Exploring metabolic interventions for CIN cancer therapy

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By

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Thesis Summary

Cancer is one the major diseases with highest mortality rate worldwide. Just in Australia, by 2020 the number of cancer patients is expected to cross 150,000. This is a huge human loss and economic burden not only on the patients and the families, but also on the government. Cancer is the outcome of cell growth anomalies, with a potential for metastasis. Cancer treatments are available but drug resistance and relapse of the disease is very common. One of the factors which promote drug resistance and relapse is chromosomal instability (CIN). CIN is a continuous process of chromosomal reshuffling, which leads to genetic heterogeneity in tumours. This may help tumours to evolve against different stresses like drug treatments. CIN is a common feature of solid and advanced tumours, and has been accepted as one of the hallmarks of cancer. CIN offers a cancer specific target because it is not present in normal cells.

In our lab we have a keen interest in targeting CIN for cancer therapy. For this purpose we have developed different CIN models in *Drosophila melanogaster*. *Drosophila* offers easy and targeted genetic manipulations at any development stage e.g. by RNAi, *in vivo* studies, tissue specific expression, and short with characterized genome. There are *Drosophila* models for several cancers, and CIN models have also been studied in *Drosophila*. CIN models have been developed inducing mitotic defects. In my lab we used *mad2*, *rad21*, *bubR1*, and *brat* inhibition to induce CIN locally in a specific tissue or ubiquitously at different stages of animal development. All these CIN candidates show high levels of CIN when compared to the normal cells and the uniform data from these candidates support the concept of targeting CIN for cancer treatment.

While screening for phosphatases and kinases to kill CIN cells, we found some interesting metabolic candidates which had not previously been described as affecting mitosis, including Pas-kinase and phosphofructokinase. This instigated us to start screening other metabolic alterations which could specifically kill CIN cells. We found metabolic candidates from several important metabolic pathways which when knocked down killed only CIN cells. Moreover, we found that these metabolic alterations cause mitochondrial hyperactivity, oxidative stress, DNA damage, and lipid peroxidation in the CIN background, showing CIN cells are sensitive to redox stress.

I extended the metabolic study to CIN tumours and found a gluconeogenic protein, PEPCK, to be essential for CIN tumour growth. PEPCK deficiency was characterized by a high NADH level, which stopped the tumour growth. Redox stress was also an important factor for PEPCK deficient tumour survival, as when redox stress was relieved the tumour grew. Our results showed that the glycerol-3-phosphate shuttle was responsible for elevated ROS production in PEPCK deficient tumours. High NADH and ROS levels are detrimental for the tumour growth.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Rashid Hussain Dated

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Special thanks to my family, here and in my home country, to bear with me all the time, and their encouragement and struggle.

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List of Publications

- Dawei Liu, Zeeshan Shaukat, Rashid Hussain, Mahwish Khan and Stephen L. Gregory, 2014. *Drosophila* as a model for chromosomal instability. *AIMS Genetics*, 2 (1), pp.1-12.

- Zeeshan Shaukat, Dawei Liu, Amanda Choo, Rashid Hussain, Louise O'Keefe, Robert Richards, Robert Saint and Stephen L. Gregory, 2015. Chromosomal Instability Causes Sensitivity to Metabolic Stress. *Oncogene*, 34, pp.4044-4055.

- Zeeshan Shaukat, Dawei Liu, Rashid Hussain, Mahwish Khan and Stephen L. Gregory, 2016. The role of JNK signalling in response to oxidative DNA damage. *Current drug targets*, 17, pp.154-163.

- Rashid Hussain, Zeeshan Shaukat, Mahwish Khan, Robert Saint and Stephen L. Gregory. PEPCK is needed to maintain glycolysis-driven growth in *Drosophila* tumour. (Submitted for publication)

Abbreviations

17-AAG	17-N-allylamino-17-demethoxygeldanamycin
AICAR	5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide
AP1	Activator protein 1
APC	Adenomatous polyposis coli
APC/C	Anaphase promoting complex/cyclosome
Asp	Abnormal spindle protein
ATM	Ataxia telangiectasia mutated
Aur	Aurora kinases
AURKA	Aurora kinase A / Aurora A
AURKB	Aurora kinase B / Aurora B
BFB	Breakage-Fusion-bridge
BRCA1	Breast cancer 1
BUB	Budding uninhibited by benzimidazoles
BubR1	Bub1-related protein kinase
CD95	Cluster of differentiation 95 (FAS receptor)
Cdc20	Cell division cycle 20 homologue
CDK1	Cyclin B dependent kinase 1
CDKs	Cyclin dependent kinases
CENP-E	Centromere linked motor protein E
Chk2	Checkpoint kinase 2
CIN	Chromosomal instability
DDR	DNA damage response
DNA	Deoxyribonucleic acid
DSB	Double strand break
FOXO	Forkhead box protein O
Fzr1	Fizzy-related protein homolog 1
G6PD	Glucose-6-phosphate dehydrogenase
HEC1	Highly expressed in cancer protein 1
HIF	Hypoxia-inducible factor
Hippo	Hippo (hpo gene), Salvador/Warts/Hippo (SWH) signaling pathway,
HSP90	Heat shock protein 90
IDH	Isocitrate dehydrogenase
JNK	c-Jun N-terminal kinases
k-MT	kinetochore–microtubule
KRAS	Kirsten rat sarcoma viral oncogene homolog

KSP/Eg5	Kinesin spindle protein
Mad2	Mitotic arrest deficient 2
MAPKKK	Mitogen-activated protein kinase kinase kinase
Mps1	Monopolar Spindle 1
MSI	Microsatellite instability
MT	Microtubules
mTORC1	Mammalian target of rapamycin complex 1
MVA	Mosaic Variegated Aneuploidy
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
Nek2	NIMA-related kinase 2
NHEJ	Non homologous end joining
NIMA	Never In Mitosis Gene A
Notch	Notch proteins: a family of transmembrane proteins
p53	Tumour protein (SDS-PAGE: 53 kDa) p53
PAS	Per Arnt Sim
PASK	PAS domain containing serine/threonine kinase
PI3K	Phosphoinositide 3-kinases
PLK1	Polo-like kinase 1
Plk4	Polo-like kinase 4
PP1	Protein phosphatase 1
PTEN	Phosphatase and tensin homolog
Rad21	RAD21 homolog (S. pombe), kleisin subunits of Cohesin Rad21
Rad54	DNA repair and recombination protein RAD54
Rae1	RNA export 1
Ras	Rat sarcoma
Rb	Retinoblastoma
RNAi	RNA interference
Rod	Rough deal
SAC	Spindle assembly checkpoint
Scc1	kleisin subunits of Cohesin Scc1
Smc1	Structural Maintenance of Chromosome 1
Smc3	Structural maintenance of chromosomes 3,
SOD1	Superoxide dismutase 1
STAG3	Stromal antigen 3
TCA	Tricarboxylic acid cycle
Trp53	Transformation-related protein 53
Wnt	From: Wingless gene (*Drosophila*), a homolog of int-1
zw10	Zeste white 10, kinetochore associated protein homolog

Thesis outline

Chromosomal instability (CIN) has been established **as** one of the hallmarks of cancer, which is prevalent in most of the solid and advanced tumours. CIN enhances genetic heterogeneity in cancer cells. This heterogeneity provides selective advantages to cancer cells against the drugs and the therapies, which are linked to poor prognosis and relapse of cancer. Altered metabolism is another hallmark of cancer, which is being targeted for cancer therapy. In this thesis, I have discussed the therapeutic effect of targeting metabolism in CIN cells and CIN tumours**.** **Chapter 1** is my introduction in which I have reviewed cancer, its therapy, CIN, its types, mechanisms, causes, and therapeutic targeting of CIN. I also review cancer metabolism, its targeting for the treatment, and targeting metabolism in CIN cells. **Chapter 2** is a published review article about *Drosophila* being a model for CIN. In this article I have discussed different CIN models and their limitations, then I described *Drosophila* as a model for CIN studies. I later discussed different *Drosophila* CIN model systems which have been studied to understand CIN and cancer. As *Drosophila* has been extensively studied for CIN and cancer therapy, our lab has focused on targeting CIN cells in *Drosophila*. In an earlier study (Shaukat et al, 2012) it was found metabolic candidates such as Pas kinase and phosphofructokinase could be crucial for CIN cell survival. **Chapter 3** is a further screening of metabolic candidates. We found few potential targets from all the major metabolic pathways whose knock down can specifically kill CIN cells. It was found, mitochondrial activity and oxidative stress was high which induced DNA damage and apoptosis in CIN cells targeted by these metabolic alterations. In **chapter 4**, I discuss the application of the selected candidates on CIN tumours. We further explain how one of my metabolic candidates stopped the tumour growth. This chapter also discusses the mechanism of ROS (reactive oxygen species) production and implications of high NADH levels in CIN cells, which was deficient in our earlier studies. **Chapter 5** is my discussion in which I have collectively discussed my results, the significant of my work, my current model, and future directions. In **appendix 1** I have presented a published review article on the role of JNK in response to oxidative DNA damage. This chapter encompasses activation of JNK by ROS, outcomes of JNK in response to ROS. **Appendix 2** has figures for SOX drug and ovary numbers of the hosts.

Chapter 1

INTRODUCTION

1.1- Cancer

Cancer is a disease of abnormal cellular growth and its invasion/metastasis into other body systems. It is a disease of complex and multiple aberrations which ultimately generate cellular neoplastic capabilities and the death of the organism (Meng et al, 2012). Cancer has been a leading cause of death worldwide, nearly 8 million every year. Each year nearly 14 million new cancer patients are reported, and it costs $1.16 trillion on cancer related medicines and treatments in the world. Australia is one of the worst cancer stricken countries. About 30% of deaths in Australia are attributed to cancer, with 4.5 billion AUS dollars spent annually on cancer treatment and management. Soon, by 2020, 0.15 million new cancer cases are expected, here in Australia (Cancer Council Australia).

The most important factor related to cancer development is our lifestyle and a whole list of environmental related agents, which we deliberately or accidentally are exposed to. For lung cancer, 90% of cases are related to tobacco smoke exposure (Biesalski et al, 1998). Liver and digestive tract cancers are related to high alcohol consumption (Dubey and Powell, 2008). Another important factor in cancer development is diet and exercise. About 30-35% of cancer deaths are found to involved physically inactive and obese people (Kushi et al, 2006). Other important factors include: infections, radiation and hormonal dysregulations. Only 10% of cancers are hereditary, including: breast cancer, ovarian cancer, and hereditary nonpolyposis colorectal cancer (Roukos 2009; Cunningham et al, 2010).

1.2- Cancer therapy

Cancer is one of the deadliest diseases across the world. The main problems are unreliable and ineffective therapies for the disease. The most prevalent treatments for cancer are surgery, radiation and chemotherapy (Caley & Jones, 2012). Early stage solid tumours can be removed by surgery but advanced and metastatic tumours cannot be eliminated by surgery. To overcome this issue radiation and chemotherapy is being used. However, radio- and chemo-therapy for cancer can adversely affect patients, sometimes even contribute to their death (Schmidt & Bastians, 2007).

Cancer cells proliferate and thus undergo mitosis a large number of times. Microtubules (MT) are part of the cytoplasmic cytoskeleton which is involved in many cellular processes including mitotic spindle formation and chromosomal separation (Howard and

Hyman, 2003). MT are targeted in some cancer therapies. The most common MT drugs are vinca alkaloids and paclitaxel. Both mitotic poisons bind and inhibit polymerization of tubulin (Ivachtchenko et al, 2007; Sudakin & Yen, 2007). However, MT are very important in other cellular functions apart from proliferation. MT are involved in intracellular transport, cellular shape and integrity. Targeting MT as a cancer therapy has not been very effective, since it produces significant haematological and neural side effects in the patients (Goa & Faulds, 1994; Lobert, 1997; Rowinsky, 1997). Cancer has also been targeted using inhibitors to other mitosis specific proteins, such as polo kinases (PLKs), aurora kinase, centromeric protein E (CENPE) (Jackson et al. 2007; Schmidt and Bastians 2007; Hosoya and Miyagawa 2014). These targets still harbor the disadvantage of drug resistance emergence following cancer therapy. Nearly 90% of the patients treated with MT drugs develop advanced tumours, despite the fact that the drug hinders their growth. Nonetheless, these mitotic drugs are still used in therapy and trials are under way to enhance their effectiveness (Harrison et al, 2009).

Another very common therapy for cancer is using drugs that induce DNA damage. Cancer cells are proliferative and are under high demand for new genetic material; using DNA damaging drugs can cause fatal damage to these highly proliferative cancer cells. These drugs usually trigger the DNA damage checkpoint and spindle assembly checkpoint, inducing cell cycle arrest or cell death in cancer cells. Alkylating agents have been used as DNA damaging drugs, however, these drugs also damage normal proliferating cells and cause permanent damage to their DNA (Hosoya and Miyagawa 2014; Tian et al. 2015). Every proliferating cell should have a DNA damage response; either to rectify it or to eliminate the cell using apoptosis. Cancer cells are a peculiar exception in this case. Most cancer cells have a compromised DNA damage response due to mutations in their DNA damage response genes (Sturm et al, 2003). The most important of these are p53 and retinoblastoma (Rb); most cancers have mutated p53 and/or Rb. Such defective DNA damage responses lead to genome instability, drug resistance and cancer development (Masuda et al, 2003). These mutations make the usage of DNA damaging drugs less favourable for cancer treatment (Bouwman & Jonkers, 2012).

Nearly all cancers can develop drug resistance and adaptation to their microenvironment. In addition to the previously discussed factors that can promote drug resistance and adaptation, another area of current interest in this group is chromosomal instability (CIN).

CIN is a continuous loss or gain of chromosome numbers or their segments. CIN is a common feature of nearly all solid tumours (Mertens et al, 1994), and helps cancer cells progress to metastasis. The presence of chromosomal instability makes these tumours more adaptive to cellular and environmental stresses as well as resistant to multiple drugs, making them difficult to target with regular therapies (Gao et al, 2007; Heilig et al, 2009; Sotillo et al, 2010). CIN, along with aneuploidy has been studied as a possible therapeutic target in cancer cells. Cancer cells can tolerate a high level of CIN and aneuploidy, a feature rare in normal cells. Tang et al (2011) identified proteotoxic stress in CIN cells and targeted them with some metabolic inhibitors, for example heat shock protein 90 (Hsp90), and found that inducing AMPK (5'AMP activated protein kinase) can restrict aneuploid cell growth.

