However, the formation of multinucleated ovarian cancer cells in control, untreated conditions is not extensively studied and hence reported. Here, (i) multinucleated status of different ovarian cancer cell lines and (ii) a mechanism for formation of multinucleated cancer cells is examined and reported, and (iii) also suggest a possible gene that could play a role in cell fusion-mediated multinucleation of ovarian cancer cells.

5.2 Ovarian cancer cells are highly multinucleated

Ovarian cancer cells are sometimes reported in the literature to be polyploid and multinucleated (Hsu *et al.*, 2005), however, the mechanisms of formation of multinucleated cells is not known. Therefore, to understand the emergence of multiple nuclei, it was imperative to verify whether the cells in the lab also presented with multiple nuclei. In order to examine that, A2780, IGROV-1, OVCAR3, OVCAR4, OVCAR5, OVCAR8 and SKOV3 cells were grown on coverslips to 60-70% confluency and nuclei and cell membrane were labelled with Hoescht 33342 and wheat germ agglutinin (WGA) AF594, respectively. The cells were fixed and imaged using confocal microscope LSM 880 and 3D Z-stacks were created. Using cell membrane labelling as a guide for cells in cluster, number of nuclei per cell were counted. Approximately 500 – 900 cells were counted per cell line from three independent experiments. As shown in Figure 5.1, all the ovarian cancer cells in stock presented with some level of multiple nuclei with some cell

lines being highly multinucleated such as A2780 (78%) and OVCAR4 (17.4%), some moderately multinucleated such as OVCAR3 (8.1%) and OVCAR5 (5.4%), while some cell lines much less multinucleated such as IGROV-1 (2.1%), OVCAR8 (1.3%) and SKOV3 (1.2%).

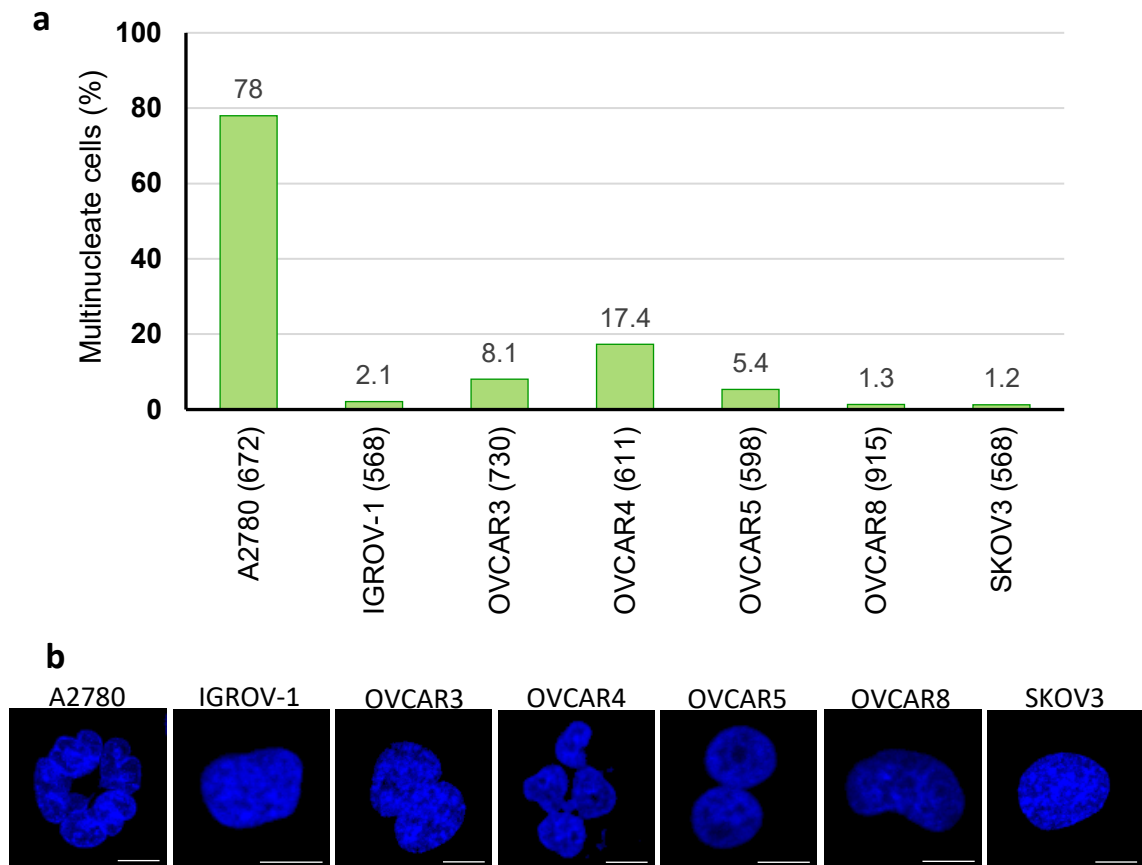


Figure 5.1. Multinucleated ovarian cancer cells. A2780, IGROV-1, OVCAR3, OVCAR4, OVCAR5, OVCAR8 and SKOV3 cells were grown on coverslips to 60-70% confluency and nuclei were labelled with hoescht 33342 (blue). The cells were fixed and imaged using confocal microscope LSM 880. 3D Z-stacks created were used to measure multinucleated cells. (a) A2780 and OVCAR4 were highly multinucleated, OVCAR3 and OVCAR5 were moderately multinucleated and IGROV-1, OVCAR8 and SKOV3 were mostly single nucleated. (b) Representative confocal images. Number in brackets indicate total number of cells counted for each cell line from three independent experiments.

5.3 Live time-lapse imaging of ovarian cancer cells shows cell-to-cell fusion of daughter cells following cell division

Presence of multinucleated ovarian cancer cells has been reported previously, however, the way in which such multinucleated cells arise is not known (Hsu *et al.*, 2005). Several studies have suggested that multinucleated or giant polyploid cells under the influence of chemotherapy or radiation could emerge as a result of endoreplication, cytokinesis failure or cell-to-cell fusion (Diaz-Carballo *et al.*, 2018; Fox and Duronio, 2013; Larsson *et al.*, 2007). However, no clear evidence is provided in untreated ovarian cancer cells. Here, it has been investigated whether multinucleate ovarian cancer cells result from cell-to-cell fusion. To study this process, ovarian cancer cell lines A2780, OVCAR3 and SKOV3 were grown in separate glass-bottomed dishes, and 24hr time-lapse images of these cells were captured using the Zeiss LSM880 confocal microscope. 24hr time lapse images of these cells showed that daughter A2780 cells of multi-nucleated parents undergo partial or complete cell-to-cell fusion with each other following cell division, giving rise to increased nuclei number and syncytium formation (Figure 5.2, Supplementary Video 1). In Figure 5.2, a bi-nucleate parent A2780 cell is going through synchronous tetra-polar mitosis followed by an apparent complete cytokinesis. Two daughter cells then fuse with other two daughter cells, giving rise to two bi-nucleate cells. In another instance, a single nucleated A2780 cell goes through cell division and both the daughter cells fuse with each other to produce a bi-nucleated cell (Figure 5.3, Supplementary Video 2). Furthermore, daughter cells of multinucleated A2780 cells following cell division were also observed to fuse with neighbouring cells (Supplementary Video 3). Similar cell fusion events were also observed in OVCAR3 cells. As shown in Figure 5.4, bi-nucleate OVCAR-3 cell upon synchronous tripolar mitosis and cytokinesis produces three daughter cells and undergo partial cell-fusion (Supplementary Video 4). Meaning, only two of the three daughter cells fuse with each other after an extended period, while the third daughter cell remained as a uni-nucleate cell until the end of the 24hr timeseries. This strengthens our observation further that the cells have gone through complete cytokinesis. On the other hand, no SKOV3 daughter cells were seen fusing with each other after cell division (Figure 5.5, Supplementary Video 5).

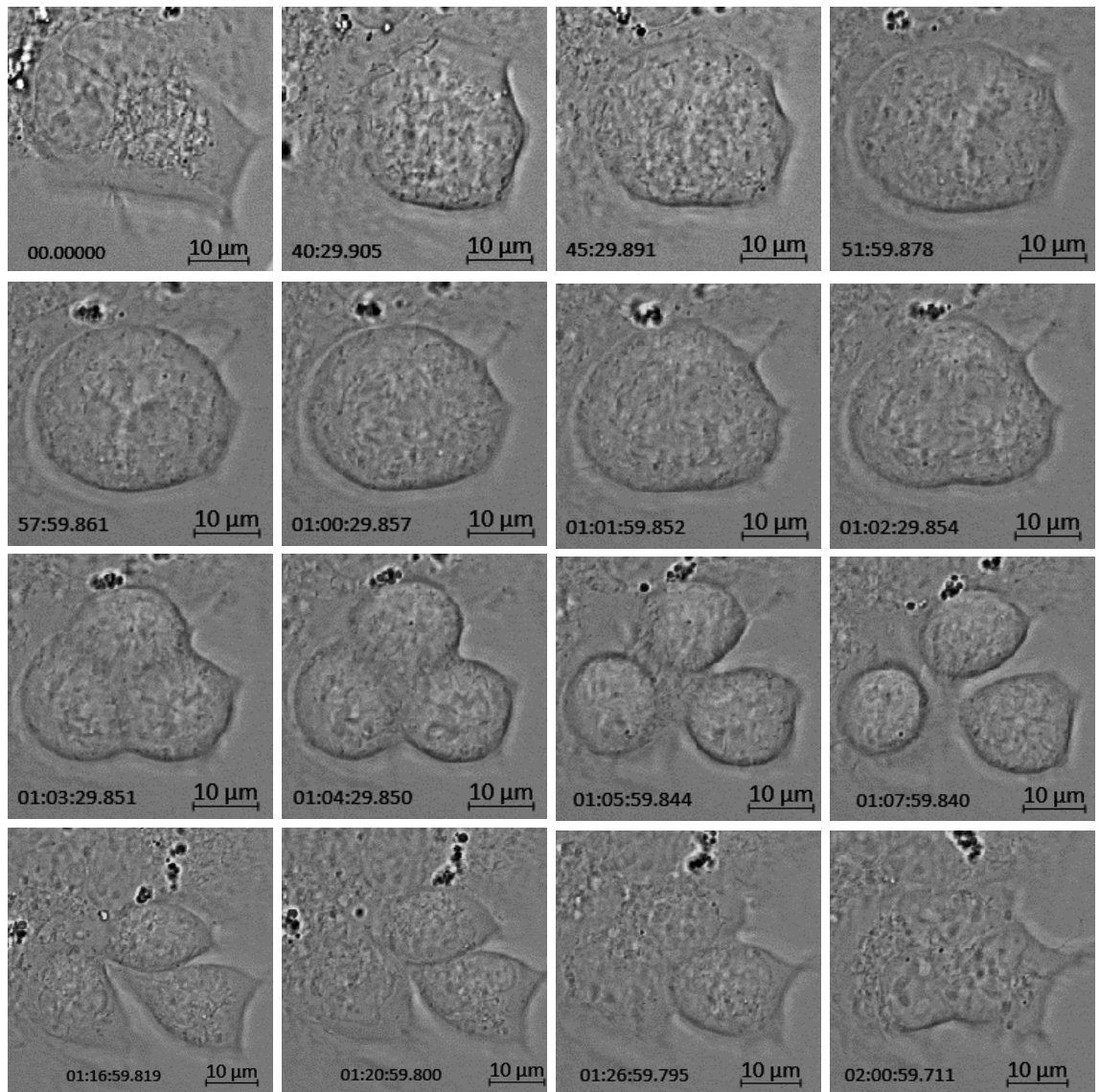


Figure 5.2. Cell fusion of A2780 cells. A bi-nucleate parent A2780 cell is going through synchronous tetra-polar mitosis followed by an apparent complete cytokinesis. Two daughter cells then fuse with other two daughter cells giving rise to two bi-nucleate daughter cells.