Altered metabolism has emerged as a new hallmark of cancer. The Warburg effect is one of the most studied metabolic characteristics of cancer cell metabolism, which describes cancer cells being glycolytic in aerobic conditions and less dependent upon oxidative phosphorylation (OXPHOS) for energy production (Heiden et al, 2009). Targeting glycolysis using glycolytic inhibitors like 2-DG (2- deoxyglucose), TEPP-46, FX11 and STF-31 (a Glu-1 inhibitor) has shown some promising results in tumour suppression. However, recent studies emphasize the importance of OXPHOS for cancer cell existence and development, which earlier had been underestimated. High levels of TCA enzymes (e.g., succinate dehydrogenase, and fumarate hydratase), mitochondrial glutaminolysis and ROS suggest a much more significant role for mitochondria than anticipated. An important factor in altered metabolism is its implications for genetic regulation in cancer cells. 70% of human cancers have over expressed glycolytic genes (Altenberg and Greulich, 2004). Mutated mitochondrial genome, p53 inducible regulation of cytochrome c oxidase (COX), higher ROS, and dysregulated HIF are some of the genetic links related to altered metabolism in cancer (Tran et al, 2016). These extensive studies suggest the possibility of targeting the altered metabolism in tumours.

Cancer cells are difficult to distinguish from normal cells, so finding a suitable target and hitting it in cancer cells has not been very fruitful. Rather, proliferative normal cells are prone to be tumourigenic with cancer treatments (Caley & Jones, 2012). Many cancer treatments increase genetic heterogeneity, promoting drug resistance and relapse of most tumours.

In this thesis, I have explored the possibility of targeting CIN tumours with metabolic therapy. I discuss CIN and altered metabolism and their possible implications for tumour treatment. I looked for possible links between CIN and altered metabolism. We already have reported the selective killing of CIN cells by a metabolic change (Pas-kinase). $Pask^{RNAi}$ not only induced apoptosis in CIN cells but also induced more DNA damage, high ROS, and more mitochondrial dysfunction (Shaukat et al, 2012). The exact mechanism for these CIN specific outcomes was not clear, still it encouraged us to target metabolism in CIN cells. For this purpose first we targeted a range of metabolic enzymes in CIN cells and then verified the results in CIN tumours. *Drosophila melanogaster* was used a model organism to target metabolism in both CIN cells and CIN tumours.

1.3- Chromosomal instability

Chromosomal instability has emerged as one of the hallmarks of cancer. It is a continuous process of shuffling and reshuffling of a genome, which helps in tumourigenesis and its progression. It can start with just an allelic imbalance or a whole chromosome missegregation (Bardi et al, 1997). CIN produces heterogeneity which is the driving fuel for tumour evolution and drug resistance as CIN is linked with poor prognosis, metastasis, and adaptability to environmental and chemical stresses (Carter et al, 2006; Choi et al, 2009).

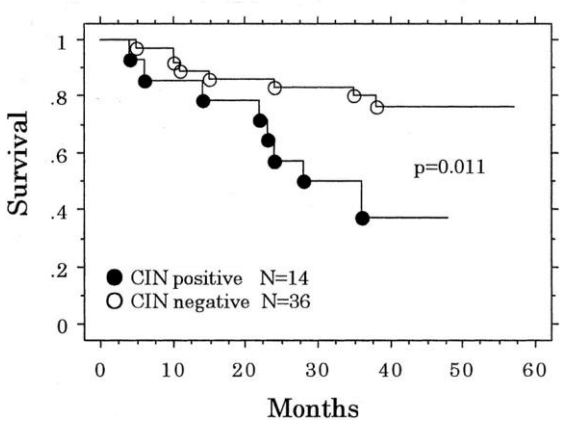

Figure 1.1: *Kaplan-Meier survival curve of NSCLC (Non-small cell lung cancer), according to CIN status.* [Taken from (Nakamura et al, 2003)]

Figure 1.1 shows survival rate of cancer patients with and without CIN. Patients with CIN positive cancer have less survival curve than the patients without CIN. Figure 1.2 describes CIN is often conflated with aneuploidy but they are different terms, although

CIN causes aneuploidy in tumours. CIN involves the constant process of genomic rearrangement while aneuploidy can describe a stable phenotype.

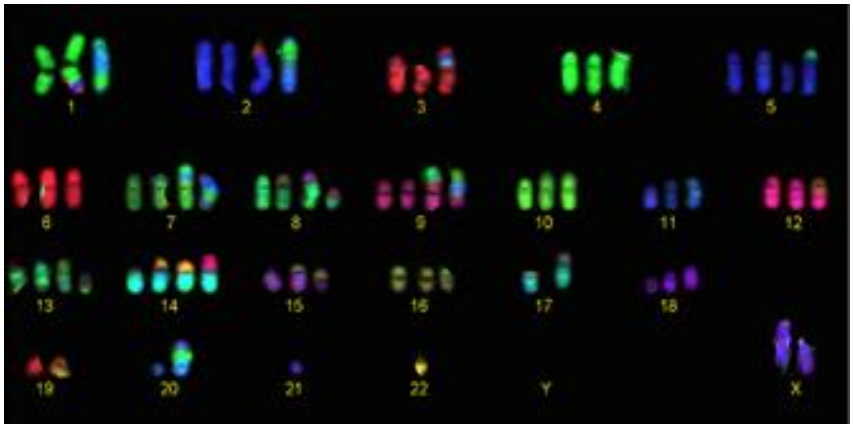

Figure 1.2: *Karyotype of a typical CIN cancer cell (osteosarcoma cell line), showing structural and numerical CIN.* [Taken from (Jansson & Medema, 2013)]

The figure 1.2 is showing two types of CIN, structural and numerical CIN. Structural CIN arises due to chromosomal alterations of different regions of chromosomes. These may occur due translocations, insertions, deletions and amplifications of DNA (Mitelman et al, 1997). Whereas, numerical CIN arises due to gain or loss of whole chromosomes (Rajagopalan et al, 2003). These events may lead to over-expression of oncogenes or knockout tumour suppressor genes which are linked to tumourigenesis (Thompson & Compton, 2011)

Data from the Mitelman database shows about 86% of solid tumours and 72% of hematopoietic cancers have chromosomal aberrations, including segmental and numerical. Zasadil *et al.,* (2014) calculated CIN and aneuploidy from this database. They calculated, about 44% of solid tumors and 14% of hematopoietic cancer cells were CIN positive while the rest of the cancer cells were aneuploid. In colorectal cancer with CIN, 10-100 times more loss or gain of chromosome segments per generation is observed (Maria and Chung, 2010).

Aneuploidy is also related to tumour outcomes but there are several aneuploid diseases that do not predispose to cancer, and tissues like some hepatocytes are naturally aneuploid. The most common outcomes of aneuploidy are proteotoxic stress and redox stress (Jefford and Irminger-Finger, 2006). Proteotoxic stress is caused by the excess of

proteins produced by additional chromosomes. This produces protein aggregates in the aneuploid cells which compromise chaperones e.g., Hsp90 (Ohashi et al, 2015; Oromendia et al, 2012). This stress initiates intricate energetic dysregulation like PI3K activation, high glucose uptake, autophagy upregulation, and high ROS which can affect cell proliferation (Jin et al, 2014). However, these data still do not show if aneuploidy could cause tumours.

1.4- Mechanisms of Chromosomal instability

Conventionally, CIN is linked to segregation errors of chromosomes during mitosis caused by aberrations in the mitotic spindle assembly checkpoint (SAC), kinetochore attachment to microtubules or centrosomal dysfunctions (Gollin, 2005; Thompson et al, 2010). Errors in DNA repair and recombination can also lead to segmental and allelic CIN in proliferative cells (Pino and Chung, 2010). In addition, DNA replication stress and the state of cellular metabolism are also reported to be related to CIN (Bristow & Hill, 2008; Burrell et al, 2013). All these CIN inducing factors are characterized by lagging chromosomes or chromosomal bridges, during anaphase of a mitosis (Thompson et al, 2010). These CIN inducing factors are related to the spindle assembly checkpoint (SAC) of mitosis which ensures proper chromatid separation. The SAC prevents an early onset of anaphase and ensures proper alignment and attachment of chromosomes to the spindles (Musacchio, 2007). It senses tension at kinetochores when spindles are pulling apart during a metaphase. Any aberration during this tension sensing process can shorten or lengthen the metaphase which can lead to apoptosis, if not resolved. This tension sensing process is ensured by proper bi-directional alignment of chromosomes on the spindle and prevents anaphase until the proper attachment is done. (Li et al, 2010; Mukherjee et al, 2011). Any deviation from alignment or the bi-directional model allows the SAC pathway to delay metaphase (Chi et al, 2009). Resolving the deviations will deactivate the SAC and the mitosis will enter anaphase by the separation of sister chromatids (Mitelman et al, 2010). The SAC has two major types of proteins: Core and Auxiliary proteins. Core proteins include a Mitotic Checkpoint Complex (MCC) which includes Mad2, Bub3 and BubR1 and an Anaphase Promoting Complex/cyclosome (APC/C), its co-factor Cdc20 and downstream proteins cyclin B, securin, 26S proteasome and separase which finally segregates two sister chromatids (Habermann et al, 2009). Auxiliary proteins include Mad1, Bub1, Mps1 and AuroraB (Choi et al, 2009). These

proteins amplify SAC activity and accelerate MCC formation. During pro-metaphase the proteins, core and auxiliary, concentrate at the kinetochore and help in the proper attachment of chromatids to microtubules. SAC activity primarily involves activation of the MCC which inhibits the interaction of cdc20 with the APC/C. APC/C without its effector Cdc20 is unable to polyubiquitinize cyclin-B and securin (Niikura et al, 2007). The presence of cyclin-B and securin keeps an optimum level of CDK1 and inactive separases during metaphase. Degradation of cyclin-B and separase will decrease the level of CDK1 and activate separases, and anaphase will be activated (Fang et al, 2006). Interestingly, both hypo- and hyper-activity of the SAC is tumourigenic. Hypo-activity of the SAC will not detect any metaphase abnormality and mitosis will enter anaphase without proper chromosomal segregation, while hyper-activation of the SAC will inhibit anaphase entry and sister chromatids will not be separated. Both hyper and hypo activation of the SAC produces CIN (Yang et al, 2012).

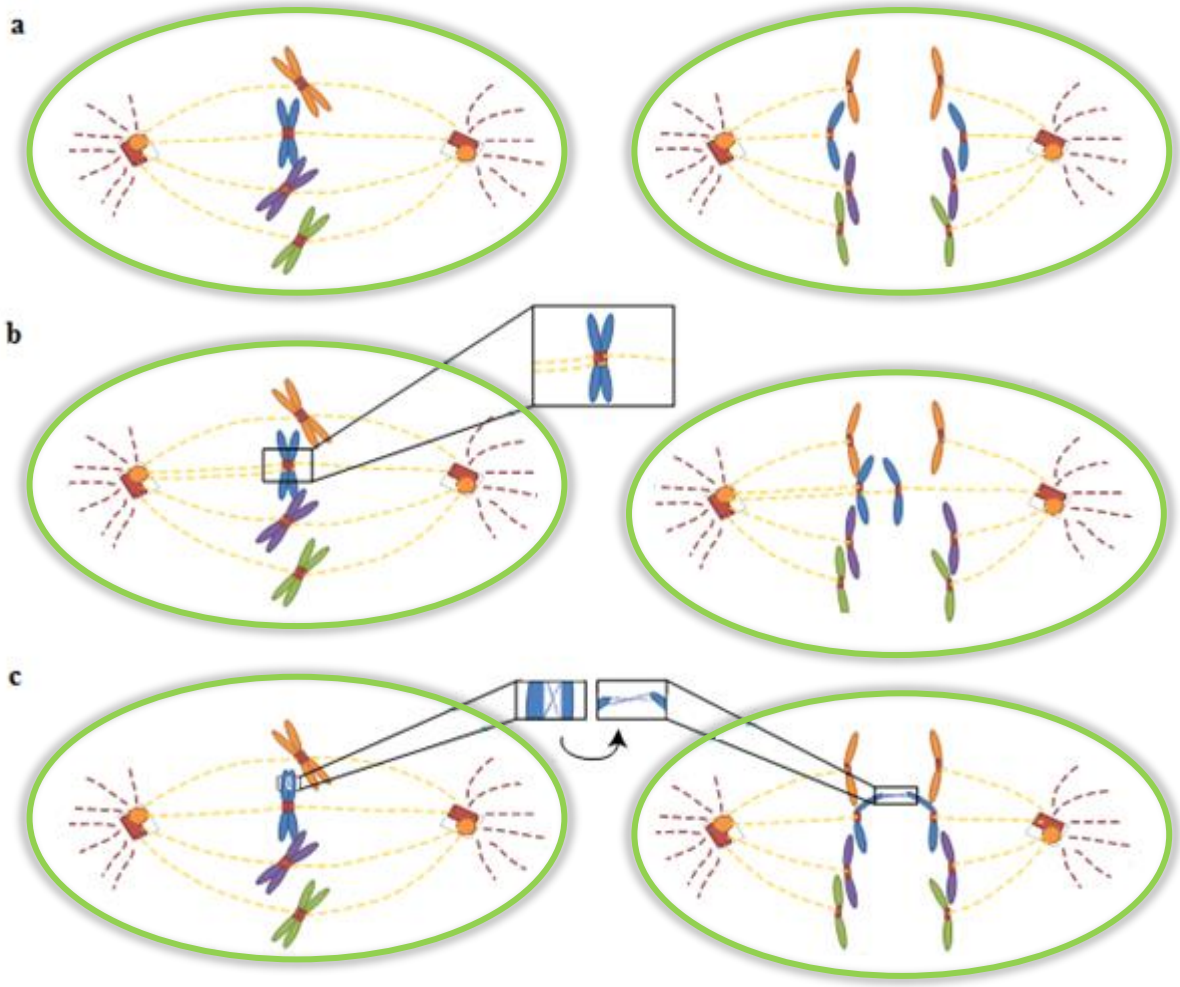

Figure 1.3: *Aberrant spindle-chromosome attachments and chromosomal bridges lead to chromosomal instability. a) Normal chromatid separation during Anaphase, b) a centromere attached to both the spindle poles may cause lagging chromosomes, c) ends of separating chromatids are unresolved, resulting in the formation of a chromosomal bridge.*

1.5- Causes of Chromosomal instability in Cancer

CIN is created by mechanisms which reduce the fidelity of mitosis (Shen et al, 1998). These mechanisms include defects in chromosome cohesion, spindle assembly checkpoint (SAC), centrosome copy number, kinetochore-microtubule attachment dynamics and cell-cycle regulation (Macklon et al, 2002). These mitotic infidelities can induce either whole chromosome segregation errors (W-CIN) or segmental chromosome segregation errors (S-CIN).

1.5.1- Cohesion Defects

Sister chromatids are attached together during mitosis. The cohesin protein complex participates in this sister chromatid cohesion (Nasmyth, 2011). Any defect in sister chromatid cohesion can either delay sister chromatid disjunction or cause a premature separation. These two aberrant processes can result in unequal chromatid distribution in the daughter cells. This partial or premature cohesion failure may results in numerical CIN (Figure 1.3), while a complete failure of the cohesion complex may result in tetraploidy in one the daughter cells (Thompson et al., 2010). Mutated cohesion genes have been identified in human cancer, and when expressed in yeast these induced CIN. These genes include *Smc1, Smc3, Scc1, Stag1, Stag2, Stag3* and *separase* which are involved in cohesion of sister chromatids (Jallepalli et al, 2001; Yu et al, 2003; Wirth et al, 2006; Barber et al, 2008; Zhang et al, 2008; Iwaizumi et al, 2009; Mannini & Musio, 2011; Xu et al, 2011). Furthermore, members of the core cohesion complex (i.e. Scc1 and Smc3) and cohesion regulators (i.e. separase) are overexpressed in CIN positive cancers (Mannini & Musio, 2011).

1.5.2- Merotelic attachments.

Lagging chromosomes are another mechanical reason for CIN development. These lagging chromosomes result due to merotelic attachment of chromosomes. During mitosis a chromatid can get attached to microtubules of both the spindle poles; ideally each

chromatid should be attached to microtubules of its respective spindle pole. These lagging chromosomes may stay on the metaphase plate without being separated. An anaphase is normally accomplished after sister chromatid separation. In these merotelic

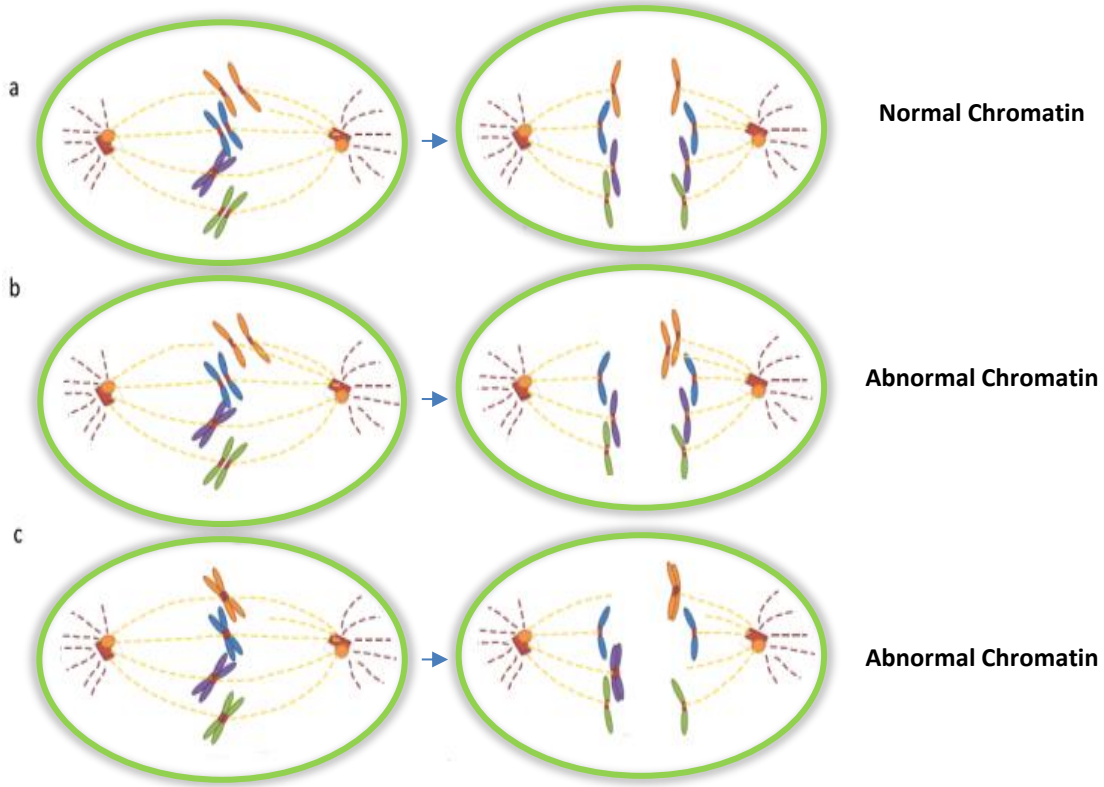

Figure 1.4: *Cohesion defects lead to chromosomal instability*; *a) normal sister chromatid separation b) a pair of sister chromatids erroneously moved to one spindle pole, causing aneuploidy c) two pairs of chromosomes wrongly moving towards one spindle pole, causing aneuploidy for the respective chromosomes.*

conditions, anaphase is not accomplished and the affected chromosomes are left at the metaphase plate waiting to be separated apart. These merotelic conditions are undetectable by the SAC, because both chromatids have some correct microtubule connections which generate some tension which is necessary to keep them on the metaphase plate waiting to be separated (see figure 1.4) (Gregan et al, 2011; Holland & Cleveland, 2012). To complete a cell cycle these abnormalities may end abruptly, with a chromatid on wrong daughter cell, making daughter cells aneuploid. However, if merotelic chromatids remain at the metaphase plate and fail to detach from each other, it may end in the formation of a micronucleus (Lampson et al, 2004; Pinsky et al, 2006). Two main causes of these merotelic attachments are kinetochore-microtubule attachment instability and centrosome amplification.

1.5.2.1- Kinetochore-microtubule attachments

The kinetochore is a protein complex at the centromere which anchor and attaches chromatids to microtubules of a spindle assembly. Attachment of kinetochores to multiple spindle poles and a weakened ability of the cell to resolve this problem can potentially lead to the merotelic condition. These hyper stable kinetochore-microtubule attachments have been a characteristic of CIN cancer cells (Thompson & Compton, 2008; Compton, 2011). Anything which weakens or hyper stabilizes kinetochore-microtubule attachments can trigger CIN. The weakening of kinetochore-microtubule attachments may shorten mitotic time to correct the errors and a delay may result in abrupt termination of anaphases without proper chromatid segregation or aneuploidy. The best example comes from *Mad2* protein. *Mad2* is a component of the spindle assembly checkpoint (SAC). Unattached kinetochores attract Mad2 to form a mitotic checkpoint complex. Its over expression and deficiency can produce merotelic attachments (Tanaka et al, 2001; Sotillo et al, 2007; Kato et al, 2011; Kabeche & Compton, 2012). Similarly, *BubR1* (Bub1-related protein kinase), *Aurora kinase B* (aurora B) and *protein phosphatase 1* (PP1) are involved in the regulation of kinetochore-microtubules dynamics (Cimini et al, 2006; Emanuele et al, 2008; Liu et al, 2009).

1.5.2.2 Supernumerary Centrosomes

Centrosomes are a major organelle in a cell which regulates the cell cycle. Centrosomes serve as the main microtubule organising centres (MTOC) which maintain microtubule organization and the bipolar symmetry of the cell, during mitosis. Defects in centrosomal activity create chromosomal segregation defects, which can lead to aneuploidy and CIN. The link between supernumerary centrosomes and tumourigenesis has long been studied, since Theodor Boveri in 1914. The most common centrosomal defect in cancers is multiple centrosomes. This supernumerary condition can lead to attachments of kinetochores to multiple centrosomes.

Extra centrosomes may extend mitotic timings, not satisfying the SAC, resulting in the CIN phenotype. (Boveri, 1914; Pihan et al, 1998; Lingle et al, 2002; Anderhub et al, 2012). Interestingly, at anaphase these multiple centrosomes typically cluster in such a way that leads to bipolar spindle formation. This increases the chances of merotelic

attachments which may cause defects in chromosomal segregation.(Brinkley, 2001; Quintyne et al, 2005; Basto et al, 2008).

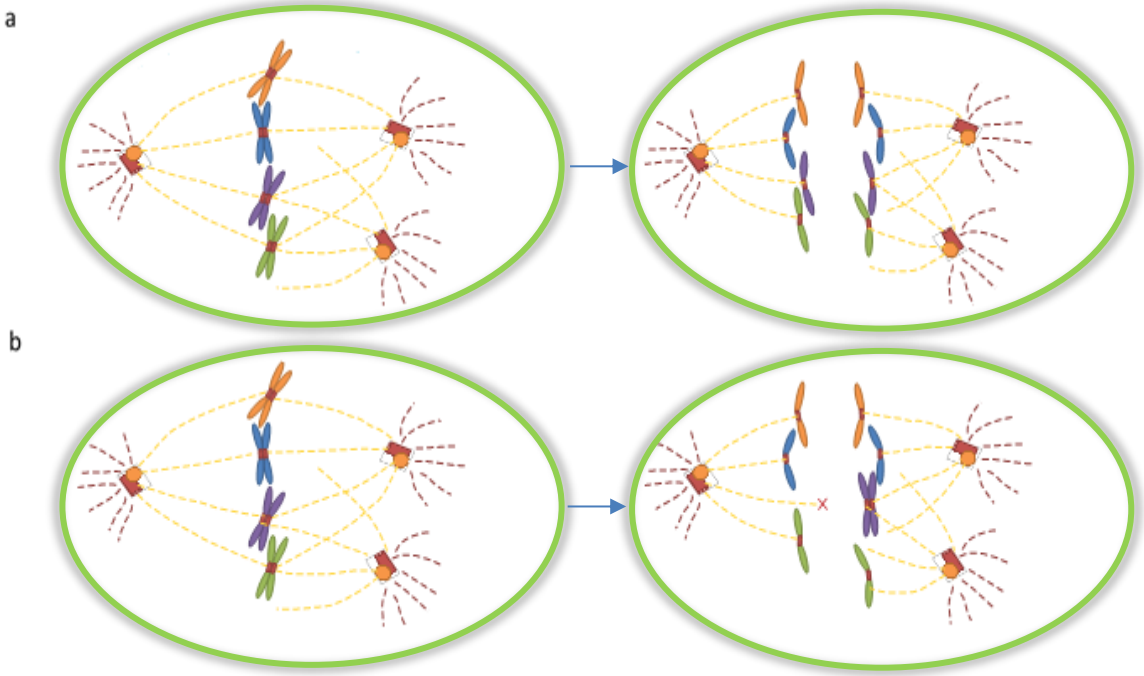

Figure 1.5: *Multi-polar spindle and merotelic attachments lead to chromosomal instability. a) Extra centrosomes that cause multiple attachments of sister chromatids to two or more centrosomes may delay mitosis but resolve normally, b) Multiple centrosome attachments may cause aneuploidy.*

1.5.4 Telomere dysfunction

Mitosis is a complicated chain of events. It not only divides cells and segregates chromosomes but also protects chromosomes from any damage. Telomeres are proteinaceous hexameric repeats which stretch along and protect the chromosome during DNA replication. Telomeres themselves are protected by shelterin proteins and telomeric encoded RNA (Pino and Chung, 2010). Loss of telomeric protection can lead to chromosomal breakage-fusion-bridge (B/F/B) cycles which if continued for many generations can lead to gross chromosomal rearrangements (O'Hagan et al, 2002). These chromosomal changes can lead to tumour formation, as is seen in mice with *Terc*$^{-/-}$ (an RNA component of telomerase) which leads to tumour formation (Rudolph et al, 2001). Colorectal cancer usually shows shortened telomeres (Gertler et al, 2004). B/F/B cycles can also lead to larger telomeres in some cases. Metastasis in some colorectal tumours, such as Dukes C and D tumours, has been attributed to longer telomeric length (Engelhardt et al, 1997).

1.5.5 DNA damage response

DNA is in constant danger of damage during replication, transcription and from non-cellular factors such as toxins. A strong DNA damage response is required to prevent further damage. If compromised, DNA damage response failure can lead to cancers. Mutations in ataxia-telangiectasia (*ATM*), Seckel (*ATR*), Li-Fraumeni (*TP53*), and hereditary breast-ovarian cancer (*BRCA1* and *BRCA2*) genes are linked to tumour development in humans (Khanna and Jackson, 2001). In vivo studies show a direct link between DNA damage response malfunctioning and CIN. Haplo-insufficiency of histone *H2AX* in a p53-deficient background can compromise genomic integrity, whereas mutants of *ATM* and *H2AX* in mice show chromosomal instability (Bassing et al, 2003; Zha et al, 2008).

1.6- Chromosomal instability as cancer target

CIN cells are genetically heterogeneous, which may provide a selective advantage to CIN tumour cells. The CIN is associated with drug resistance, metastasis and relapse of tumours. This makes curing CIN tumours a challenging job. However, it also provides an opportunity for CIN specific cancer therapy, because CIN is specific to cancers, and found with a high frequency in cancer cells (Swanton et al, 2011).

CIN can also induce apoptosis in cancer cells. Further exacerbating CIN levels in already CIN cancer cells can kill CIN cancer cells (Costa and Lengauer, 2002). The anti-mitotic drug paclitaxel may accomplish this task by inhibiting Mps1 and Bub1b in CIN cancer cells. This drug increases segregation errors in CIN cancer cells and then induces apoptosis in cancer cells with a higher mis-segregation rate (Janssen et al, 2009). Aurora B has similarly been used in targeting CIN cancer therapy (Payton et al, 2010; Tsuboi et al, 2011). Its deficiency induces more CIN which leads CIN cancer cells to cell death (Torres et al, 2007; Weaver & Cleveland, 2007; Birkbak et al, 2011; Pfau & Amon, 2012).

As discussed previously, in cancer cells CIN may produce genetic heterogeneity. Cancer cells can take advantage of this genetic diversity to evolve against drugs to acquire resistance. This drug resistance offers better survival to the cancer cells against the drugs and makes it difficult to target the cancer (Swanton et al, 2009; Sotillo et al, 2010). CIN levels are higher in malignant tumours than in benign ones (Yunis, 1983; Mitelman et al,

1997), and cancer cells can tolerate high levels of CIN compared to normal cells (Mitelman et al, 1997; Campbell et al, 2010; Janssen & Medema, 2011), so it offers an attractive target for a cancer-specific therapy.