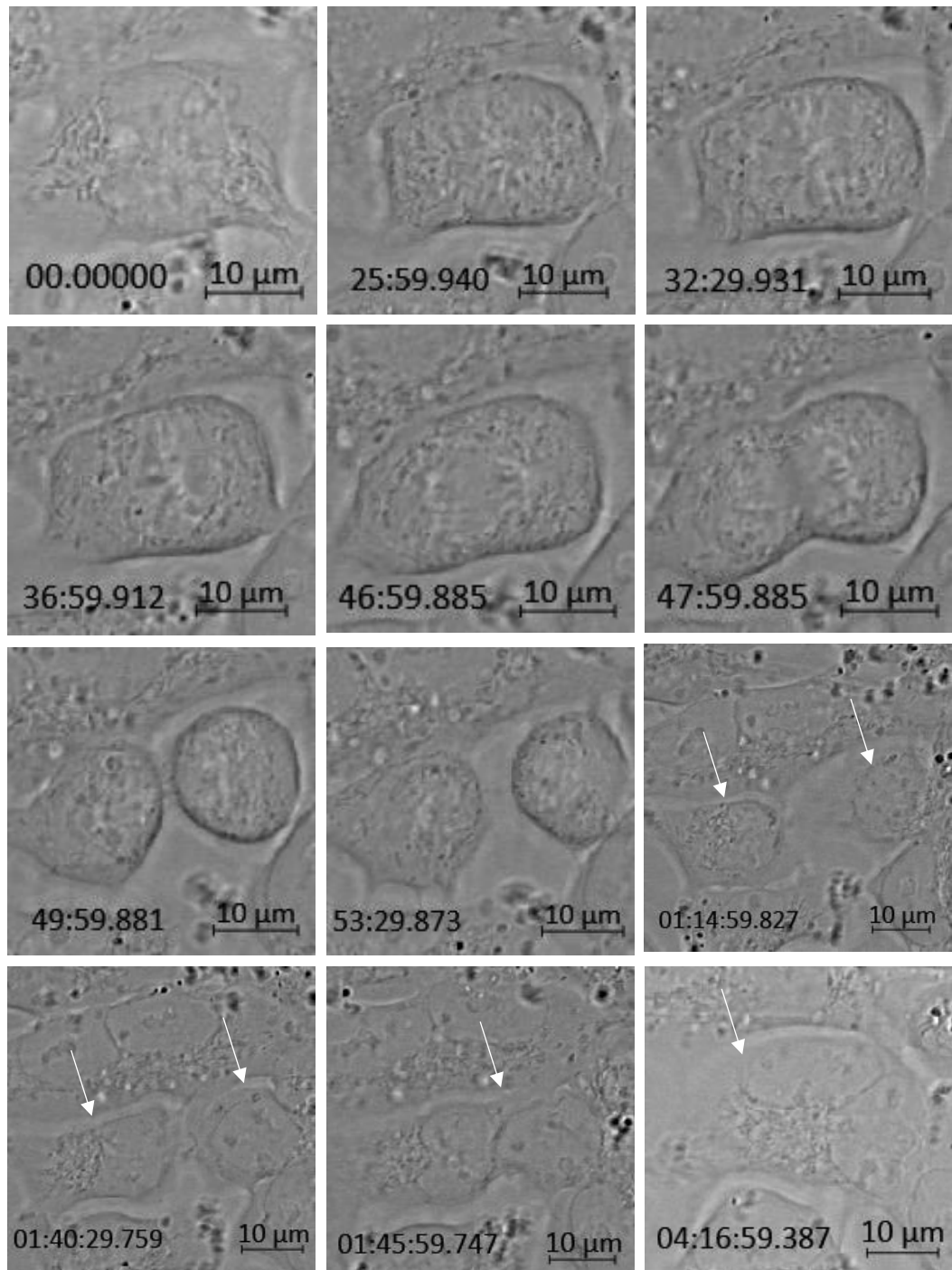


Figure 5.3. Cell fusion of A2780 cells. A single-nucleated parent A2780 cell goes through bi-polar mitosis producing two daughter cells which later fuse with each other producing a bi-nucleated cell.

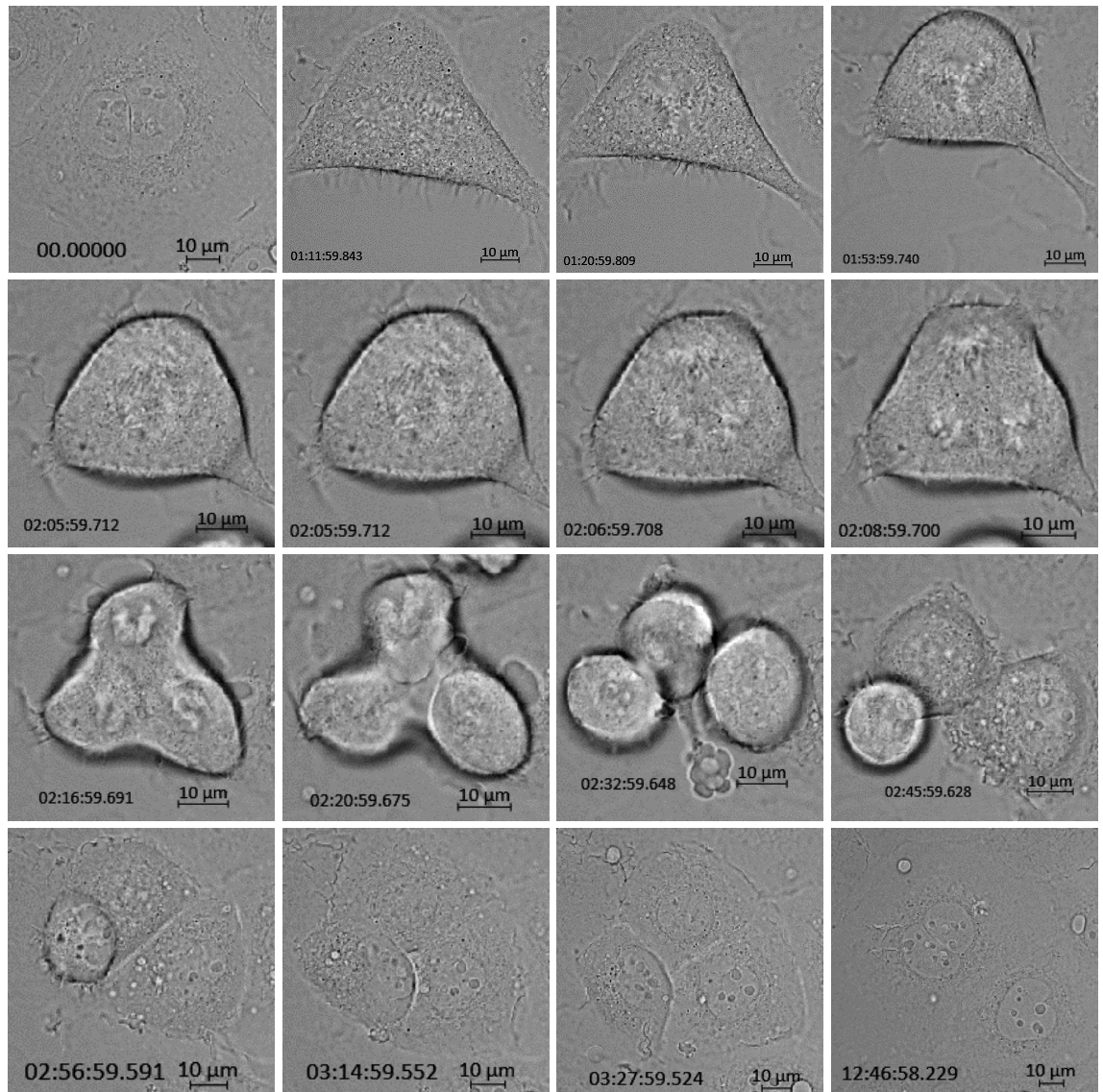


Figure 5.4. Cell fusion of OVCAR3 cells. A bi-nucleate OVCAR-3 cell upon synchronous tripolar mitosis and cytokinesis produces three daughter cells and undergo partial cell-fusion whereby two of the three daughter cells were seen fusing with each other while the third daughter cell remained single nucleated.