Many approaches for targeting chromosomal instability for the treatment of cancer are currently in preclinical stages (Cimini et al, 2006; DeLuca et al, 2006; Bakhoum et al, 2009; Thompson et al, 2010). These include the manipulation of CIN levels in order to get a better prognosis and, to generate sensitivity to current therapies. Alternatively, CIN cells can be targeted by altering the mechanisms which cancers use to tolerate high levels of CIN or CIN related stresses (Manchando et al, 2012).

Kinetochores as a regulator of microtubule attachments to chromosome have been considered as a potential therapeutic target to modify levels of missegregation in cancer (Cimini et al, 2006; Bakhoum et al, 2009; Bakhoum et al, 2009b). Alterations in the stability of microtubules also affect the process of segregation. Hyper-stability of microtubules results in an increase of the chromosomal missegregation rate (Liu et al, 2009). In contrast, destabilization of attached microtubules up to a certain extent leads to a decrease in chromosomal instability which results in better prognosis (Bakhoum et al, 2009; Bakhoum et al, 2009b).

Although induction of CIN could induce apoptosis in cancer cells, it could also promote the evolution of drug resistance and relapse. A few studies have been done to manipulate CIN levels in tumours for cancer therapy, with other cancer therapies. Telomerase inhibition can increase CIN up to moderate levels and increases the efficacy of conventional anti-cancer drugs (Dome et al, 2005; Stewenius et al, 2007). CENP-F (Centrosome protein-F) deficiency also increases the efficiency of anti-cancer drugs, although its deficiency alone causes spleen and lung tumour formation. Similarly, PTEN exacerbates the CIN condition in Bub1 deficient mice (Weaver et al, 2007). The SAC malfunction increases the CIN rate, increasing tumourigenesis in cancer. Other major CIN induction mechanisms involve supernumerary centrosomes, which increase the chances of missegregation and thus the CIN rate. Mutations that alter the centrosomal duplication or bipolar clustering of extra centrosomes have been used to target cells with pre-existing centrosome-related CIN (Rebacz et al, 2007; Kwon et al, 2008; Mazzorana et al, 2011; Kawamura et al, 2013; Korzeniewski et al, 2013).

These studies support targeting CIN for cancer therapy by manipulation of the CIN levels in tumours. However, this strategy has a basic flaw. Although drugs used to induce CIN can be pro-apoptotic in severely affected CIN cells, these drugs can be beneficial to non-CIN cancer cells which are present in a tumour. These non CIN cells can become CIN cells over a time period and may adapt to the environment and the drugs, resulting in relapse and drug resistance (Gao et al, 2007; Heilig et al, 2009; Sotillo et al, 2010). These drugs can also induce missegregation in normal cells, increasing further chances of tumourigenesis (Goa & Faulds, 1994; Lobert, 1997; Rowinsky, 1997).

CIN cancer cells can be studied to identify mechanisms which help them to tolerate high levels of CIN. CIN cells have proteotoxic stress, with elevated levels of HSP90 (Chen et al, 2012; Donnelly and Storchova, 2015). An inhibitor of HSP90, 17-AAG, suppresses tumour growth in colon cancer. In a study to find targets to kill CIN cells specifically, a large number of genetic candidates were selected which when knocked down in CIN cell killed them, leaving normal cells intact. The genes identified included centrosomal, JNK pathway, DNA damage, Wnt signalling pathway, histone kinases, MAPK, metabolic and apoptotic genes (Shaukat et al, 2012).

CIN is present in most solid and advanced tumours and has the potential to be a therapeutic target in cancer cells. Inducing CIN by itself in tumours may increase the chances of drug resistance and relapse. If CIN cells are studied for their biochemical and genetic changes and these changes are targeted in CIN cells, this may increase the chances of cell death in CIN tumours. This would be more accurate and specific than simply increasing the CIN systemically.

1.7- The outcomes of chromosomal instability

Chromosomal instability has become a hallmark of cancer since the identification of aneuploidy in cancer cells (Bakhoum et al, 2012). About 85% of solid tumours, and 90% of advanced tumours show the CIN phenotype (Mertens et al, 1994). The accepted outcome of CIN is tumourigenesis but the explanation of how cancer is induced by the CIN is still under investigation (Giam et al, 2015).

Experimental evidence shows the involvement of CIN in tumour evolution and development. The overexpression of spindle assembly checkpoint proteins like Mad2 leads to CIN. Both hypo- and hyper-activation of Mad2 are CIN inducing. A mouse with

either genotype has shown an increased rate of tumourigenesis (Birkbak et al, 2011). Neoplastic tumours developed in mice with Mad2 over-expression (Schvartzman et al, 2011). A large number of mouse models have been tested for CIN induced tumourigenesis, such as Bub1, Bub3, BubR1 and Rad21 (Schliekelman et al, 2009; Solomon et al, 2014). Furthermore, in another mouse study, over-expression of hec1 (a kinetochore-microtubule stabilizing protein) also induced tumours in lung, liver, and pancreas (Diaz-Rodriguez et al, 2008). However, most of the studies were done in deletion of tumour suppressor background, e.g., loss of p53, p21. Loss of tumour suppressor genes can provide tolerance to aneuploidy in CIN cells (Bakhoum et al, 2012).These results support the role of CIN in tumourigenesis but the question whether CIN initiates a tumour is still open.

Tumour initiation investigations in human patients with mosaic variegated aneuploidy syndrome (MVA),which is related to defects in either BubR1, a SAC protein or Cep57, a centrosomal protein, and they show aneuploidy, developmental defects and childhood cancer (Hanks et al, 2004; Snape et al, 2011; Garcia-Castillo et al, 2008; Suijkerbuijk et al, 2010). Tumourigenesis induced by MVA supports the notion of CIN initiating cancer but the high aneuploidy rate and other non-cancerous defects in MVA patients demand more explanation than just the involvement of the CIN. Unlike the question about CIN initiating cancer, the question about CIN's role in tumour progression is generally straight forward; CIN increases tumour growth rate as along as apoptosis is inhibited (Bakhoum et al. 2012). Lung tumours driven by the Kras oncogene did not stop growing when the oncogene was inhibited if CIN was being driven by Mad2 overexpression (Thompson et al, 2010).

The fact that CIN is involved in tumourigenesis is being challenged by its anti-tumourigenic role. CIN kills liver tumours, though this might be due to the fact that liver cells are already aneuploid and by adding CIN it killed them (Weaver et al, 2007). This makes CIN more tissue specific and suggests it might kill tumours that are already moderately or highly aneuploid by increasing the deleterious effects of chromosome missegregation (Bakhoum et al, 2012).

Another important outcome of CIN is drug resistance (Giam and Rancati, 2015). This is an important aspect of cancer clinical practices which leads to relapse of the tumour. CIN+ and CIN- colorectal cancer cell (CRC) lines have been studied for their drug

resistance. CIN+ CRC lines were multi-drug resistant compared to the CIN- CRC lines. Similarly CIN+ CRC represented worse progression free survival relative to CIN- CRC disease (Lee at al, 2011). In a study to investigate drug resistance in CIN cells it was found that NEK2 (a centrosomal protein) over expression is involved in drug resistance. NEK2 overexpression enhances CIN and cell proliferation (Zhou et al, 2013).

Mps1 mutants in a p53 heterozygous background increase metabolic stress response proteins. Although the authors did not specifically look into the metabolic profiles, they found high transcriptome levels of biosynthetic processes, RNA metabolism, cellular metabolic processes and gene regulatory processes (Foijer et al, 2014). CIN can lead to unbalanced karyotypes which can affect cellular physiology and the disease status (Oromendia et al, 2012). These unbalanced karyotypes can initiate a proteotoxic stress response in aneuploid cells because loss or gain of chromosomes may affect protein folding, assembly of multiprotein complexes and the proteasome, and hence the protease capacity of aneuploid cells (Tyedmers et al, 2010; Houck et al, 2012). It was found that aneuploid cells have high activity of Hsp 104 - a chaperone, which was also true for the aneuploid cell arising from meiotic or mitotic chromosomal missegregation. Similarly some disomic yeast strains were prone to more death when Hsp90 inhibitors were used (Oromendia et al, 2012). This extra load of proteotoxic effect requires ATP and this demands more efficiency of metabolic processes (Ohashi et al, 2015). A critical factor which increases with these metabolic stresses is redox stress (Oromendia et al, 2012).

Metabolism is one of the hallmarks of cancer (Shaukat et al, 2014). Trisomic mouse embryo fibroblasts (MEF) do not show a high degree of change in glucose consumption and lactate production but glutamine consumption is high in these CIN cells. Similarly, amino acid production increased in all trisomic forms of MEF. (Williams et al, 2008). This supports the notion that these CIN cells were depending upon the Krebs cycle for their energy and biosynthetic pathways. Contrary to these findings in vertebrate cells, carbohydrate metabolic genes were upregulated in aneuploid yeast cells. When glucose consumption measured it was also increased for yeast CIN cells (Torres et al, 2007). In an attempt to kill CIN cells, Pask and Pfrx were identified as metabolic targets. Loss of Pask, a regulator of glucose metabolism, led to elevated mitochondria activity, ROS generation and apoptosis in CIN cells (Shaukat et al, 2012).

In a study on lung cancer associated fibroblasts (LCAF) it was shown that LCAF were under stress for overall metabolites compared to normal cell lines. About 203 metabolites were identified and measured using mass spectrophotometry. Peptides and amino acids were the factors raised in LCAF while carbohydrate and nucleotides were lower in LCAF than the normal cell lines (Chaudhri et al, 2013). This data suggest LCAF might be glucose deficient and using glutaminolysis for their metabolism. A similar kind of data was developed for colorectal cancer tissue in which there was a difference of metabolites between normal and the cancer cells. Nucleotides like adenine were lower in the colorectal tumour, primary and metastatic cancer samples. Amino acid derivatives like carnitine were two times higher in the colorectal tissue. Importantly, glutathione levels were high in the cancer tissue (Williams et al, 2015), suggesting a need for anti-oxidants. CIN cells are under redox stress (Kopes et al, 2015) which instigates proteo-toxicity (Oromendia et al, 2012) which further increases ROS production activating various metabolic pathways like JNK (Vallerie and Hotamisligil, 2010).

1.8- Cancer metabolism

Otto Warburg witnessed a high glycolytic rate with increased lactic acid fermentation in cancer cells (Warburg, 1956). This discovery paved the way for metabolic studies in cancer cells. Continuous research over a long period of time has shown metabolic alterations/rewiring in cancer cells. Although these metabolic alterations are not uniform, nonetheless altered metabolism is one of the hallmarks of cancer. These metabolic alterations encompass a wide range of cellular biochemistry, however, their main target is to maintain energy, biomolecules, and redox balance for the proliferation of cancer cells (DeBerardinis and Chandel, 2016).

Higher metabolic rates are linked to higher likelihood of cancers. Major metabolic pathways are depicted in Figure 1.6. According to Peto's paradox (Caulin and Maley, 2011) big animals like whales and elephants having lower metabolic rates have a lower incidence of cancer, while the mouse, on the other hand, having a higher metabolic rate, has a 40% lifetime frequency of cancer despite its short lifespan. The most obvious explanation is the occurrence of somatic mutations in oncogenes and tumour suppressor genes in response to a more rapid metabolism. Higher ROS levels may be the main source of these mutations, as an outcome of higher metabolic rates (Dang, 2012). Beside ROS, insulin insensitivity is a normal physiological process which can lead to tumourigenesis,

by dysregulating glucose and its associated metabolism (like the PI3K-AKT pathway) (Khandekar et al, 2011). In a number of different animal models, lowering metabolic rates by restricting caloric intake inhibits tumourigenesis (Hursting et al, 2010). Nevertheless, mutations in the genes of some of the metabolic pathways, e.g., PI3K, can make the tumour cell resistant to the caloric restriction by the over-activation of the energy pathway (Kalaany and Sabatini, 2009).

Whether or not metabolic changes lead to the initiation of cancer is still an open question, but metabolic changes have become one of the hallmarks of cancer. Here, I will be discussing different metabolic platforms which cancer cells use for their efficient survival and replication.

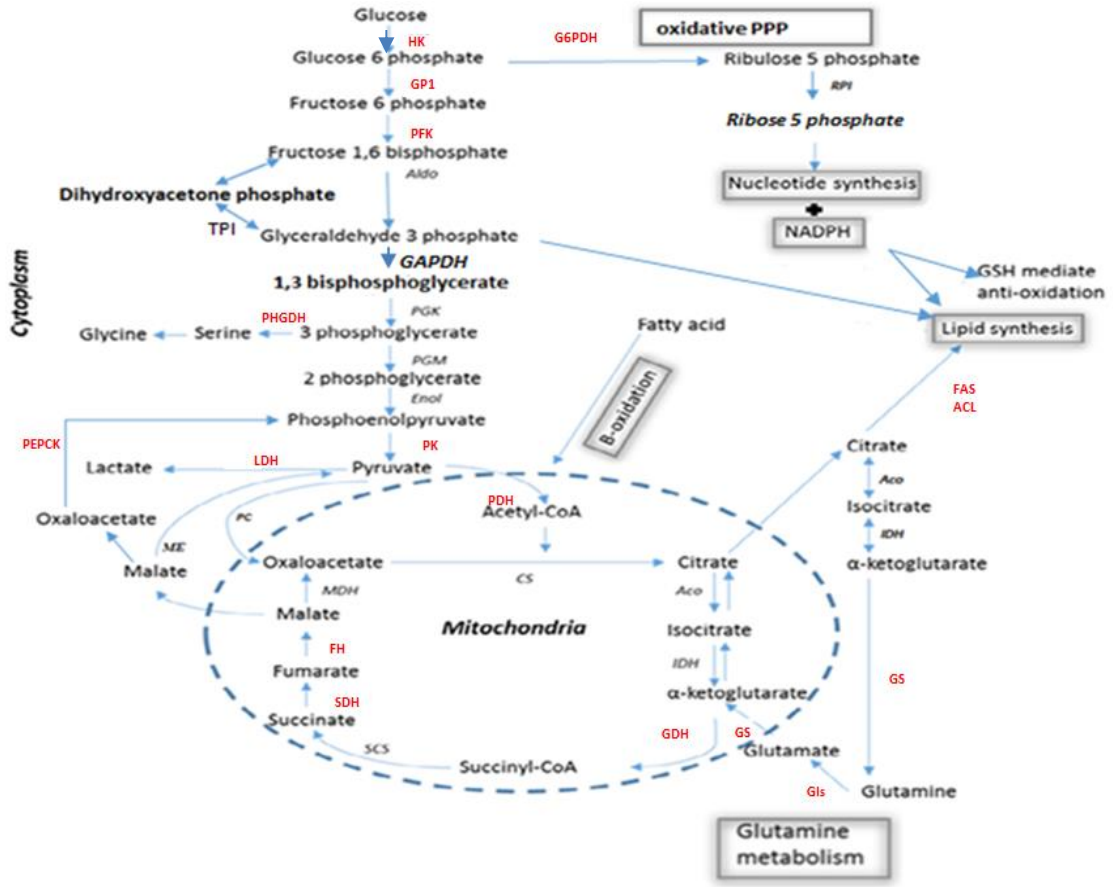

Figure 1.6: *Major pathways of central carbon metabolism and key enzymes commonly dysregulated in cancer cells. Important pathways implicated in cancer cell proliferation are indicated, including glycolysis, TCA cycle, nucleotide synthesis, anti-oxidant, lipid synthesis, glutamine metabolism and beta oxidation. Enzymes known to be dysregulated in some cancers are shown in red text. Abbreviations: Aco = Aconitase, KGDH=α - ketoglutarate dehydrogenase, Aldo = Aldolase, ALT = Alanine transaminase, AST = Aspartate transaminase, CS = Citrate synthase, Enol = Enolase, FAS = Fatty acid*