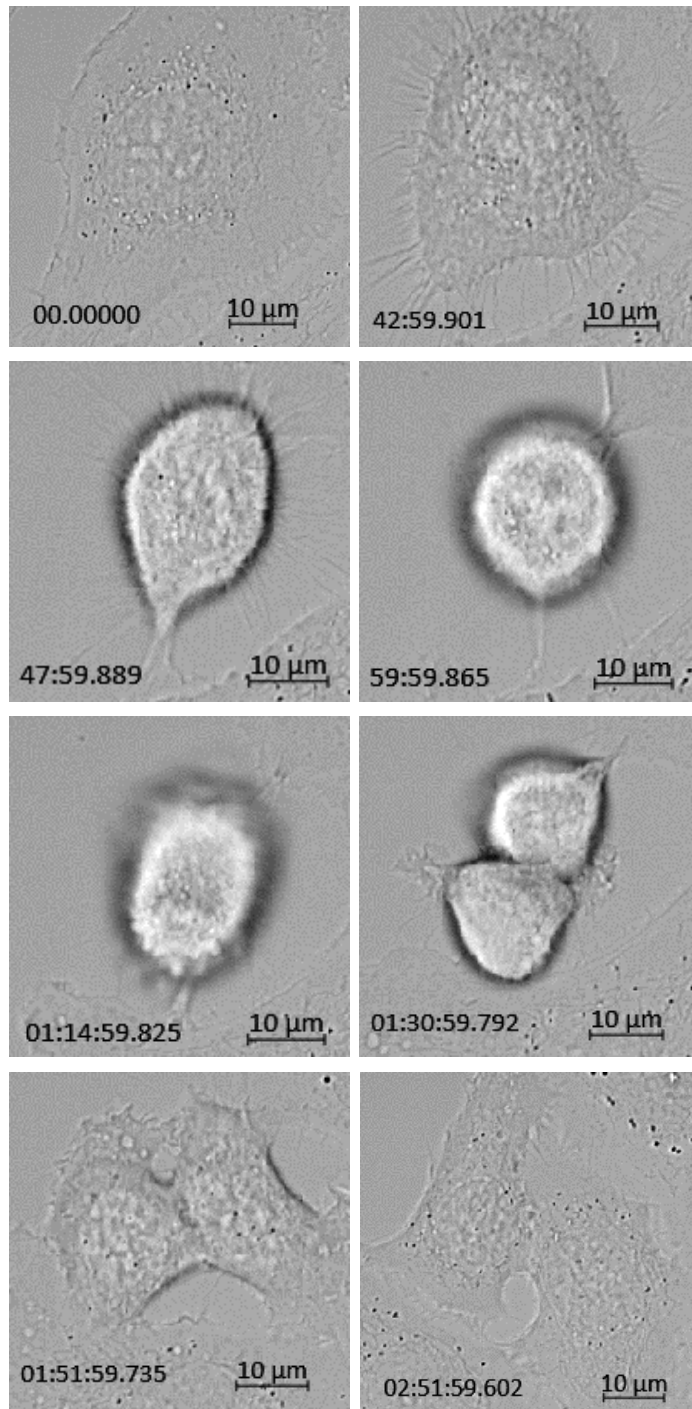


Figure 5.5. Cell division of SKOV3 cell. A single nucleated SKOV3 cell produces two daughter cells following mitosis. The daughter cells did not fuse with each other after cell division.

Cell fusion events observed in these cells were also quantified using the following criteria: (i) whether it was a multipolar or bi-polar division indicating whether the parent cell was multinucleated or single-nucleated and (ii) whether daughter cells fused or not after cell division. In A2780 cells, the total number of cell divisions observed in three different 24hr time-series were 14, 20 and 20, with 50%, 65% and 40% of the cell divisions observed being multipolar (or from multi-nucleated cells), respectively (Table 5.1). Interestingly, 100% of these multipolar divisions led to fusion of daughter cells, whereas only 43%, 57% and 25% of daughter cells from single-nucleated parent cells fused following cell division. In OVCAR3 cells, 37 and 12 total cell divisions were observed in two different 24hr time-series. 22% and 17% of cell divisions were multipolar with 75% and 100% of these daughter cells fusing (partial or complete) following cell division, and 3% and 20% of daughter cells from single-nucleated parent cells fusing either with each other or with neighbouring cells. On the contrary, out of 51 cell divisions observed from one 24hr time-series of SKOV3 cells, only 1 cell division was multi-polar and neither of the daughter cells fused by the end of the timeseries (Table 5.1). These results and observations suggest that at least one of the ways that ovarian cancer cells form multinucleated cells is through direct cell-to-cell fusion of daughter cells following mitosis.

Table 5.1. Quantified cell-fusion events as observed during 24hr time-series in A2780, OVCAR-3 and SKOV-3 cells

CELL LINE	BIOLOGICAL REPS.	CELL DIVISIONS	MULTI-POLAR (multinucleate parent cell)	BI-POLAR (uni-nucleate parent cell)	DAUGHTER CELL FUSION (%)	
					Multi-polar	Bi-polar
A2780	1	14	7 (50%)	7 (50%)	100	42.9
	2	20	13 (65%)	7 (35%)	100	57.1
	3	20	8 (40%)	12 (60%)	100	25
OVCAR-3	1	37	8 (21.6%)	29 (78.4%)	75	3.4
	2	12	2 (16.7%)	10 (83.3%)	100	20
SKOV3	1	51	1 (2%)	50 (98%)	0	0

5.4 Gene expression correlation analysis

Gene expression dataset generated by Reinhold *et al.* (2015) (CellMiner GSE32474) comprising of NCI-60 ovarian cancer cell line panel was used to perform gene expression correlation analysis of ovarian cancer cells (IGROV-1, OVCAR3, OVCAR4, OVCAR5, OVCAR8 and SKOV3) and their multinucleation count from section 5.2. Following Benjamini-Hochberg corrections, top ten genes were identified (Table 5.2) and *TADA3L* was considered a candidate gene involved in multinucleation of ovarian cancer cells (Pearson correlation $r = 0.99$, $p < 0.0001$; Figure 5.6).

Table 5.2. Top 10 genes identified from gene expression correlation analysis. Expression of genes in NCI-60 ovarian cancer cell line panel from GSE32474 dataset and their respective multinucleation count were used to perform Pearson correlation analysis.

Gene	Gene description	Pearson correlation r (absolute)	p-value	Adjusted p-value (Benjamini-Hochberg correction)
<i>ITPKB</i>	Inositol-triphosphate 3-kinase B	0.99	3.24E-06	0.14
<i>TADA3L</i>	Transcriptional adapter 3	0.99	4.27E-06	0.09
<i>ZBTB11</i>	Zinc finger and BTB domain-containing protein 11	0.99	2.34E-05	0.34
<i>KIAA1217</i>	Sickle tail protein homolog	0.99	4.68E-05	0.51
<i>ARIH1</i>	Ariadne RBR E3 ubiquitin-protein ligase	0.99	5.7E-05	0.50
<i>KLK2</i>	Kallikrein Related Peptidase 2	0.99	7.36E-05	0.54
<i>ELOVL3</i>	Elongation of very long chain fatty acids-like 3	0.99	9.59E-05	0.6
<i>BOLA2</i>	BolA family member 2	0.99	9.6E-05	0.53
<i>ZDHHC1</i>	Zing finger DHHC domain-containing protein 1	0.99	1.19E-04	0.58
<i>C22orf36</i>	Leucine-rich repeat containing protein 75B	0.99	1.29E-04	0.56