synthase, FH = Fumarate hydratase, G6PDH = Glucose-6-phosphate dehydrogenase, GAPDH = Glyceraldehyde-3-phosphate dehydrogenase, GDH = Glutamate dehydrogenase, Gls = Glutaminase, HK = Hexokinase, IDH = Isocitrate dehydrogenase, LDH = Lactate dehydrogenase, MDH = Malate dehydrogenase, ME = Malic enzyme, PC = Pyruvate carboxylase, PDH = Pyruvate dehydrogenase, PEPCK = Phosphoenolpyruvate carboxykinase, PFK = Phosphofructokinase, PGI = Phosphoglucose isomerase, PGK = Phosphoglycerate kinase, PGM = Phosphoglycerate mutase, PHGDH = Phosphoglycerate dehydrogenase, PK = Pyruvate kinase, RPI = Ribulose phosphate isomerase, SCS = Succinyl-CoA synthetase, SDH = Succinate dehydrogenase, TPI = Triose phosphate isomerase.

1.9- Energy regulations

Cancer cells are found to be very flexible metabolically. They have developed different mechanisms to maintain ATP levels, predominantly fuelled through glycolysis, the TCA cycle, and providing oxidative molecules to the electron transport chain (ETC). A large number of cancer cells have a high glycolytic rate and produce significant amounts of lactate, as observed by Otto Warburg - the Warburg effect. This has led to the hypothesis of ATP generation by aerobic glycolysis in cancer cells, instead of oxidative phosphorylation (OXPHOS) (DeBerardinis and Chandel, 2016). Substantial evidence suggests that the Warburg effect is important for anabolic pathways, not just energy production, and the elevated glucose uptake is due to dysregulation of oncogenes and tumour suppressor genes, and energy regulatory pathways like PI3K pathway (Pavlova, 2016; Dang, 2012). Slowing down glycolysis in cancer cells has not been very useful to stop tumourigenesis (Joshi, 2015). Other studies in cancer cells reveal utilization of OXPHOS for ATP production, and mitochondrial utilization in tumourigenesis (Weinberg et al, 2010; Martinez-Reyes et al, 2016). This evidence suggests the importance of OXPHOS in glycolytic cancer cells.

Cancer cells also fuel their mitochondria with fatty acids and amino acids, these nutrients supply different substrates to the TCA cycle and ETC (electron transport chain) to maintain the energy demands of cancer cells. Fatty acid breakdown produces acetyl-CoA, and NADH and $FADH_2$, which are involved in ATP production in mitochondria (Ulrike and Joanna, 2016). Furthermore, glutaminolysis through glutamine can fuel the TCA cycle to facilitate energy production. Besides glutamine, valine, leucine and isoleucine levels are elevated in some cancers. These can help cancer cells with ATP production by their conversion to acetyl-CoA (Martinez-Reyes et al, 2016).

Interesting metabolic remodelling in cancer cells is done under hypoxic and nutrient deprived conditions (Fan, 2013). Tumour cells may also survive in hypoxic condition by lowering their ATP demand, thus maintaining their ATP/ADP ratio. If the ratio is still difficult to maintain, ADP can activate AMPK (AMP Kinase) through AMP, which triggers catabolic processes in tumour cells to generate ATP for survival (Hardie and Schaffer, 2016). During nutrient depletion, cancer cells use extracellular nutrients to produce glutamine and other amino acids as fuel for their TCA cycle. If the nutrient deprivation condition persists, the ATP/ADP ratio can also be maintained by inducing autophagy through mTOR deactivation.

Metabolic studies in cancer suggest diverse metabolic phenotypes. This very fact is making it hard to target a specific metabolic dysregulation in cancer therapy (Martinez-Outschoorn et al, 2016). However, cancer cells for their growth, proliferation, and metastasis need higher concentrations of glucose, lactate, pyruvate, acetate, glutamine, and fatty acids than the normal cells. These common metabolic features of cancer can be targeted to treat cancer (Martinez-Outschoorn et al, 2016).

1.10- Macromolecule requirements

While metabolic rewiring for energy generation is the fundamental metabolic alteration in cancer cells, cancer cells also need an ample supply of biomolecules for proliferation. The biomolecule metabolic alterations are not only used in cell proliferation, but also in energy production, DNA repair, angiogenesis, hypoxic response, tumourigenesis and autophagy (Galluzzi et al, 2014) In this metabolic biosynthesis, simple nutrients (like sugar and amino acids) are taken from the extracellular space and then are converted to more complex molecules through the main metabolic pathways like glycolysis, the TCA cycle, and pentose phosphate pathway (PPP). These processes are energy dependent and are regulated through energy sensing pathways like the PI3K-mTOR pathway (DeBerardinis and Chandel, 2016).

Cancer cells can proliferate rapidly which require higher lipid levels for membrane synthesis, and cellular signalling (Yoo et al, 2004). Acetyl-CoA, and NADPH, required for fatty acid synthesis are well maintained in cancer cells. High pyruvate levels and pentose phosphate pathway activity supports fatty acid synthesis. Glutamine, acetate, and leucine can provide the carbon skeleton for acetyl-CoA, in addition to glucose when

required (Mullen et al, 2012; Yoo et al, 2004). Fatty acid synthesis is also regulated to maintain the fatty acid pool in the cell for faster proliferation. SREBP-1, a transcriptional factor, has been found in elevated amounts in cancer cells with a high rate of fatty acid synthesis. SREBP-1 has been shown to regulate acetyl-CoA conversion to fatty acids, and the conversion of acetate and glutamine to acetyl-CoA (Horton 2002). Fatty acid uptake can be increased and its oxidation can be decreased under PI3K signalling. This maximizes lipogenesis under high lipid demand in cancer cells (Deberardinis, 2006).

Nucleotides are required for replication and cellular signalling and their production involves various metabolic pathways. Cancer cells have been targeted with nucleoside analogs and anti-folate drugs for chemotherapy for a long time, but still very little is known about the effect of cancer metabolism on nucleotide biosynthesis (DeBerardinis and Chandel, 2016). A major pool of nucleotides comes from the PPP pathway, and a high glycolytic flux in cancer cells can increase nucleotide production by increased use of the PPP pathway. The TCA cycle also contributes to nucleotide synthesis through oxaloacetate, which is converted to nucleotides via aspartate. Cancer cells using the TCA cycle for biomolecule production can divert it to nucleotide synthesis, when replication is required at a high rate. Apart from these intermediates and pathways, NADPH also helps in nucleotide synthesis by regulating the oxidative environment in the cells (Stincone et al, 2014).

1.11- Redox maintenance

Cancer cells have been associated with high ROS production (Liou and Storz, 2010). Although ROS has been considered as a lethal by-product of cellular metabolism, now ROS is implicated in the modulation of cellular signalling (such as PI3K and MAP Kinase pathways) and transcription factors (such as HIF), whose dysregulation can be helpful for tumourigenesis (DeBerardinis and Chandel, 2016). Apart from these effects, ROS can also oxidize cysteines in proteins; this can induce the unfolded protein response and put a cell under metabolic stress (Finkel, 2012). ROS includes different molecules such as superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), or hydroxyl radicals ($OH^\cdot$). These chemicals can induce cellular dysregulation, including cell death. Different anti-oxidant systems are present in a cell to cope with elevated ROS levels. NADPH oxidase (NOX), superoxide dismutase 1 & 2, peroxiredoxins (PRXs), thioredoxin (TXN), thioredoxin reductase (TrxR), glutathione peroxidase (GPXs), catalase (Cat), and glutathione are

some of the most important anti-oxidants (Rhee et al, 2012; Murphy, 2012). These anti-oxidants ultimately convert ROS to H_2O and O_2, to detoxify a cell with elevated ROS levels. Cancer cells have higher levels of ROS, which not only induces cell damage but also anti-oxidant enzymes to avoid cell death. One of the important genetic tools to increase anti-oxidants in cancer cells is a nuclear factor (erythroid-derived 2)-related factor-2 (NRF2) (Jaramillo and Zhang, 2013). NRF2 once activated can induce higher production of GPXs, TXNs, GSH synthesis, and cytosolic NADPH, which can evade cell death by suppressing ROS levels (Ye at al, 2014).

1.12- Metabolic targeting of cancer

Therapeutic targeting of cancer cells has always been complicated but it is critical for effective therapy. Large scale research has been done to systemically target cancer cells, which may block some critical metabolic pathways that have been dysregulated in cancer cells (Le et al, 2010). These therapies have never been very successful possibly because cancer cells have all the normal functional metabolic pathways. Although some may be dysregulated, their metabolism is very similar to other body tissues e.g., stem cells or, immune cells. Cancers also undergo a high degree of metabolic reprogramming at different stages of the cancer development, like drug resistance development, metastasis and tumourigenesis (Pearce et al, 2013). These facts make it difficult to target a specific metabolic pathway in cancer cells, however, it may be more successful to address multiple metabolic targets in cancer cells (Gorrini and Harris, 2013).

Many studies have targeted glycolysis in cancer cells (DeBerardinis and Chandel, 2016). Hexokinase 2 (HK2) is involved in the first step of glycolysis, and its overexpression is described in many cancers. When inhibited, cancer models like NSCLC and breast cancer tissue showed delayed tumour progression, moreover, systemic inhibition of HK2 did not show adverse effects on normal tissues. The other most obvious glycolytic target has been lactate dehydrogenase (LDH), which converts pyruvate to lactate. Higher glycolysis tends to generate high lactate levels for either conversion to other metabolites or to be secreted out of the cancer cells (Heiden, 2011).Targeting LHD-A in MYC driven tumours, NSCLS tumours, and myeloid leukaemia shows either regression of the tumour or delayed progression (Xie et al, 2014). Glycolysis is regulated by growth factors and the energy sensing mechanisms of the cell, such as the PI3K-AKT, mTOR, and HIF pathways. These pathways also affect other cellular processes like autophagy, translation

of specific genes, apoptosis, and ribosomal biogenesis. The effect of targeting metabolism on these cellular processes in cancer is still under investigation (Heiden, 2011).

Mitochondria are the other potential metabolic target for cancer therapy. Although cancer cells are supposedly glycolytic, still these cells show an abnormal mitochondrial metabolism. Mitochondrial metabolism provides some important metabolites to promote macromolecule, ATP, and NADPH synthesis for cancer cell proliferation (Ward and Thompson, 2012). Utilization of citrate for lipid biosynthesis and nucleotide production makes it essential for the cancer cells to replenish the TCA cycle effectively (DeBerardinis and Cheng, 2010). This makes intermediate TCA steps critical for cancer proliferation. Intermediates like α-ketoglutarate, succinate and fumarate are often elevated in lungs, liver and colon cells (Sullivan et al, 2016). Elevated mitochondrial activity makes additional ROS. Cancer cells are known to have elevated ROS levels for their proliferation, tumourigenesis and metastatic activities. ROS, being highly reactive oxygen species, may activate oncogenes, mutate mitochondrial genome, induce hypoxia, and loss of tumour suppressor genes (Sullivan et al, 2013; Sullivan et al, 2014; Porporato et al, 2014).

1.13- Targeting metabolism in CIN cells and CIN cancers

About 90% of solid tumours and 50% of haematopoietic tumours are CIN positive (Mitelman et al, 2012). CIN being a hallmark of cancer is present in 70% of sporadic colorectal and 70% of lung cancers (Pino and Chung, 2010; Masuda and Takahashi, 2002). CIN is known to cause proteotoxic stress and metabolic changes in the cells (Oromendia et al, 2012) which has been exploited to target cancer cells (Tang et al, 2011). Hepatocarcinomas, which are CIN positive, are dependent upon arginine. Depletion of arginine by using pegylated arginine deiminase (ADI) increased the life time of patients with hepatocarcinoma. The drug is under phase II for clinical trials (Glazer et al, 2010). Metformin is probably one of the most discussed metabolic drugs for cancer. It is a diabetic drug and is recommended for ovarian cancer (Kasznichi et al, 2014). In a study on advanced pancreatic cancer, patients were treated with metformin. Although results with metformin were not statistically significant still some patients survived with metformin. Moreover, the tumours were at the latter and advanced stage which may have compromised the effect of metformin treatment (Kordes et al, 2015).

Mitochondria are metabolically very active and diverse organelles and are involved in the TCA cycle, OXPHOS, to provide and metabolic intermediates to a cell (Tzameli, 2012). Mitochondria in cancer cells are known for their anabolic precursor production which cancer cells can use for their replication and metabolic adaptions. Trials are now under investigation to target cancer mitochondria for their cancer specific activities (Zong et al, 2016). Pardee et al., (2014) used an anti-mitochondrial agent CPI-613 against hematologic malignancies. CPI-613 was well tolerated by the patients and some of the patients (about 29%) achieved their targeted response.

In a comprehensive study by Tang et al (2011) to target CIN and aneuploidy, the cells were hit with various metabolic drugs. They found that in a combination, an energy stress inducer (AICAR), and a protein folding inhibitor (17-AAG) were helpful to limit the growth of trisomic yeast cell lines and aneuploid cancer cell lines. However, the effect of the drugs was limited, and different for different lines. Trisomic yeast lines were significantly affected by the drugs but CIN cancer cell lines such as colorectal cancer were moderately affected and lung cancer cells were least affected by the drugs. It was found these drugs induced AMPK dependent, and p53 dependent as well as independent apoptosis in an aneuploid cell. The mechanism by which these drug induced apoptosis in the CIN and euploid cells could be not explained. Moreover, AMPK is both pro- or anti-tumourigenic (Faubert et al, 2014), and p53 is already deregulated in more 50% of human cancer (Surget et al, 2013).

In my lab we targeted CIN to kill cancer cells, as CIN is found in advanced tumours. CIN offers cancer specific target for cancer therapy. Initially we targeted kinases and phosphatases because these regulatory enzymes are involved in a lot of cellular processes such as, cell growth, survival, proliferation (Hunter, 2000). We found inhibition of Pask (Pas domain-containing serine/threonine protein kinase), pfrx (phosphofructokinase) killed CIN induced progeny and CIN cell in *Drosophila* wing-disc epithelial cells (Shaukat et al, 2012). It was found that the cell death was caspase dependent with high ROS levels, and elevated mitochondrial activity. These metabolic candidates had not previously been implicated as a potential therapy for targeting CIN cells. Pask is a metabolic nutrient sensor and has no known direct effect on glucose consumption, so it was surprising to have a CIN specific effect with Pask.

Evidence from the literature and our previous findings in the lab suggested further screening of metabolic candidates in CIN cells would be useful in identifying further CIN-specific metabolic vulnerabilities. All major metabolic candidates were depleted to identify a mechanism which could be described to specifically kill CIN cells. In this thesis I will be focusing on targeting metabolism in CIN cells and CIN tumours

1.14- Drosophila: a CIN and tumour model

CIN is linked to tumourigenesis and is involved in drug resistance and relapse in cancer by increasing the genetic diversity of cancer cells (Bakhoum et al, 2012). About 90% of solid tumours are CIN positive (Weaver and Cleveland, 2006). Working on CIN in cancers has been difficult because by the time cancer is identified, CIN has already induced high genetic heterogeneity which makes it difficult to identify the changes which led to CIN (Burrell et al, 2013). For this reason induced-CIN models are employed to study CIN phenomena. Quite a range of models is available to study CIN from cell cultures, yeast to mouse with their advantages and disadvantages (McGranahan et al, 2012). Cell lines are easy to work with but their huge genetic diversity (every cell is genetically different) makes it difficult to get reproducible results and generalized CIN therapy (Roschke et al, 2005; Wallqvist et al, 2005). Yeast is another significant CIN model, and important metabolic information relating to aneuploidy has been inferred from yeast, like high transcriptional profiles and proteotoxic stress in aneuploid yeast cells (Oromendia et al, 2012), but yeast is genetically and physiologically far from humans. Even some proteins orthologs are not present in yeast, and yeast does not have cancer. The only mammalian animal model is a mouse and is employed in cancer studies due to genetic and physiological homologies to humans, but difficult genetic manipulations, extra time consumption, and difficulty to replicate human gene functions have made it a hard choice for genetic experiments (McGranahan et al, 2012).

Drosophila has been a genetic model for more than a century. It has been completely sequenced and a huge set of genetic and biochemical information is available (Fortini et al. 2000; Bier 2005). *Drosophila* is a fairly simple model with substantial genomic similarity to human genome: about 60% of genes are conserved, and 75% of human disease-causing genes have Drosophila homologues (Pandey and Nichols, 2011). *Drosophila* has a short life cycle, is cost effective and, easy to rear, as well as being easy to manipulate genetically, at any stage of its life cycle. More importantly, tissue and stage

specific genetic manipulation are feasible in *Drosophila* (Shaukat et al, 2012). Cancer modelling has long been done in *Drosophila* and several key pathways involved in a cancer like Ras, Hippo, Wnt, and Notch were discovered in the fruit fly (Bier, 2005). *Drosophila* has diverse models for CIN study. Mad2, BubR1, Rad21, polo are some of the proteins which have been utilized in *Drosophila* CIN modelling (Liu et al, 2014). Tumour studies in *Drosophila* have been going on for more than a decade in which *Drosophila* explants are implanted into a host fly to analyse tumours and the molecular mechanisms of the disease (Caussinus and Gonzalez, 2005). As I also planned CIN tumour studies, I considered *Drosophila* as my first choice as it would be possible to perform tissue specific gene manipulation, induce a tumour, explant a tumour in a host fly and then follow it for a time period.

1.15. Key points

The key points of the introduction are as follows:

1. CIN is a hallmark of cancer.
2. CIN can contribute to acquisition of other hallmarks of cancer
3. CIN leads to chromosomal rearrangements and aberrations which help cancer cells to evolve drug resistance, metastasis, relapse, and poor prognosis.
5. CIN cancer cells can be metabolically different than normal cells.
6. Targeting metabolism in CIN cells can have therapeutic significance.

1.16. Aims of the study

The main object of this project has been to identify metabolic targets in CIN cells, which could kill CIN cells specifically. We extended the project to evaluate metabolic targeting to CIN cancers. The study was done to understand the mechanisms which could be involved in killing CIN cells.

Aim 1: To screen for metabolic candidates whose knockdown can trigger apoptosis in CIN cells in *Drosophila*.

Aim 2: To implement CIN specific metabolic targeting in CIN tumours, to assess the effects of metabolism on cancer therapy.

Aim 3: Characterization and identification of the metabolic pathways which kill CIN cancer cells.

Aim 1 is described in **Chapter 3**. **Aim 2** and **Aim 3** are described in **Chapter 4**.

Chapter 2

Drosophila as a model for chromosomal instability

Chromosomal instability (CIN) is a characteristic of most solid tumours, and is involved in the loss or gain of whole or fractions of chromosomes. CIN has the potential to initiate tumorigenesis and may help in tumour evolution against drugs by generating genetic diversity (Geigl et al, 2008). Although CIN is a hallmark of cancer, but it also offers a cancer specific therapy. CIN cells can be targeted specifically leaving normal cells intact (Shaukat et al, 2012). This targeted study can be done on CIN cells like CIN cancer lines, however, limitations such as the different genetic makeup of the individual CIN cells, the *in vitro* environment, and reproducibility of the results restrict cancer therapy studies. (Roschke et al, 2005; Roschke and Kirsch, 2010). Contrary to CIN cell lines, models for induced CIN are more reliable because they start from a common genetic background.

In this chapter, I have discussed *Drosophila* as a model for CIN. *Drosophila* tumour tissues develop CIN quickly over a short span of time (Caussinus and Gonzalez, 2005). This provides an opportunity to analyse the effect of CIN not only on the tumour tissue but also to compare it to normal tissue which develops CIN. I have described limitations of CIN cell lines, and the advantages of using *Drosophila* in CIN tumour studies. Aberrations in mitotic processes, like the spindle assembly checkpoint, cytokinesis, and DNA repair which were induced in *Drosophila* to develop different CIN models are also discussed in this chapter.

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Chapter 3

Chromosomal instability causes sensitivity to metabolic stress

Otto Warburg coined a term “aerobic glycolysis” for cancers with high glucose absorption, that produce lactate in presence of oxygen (Warburg. 1925). Herbert Crabtree (1929) further extended the Warburg effect and found variability in fermentation in tumours. These two studies opened the field of cancer metabolism, which still to this day is a very exciting research field to study cancer. Later studies found different metabolic changes in cancer, such as the use of glycolysis and Krebs cycle intermediates for biosynthesis, amino acid addictions-e.g., glutamine addiction, increased nitrogen demand, elevated oxidative stress, and metabolism driven gene regulation (Pavlova and Thompson, 2015; Liou and Storz, 2010). These findings have made altered metabolism one of the hallmarks of cancer (Hanahan et al, 2011).

As altered metabolism is a hallmark of cancer it thus offers a therapeutic target to treat it. However, metabolic therapy in cancer treatment is still in a preliminary phase because cancer cells also possess other metabolic pathways which are present in normal cells (Pearce et al, 2013). Moreover, metabolism is a highly integrated and interlinked process, so affecting one pathway is sure to have an effect on other related pathways. These and other factors, such as the effect of oncogenes on metabolism, have restricted the effectiveness of metabolic treatment of cancer (Vander Meiden 2011). This promotes the notion of combination therapy for cancer treatment.

In this work we tested metabolic treatment of chromosomally instable (CIN) cells. CIN is another hallmark of cancer and has the potential to induce tumorigenesis (Hanahan et al, 2011). Poor prognosis, metastasis, and relapse are common outcomes of CIN in cancer (Choi et al, 2009). About 70-80% of solid tumours like colorectal, and lung cancers, have CIN (Zasadil et al 2014; Pino and Chung, 2010; Masuda and Takahashi, 2002).

In this chapter I will be explaining the effect of metabolic disruption on CIN cells. Metabolic candidates from several metabolic pathways (glycolysis, Krebs cycle, oxidative phosphorylation, gluconeogenesis, glucose metabolism, and PP pathway), were found which could induce apoptosis in CIN cells. These metabolic disruptions did not adversely affect normal cells. It was also evident that mitochondria were behaving abnormally and oxidative stress was high. Oxidative stress was a major cause of apoptosis. We could not fully explain the reasons for the apoptosis of CIN cells with metabolic disruption. However, the study proved the beneficial outcomes of metabolic targeting of CIN cells.

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Chapter 4

Phosphoenolpyruvate carboxykinase is needed to maintain glycolysis driven growth in Drosophila tumours

In the previous chapter (Chapter 3) I discussed the metabolic targeting of CIN cells. We found metabolic proteins which when down regulated in a CIN background, killed the CIN cell and did not affect normal cells. However, we could not explain the ROS generation mechanism in CIN cells. Moreover, we were not sure about the effectiveness of metabolic treatment on CIN tumours. In this chapter, "PEPCK is needed to maintain glycolysis-driven growth in *Drosophila* tumours", I have addressed these questions.

Both, CIN and altered metabolism are hallmarks of cancer (Hanahan et al, 2011). CIN by itself can cause proteotoxic stress and metabolic changes which can be targeted to treat cancer (Oromendia et al, 2012). CIN cancers have been treated with metabolic interventions and studies have been reported in which these metabolic interventions restrict the CIN tumour growth (Tang et al, 2011; Glazaer et al, 2010). However, specific metabolic signatures which are changed in CIN cancer cells are still under investigation.

To overcome the issue of CIN specific targeting, my lab conducted screening of phosphatases and kinases. Candidates, including metabolic candidates, were found which could kill CIN cells specifically. Depletion of Pas-kinase, a glucose regulator, and, phosphofructokinase (*pfrx*), a glycolytic enzyme, were found to give CIN specific cell death (Shaukat et al, 2014).

In this chapter I address the above mentioned question of ROS generation and test the effectiveness of metabolic interventions to stop CIN tumour growth. A CIN tumour model was developed using *brat-RNAi* neuroblast tissues. Explants of the tissue were transplanted in *Drosophila* hosts. The growth of the tumours was manipulated by genetic and chemical interventions. I found PEPCK was essential for CIN tumour growth. In my previous and the current study (Chapters 3 and 4), I concluded that PEPCK is involved in glyceroneogenesis, and not gluconeogenesis. The study also shows the importance of NADH, which can stop glycolysis and the tumour growth if not oxidized. It was also found that the glycerol-3-phosphate shuttle was involved in ROS production which could lead to apoptosis. The study emphasises the benefit of unbiased screening for metabolic candidates in CIN tumours. This work explains at least one mechanism by which a specific metabolic pathway may kill a CIN tumour.

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Chapter 5

DISCUSSION

In this work, major metabolic pathways were inhibited from inducing apoptosis in CIN cells. Glycolysis, gluconeogenesis, TCA cycle, PPP pathway, fatty acid metabolism, and oxidoreductase genes were initially screened in a *mad2*^{RNAi} CIN background. The selected metabolic candidates were subsequently used in a CIN tumour model, *brat*^{RNAi}, to test if the candidates were able to suppress CIN tumour growth. *mad2* is spindle assembly checkpoint protein, and its dysregulation can induce CIN in normal cells (Schvartzman et al, 2011), whereas *brat* regulates neuronal differentiation and neuroblasts self-renewal (Lee et al, 2006). I selected *brat* also because the deregulation of its human ortholog TRIM3 leads to malignant gliomas (Boulay et al, 2009), especially glioblastoma (GBM) (Chen et al, 2014). Thus the findings for *brat* tumours may have implications for human TRIM3 induced cancers. Deficiency of *mad2* can induce CIN up to 24% when compared to the wild type brain tissue in *Drosophila* (Shaukat et al, 2014). *mad2* dysregulations are also related to human cancers, such as colorectal cancer (Li and Zhang, 2004). However, tumorigenic effects of *mad2* dysregulations are yet to be determined (Ricke et al, 2008). In contrast to *mad2* deficiency, knockdown of *brat* can initiate tumorigenesis in *Drosophila* (Basto et al, 2008). I measured CIN rate in *brat* deficient tissue, and it was high about 20% compared to the wild type (Chapter 4, Figure 1g). *brat* deficiency with high proliferative effects and high CIN level gave me an opportunity to test my selected metabolic candidates on the tumour with high CIN rate.

The results were encouraging, with a wide range of candidates killing CIN cells (Chapter 3-TableS1). Metabolic candidates like PEPCK, Men, Mdh1, and GPDH were also able to restrict CIN tumour growth (Chapter 4- Figure 3a, 3b). All the effective candidate's induced mitochondrial hyperactivity, high levels of lipid peroxidation, and DNA damage in the CIN cells, while a few candidates affected glycolysis to restrict CIN tumour growth (Chapter 4-Figure 3b, 3c, 3d, 3e, S2b). I found metabolic candidates were inducing oxidative stress in CIN cells, where they might be activating caspase pathways to induce apoptosis (Chapter 3-Figure 1, 4, 6; Chapter 4-Figure 1)

The most prominent factor was oxidative stress, which already is higher in CIN cells (Chapter 3-Table 1), and in cancer cells (Liou and Storz, 2010). When some of the anti-oxidant enzymes were knocked down in CIN cells, an increase in ROS levels was observed, whereas no change in ROS levels was detected when anti-oxidant enzymes were knocked down in normal cells. ROS can easily damage active macromolecules, such

as DNA, lipid membranes, and affect cellular signalling, inducing apoptosis (Day and Suzuki, 2006). Over expression of anti-oxidant enzymes (SOD1, catalase) reduced ROS levels and rescued apoptosis in metabolically defective CIN cells. This suggests that CIN cells are sensitive to ROS. This finding is in accordance with our previous findings that candidates of different pathways like JNK, centrosomal, DNA repair and histone kinases increased ROS levels in CIN cells (Shaukat et al, 2012). I further tried to rescue $PEPCK^{KD}$ CIN tumours with propyl gallate (an anti-oxidant), which allowed some of the tumours to grow. This suggests oxidative damage could be a limiting factor in the growth of these tumours. The idea to kill cancer cells by inducing more ROS (or by inhibiting anti-oxidants) is not new and is currently in trials (Cabello et al, 2007; Fry, 2006). However, moderately elevated ROS can contribute to cancer cell proliferation, cancer survival, angiogenesis and metastasis, and metabolic alterations (Dong et al, 2016; Trachootham et al, 2009; Ramanathan et al, 2005; Attia et al, 2008). All these characteristics are hallmarks of cancer which can be instigated by ROS. Nonetheless, inducing ROS in cancer cells could have a therapeutic advantage (Trachootham et al, 2009) due to the fact that cancer cells are already living with moderate ROS levels, which, when increased, may kill cancer cells.