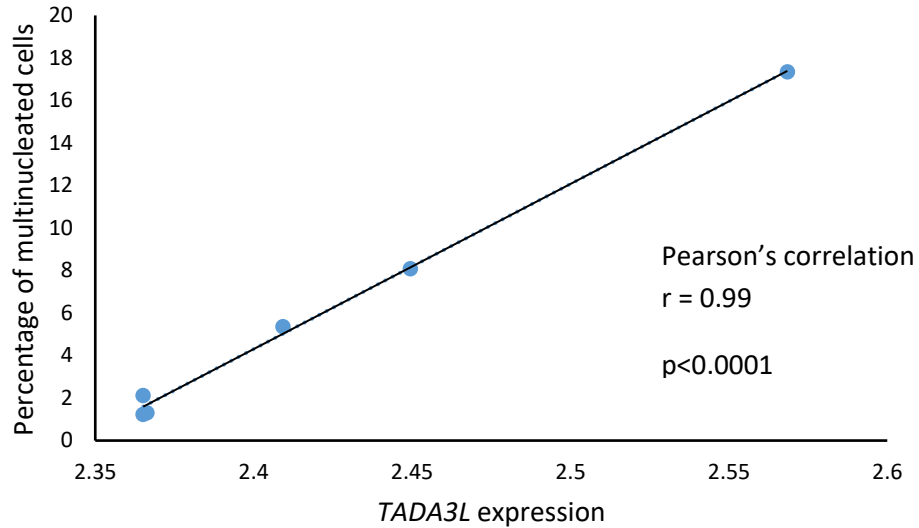


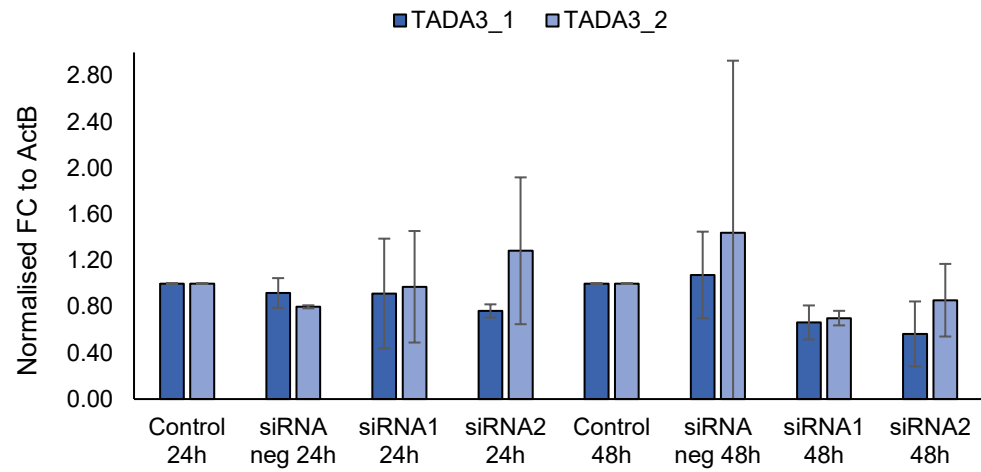
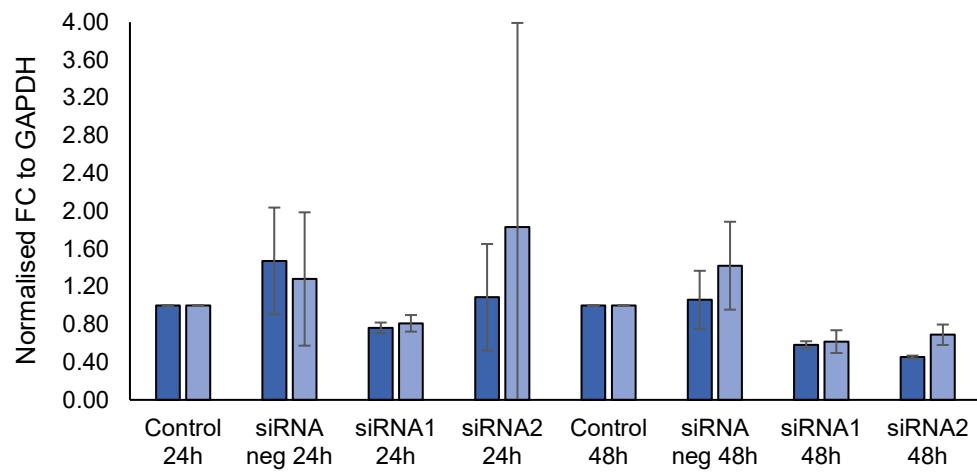
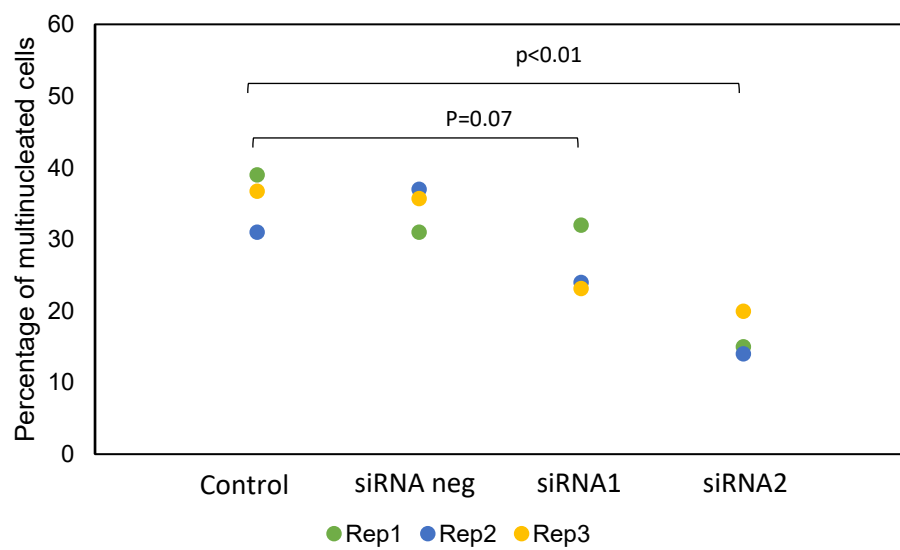
Figure 5.6. Gene expression correlation analysis of NCI-60 ovarian cancer cell line panel. Correlation was performed between percentage of multinucleated ovarian cancer cells and expression of *TADA3L* (Pearson's correlation $r = 0.99$; $p < 0.0001$).

5.5 *TADA3/TADA3L* identified as possible gene involved in cell-to-cell fusion of ovarian cancer cells

Transcriptional adapter 3 (*TADA3*) or *TADA3*-like (*TADA3L*) acts as a coactivator for p53/TP53-dependent transcriptional activation in mammalian cells and plays a crucial role in chromatin modulation and cell cycle progression (Mohibi *et al.*, 2015). Its fungal homologue, *ada-3*, has been identified as one of the transcription factors required for cell fusion in *Neurospora crassa* (Fu *et al.*, 2011). Here, it has been investigated whether *TADA3/TADA3L* plays a role in cell fusion observed in ovarian cancer cells by silencing this gene through RNAi and quantifying number of multinucleated cells following gene silencing.

Firstly, *TADA3* was silenced using siRNA and its expression was validated through qPCR. A2780 and OVCAR3 were transfected with two different siRNA (siRNA1 and siRNA2) targeted towards *TADA3*. Mock siRNA (siRNA neg) was used as negative control. mRNA levels for *TADA3* were then quantified 24hr and 48hr after transfection. Two different primer pairs (*TADA3_1* and *TADA3_2*) targeted towards *TADA3* were used to measure its expression levels. *TADA3* was silenced following transfection with siRNA1 and siRNA2 in A2780 cells after 48hr with respect to housekeeping genes *ActB*

(Figures 5.7a) and *GAPDH* (Figure 5.7b). Counting the percentage of multinucleated cells in A2780 showed that 48hr following *TADA3* silencing using siRNA2, the percentage of multinucleated cells significantly reduced ($p<0.01$), while siRNA1 showed non-significant reduction in number of multinucleated cells ($P=0.07$; Figure 5.7(c and d)). These results suggest that silencing *TADA3* reduced the number of multinucleated A2780 cells formed. Transfection of OVCAR3 cells with siRNA1 and siRNA2 resulted in silencing of *TADA3* after 48hr with respect to housekeeping gene *ActB* (Figure 5.8a) and *GAPDH* (Figure 5.8b). Percentage of multinucleated OVCAR3 cells were significantly reduced ($p<0.05$) 48hr following silencing using siRNA2 ($p<0.05$; Figure 5.8(c and d)), while silencing using siRNA1 showed no difference in percentage of multinucleated cells. Similar to A2780 cells, silencing of *TADA3* also reduced the number of multinucleated OVCAR3 cells formed. These results together indicate that *TADA3* plays a role in formation of multinucleated ovarian cancer cells.

a**b****c**

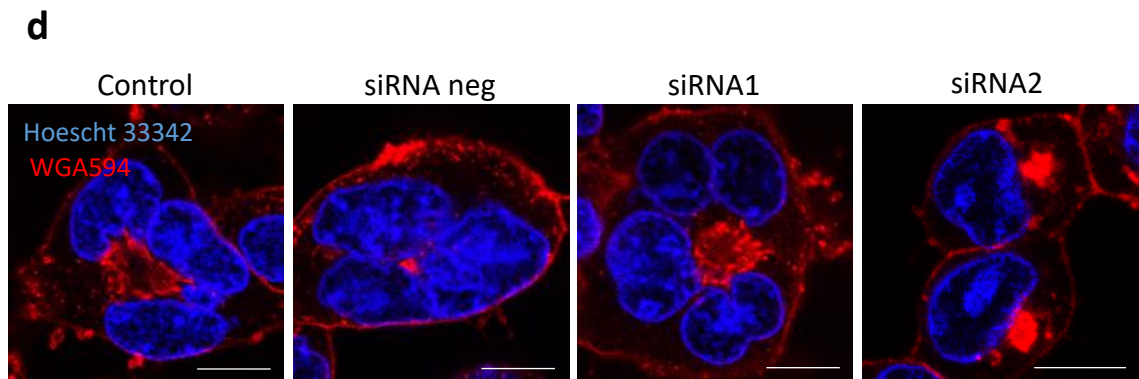
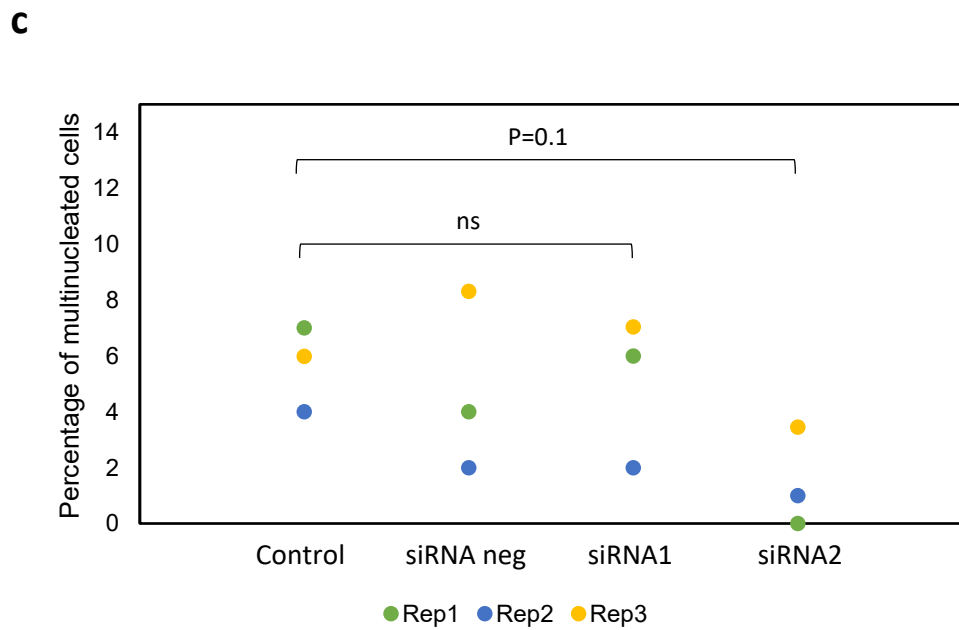
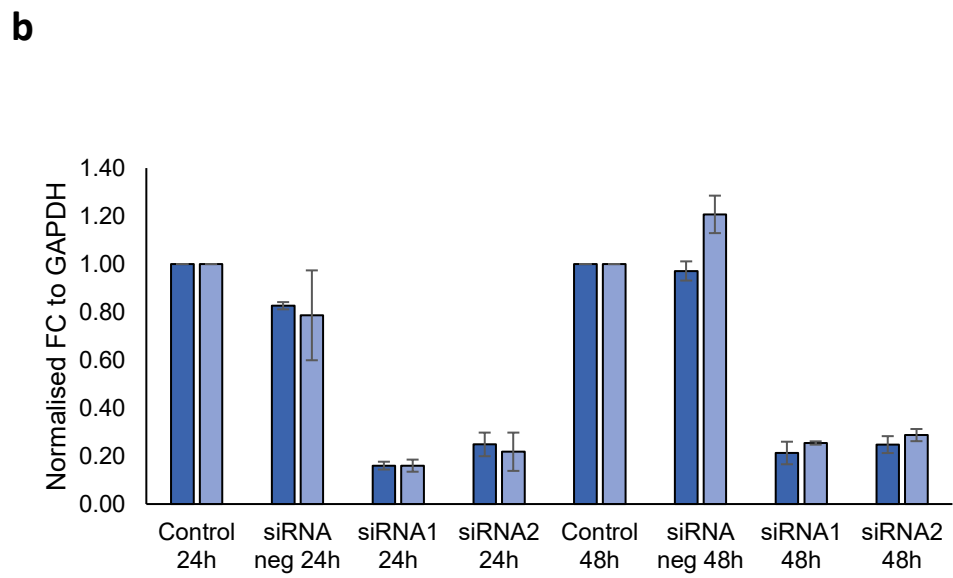
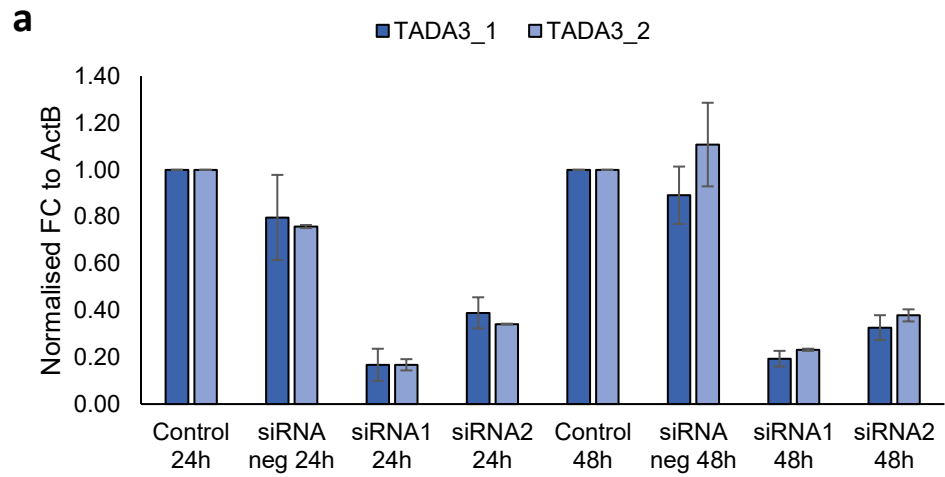


Figure 5.7. Validating *TADA3* expression and percentage of multinucleated A2780 cells following *TADA3* silencing. (a, b) normalised expression for *TADA3* with respect to housekeeping genes *ActB* and *GAPDH* following RNAi. Expression of *TADA3* compared to *ActB* and *GAPDH* was reduced 48 hours post transfection. qPCR data was generated from one independent experiment with two technical replicates. Error bars represent +1 SD. (c) Percentage of multinucleated cells non-significantly reduced following *TADA3* silencing using siRNA1, while number of multinucleated cells significantly reduced following silencing using siRNA2 ($p < 0.01$). 600 cells in total were analysed from three independent experiments performed with two technical replicates. One-way ANOVA followed by Tukey's test was used to determine significance ($p < 0.01$). (d) Representative confocal images. Nuclei were labelled with Hoechst 33342 (blue) and cell membrane labelled with wheat germ agglutinin (WGA)-594 (red).



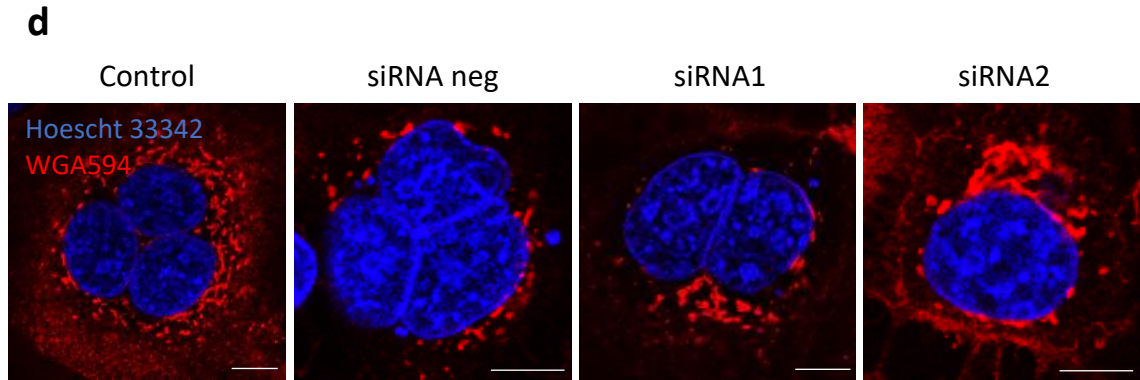


Figure 5.8. Validating *TADA3* expression and percentage of multinucleated OVCA3 cells following *TADA3* silencing. (a, b) normalised expression for *TADA3* with respect to housekeeping genes *ActB* and *GAPDH* following RNAi. At 48hr post transfection, expression of *TADA3* was reduced. qPCR data was generated from one independent experiment with two technical replicates. Error bars represent +1 SD. (c) Percentage of multinucleated cells were reduced non-significantly following silencing using siRNA2 ($P=0.1$). 600 cells in total were analysed from three independent experiments performed with two technical replicates. One-way ANOVA followed by Tukey's test was used to determine significance ($p<0.05$). (d) Representative confocal images. Nuclei were labelled with Hoechst 33342 (blue) and cell membrane labelled with wheat germ agglutinin (red).

5.6 Discussion

Multinucleated ovarian cancer cells result from cell-to-cell fusion of daughter cells

Ovarian cancer cells are reported to be highly multinucleated and polyploid (Hsu *et al.*, 2005; Telleria, 2013). Several possible theories for how they become multinucleated have been proposed including endoreplication, asynchronous mitosis, cytokinesis failure and syncytin-mediated cell to cell fusion producing a syncytium (Diaz-Carballo *et al.*, 2018; Larsson *et al.*, 2007; Sheehy *et al.*, 1974). These possible mechanisms have been proposed for cells subjected to mutagenic treatment such as chemotherapy or radiation. However, the emergence of multiple nuclei in untreated ovarian cancer cells is not extensively studied. Here, the formation of multinucleated ovarian cancer cells has been examined. Firstly, multinucleated status of ovarian cancer cell lines A2780, IGROV-1, OVCAR3, OVCAR4, OVCAR5, OVCAR8 and SKOV3 was quantified. It was found that ovarian cancer cells presented with different level of multinucleation with A2780 cells in particular being the most multinucleated followed by OVCAR4, OVCAR3 and OVCAR5 cells, and IGROV-1, OVCAR8 and SKOV3 being the least multinucleated. Based on these results, cell lines that were most (A2780), medium (OVCAR3) and least (SKOV3) multinucleated were selected for 24hr time lapse imaging. It was observed that multinucleated A2780 parent cells underwent multipolar mitosis, and their daughter cells fused with each other following cytokinesis 100% of the time (Table 5.1). This trend was observed consistently for binucleated and trinucleated A2780 parent cells as well, producing and leading to even more multinucleated cells. Daughter cells of single nucleated parent cell fused with each other approximately 25-50% of the time. It appears that formation of multinucleated cells would have initiated with daughter cells of single-nucleated parent cells fusing with each other producing binucleated cells. Because multinucleated cells (including binucleated cells) are prone to fusing with each other following cell division, that would have further prompted subsequent fusion events. Similar results were also observed for multinucleated OVCAR3 parent cells except that these cells were found to sometimes engage in partial fusions – for instance, only two of three daughter cells would fuse following cell division or one of the daughter cells would fuse with a neighbouring cell. Nevertheless, daughter cells of multinucleated parent cells 75-100% of the time engaged in cell fusion. Daughter cells of single nucleated OVCAR3

cells were found to fuse with each other or with neighbouring cell 3-20% of the time. SKOV3 cells that were found to be mostly single nucleated never went through cell fusion of daughter cells following mitosis. These results and observations suggest that at least one of the ways that ovarian cancer cells form multinucleated cells is through direct cell-to-cell fusion of daughter cells following mitosis.

Bjerregaard *et al.* (2006) suggests that in case of breast cancer cell fusion with endothelial cells was prompted by the expression of fusion protein syncytin-1 on breast cancer cells and corresponding D-type retroviral receptor on the endothelial cells. The process of generating multinucleated cells is described and documented well in Reed-Sternberg cells of Hodgkin's lymphoma. Salipante *et al.* (2009) identified mutation in cytokinesis regulator KLHDC8B in individuals with Hodgkin's lymphoma. They found that depletion of KLHDC8B produced binucleated cells through endoreplication (failed cytokinesis) and accumulation of binucleate Reed-Sternberg cells was a signature for disease progression. Although A2780 and OVCAR3 cells do appear to go through cytokinesis, KLHDC8B would be an interesting candidate gene to investigate. Rengstl *et al.* (2013) identified that in Hodgkin lymphoma, multinucleated giant Reed-Sternberg cells were generated by fusion of daughter cells following mitosis. They further confirmed incomplete cytokinesis by observing microtubule connection between daughter cells. Majority of A2780 cells observed fused with each other following cell division. Although microtubule marker was not used to determine any connection between daughter cells, it is possible that the presence of microtubule linker drove the fusion of daughter cells with each other. Future experiments involving labelled microtubules would help understand this better. In case of OVCAR3 cells, this does not seem to be true because of two reasons: 1 – daughter cells did not readily and necessarily fuse with each other. There were instances where one of the daughter cells fused with a neighbouring cell. This would indicate that a microtubule linker was not present and did not hold the two daughter cells together following cytokinesis. 2 – Although no cell membrane markers were used, formation of a cell membrane is clearly evident from time-lapse images captured. Moreover, the cells imaged here were not in a mechanically confined environment, as shown by Tse *et al.* (2012) in case of HeLa cells that induced cell fusion. Sheehy *et al.* (1974) suggested generation of multinucleated ovarian cancer cells through asynchronous mitosis where one nucleus goes through mitosis while the other remains dormant. We observed that all the nuclei simultaneously undergo mitosis,

further eliminating this option. Based on process of elimination, it can be concluded that multinucleated ovarian cancer cells are generated through direct cell fusion of daughter cells following mitosis. These results also indicate presence of other underlying factors that possibly regulate cell fusion.

TADA3 plays a role in cell fusion of ovarian cancer cells

To better understand the process of cell fusion in ovarian cancer cells, *TADA3/TADA3L* was identified through gene expression correlation analysis between NCI-60 ovarian cancer cell panel and multinucleated status of ovarian cancer cells (Table 5.2, Figure 5.6), and has been suggested to play a role in cell fusion. *TADA3/TADA3L* has been mostly studied for its role as a coactivator for p53/TP53-dependent transcriptional activation in mammalian cells and plays a crucial role in chromatin modulation and cell cycle progression (Mohibi *et al.*, 2015). Also known in the literature as Ada3, depletion or silencing of Ada3 has been shown to result in the formation of multinucleated MEF (Mohibi *et al.*, 2012) and mouse NIH3T3 cells (Orpinell *et al.*, 2010). Its role in mammalian cell fusion is not known. Its fungal homologue, *ada-3*, has been identified as one of the transcription factors required for cell fusion in *Neurospora crassa* (Fu *et al.*, 2011). Based on these evidences, *TADA3/TADA3L* was silenced using two different siRNAs (siRNA1 and siRNA2) in A2780 and OVCAR3 cells and percentage of multinucleated cells was measured again. A2780 cells showed significantly lower number of multinucleated cells following *TADA3/TADA3L* silencing, while number of multinucleated cells in OVCAR3 were non-significantly reduced (Figure 5.7 and 5.8), suggesting a role for this gene in cell fusion. Fu *et al.* (2011) identified *ada-3* as a possible transcription factor that is required for the transcription of cell fusion genes in *Neurospora crassa*. It is possible that *TADA3/TADA3L* also acts as transcription factor that regulates activation of other unidentified cell fusion genes in ovarian cancer cells.