In the initial screening, a diverse range of cellular metabolic candidates was identified that made CIN cells susceptible to cell death (Chapter 3-Table 1). Many of these metabolic candidates were related to glucose metabolism, like Fructose 1,6 bisphosphatase, PASK, Glucose 6 phosphatase, G6PD, Wwox, PEPCK, and Galactose 1-phosphate uridyltransferase. A few were related to the TCA cycle (Idh and Malic enzyme b), or beta-oxidation in peroxisomes and mitochondria (Mtpα, Mfe2 and AcetylCoA oxidase at 57D).

Glucose plays a pivotal role in cellular metabolism. A number of biological factors regulate glucose homeostasis: Pas-kinase (PASK), a glucose sensing regulator, is one of them (DeMille and Grose, 2013). Pask was a strong hit in the initial metabolic CIN screening (Shaukat et al, 2012). Its inhibition in CIN cells promoted apoptosis, when compared to Pask inhibition in normal cells though Pask has never previously been discussed in relation to cancer metabolism. It was found that limiting Pask induced CIN-specific apoptosis (Shaukat et al, 2012). Higher expression of Pask has been linked to higher glucose and glucagon-like peptide-1 (GLP-1) levels in normal human cells

(Hurtado-Carneiro et al, 2013), whereas Pask inhibition inhibits glucose stimulated insulin activity (Hao et al, 2007). Pask knockdown in mice shows elevated mitochondrial oxidation in beta-pancreatic cells (Hao et al, 2007) and low lipid storage in hepatocytes (Wu et al, 2014). This can help to explain Pask's role in CIN cells: decreasing Pask levels can increase mitochondrial activity, producing more ROS which can induce cell death when it exceeds the threshold (Chapter 3-Figure 3, 4). The molecular targets of PASK in glucose metabolism are still poorly understood. It is possible that CIN cells with reduced Pask function may sense a glucose shortage and up-regulate mitochondrial oxidization, increasing ROS to lethal levels (Demille and Grose, 2013). Pask deficiency can also induce lipid metabolic defects, as seen in hepatic tissue (Hao et al, 2007; Wu et al, 2014), to induce cell death in CIN cells. Cell death was found to be p53 dependent and positive for caspase-3 staining in Pask deficient CIN cells (Shaukat et al, 2012). These findings suggest ROS and DNA may have acted through p53 to induce apoptosis. However, the reason ROS is generated in Pask deficient CIN cell could not be concluded. The main reason was we could not draw any appropriate molecular mechanism which would explain ROS generation in Pask deficient CIN cells. This is still an open question. As Pask is not a direct regulator of glucose metabolism and it is still confined to nutrient sensing (Zhang et al, 2015) we found some other glucose regulators (G6PD, PEPCK) which have a better characterized effect on glucose consumption than Pask, and can be targeted to more easily study metabolism in CIN cells.

G6PD is involved in the pentose phosphate (PP) pathway which generates NADPH (an anti-oxidant) and ribose 5 phosphate (for nucleotide synthesis) (Stanton, 2012). Multiple tumours have elevated levels of G6PD. These include leukemia, gastrointestinal cancer, renal cell carcinoma, colon cancer, breast cancer, endometrial cancer, liver cancer and prostate cancer (Hu et al, 2013). This indicates that G6PD up-regulation supports tumour survival, being an anti-oxidant and cell proliferation regulator. Hu et al. (2013) saw the lowest growth rate in G6PD deficient tumours in mice. They also observed down-regulation of cell cycle proteins (cyclin D1, cyclin E, p53 and S100A4). Similarly, when G6PD was targeted using inhibitors (Bay 11-7082 and DMF) in erythrocytes, this depleted glutathione (GSH) in erythrocytes and induced eryptosis in the cells (Ghashghaeinia et al, 2016). CIN cells already suffer redox and replication stress (Shaukat et al, 2012). When CIN cells were targeted for G6PD, apoptosis was evident in *Drosophila* wing discs (Chapter 3-Figure S1c). I further checked G6PD in $brat^{RNAi}$

induced CIN tumours. Unexpectedly, G6PD^{RNAi} could not stop CIN tumour growth. This resembles the results of Hu et al. (2013), in which mutant G6PD slowed down the tumour growth but did not completely stop it. In our study, the effect of G6PD deficiency on CIN tumour growth was not dramatic. G6PD inhibition would be predicted to affect ribose 5 phosphate (nucleotide synthesis) or NADPH (anti-oxidant) production (Hay, 2016). However, haemolysis in G6PDH deficient patients supports high oxidative stress being more detrimental rather than the nucleotide biosynthesis defects (Frank, 2005). Similarly, brat^{RNAi} tumours in passage 1 grew very efficiently despite having higher ROS levels than the controls (Chapter 4-Figure 1f). In a study to evaluate the role of G6PDH in tumour cell proliferation and apoptosis, G6PDH inhibition despite decreasing cell cycle proteins like cyclin E, cyclin D1, and increasing apoptotic factors like Bcl-2 and Bcl-xl, also down-regulated apoptosis factor Fas (Hu et al, 2013). Moreover, some cancers have already down-regulated cyclin E levels (Mazumder et al, 2004) which can reduce the effect of G6PDH inhibition. Moreover, inhibition of G6PD can be compensated by non-oxidative PPP pathways using fructose 6 phosphates and glyceraldehyde 3 phosphate to produce ribose 5 phosphate (Hay, 2016). I also found high glucose consumption in CIN tumours (Chapter 4-Figure 3 and S2) which can support the use of the non-oxidative PPP pathway in G6PD deficiency. Therefore, I could not establish the role of G6PD depletion in tumour suppression in our model despite its well characterized role in tumour progression and development (Kuo et al, 2000; Patra and Hay, 2014). The PP pathway may be an effective target in cancer therapy if candidates more directly involved in nucleotide synthesis, such as transketolase, are targeted, as this will also block the non-oxidative PP pathway.

JNK was another interesting candidate for targeting CIN cells. In our earlier studies we found that chromosomal instable cells required JNK for their survival (Shaukat et al, 2012; Wong et al, 2014). We found the loss of JNK led to DNA damage, increased ROS, mitochondrial hyperactivity and apoptosis (Shaukat et al, 2012; Wong et al, 2014). An over-expression of JNK has been reported in various cancers, including, T-cell leukemia, acute myeloid leukemia, breast cancer, and tumour growth in different mouse models (Bode and Dong, 2007). JNK is a stress response protein which is involved in DNA damage repair during pre-mitotic cell cycle phases like G1 and G2, so inhibiting JNK reduces the DNA damage response and unrepaired DNA damage can lead to caspase dependent apoptosis (Wong et al, 2014). Thus I knocked down JNK to induce apoptosis in my CIN tumours in response to DNA damage and ROS as seen in CIN wing discs

(Shaukat et al, 2014). Contrary to this, JNK^{KD} CIN explants grew normally compared to brat mutant explants (Chapter 4 Figure 2). This suggests a loss of a pro-apoptotic function of JNK. JNK^{KD} allowed the survival of more aneuploid cells in the tumour explants and yet the tissue grew as explants (Chapter 4 Figure 1). JNK also has a pro-apoptotic role. It is involved in both extrinsic and intrinsic apoptotic pathways, targeting TNF-a, TRAIL, FAS-2 and L, and mitochondrial initiated apoptosis (Elmore, 2007). It was possible that depleting JNK would stop the explant growth by inducing apoptosis but the CIN cells losing JNK might alternatively have lost apoptotic signals from DNA damage which helped the explant to grow. JNK has both apoptotic and anti-apoptotic role in cells (Wong et al, 2015). JNK has three isoforms in the mammal, JNK1, JNK2, and JNK3 (Bode and Dong, 2007). In a mammalian study JNK1 inhibition increased p53 levels while JNK2 decreased p53 levels (Tafolla et al, 2005). Acute activation of JNK can help in cell survival while either prolonged activation or severe deficiency can promote cell death (Wu et al, 2014). The other factor which may be involved in JNK deficient tumour growth could be levels of JNK, as I have mentioned above. I have been using RNAi lines which do not completely abolish the expression of a target gene. There could be a possibility of just enough levels of JNK protein to start the DNA damage repair response. In a time course study, JNK activation of less an hour increased cell survival, whereas a longer activation of JNK or a complete loss of JNK induced apoptosis (Ventura et al, 2006; Bode and Dong, 2007). My results with JNK deficiency strongly suggest for a study to look into to both pro- and anti-apoptotic roles of JNK CIN tumours.

The most exciting results came from *mfe2* and *PEPCK*, which, when knocked down in CIN tumour cells, stopped the growth of the explants (Chapter 4-Figure 2d). *mfe2* (multifunctional enzyme 2) is a peroxisomal beta-oxidation enzyme. It is involved in the third step of beta oxidation and converts 3-hydroxytetracosanoyl-CoA to 3-ketotetracosanoyl-CoA and reduces NAD+ during the reaction (Poirier et al, 2006). Inhibiting Mfe2 compromises the cell's ability to breakdown long chain fatty acids to short chain fatty acids, which are then involved in the acetyl-CoA production (Wanders et al, 2016). Fatty acid metabolism is becoming a popular therapeutic target in cancer studies (Djefaflia et al, 2016). Well known metabolic and neurological disorders are related to fatty acid metabolism defects (Zhou et al, 2016). Fatty acid oxidation provides cells with acetyl-CoA, a metabolite which can be used in the TCA cycle for energy production, or can be used for the biosynthesis of cellular membranes for cell proliferation

(Poirier et al, 2006). We expected beta oxidation and the production of acetyl-CoA to be an important factor in the effect of *mfe2* deficiency on CIN tumours. Surprisingly, when explanted $brat^{RNAI}$ CIN tumours were treated with Etomoxir and Trimetazidine (inhibitors of beta-oxidation), I could not find any explant growth restriction. This suggested a novel role for peroxisomal beta-oxidation, but exploring this needs further investigation. Studying peroxisomes and their metabolites as a whole might help to understand this restrictive effect on CIN tumours. Unlike *mfe2, PEPCK* depletion was consistent with PEPCK inhibitors in their ability to stop tumour growth (Chapter 4). Thus, we focused our attention on explaining the mechanism by which *PEPCK* might be affecting CIN cells.

PEPCK is involved in gluconeogenesis in the liver and the kidney (Burgess et al, 2004). However, its presence in non-gluconeogenic tissues like epithelial tissue suggests a non-gluconeogenic role in metabolism (Previs et al, 2009). In addition, cancers like neuroendocrine prostate cancer, and uterine carcinoma express higher levels of PEPCK despite having high hexokinase activity for glycolysis (CBioPortal). Similarly, whether PEPCK is a rate limiting factor in gluconeogenesis is still under debate. Rognstad (1979) found that inhibiting PEPCK with mercaptopicolinic acid stops gluconeogenesis, but later studies found a limited effect of PEPCK inhibition on gluconeogenic rate (Groen et al, 1986; Argaud et al, 1991). Some recent studies found that PEPCK is involved in glucose metabolism in the tumour growth and it's proliferation (Montal et al, 2015; Vincent et al, 2015; Leithner et al, 2015). The studies showed that PEPCK is involved in glutaminolysis in the absence of glucose. It was observed that PEPCK converts oxaloacetate (OAA) to phosphoenolpyruvate (PEP) which can support TCA and biosynthetic intermediates for tumour growth and survival in glucose limited conditions (Vincent et al, 2015; Leithner et, 2015), and when glucose is available, PEPCK enhances glycolysis. Major metabolic intermediates like pyruvate, glutamate, citrate, fumarate, phosphoenolpyruvate, and malate were decreased with PEPCK deficiency (Montal et al, 2015; Vincent et al, 2015; Leithner et al, 2015). However, these studies did not address the glycerogenic role of PEPCK. PEPCK is one of the rates limiting steps in glyceroneogenesis (Hanson and Reshef, 2003; Bartok et al, 2016). Glyceroneogenesis produces glycerol 3 phosphates from sources other than glucose like pyruvate, amino acids, and metabolic intermediates such as OAA to generate triacyglycerol (Hanson, 2005). When PEPCK was knocked down, *Drosophila* larvae had lower lipid levels (Chapter 3 Figure S3). Bartok et al.,

(2016) also found PEPCK inhibition lowered glucose, glycerol and TAG levels in the animal which suggests the effect of PEPCK on lipid metabolism. From my results I predict that the glyceroneogenic role of PEPCK is an important factor to restrict tumour growth by affecting ROS production and manipulating the glycolytic rate by affecting levels of cytoplasmic NADH.

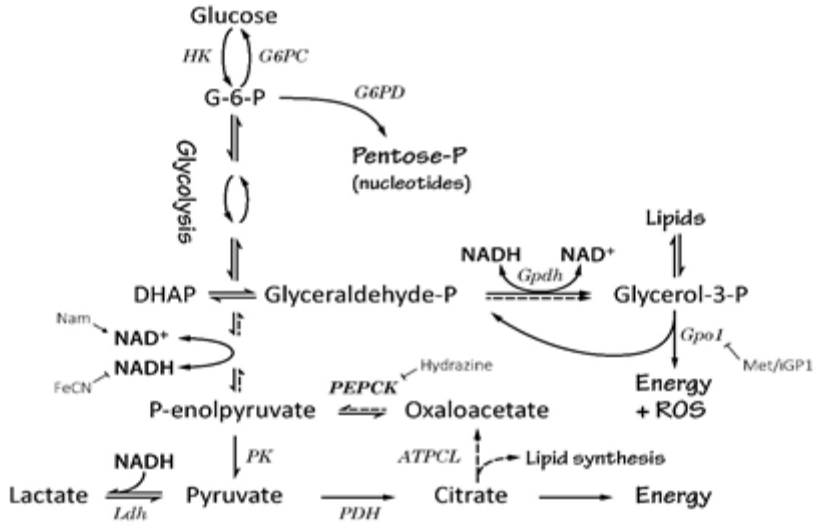

Figure 5.1; *A proposed model for PEPCK role in CIN cancer cells*

Important results came from my ROS experiments. ROS levels were very high in PEPCK^{KD} CIN cells and tumours (Chapter 3-Figure 4; Chapter 4-Figure 1f). ROS is expected to be produced in mitochondria due to hyper-mitochondrial activation in PEPCK^{KD} CIN cells (Chapter 3 figure 2). Mitochondria can produce ROS using the glycerol-3-phosphate (G3P) shuttle (Miwa et al, 2003). The G3P shuttle involves both cytoplasmic and mitochondrial Glycerol-3-phosphate dehydrogenases, cGPDH and mGPDH respectively (Mracek et al, 2012). mGPDH produces ROS from complex I and II by reducing ubiquinone in the mitochondrial oxidative phosphorylation process (Miwa et al, 2003). When I inhibited mGPDH using an inhibitor (metformin) it reduced ROS levels in both CIN epithelial cells and CIN tumours (Chapter 4, figure 3e). This was consistent with high mGPDH activity producing high ROS. This result also suggests that glycerol-3-phosphate is not being heavily used to make more TAG and glycerol. Rather, the G3P shuttle is involved in NADH oxidation (Shen et al, 2006). NADH is produced during glycolysis, and if not oxidized, NADH can hamper glycolysis because no more

NAD+ is available for the process to proceed further (Mracek et al, 2012). Thus G3P shuttle works as a sink for NADH oxidation, as well as producing glyceraldehyde-3-phosphate for glycolysis, and ROS in the mitochondria. When the G3P shuttle was targeted in CIN tumours it decreased ROS but the tumours could not grow. This is in accordance with the biochemical studies showing that elevated levels of mGPDH are required in fast growing undifferentiated tumours (Peron et al, 1974; Karsten et al, 1971; Dionisi et al, 1970). In PEPCK^{KD} CIN tumours, the G3P shuttle might not be expected to be highly active, as PEPCK inhibition lowers triacyglycride (TAG) and glycerol levels in *Drosophila* hemolymph (Bartok et al, 2016). In this scenario, data about glycerol kinase which converts glycerol to G3P, and GPDH is still miss

ing and needed to be addressed as high ROS production in PEPCK^{KD} CIN cells through G3P shuttle would then be difficult to explain (Chapter 4 Figure 4). However, limiting G3P shuttle reduced ROS levels in CIN cells (Chapter 4, Figure 3e). The interesting factor could be NADH level which should be high in PEPCK^{KD} but still needs to be measured to evaluate the importance of NADH oxidation in PEPCK deficient CIN cells. As limiting PEPCK should reduce the conversion of OAA to PEP, so less PEP should be available to glyceraldehyde-3-phosphate dehydrogenase (Gapdh) to be converted to glyceraldehyde phosphate, a precursor of glycerol-3-phosphate (Chapter 3 Figure 4; Miwa et al, 2013). This would increase NADH levels and restrict glucose consumption (Chapter 4 figure 4; Bartok et al, 2016). This NADH can be cleared by G3P thus producing ROS. When PEPCK^{KD} CIN tumours were supplemented with anti-oxidants and agents lowering NADH levels, the tumours grew (Chapter 4 figure 3b,c). The G3P shuttle seems to be more critical in CIN tumours than the other possible sinks for NADH, such as lactate dehydrogenase (LDH) and the aspartate/malate shuttle (Vemuri et al, 2006). Under high energy and proliferation demands, more pyruvate is needed for energy or lipid synthesis; this may make LDH less likely to be active (Fan et al, 2011). Consistent with this, CIN tumours did not stop their growth when treated with sodium oxamate, an LDH inhibitor. (Appendix-Figure 1a). I also targeted the aspartate/malate shuttle with amino-oxyacetate (AOA) and it too did not stop the CIN tumour growth (Chapter 4 figure 3a), highlighting the significance of the glycerol phosphate shuttle in clearing NADH.

This study is an initial work in our lab to demonstrate the importance of metabolism in CIN tumour therapy. I believe, if explored further we might have a better mechanistic

understanding of this vast metabolic interactive world in the cell to be able to target cancer.

I was also interested in temporal tumour development characteristics, like the tumour growth rate, cell death, ROS levels, CIN rate (Chapter 4 Figure 1d, 1e, 1f, 1g), and metastasis (Appendix-Figure 1b). I planned for three successive explants of *brat*^{RNAi} tumour tissue for the same time periods. Passage 1 tumours were distinctive in having the highest CIN rate but the lowest cell death rate. The factor responsible for this could be the ROS levels, which were lowest in Passage 1 (when compared to the other passaged tissues) (Chapter 4-Figure 1f). I observed higher apoptosis in successive passages with increasing oxidative stress (Chapter 4-Figure 1e,f). It is known that CIN cells are already facing cellular stress due to their aneuploidy, and if ROS is further elevated, these CIN cells can undergo apoptosis (Shaukat et al, 2012). It was possible that the high cell death and ROS generation in the progressively passaged explanted tissues could be hazardous for the host. A high rate of cell death can induce phagocytosis and inflammatory responses in the neighbouring normal tissue, at least in vertebrates (Gregory and Pound, 2011). I speculate that there is a similar effect in the explant hosts, because I observed successively fewer ovarioles in the passaged hosts. Hosts carrying Passage 3 explants (with the highest ROS levels and cell death) had the least number of ovary (Appendix-Figure 1b). Ovariole number has already been related to environmental stress in *Drosophila* (Sisodia et al, 2012; Wayne et al, 2005). These results support the model that excessive CIN can induce cell death via higher ROS levels (Liu et al, 2016). My data did not allow me to address whether high CIN could contribute to tumour relapses following therapy, because of the low number of passages and the limited time given to each explant (13 days) which limits the effect of CIN on tumourigenesis. This issue can be solved by anti-oxidant treatments or inhibiting apoptosis in the explanted *brat*^{RNAI} passages, long enough for the passaged explant to grow, and to increase the passage numbers.

CIN cells are already under stress such as redox and proteotoxic stresses (Tang et al, 2011; Shaukat et al, 2012). I have tried to target CIN cells by manipulating the metabolism of the cells. I have shown that metabolic interventions can exacerbate stress conditions in CIN cells to kill them. Metabolic candidates from major metabolic pathways like glycolysis, TCA cycle, OXPHS, PP pathway, and gluconeogenesis were able to induce apoptosis in CIN cells. I further explored this metabolic screening in CIN tumours. Most

of the screened and selected candidates could not stop the tumour growth. Of all the candidates, PEPCK was the strongest as it stopped the CIN tumour growth. PEPCK loss increased NADH levels in CIN cells and to reduce NADH, the glycerol-3-phosphate shuttle produced ROS. These two factors, elevated levels of NADH and ROS, may have caused apoptosis and limited the tumour growth.

Future directions

With this study there are still some open questions which need to be addressed.

1- The role of JNK in CIN tumours is still unclear. JNK inhibition increased CIN in CIN tumours but could not induce much apoptosis to stop CIN tumour growth. CIN induces DNA damage, which should initiate apoptosis a part of JNK's pro-apoptotic role. JNK activation upon DNA damage is well known. It is accepted that small and acute activation of JNK can be pro-survival while persistent and strong activation of JNK can be pro-apoptotic (Picco and Pages, 2013). As JNK expression was not fully silenced in my RNAi model it is possible that the low level of JNK was functionally anti-apoptotic. Using complete knockout of JNK may resolve this issue. Moreover, overexpression of JNK might be helpful to look into the details of this phenotype.

2- The effect of PEPCK on lipid metabolism in CIN tumours needs to be investigated. We do not know how PEPCK is affecting lipid metabolism. Is it affecting the energy generation capabilities of lipids, structural investment of lipids like membrane production, cellular signalling or all of these factors? To explore this further, levels of glycerol 3 phosphate and related lipid metabolism enzymes like glycerol kinase, acylCoA transferase should be measured. Lipid profiling should also be done to investigate what kinds of lipid are dysregulated.

3- Why is there ROS production in $PEPCK^{KD}$ CIN tumours? Although we suggest that the G3P shuttle is involved in ROS production, we do not know the detailed effect of the G3P shuttle on ROS generation.. The basic experiment can be glycerol -3-phosphate analysis in normal and CIN cells, to see if the shuttle is still active in the absence of PEPCK.

4- Clearance of NADH is the main driving force of the glycerol 3 phosphate shuttle. The level of NADH has not been measured in a PEPCK depleted context and NADH measurement would provide valuable confirmation of the effect of

the shuttle. Similarly measurements of phosphoenolpyruvate (PEP), and oxaloacetate (OAA) can also help to draw a conclusion about the inhibitory role of NADH in PEPCK deficient CIN cells.

5- The role of nucleotide synthesis in CIN tumours has not been tested. CIN cells need nucleotides for their DNA repair and replication. We still do not know what nucleotide synthesis interventions can kill tumours. G6PDH was selected because it could affect nucleotide synthesis. Alternatives of G6PDH, such as transketolase, can be investigated which are directly involved in nucleotide synthesis. This would also help to evaluate the possible role of G6PDH as an antioxidant or a nucleotide synthesiser.

Significance

Cancer is a leading cause of death worldwide. About 15.5 million people are currently diagnosed with cancer in the USA (Miller et al, 2016). Cancer treatment may enhance a patient's life but poor prognosis, drug resistance, relapse, and expensive treatments make it difficult to treat the cancer patients. One of the many factors which cause poor prognosis, drug resistance, and relapse of the disease is chromosomal instability (CIN). CIN may help cancer cells to develop genetic diversity which may provide a selective advantage against the drugs to the relapse the disease. However, CIN can also be targeted to kill cancer cells. In my lab we have targeted CIN specifically by inhibiting different proteins in CIN cells. We found inhibition of certain metabolic proteins resulted in the death of CIN cells specifically. I investigated the mechanism involved in the selective killing of the CIN cells with metabolic interventions. I found that CIN cells were sensitive to metabolic stress. I further extended my results to CIN tumours and found deficiencies of metabolic proteins involved in glycerogenesis could stop the CIN tumour growth. However, the exact mechanism by which glycerogenesis disruption could stop the tumour growth is still in a preliminary phase. Nonetheless, I found NADH and ROS levels could be critical for the CIN cells, for their survival. CIN cells already have high ROS levels, so increasing ROS in CIN cells can induce apoptosis in CIN cells while not killing normal cells. However, the level of NADH levels in CIN cells needs to be addressed. If NADH is also high like ROS then we can also target NADH levels to kill CIN cells specifically.

Conclusion

My aims were to identify metabolic targets in CIN cells, to replicate their capacity to kill CIN cells in a tumour background, and to characterize the mechanism which could be involved with the selected candidates. My initial work identified metabolic candidates related to all aspects of metabolism, including glycolysis, Krebs cycle, electron transport chain, gluconeogenesis, redox maintenance, and lipid oxidation. When tested in a tumour background, only PEPCK and Mfe2 gave me promising results. I focused my attention on PEPCK because of its important role in gluconeogenesis and glycerogenesis. I suggest a critical role for PEPCK in regulating the glycerol-3-phosphate shuttle in CIN tumours. I found that glycerol 3 phosphate is involved in ROS production in CIN cells. I also found that PEPCK and the glycerol 3 phosphate shuttle affect glycolysis by affecting NADH levels in CIN tumours.

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Appendix 1

The role of JNK signalling in response to oxidative DNA damage

Cancer cells are characterised by elevated oxidative stress. In cancer cells, there is an intricate relationship between oxidative stress and the anti-oxidant response to allow their survival (Liou and Storz, 2010). Oxidative stress exceeding a threshold limit can kill a cell (Shaukat et al, 2014). This threshold limit is hard to define because every cancer cell is genetically different, and metabolic and environmental requirements of the individual cells induce a different response to a common stress (Sossa et al, 2012; Reuter et al, 2010). Oxidative stress is produced by excessive reactive oxygen species (ROS), which are produced during cellular metabolism. Any aberration in cellular metabolism can increase ROS levels pushing the cell towards oxidative stress (Barzilai and Yamamoto, 2004).

Oxidative stress is related to a wide range of diseases, including, Alzheimer, Parkinson, inflammatory, cardiovascular, diabetes, ageing and cancer (Sosa et al, 2013). One of the most common products of oxidative stress is oxidative DNA damage (Cooke et al, 2003). Reactive oxygen species (ROS), interact with DNA to produce modified DNA bases. These modifications can result in chromosomal aberrations, such as DNA breaks, over a period of time (Maser et al, 1997). To overcome these aberrations, the cell activates various repair mechanism, which may include the activation of stress responses (Barzilai and Yamamoto, 2004). JNK is one of the many stress response proteins which are activated in response to DNA damage (Pearce and Humphrey, 2001).

JNK can regulate NF-κB, p53, AP-1, H2AX protein to induce a wide variety of cellular responses to the stimulus (Picco and Pages, 2013). We have found that JNK is an important factor for CIN cells survival. When knocked down in CIN cells, loss of JNK induces apoptosis (Shaukat et al, 2014). We also found that JNK is important for DNA repair in the G2 phase of mitosis (Wong et al, 2015).

In this chapter "The role of JNK signalling in response to oxidative DNA damage", we will be discussing the effect of JNK in response to oxidative DNA damage. We have described various oxidative stress response which can activate JNK. We have also discussed JNK activated responses which can regulate cellular behaviour. These responses include DNA repair, autophagy and cell death. This chapter may help us to review the therapeutic implications of JNK.

Statement of Authorship

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Appendix 2

Figures

Figure A-2a

	$Brat^{RNAi}$	$Brat^{RNAi}$ SOX
Tumour Size (mm^2/week)	0.445923	0.39897469
	0.300769	0.442861906
	0.411308	0.355087474
	0.598923	0.634369757
	0.502462	0.313480114
		0.591479978
		0.360787113
		0.302080837
Average Growth Rate	0.451877	0.442434433

Figure 2a: *Effect of SOX on tumour growth*

Figure A-2b

Passage 1	Passage 2	Passage 3
2	2	1
2	1	1
2	1	0
2	1	0
2	1	0
2	2	0
2	1	0
2	1	1
2	1	1
2	1	0
2		0
2		

Explant Hosts

Figure 2b: *Number of ovary in the tumour host fly*