TADA3/TADA3L is a subunit of histone acetyl transferase complex, ATAC (Ada Two A containing) and controls mitotic progression by regulating activity of non-histone targets (Mohibi *et al.*, 2015). Orpinell *et al.* (2010) showed that silencing ATAC subunits Ada2a/Ada3 resulted in delayed M/G1 transition, centrosome multiplication, defective

spindle and midbody formation and generation of binucleated NIH-3T3 cells. They suggested that ATAC subunit Gcn5 and Ada3 localises to the mitotic spindle and controls cell cycle progression through direct acetylation of Cyclin A/Cdk2. Specifically, ATAC subunits Ada2a and Ada3 strongly co-localised with the microtubule network from early mitotic stages to anaphase. Griffin *et al* (2016) showed that overexpression of Ada3 in ER+ breast cancer cell lines enhanced cell proliferation. Kaplan Meier analysis showed low Ada3 and high c-MYC expression predicted poor survival in patients independent of each other (Griffin *et al.*, 2016). Deletion of Ada3 also affected several mitotic and DNA replication genes based on microarray analyses (Mohibi *et al.*, 2015). Moreover, cells depleted of Ada3 showed increased accumulation of cells with abnormal nuclei including fragmentation, lobulation and multinucleation. These cells also failed to divide normally, with some cells snapped back while attempting to undergo cytokinesis leading to the formation of binucleated cells. Cells that had normal nucleus before mitosis showed fragmented nuclei afterward and were unable to divide. Their findings corroborated with that of Orpinell *et al.* (2010). The effect of *TADA3* or *Ada3* silencing observed and reported for ovarian cancer cells in this study are completely opposite of what other studies have reported. Although the reason is unclear, the discrepancy in results could partly be attributed to: firstly, the cell lines used are completely different, and hence the role the gene plays could also be different. And secondly, siRNA technique was employed for silencing *TADA3* in ovarian cancer cells, while other studies have used shRNA (Orpinell *et al.*, 2010) or Cre-mediated deletion of Ada3 (Mohibi *et al.*, 2012). Furthermore, Nag *et al.* (2007) identified that Ada3 stabilises and enhances activity of p53 protein by promoting its acetylation. While, mutations in p53 are associated with cytokinesis failure and chromosomal instability (Tarapore and Fukasawa, 2000). A2780 cells are wild type for p53 while it is mutated in OVCAR3 cells, as reported by Hernandez *et al* (2016). Here it has been reported that while A2780 are most prone to fusion, OVCAR3 are somewhat prone to fusion. This is opposite of what is expected based on the role that p53 plays. Future investigation in this area will help understand the relationship between *TADA3*, p53 and cell fusion.

One of the main limitations of this study is the variability in percentage of multinucleated cells counted. It is possible that A2780s experienced a growth spurt resulting in lack of nutrients which may stressed the cells out resulting in fusion behaviour. It is also possible

that due to their growth spurt, cells did not have enough space to grow and that confined environment caused them to fuse with each other. There is evidence for confined environment inducing fusion behaviour in HeLa cells (Tse *et al.*, 2012). Variability in OVCAR3 could be the result of cells at a different (later) passage, although there is no direct evidence that shows that later passage cells are more multinucleated, it is believed that later passage cells accumulate genetic mutations that can alter behaviour of cells. This also highlights the limitations posed by using gene expression datasets. It is very likely that the growth conditions and passaging of cells done in one lab varies from the other.

CONCLUDING DISCUSSION

CHAPTER 6

6. CONCLUDING DISCUSSION

Despite the risk of developing resistance, cisplatin has been used for decades to treat ovarian cancer. It is also one of the only drugs that has been shown to effectively treat cancer cells. This study proposes a novel mode of action for cisplatin-induced cytotoxicity in cancer cells. Understanding its biology can open avenues for future treatment options.

6.1 Alterations to Golgi apparatus morphology an early stress response to cisplatin

Cisplatin-induced alterations in cellular organelles such as the ER, mitochondria, lysosomes and the cytoskeleton are well known, but its effect on the Golgi apparatus are not yet known. In this study, structural and functional alterations of the Golgi apparatus in ovarian cancer cells in response to cisplatin were examined. Firstly, cisplatin treatment was shown to cause increased fragmentation of Golgi apparatus in several different ovarian cancer cell lines, indicating this to be a universal response to cisplatin. Fragmented state of the Golgi is indicative of a diseased-state – several neuronal conditions including Alzheimer's and Parkinson's, show fragmented Golgi. In response to lot of different stressors such as chemotherapy, heat-shock and ionizing radiation, Golgi is shown to be fragmented. Welch and Suhan (1985) demonstrated that heat-shock of rat embryo fibroblast cells for 3 hours resulted in vesiculation in the perinuclear region which was confirmed to be the Golgi apparatus through electron microscopy. Petrosyan and Cheng (2014) also showed that heat-shock of Panc1 cells resulted in Golgi disorganisation due to increased interaction between non-muscle myosin IIA and glycosyltransferase (core 2 N-acetylglucosaminyltransferase-M). Farber-Katz *et al.* (2014) provided with more mechanistic detail to stress-induced Golgi fragmentation. They showed that treatment of primary and immortalised cell lines with either chemotherapy drugs (doxorubicin or camptothecin) or ionizing radiation resulted in Golgi dispersal through activation of DNA-Pk/GOLPH3/MYO18A/F-actin cascade. In response to DNA damage, DNA-PK phosphorylated overexpressed GOLPH3 which resulted in recruitment and increased interaction with unconventional myosin MYO18A to the *trans*-Golgi where it bound to F-actin. This complex applied a tensile force that

resulted in excessive vesicle budding and deformation. Furthermore, apoptosis induction involves cleaving of several proteins implicated in Golgi structure including golgin-160 (Mancini *et al.*, 2000), GRASP65 (Gervais *et al.*, 2002), GM130 (Chiu *et al.*, 2002; Walker *et al.*, 2004) and giantin (Lowe *et al.*, 2004) by caspases that produces disassembled Golgi. Therefore, it seems that one of the ways cisplatin exerts its cytotoxicity on cancer cells is by fragmenting Golgi.

Although Farber-Katz *et al.* (2014) suggested Golgi dispersal a downstream effect of stress-induced DNA damage, Golgi fragmentation in ovarian cancer cells was detected between 3 and 6 hour post cisplatin treatment while maximal DNA damage as shown by γ H2A.X labelled nuclear foci was detected between 12 to 24 hour post treatment. Only 1% of cisplatin is reported to bind to DNA and cause DNA damage, while the rest is hydrolysed in the cytoplasm where it exerts its effects on other cellular organelles and initiates apoptotic response (Yu *et al.*, 2008). Based on this it has been suggested that Golgi fragmentation observed could be a combined effect of stress-induced apoptotic response from other organelles. Furthermore, caspase-2 was shown to be activated in response to cisplatin in renal tubal epithelial cells (Kaushal *et al.*, 2001), and procaspase-2 is localised at the Golgi complex (Mancini *et al.*, 2000). More importantly, golgin-160, implicated in Golgi structure, acts as a substrate for caspase-2 and interacts with upstream apoptotic regulators that results in its cleavage (Mancini *et al.*, 2000). It is possible that cisplatin-induced fragmentation could be the result of cleaving of Golgi structure proteins. Thus, it seems likely that alteration of Golgi structure is more of a combined effect of different cellular responses to cisplatin leading to its fragmentation.

6.1.1 Cisplatin-induced structural alterations mediated by JNK signaling

A mechanism to understand cisplatin-induced Golgi fragmentation was also investigated in this study. Cisplatin treatment activates a series of signaling cascades including JNK and ERK. JNK activation leads to apoptotic response while ERK activation can either enhance survival or mediate apoptosis by initiating the apoptotic signal in cisplatin treated cells (Persons *et al.*, 1999; Hayakawa *et al.*, 2000; Yan, An and Kuo, 2016). Coincidentally, these two pathways are also activated during mitosis that lead to Golgi

disassembly and vesiculation. JNK and ERK activation leads to phosphorylation of GRASP65 and GRASP55, respectively, on Golgi complex and prevents *trans*-oligomerisation resulting in unlinking of Golgi stacks (Cervigni *et al.*, 2015; Feinstein and Linstedt, 2008). Independent inhibition of ERK and JNK using a chemical inhibitor in ovarian cancer cells showed that ERK inhibition in A2780 cells resulted in significantly more fragmentation of the Golgi in cisplatin treated cells, while ERK inhibition in cisplatin treated OVCAR3 and SKOV3 cells showed no statistically significant difference in Golgi fragmentation. On the other hand, JNK inhibition in cisplatin treated A2780 and OVCAR3 cells showed significantly reduced fragmentation of the Golgi (SKOV3 cells showed trending significance for the same). Thus, it seems that cisplatin-induced Golgi fragmentation is mediated by JNK, not ERK, pathway. It is therefore suggested that similar to mitotic JNK activity, cisplatin-induced JNK possibly phosphorylates GRASP65 on Golgi preventing *trans*-oligomerisation and causing unlinking of Golgi stacks (Figure 6.1). Future phosphorylation studies of JNK-mediated GRASP65 phosphorylation will provide with more mechanistic detail.

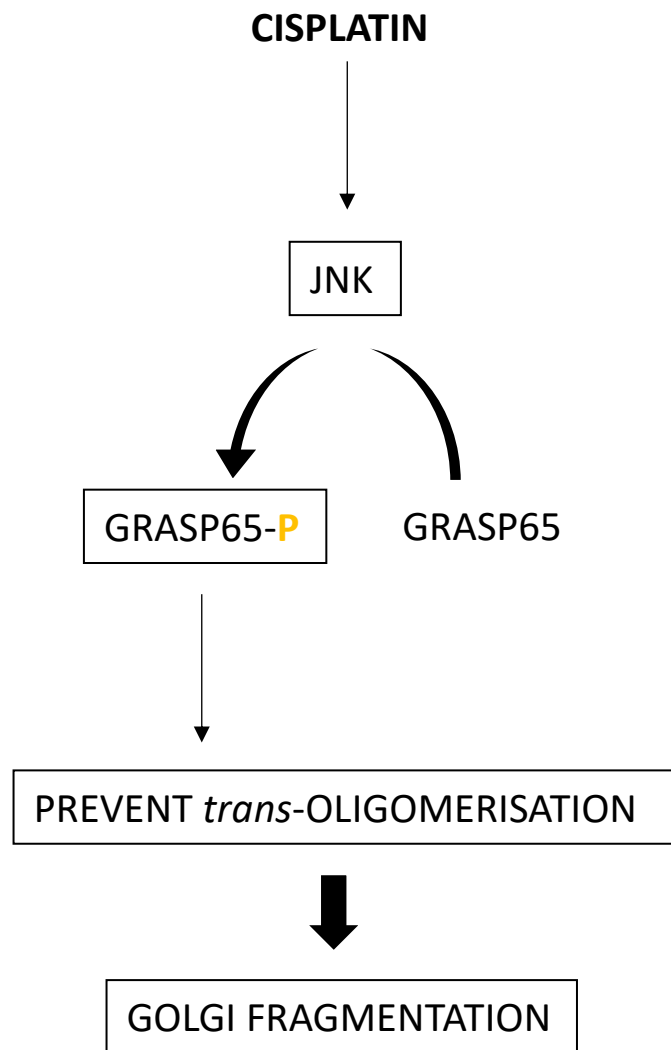


Figure 6.1 Mechanism for cisplatin-induced Golgi fragmentation through JNK signaling.

6.2 Cisplatin-induced alterations of the Golgi functions

6.2.1 Cisplatin interferes with Golgi polarisation during cancer cell migration

It was hypothesised that alterations in structure of the Golgi might also have an effect on its functions. In order to study that, firstly, Golgi polarisation during cancer cell migration in response to cisplatin treatment was tested. Pericentriolar localisation of the Golgi apparatus plays an important role in establishing and maintaining cell polarity (Yadav, Puri and Linstedt, 2009). Its orientation further guides membrane traffic towards the plasma membrane which lays the cornerstones for directional protein secretion required for cell migration (Bisel *et al.*, 2008; Yadav, Puri and Linstedt, 2009; Preisinger *et al.*, 2004). Cell migration is also the driving force behind cancer cell invasion and metastasis (Hanahan and Weinberg, 2011), and several Golgi-associated proteins have been implicated to play a role (Xing *et al.*, 2016; Halberg *et al.*, 2016). Based on these evidences, effect of cisplatin on Golgi polarisation during ovarian cancer cell migration was tested using a scratch/wound healing assay and Golgi orientation angle measured. As expected, control A2780, OVCAR3 and SKOV3 cells that had migrated showed polarised Golgi ($\leq 60^\circ$). In the presence of cisplatin, the Golgi was significantly depolarised with the orientation angle of Golgi measured in migrated cells was $>60^\circ$ in A2780 and OVCAR3 cells (SKOV3 cells showed trending significance for the same). Based on this, it was hypothesised that since Golgi failed to correctly polarise in the presence of cisplatin, the drug treatment would also affect the migration speed of cells. Further testing this in A2780 and SKOV3 cells showed that cell speed of cisplatin treated cells was significantly slower than their control counterpart. Moreover, the wound healing capacity of cisplatin treated OVCAR3 and SKOV3 cells was also significantly slower compared to control cells (A2780 cells showed trending significance for the same). Golgi orientation during cell migration depends on actin and microtubule network whereby the motor proteins position Golgi by generating forces and mechanical tension (Egea *et al.*, 2013). Cisplatin causes depolymerisation of the actin (Zeng *et al.*, 1996). It is thus believed that in the presence of cisplatin depolymerised actin fails to generate necessary forces required for correct orientation of the Golgi during cell migration. More importantly, this highlights that as an early response, cisplatin prevents metastatic spread of cancer cells

by interfering with directional cell migration. It achieves this by interfering with Golgi polarisation, which is key for directional cell migration. And this proves lethal to cancer cells as they cannot escape the initial tumour. The cell speed was also found to be slow in response to cisplatin further indicating that their migratory capacity is reduced. Future experiments directly investigating the effect of cisplatin-induced actin-cytoskeletal disruption on Golgi polarisation would provide with more mechanistic detail.

6.2.2 Ovarian cancer cells aberrantly glycosylated in response to cisplatin

Next, effect of cisplatin on glycosylation profile of ovarian cancer cells was tested. The Golgi apparatus upregulates expression of genes involved in mediating glycosylation in response to stress (Oku *et al.*, 2011). Secreted and cell-surface glycoconjugates play an integral role in mediating key cellular processes including cell-to-cell communication, cell adhesion, motility and cell-signalling pathways during normal development (Fuster and Esko, 2005). Alterations in glycoconjugate profiles, specifically overexpression of cytoplasmic and secreted GalNAc epitopes have been associated with disease progression of cancer cells (Rambaruth, Greenwell and Dwek, 2012). HPA lectin from *Helix pomatia* has a binding specificity for GalNAc and presence of HPA-binding glycans (HPA positivity) in tumour tissue samples was associated with poor patient prognosis (Brooks, Hall and Buley, 2001). Based on these evidences, changes in HPA binding profiles in response to cisplatin were tested. Cisplatin treated ovarian cancer cell lines (A2780, IGROV-1, OVCAR3, OVCAR4, OVCAR5, OVCAR8 and SKOV3) showed differential alteration of cell-surface GalNAc. Meaning, cisplatin increased expression of GalNAc for A2780, OVCAR3, OVCAR4 and OVCAR8 cells measured as increased HPA positivity, while its expression was reduced in IGROV-1 and SKOV3 cells, and OVCAR5 showed no change in binding profile following cisplatin treatment. Krushkal *et al.* (2017) reported that expression of multiple *GALNT* encoding multiple N-acetylgalactosaminyltransferases was downregulated by cisplatin treatment. Based on this, it seems that effect of cisplatin on glycosylation is cell line specific. Each cell line has originated from different tumour sites and have different genetic alterations and metastatic capacity. It is also possible that although cisplatin reduced expression of *GALNT*, further alterations in activity of N-acetylgalactosaminyltransferase in response

to cisplatin could amount to increased or decreased GalNAc expression. Fragmented state of the Golgi in cisplatin treated cells could have also accounted for overexpression of GalNAc epitopes in some cell lines and not in others.

HPA binding profile during cell migration was also measured. Ample evidence in literature reports that HPA positive cells are more invasive, form metastatic tumours when injected in SCID mice and are associated with poor patient prognosis (Schumacher *et al.*, 1996). Cell migration assay on A2780 cells showed that in the presence of cisplatin comparatively less GalNAc was expressed during cell migration. Because A2780s are reported in literature to have medium invasive capacity *in vivo* (Hernandez *et al.*, 2016), suggests that cisplatin exerted its effects by reducing metastatic capacity of these cells. In case of OVCAR3 and SKOV3 cells, although cisplatin increased expression of GalNAc epitopes during migration, overall more HPA negative cells were present at the wound edge. This is opposite of what was expected for these cells. OVCAR3 cells are characterised to have low invasion capacity (Hernandez *et al.*, 2016), and the fact that overall more HPA negative cells were present at the wound edge supports this. SKOV3 cells on the other hand are characterised to have high invasion capacity (Hernandez *et al.*, 2016), and completely opposite results were observed with more HPA negative cells being present at the wound edge. The discrepancy in results can be attributed to different sample preparation and staining techniques used in this study. Traditionally, cancer cells have been labelled with streptavidin-biotin based HPA labelling followed by imaging using light microscopy. Nevertheless, performing further experimentation involving invasion assays will provide with in depth understanding of the effect of cisplatin on alterations in glycosylation and GalNAc expression levels.

6.2.3 Protein secretion and trafficking reduced in response to cisplatin

Finally, protein trafficking from the ER/Golgi to the plasma membrane was tested in the presence of cisplatin. One of the main functions of the Golgi apparatus is to ensure efficient transfer of modified protein cargo coming from the ER and its secretion to the plasma membrane. Given that treatment of cells with cisplatin resulted in fragmented and depolarised Golgi, it was imperative to examine its effect on protein trafficking in ovarian

cancer cells to understand whether alterations in morphology also affect other Golgi functions. Temperature sensitive protein trafficking reporter VSVG has been used extensively in the past to study protein transport (Presley *et al.*, 1997; Farber-Katz *et al.*, 2014). OVCAR3 and SKOV3 cells were transfected with VSVG-EGFP and its rate of recovery after photobleaching at permissive temperature was used to determine protein transport. Cisplatin treated cells showed significantly reduced recovery of VSVG with VSVG visibly appeared to be restricted in ER/Golgi region compared to control cells which showed more VSVG trafficking. These results were similar to that reported by Farber-Katz *et al.* (2014). Moreover, smaller mobile protein fraction was measured for cisplatin treated OVCAR3 cells. Cisplatin is reportedly sequestered in *trans*-Golgi and in Golgi-derived vesicles of the secretory pathway (Safaei *et al.*, 2005). Cisplatin also causes ER stress and activates the unfolded protein response and apoptotic signaling (Mandic *et al.*, 2002). These observations together possibly suggest that activation of ER stress sequesters VSVG in the ER/Golgi region (mostly ER) preventing its transport and sequestration of heavy platinum-based compound in Golgi-derived vesicles further reduces the rate of transport of VSVG to the plasma membrane, which is also reflected in slower mobile protein fraction measured for these cells. Reduced protein trafficking thus seems to be one of the ways that cisplatin exerts its cytotoxic effect on cancer cells.

6.3 Limitations and future directions

One of the main limitations of this study was the lack of a primary, non-cancer cell line to compare all the results to. Studying the effects of cisplatin on a non-cancerous cell line would have provided with better insight and the extent to which cisplatin effects could be observed. Secondly, cisplatin-induced DNA damage could be measured differently. In this study, number of γ H2A.X labelled nuclear foci were measured through single cell analysis which can be very time-limiting, inefficient and somewhat biased approach. Instead, using high-throughput approach like flow-cytometry will allow larger number of cells to be analysed without any bias and will also be more efficient. Thirdly, pathway inhibition should be validated using western blot and RNAi could be used for more targeted inhibition. Finally, to determine GalNAc expression using HPA labelling, instead of using the traditional approach to score cells simply positive or negative by eye,

a more quantitative method should be used such as measuring fluorescence or intensity of labelled cells. Doing so will give more robust understanding of HPA binding profile.

Furthermore, no cell cycle regulation drugs (or techniques) were employed in this study to synchronise the cells. This was due to several reasons: firstly, the main aim of this part of the thesis was to simply study the effect of cisplatin treatment on Golgi apparatus barring any additional treatments. Cisplatin alone is known to induce an extreme stress response in cells. There was a possibility that treatment of cells with any additional drugs would have elicited an additional stress response and that would have confounded the results reported in this study. Secondly, low doses of cisplatin directly affect and arrest the cell cycle (Velma, Dasari and Tchounwou, 2016). To account for this effect in this study, cells only in interphase stage of cell cycle were selected and imaged for analyses and mitotic cells were explicitly excluded. DAPI staining was used as a guide to judge the cell's cell cycle stage. It is not known how cisplatin would have interacted with a cell cycle regulation drug and whether the interaction would have been compatible to use for the experiments performed in this study. Finally, the Golgi apparatus itself acts as a cell cycle checkpoint and goes through structural and functional alterations during mitosis. Use of cell cycle regulation drug with or without cisplatin treatment could have affected the function and behaviour of the Golgi and could have masked the effects of cisplatin observed in this study. To overcome these issues in future, cell cycle regulation drug compatible with cisplatin could be used to synchronise cell cycle of cells allowing for inclusion of more non-mitotic cells in the same stage of cell cycle into the study.

Future experiments should firstly aim to study downstream target of JNK specifically GRASP65 to better understand the mechanism for cisplatin-induced Golgi fragmentation. Secondly, electron microscopy-based analysis should be performed to determine the extent of Golgi fragmentation – whether cisplatin causes Golgi ribbon to unlink only or whether individual Golgi stacks are also unstacked in response to cisplatin. Thirdly, performing studies investigating the effect of cisplatin activated caspase-2-induced cleaving of golgins and studying effect of golgin cleaving could help determine whether cisplatin causes fragmentation of Golgi by acting directly on Golgi itself. Fourthly, during mitosis, Cdk1 and Plk also phosphorylate GRASP65 that prevent *trans*-oligomerisation. Investigating whether these pathways are also activated in response to cisplatin will also provide with more insight into cisplatin's mode of action. Finally, changes in actin-cytoskeletal dynamics in response to cisplatin, especially during cell migration should be

investigated. Doing so will help understand better the mechanism for Golgi depolarisation during migration.

6.4 Ovarian cancer cell fusion mediated by TADA3/TADA3L

Ovarian cancer cells are reported to be highly multinucleated and polyploid (Hsu *et al.*, 2005; Telleria, 2013). Several possible mechanisms have been proposed in the literature for cells subjected to some kind of mutagenic treatment that lead to multinucleated cells including endoreplication, asynchronous mitosis, cytokinesis failure and syncytin-mediated cell to cell fusion producing a syncytium (Diaz-Carballo *et al.*, 2018; Fox and Duronio, 2013; Larsson *et al.*, 2007; Sheehy *et al.*, 1974). However, the emergence of multiple nuclei in untreated ovarian cancer cells has not been reported. In this study, the formation of multinucleated ovarian cancer cells was examined and reported. It was hypothesised that multinucleated cells were a result of fusion between cancer cells.

6.4.1 Cancer cell fusion leads to multinucleation of ovarian cancer cells

Firstly, because ovarian cancer cells are sometimes reported to be multinucleated *in vivo*, ovarian cancer cells lines available in the lab were examined to determine their multinucleated status. It was found that ovarian cancer cell lines presented with different level of multinucleation with A2780 cells in particular being the most multinucleated followed by OVCAR4, OVCAR3 and OVCAR5 cells, and IGROV-1, OVCAR8 and SKOV3 being the least multinucleated. Through 24 hour time-lapse imaging performed on most (A2780), medium (OVCAR3) and least (SKOV3) multinucleated cell lines showed that multinucleated cells were a result of fusion of daughter cells following cell division. Specifically, multi-(bi-, tri-, tetra- etc.) nucleated parent cells underwent multipolar mitosis and following cytokinesis, their daughter cells fused with each other every single time. In case of A2780 cells, the daughter cells were sometimes observed to fuse with neighbouring cell or in case of OVCAR3 cells sometimes only two out three daughter cells were observed to fuse with each other. Whereas daughter cells of single-

nucleated parent cells fused with each other some (25-50% for A2780s and 3-20% for OVCAR3s) of the time. SKOV3 cells which were observed to be single-nucleated did not fuse with each other. It appears that formation of multinucleated cells would have initiated with daughter cells of single-nucleated parent cells fusing with each other producing binucleated cells. Because multinucleated cells (including binucleated cells) are prone to fusing with each other following cell division, that would have further prompted subsequent fusion events. It is unclear what drives this fusion behaviour, but it is possible that expression of fusion proteins on the cell membrane of one or all daughter cells would prompt and facilitate the fusion of daughter cells with each other. Further experimentation in this area would help understand this process better. These results and observations suggest that at least one of the ways that ovarian cancer cells form multinucleated cells is through direct cell-to-cell fusion of daughter cells following mitosis. Although there is a possibility that the presence of microtubule linker could have driven the fusion of daughter cells with each other as suggested by Rengstl *et al.* (2013) in case of multinucleated giant Reed-Sternberg cells, this does not seem to be the case for ovarian cancer cells. In case of OVCAR3 cells, the daughter cells did not readily and necessarily fuse with each other. There were instances where one of the daughter cells fused with a neighbouring cell. This would indicate that a microtubule linker was not present and did not hold the two daughter cells together following cytokinesis. Moreover, although no cell membrane markers were used, formation of a cell membrane was clearly evident from time-lapse images captured. Future experiments labelling microtubules and cell membranes would further confirm these theories. Sheehy *et al.* (1974) suggested generation of multinucleated ovarian cancer cells through asynchronous mitosis where one nucleus goes through mitosis while the other remains dormant. We observed that all the nuclei simultaneously undergo mitosis, further eliminating this option. Based on process of elimination, it can be definitively concluded that multinucleated ovarian cancer cells are generated through direct cell fusion of daughter cells following mitosis. These results also indicate presence of other underlying factors that possibly regulate cell fusion.

6.4.2 A role for *TADA3/TADA3L* in cancer cell fusion

Gene correlation expression analysis based on multinucleated status of ovarian cancer cell lines identified *TADA3/TADA3L* as a candidate gene. *TADA3/TADA3L*, also known as *Ada3*, controls mitotic progression by regulating activity of non-histone targets and has been shown to play a role in the formation of multinucleated MEF and mouse NIH3T3 cells (Mohibi *et al.*, 2012; Orpinell *et al.*, 2010). Its fungal homologue *ada-3* has been identified as a transcription factor required for cell fusion in *Neurospora crassa* (Fu *et al.*, 2011). Based on these evidences, using RNAi expression of *TADA3/TADA3L* was silenced and silencing verified through qPCR. Percentage of multinucleated cells 48 hours after silencing was significantly lower in A2780 and non-significantly lower in OVCAR3 cells, suggesting a role for *TADA3/TADA3L* in cancer cell fusion. In *Neurospora crassa*, *ada3* mutants produced abnormal developmental phenotypes (Fu *et al.*, 2011). It is possible that *TADA3/TADA3L* also acts as transcription factor that regulates activation or expression of cell fusion genes and proteins in daughter cells of ovarian cancer parent cells that drive the cell fusion following mitosis. Further investigation in this area will help identify downstream targets (cell fusion proteins) of *TADA3*. These results, however, contradict those reported by Mohibi *et al.* (2012). Cells depleted of *Ada3* generated cells with multiple, fragmented and lobulated nuclei (Mohibi *et al.*, 2012). Although the reason is unclear, the discrepancy in results could partly be attributed to: firstly, the cell lines used are completely different, and hence the role the gene plays could also be different especially in cancer cells where all the ‘normal’ pathways tend to work in abnormal ways. And secondly, siRNA technique was employed for silencing *TADA3* in ovarian cancer cells, while other studies have used shRNA (Orpinell *et al.*, 2010) or Cre-mediated deletion of *Ada3* (Mohibi *et al.*, 2012).

6.5 Limitations and future directions

One of the main limitations of this study was capturing time-lapse imaging in bright field mode. Creating stable cell lines with fluorescent-protein labelled nuclei would have enabled observing the process of cell fusion more clearly. Also, instead of measuring

multinucleated cells by eye, a more high-throughput approach such as fluorescence activated cell sorter or flow cytometry would have accelerated the analysis process with larger sample size.

For future experiments, microtubule-labelling should be employed to eliminate the possibility of presence of microtubule linker between daughter cells. Also, further studies could aim to test whether *TADA3* affects expression of already characterised fusion proteins. Metastatic and drug resistance studies could be performed on single and multinucleated cells sorted using a cell sorter. Doing so will help determine *TADA3* as a therapeutic target.

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Supplementary Information

Please note: Supplementary videos from Results - Chapter 5 are available on electronic devices (USB flash drive or CD) provided along with this thesis

Supplementary Video 1 – Cell fusion of A2780 cells. A bi-nucleate parent A2780 cell is going through synchronous tetra-polar mitosis followed by an apparent complete cytokinesis. Two daughter cells then fuse with other two daughter cells giving rise to two bi-nucleate daughter cells (see Figure 5.2 in Thesis).

Supplementary Video 2 – Cell fusion of A2780 cells. A single-nucleated parent A2780 cell goes through bi-polar mitosis producing two daughter cells which later fuse with each other producing a bi-nucleated cell (see Figure 5.3 in Thesis).

Supplementary Video 3 – Fusion of daughter A2780 cell with neighbouring cell. A multinucleated parent A2780 cells undergoes multipolar mitosis producing five to six daughter cells. Four daughter cells were readily seen fusing with each other producing multinucleated daughter cell while the other two daughter cells remained single nucleated. The multinucleated daughter cell later appeared to fuse with a neighbouring cell.

Supplementary Video 4 – Cell fusion of OVCAR3 cells. A bi-nucleate OVCAR3 cell upon synchronous tripolar mitosis and cytokinesis produces three daughter cells and undergo partial cell-fusion whereby two of the three daughter cells were seen fusing with each other while the third daughter cell remained single nucleated (see Figure 5.4 in Thesis).

Supplementary Video 5 – Cell division of SKOV3 cell. A single nucleated SKOV3 cell produces two daughter cells following mitosis. The daughter cells did not fuse with each other after cell division (see Figure 5.5 in Thesis).